
THE MORPHOMETRIC AND IMMUNOCYTOCHEMICAL ANALYSIS

ON THE HUMAN CAROTID BODY

Thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Muzaffer Seker, "THE MORPHOMETRIC AND IMMUNOCYTOCHEMICAL ANALYSIS ON THE HUMAN CAROTID BODY"

The carotid bodies are paired organs located at the bifurcation of the common carotid arteries. Together with the aortic bodies they constitute the major arterial chemoreceptors that monitor the partial pressure of oxygen, carbon dioxide, and pH of arterial blood. The carotid body consists of groups of specific cells located in a rich vascular connective tissue including many nerve fibres. The functional unit of the organ: the cell clusters or glomoids, consists of two different cell types, namely the Type I cells and Type II cells.

The thesis reviews previous work on the structure of human carotid body (HCB) and experimental studies so far on the organ including histopathological studies. In this thesis the following four types of study are reported: 1-General histology and ultrastructure of the normal human carotid body; 2-Postmortem changes in rat and human carotid bodies, the distribution of Type I cells in different pathological conditions and after different postmortem delays in fixing the tissue; 3-Quantitative studies of the human carotid body using morphometric and stereological techniques in various pathological conditions especially chronic obstructive pulmonary diseases "COPD", Essential hypertension (EH), Diabetes mellitus (DM), Thoracic carcinoma (THC); 4-The analysis of cell constituents of human carotid body using immunocytochemical (ICC) techniques. The following antibodies were studied: Neurofilament (for nervous constituents), S100 protein (for Type II & Schwann cells), Synaptophysin (for Type I cells), Von Willebrand Factor (to outline blood vessels), and Vimentin (for cells of mesenchymal origin). Their immunoreactivity in different pathological conditions are also discussed. The carotid bodies in experimental animals contain only one variety of Type I cells whilst in the human carotid body three varieties of this cell type have been described (namely light, dark and pyknotic cells). The present study provides data, obtained from different postmortem human material, showing that the three different varieties of Type I cell are the effect of delays in fixation (autolytic changes) on the cell structure. The quantitative study of the distribution of cells, ultrastructural observation, and ICC analysis of the organ all together provide data that to perform an adequate human carotid body study it is essential that specimens should be obtained within 8 hours for light microscopy and immunocytochemistry and within 3 hours for electron microscopy. It has been reported that in chronic hypoxia & EH there is an increase in the size of the organ. The present work confirms these reports and in addition, provides stereological data that DM, THC, and chronic infection also alter HCB structure (hypertrophy and/or hyperplasia). The ICC study of COPD cases shows very variable results: in some cases the classic picture of the structural changes could not be observed whilst in others it was very strong. It was observed that there is an increase in the number of blood vessels and the volume of vasculature in COPD, EH, and DM. The morphological and ICC data presented provide new insight into the structure of human carotid body and advance the postmortem study of the organ. The changes in the structure of the human carotid body in different pathological conditions also provide better understanding of the condition of the organ in health and disease. It is also postulated that the previous experimental animal studies which were exposed to "long term hypoxic conditions" may not have been sufficiently exposed. This is essential to obtain similar structural changes that match human observations. It might be interesting to study much longer periods of hypoxia on experimental animals in order to see whether these longer exposures result ultimately in Type II cell hyperplasia.

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DEDICATION

TO MY FAMILY;

My wife, Nurten

My daughters, Hatice Seher & M.Elif

for their patience, help and understanding

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It is great pleasure to acknowledge the support of colleagues too numerous to mention during the four years. I have been fortunate to work in three different countries. I wish to place on record the invaluable help of the academic and technical staff throughout my stay in all places. Especially, in Leicester, Mr. Glyn Bottomley, Mr. Simon Byrne, Mr. Chris D'Lacey, Ms. Judy McWilliam, Ms. Judy Ravenhill, Miss M.Reeve, and other colleagues and postgraduate students in the Anatomy department, In Tel-aviv; Dr. I.Hammel, Dr. I. Abramovici, In Al-Ain; Dr. Ernest Adeghate, Mr. Ijaz Ahmed, M. Hussein, A.S. Ponery, Ashok Prasad, Fabian D'Souza, Mrs Suman Wahi and academic colleagues who promptly supplied anything I asked and also supported me during the my presence in their department. It was a great pleasure to work in the same department or laboratory with them.

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CHAPTER ONE

INTRODUCTION

The carotid bodies are paired organs located at the bifurcation of the common carotid arteries. Together with the aortic bodies they constitute the major arterial chemoreceptors that monitor the partial pressures of oxygen and carbon dioxide and also the pH of arterial blood. These organs play a major role in the reflex control of ventilation and, in the case of the aortic bodies particularly, in the reflex control of the cardiovascular system. Although the major physiological role of the chemoreceptors is well established, the nature of the chemosensor is largely unknown.

The carotid body has a rich history. It was referred to as a ganglion, a gland, a vascular glomus and paraganglion before its true nature as an arterial chemoreceptor was elucidated. The changes in thinking about structure-function relationships and the pathology of the carotid body have been discussed and reviewed in numerous international symposia on the chemoreceptors; especially more recently the regular meetings held by the International Society for Arterial Chemoreceptors (Stockholm 1951, Oxford 1966, Bristol 1973, Kashmir 1974, Dortmund 1976, Valladolid 1979, Leicester 1982, Lisbon 1985, Salt Lake City 1988, Warsaw 1989, Chieti 1991, Dublin 1993). A number of books and reviews on the carotid body have also appeared (Adams, 1958; Biscoe, 1971; Verna, 1979; McDonald, 1981; Acker & O'Regan, 1983; Pallot, 1983b, 1987, and Heath & Smith, 1992); these have provided an insight into the literature for which the present author is indebted.

Initially, I will summarize the early literature on carotid body, from its discovery to its establishment as a peripheral arterial chemoreceptor.

FROM GANGLION TO CHEMORECEPTOR

There is little doubt that the carotid body was first described by Albert von Haller (1747,1749). According to his pupil Berckelmann (1744) Haller demonstrated nerve rami leaving a ganglion at the carotid bifurcation and called this structure the ganglion exiguum. A further pupil of Haller's, H.W.L. Taube called this structure in the angle between the external and internal carotid arteries, the ganglion minutum and noted its connections to these two arteries (Taube 1743).

Andersch (1797) believed he was the first to describe the ganglion and its rich blood supply. He introduced the name ganglion intercaroticum and provided the first description of the innervation of the organ. He recorded, correctly, that the sympathetic chain, and the glossopharyngeal nerve sent branches into the organ and was the first of a number of authors to believe that the vagus also innervated the organ. The work of Knoche and Kienecker (1977) established that this latter belief is incorrect as they observed that after vagotomy no structural changes occurred in the carotid body tissue.

Despite this work the carotid body remained forgotten for over forty years until Mayer (1833,1834) rediscovered the structure and remarked upon the branches of the sympathetic chain, the glossopharyngeal and vagus nerves which he believed were the sources of a nerve plexus innervating the organ. In addition to this description of innervation Mayer also noticed the attachment of the ganglion intercaroticum to the angle of the internal and external carotid arteries by a fibrous ligament which is now known as the ligament of Mayer. Valentine, in the same year, also recorded that small vessels apparently entered the organ and that these small vessels arose from the bifurcation of the external and internal carotid arteries. Valentine (1833) believed that the ganglion intercaroticum was part of the sympathetic nervous system despite the fact that the vagus and glossopharyngeal nerves were described by him as contributing to its innervation.

There was another gap on carotid body studies until the use of the microscope became more common. The first microscopic study of the carotid body was performed by Luschka (1862). He renamed the organ the glandula carotica and was thus the first to recognize the similarity of the carotid body to other glandular

structures. It possessed a very rich nerve supply and numerous blood vessels. In addition to this he provided the first accurate measurements of the size of the organ, his records suggesting that on average in man it was 5 mm long and 2.5 mm wide. By recognizing the structural similarities of the carotid body with structures like the adrenal and pituitary glands he started a line of thought and argument which has continued through to recent times.

The next important contribution was made by Svitzer (1863) who reported that the carotid body was innervated solely by the glossopharyngeal nerve. Despite the descriptions of the innervation of the carotid body, Arnold (1865) considered that the organ was no more than a vascular glomerulus and renamed it *glomeruli arteriosi intercarotica*. He thus refused to accept the view of Luschka that the organ might be glandular in nature and added that the chief cells were derived from endothelial cells lining the blood vessels.

The next stage in the development of ideas concerning the structure of the carotid body involved a number of studies associated with its origin. Stieda (1881) noticed a thickening of the epithelium on the third branchial arch and considered this to be the *anlage* of the carotid body. This idea was supported by Prennant (1894). A number of other workers e.g. Fischelis (1885), Rabl (1886), de Meuron (1886), Maurer (1899) considered this view to be correct. It remained for Jacoby (1895, 1896) to demonstrate that the work of Prennant was based on a fundamental error, for what he had regarded as the carotid body was in fact the primordium of parathyroid III, as had been previously suggested by Born (1883). Kastschenko (1887) came nearer to what is now regarded as the truth when he said that the carotid was not a derivative of the branchial arches but came from a proliferation of the wall of the internal carotid artery and close contact between the glossopharyngeal nerve, the vagus nerve and the cervical sympathetic trunk.

Stilling (1892) attempted to reconcile the two view points on the origin of the carotid body. He was the first to notice a chromaffin reaction in the cells within the carotid body which, since the time of Henle (1865) had been thought to be a specific technique for the histochemical detection of adrenaline and other amine-like substances. Subsequent to this report of Stilling the chromaffinity, or otherwise, of carotid body cells has been one of the most contentious issues in the anatomical study of chemoreceptors. Thus Kose (1902,1907) was unable to see a

chromaffin reaction in the cells of the carotid body of birds and as a result of this referred to them as clear chromaffin cells (farbloose Chromaffin Zellen). The other major contribution of the early part of the twentieth century to the study of the carotid body was that of Gomez (1907,1908), who described two specific cell types within the organ.

There followed another gap so common in the history of the carotid body where apart from the odd mention in textbooks of histology under various headings such as endocrine tissue, chromaffin tissue or paraganglion caroticum, little more was published about the organ for some twenty years..

The most significant contributions to the understanding of the function and structure of the carotid body have been the studies of Fernando De Castro (1926, 1928). He performed the first systematic studies upon the innervation of the organ and described the numerous nerve fibres and terminals associated with the parenchymal cell using classical methylene blue and silver techniques. De Castro (1926) demonstrated experimentally that the carotid body was innervated by a branch of the glossopharyngeal nerve and that this innervation was sensory in nature. The technique employed is worthy of further description, for this question of the nature of the innervation of the carotid body has been debated ever since. De Castro sectioned the rootlets of the glossopharyngeal nerve between their exit from the brain stem and its sensory ganglion. Twelve days after this operation the carotid bodies were examined and, as there were no degenerative changes within the organ, he assumed these nerve endings were sensory in nature (see also below). Overall the results of the classic experimental works of De Castro showed that (1) nerve fibres and terminals in the carotid body were derived primarily from the carotid sinus nerve (branch of the glossopharyngeal nerve), (2) individual nerve fibres branched within the organ to innervate many cells which were often located in different parts of the organ, (3) some cells were innervated by more than one nerve fibre and (4) that a single nerve fibre could give rise to terminals displaying variable morphologies including small boutons, cup-shaped (calyceal) endings and large plate-like endings,

De Castro suggested that the parenchymal cells appeared to possess polarity, one pole located close to blood vessels (pole sanguin) and the opposite pole associated with the nerve endings (pole nerveux) and from this observation De Castro (1940, 1951) suggested that the carotid body was a chemoreceptor which

functioned to perceive the concentration of oxygen and carbon dioxide within blood. Hence the carotid body was declared as a sensory end organ rather than a mere paraganglion.

Heymans and Heymans (1927) and Heymans et al. (1933,1958) provided evidence that the area in the region of the bifurcation of the common carotid artery was a peripheral reflexogenic area which was sensitive to the chemical composition of the blood and was stimulated by hypoxia, hypercapnia and blood pressure changes. They recorded activity from the whole carotid sinus nerve which showed an increase in the neural discharge in response to asphyxia. They thus confirmed that a structure innervated by this nerve was a sensory organ which responds to changes in the blood chemistry, and provided support for the theories of De Castro. Since the discovery of the chemoreceptive functions of the carotid body, almost every structure contained within the carotid body has been suggested as the "chemoreceptor".

THE GROSS ANATOMY OF THE CAROTID BODY

The carotid body is a small organ located bilaterally in the neck, at the bifurcation of the common carotid arteries (Fig 1.1). Reddish-brown in fresh specimens due to the amount of blood in the organs, they are ellipsoid structures related to the carotid sinuses. In the human each carotid body is approximately 18-30 mg in weight, 5-7 mm in length and 2.5-4 mm in width (Luschka, 1862; Adams 1958) and lies either posterior to the carotid bifurcation or between the starting point of its branches being attached to, or sometimes partly embedded in, their adventitia. The classic carotid body is a simple ovoid however it has several anatomical variants that are in descending order of frequency, the bilobed (including the V form), the double and the leaf shaped (Adams, 1958; Smith et al., 1982; Khan et al., 1988, see Fig 1.1). The carotid body receives a very rich blood supply but the origin of this blood supply varies. It may be derived from the internal or external carotid arteries or from branches of the latter vessel. The carotid body is innervated by two sources; the carotid sinus branch of the glossopharyngeal nerve (IX CN.) and a direct branch from the superior cervical ganglion (Fig 1.2).

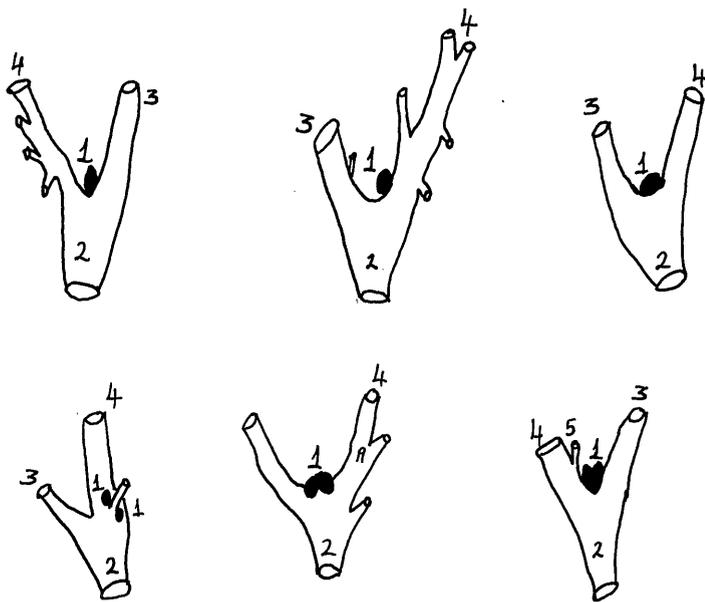


Fig 1.1. illustrates the variations of the human carotid body location and shape. 1: Carotid Body, 2: Common Carotid Artery, 3: Internal Carotid Artery, 4: External Carotid Artery, 5: Ascending Pharyngeal Artery (Gomez, 1908; Adams, 1958)

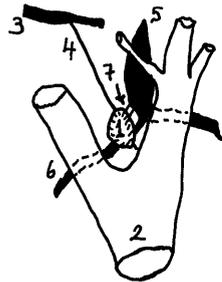


Fig 1.2. illustrates the location and innervation of the carotid body: 1:Carotid body, 2:Common Carotid Artery, 3:Petrosal ganglion, 4:Glossopharyngeal nerve (IX.C.N), 5:Superior cervical ganglion, 6:Sympahetic trunk, 7:Ganglioglomerular nerves

THE HISTOLOGY OF THE CAROTID BODY

The carotid body is surrounded by a capsule which, depending on the species, varies in thickness. Septa from this connective tissue capsule divide the carotid body into a number of lobes. Each lobe consists of several lobules, glomoids or cell clusters, separated by variable amounts of connective tissue and made up of the two specific cell types first described by Gomez (1908) (see fig 1.3a,b). Grimley and Glenner (1967, 1968), Seidl (1975) and Lubbers et al. (1977) have suggested that the cell clusters or glomoids are the functional units of the organ. The carotid body is immensely vascular (up to 25% of the total organ volume may be vasculature; Pallot et al. 1985, 1986), and the connective tissue septa of the organ provide a route of access for the numerous nerve fibres which innervate the specific cells.

The connective stroma contains fibroblasts, plasma cells, eosinophilis, collagen fibres, mast cells and occasional ganglion cells. It has been suggested by Chiocchio et al. (1967, 1971a,b) that mast cells may be responsible, at least in part, for the serotonin content. The occurrence of ganglion cells, which are usually located at the organ periphery, have been reported in the carotid body for many years (e.g. Kohn 1900), according to Adams (1958) ganglion cells are more numerous in some species than in others. They are usually larger than Type I cells (cell bodies of 20-40 nm in diameter, Pallot 1987) with large, round vesicular nuclei; their copious cytoplasm is finely granular and basophilic. In the rat carotid body some ganglion cells appear degenerate during maturation of the organ. It is more common in the embryo than in the adult. According to Biscoe and Silver (1966) there are three to five ganglion cells in the cat carotid body. de Kock (1956) in the pilot whale, Abraham (1966, 1968) in the Hedgehog and Kondo (1976a,b) in the rat have described that the cell clusters made of Type I cells, Type II cells and ganglion cells. However, according to Adams (1958) and Verna (1979) this kind of association within the cluster cell structure appears rather exceptional. The study of McDonald and Mitchell (1975) indicate that ganglion cells probably innervate the carotid body blood vessels and hence may regulate vascular tone. Apart from the vascular distribution some ganglion cells also innervate the Type I cells. Although most ganglion cells are at the periphery of the organ there is evidence that some are in contact with Type I cells.

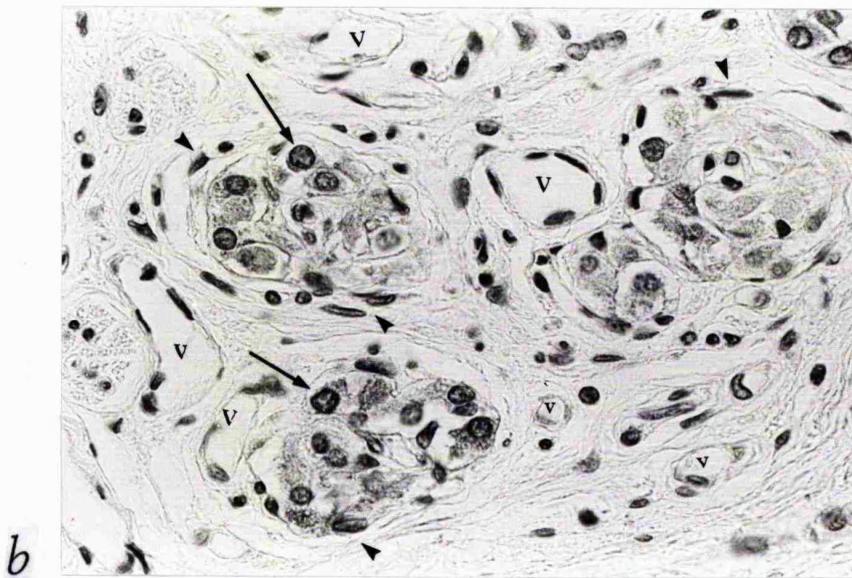
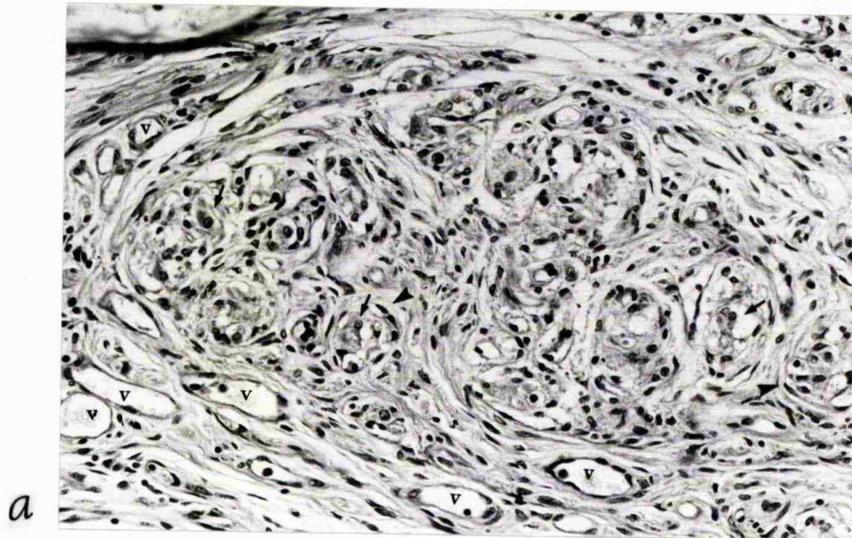


Fig 1.3a, and b show the cell clusters which consist of Type I (arrows) and Type II (arrow heads) cells set in a vascular connective tissue. (V: blood vessels, a:x20, b:x40)

However, McDonald's observation is that it seems likely that the proportion of Type I cells which contact ganglion cells is very small (McDonald, 1981; Pallot, 1987). Wharton et al. (1980) have reported some vasoactive intestinal polypeptide (VIP) immunoreactivity in some ganglion cells of cat carotid body. It has been suggested that carotid body ganglion cells may be of two varieties; orthosympathetic and parasympathetic (Smith, 1924; Watzka, 1943; McDonald and Mitchel, 1975a,b).

The main structural and functional units of the carotid body are the cells clusters. On the basis of nuclear morphology and cytoplasmic staining patterns there are two different cell types within each cell cluster. For different reasons all authors are using different terms for the cell type of the cell clusters (see table 1.1). Using the terminology of Biscoe (1971) I shall use the terms Type I and Type II cells for these two types of specific cells (Gomez, 1908; de Kock and Dunn, 1964,1968; Biscoe, 1971; & Biscoe, 1977; Al-Neamy, 1983, and Pallot 1987).

Authors	Type I cell	Type II cell
De Castro (1951)	Epitheloid cell	
DeKock & Dunn (1951;1954;1966)	Glomic, Specific, Typical, Epitheloid	Interstitial, Type II
	Glomus, Type I, & Type I cell	
Adams (1958)	Chief cell	Accessory Cells; (Glomus cell type II, Interstitial & Fuchs-Inagaki cell)
Ross (1959)	Chemoreceptor cell	Sustentacular cell, Supporting cell
Eyzaguirre & Uchizono (1961)	Chief cell	Sustentacular cell
Blumcke et al. (1967)	Receptor cell	Enclosing cell
Kraus & Martinek (1967)	Glomus Cell, Chemoreceptor cell, A & B cells	Capsular cell
Grimley & Glenner (1967)	Chief cell	Sustentacular (Schwann-like) cell
De Castro & Rubio (1968)	Chemoreceptor cell	Satellite cell
Bottaglia (1968)	Specific Cell	Satellite cell
Hess (1968)	Glomus cell	Capsule, Supporting Cell
Al-Lami & Murray (1968)	Enclosed, Glomus Cell	Enclosing Cell
Morita et al. (1969)	Main cell	Sustentacular cell
Biscoe (1971)	Type I cell	Type II cell
Kobayashi (1971)	Chief cell	Sustentacular cell
McDonald & Mitchell (1975,1983)	Glomus cell	Sheath cell
Heath et al. (1982, 1992)	Chief cell	Sustentacular cell
Pallot et al. (1982,1987,1992)	Type I cell	Type II cell

Table 1.1 showing the different terms of the carotid body cells used by different authors.

TYPE I AND TYPE II CELLS

The parenchymal cells consist of two varieties, Type I and Type II cells. A variable number of polygonal Type I cells are enveloped by the Type-II cells except in some places where the Type I cell membrane is separated from extracellular spaces by a basement membrane only. The Type-I cell is a large, oval, round or polygonal cell, and has a round or ovoid nucleus with abundant light euchromatin. In the electron microscope the nuclear envelope of Type-I cells show numerous pores and the cytoplasm have numerous small, membrane-bound, dense core granules (Figs. 1.4a, & b).

In the light microscope, Type I cells are seen to possess more or less spheroidal cell bodies up to 20 μm in diameter. In the electronmicroscope the cell is seen to contain the usual complement of organelles, which are equally distributed throughout the cytoplasm and hence the cells show no polarity. Type I cells possess a good complement of mitochondria with transversel cristae which are randomly distributed in the cytoplasm of the cell body. However, according to Seidl et al. (1977), the percentage of the cytoplasm volume occupied by mitochondria is smaller in Type-I cells than in liver cells. This feature is surprising considering the high oxygen consumption of the carotid body (Purves, 1970a, b; Leitner and Liaubet, 1971). Prior ventilation of the animals with 10% O_2 has been reported to lead to an increase in the volume percentage (Vv%) of mitochondria and a decrease in the Vv of electron dense-cored vesicles of the cat carotid body Type I cells as compared to the values after ventilation with 100% O_2 (Pallot et al. 1985). However, there was no change in the rat carotid body in mitochondrial Vv% but there was a significant increase in the volume of cytoplasm occupied by electron dense-cored vesicles. In the Type I cells, like other secretory cells, the Golgi apparatus is well developed, sometimes prominent and in the typical juxtannuclear position.

Some small clear vesicles, similar to synaptic vesicles, are present in the cytoplasm of Type I cells. According to Verna (1979) these vesicles, about 60 nm in size, are located just below the plasma membrane when the latter is exposed to extracellular spaces (without a Type II cell cover). It has been postulated that they are involved in cell to cell interactions between Type I cells and nerve endings. McDonald and Mitchell (1975a,b) have shown that approximately 40 % of these synaptic vesicles in Type I cells have a dense core after 5-

hydroxydopamine administration, suggesting that some of these vesicles may store a catecholamine (Verna 1979).

The most prominent and distinguishing feature of Type I cells is the presence of numerous electron dense-cored vesicles which were first described by Lever and Boyd (1957). Several extensive descriptive ultrastructural studies of the dense-cored vesicle have been published by Garner and Duncan (1958), de Kock and Dunn, (1966, 1968), Al-Lami (1964), Biscoe (1971), Matthiessen et al. (1973). These vesicles are usually spherical, more rarely irregular, in shape, each consisting of a core of variable electron density surrounded by a lighter clear zone and encased by a single trilaminar membrane. Their size, distribution and number vary greatly from cell to cell and area to area within the cell. According to Garner and Duncan (1958), and Lever et al. (1959) the electron-dense core is usually more prominent in a glutaraldehyde-fixed tissue which is post-fixed in osmium tetroxide than in the material fixed without glutaraldehyde. The Type I cells are known to originate from the neural crest cells in the bird. Le Douarin et al. (1972), and Abramovici et al. (1991) have noticed that Type I cells show similar cell markers to neuronal cells such as neuron specific enolase and neurofilament protein, as well as acting as a storage site of various neuropeptides and catecholamines.

The Type II cell is a flat branching cell with an oval nucleus rich in dense heterochromatin (therefore staining more densely) which is very difficult to distinguish from Schwann cells. Indeed, the Type II cells, like Schwann cells, envelop nonmyelinated fibres within the carotid body and provide a passage for these fibres to their target cells. Type II cells are much less numerous than Type I cells. According to Biscoe and Pallot (1972) there are about four or five times as many Type-I cells as Type II cells in the cat carotid body whilst McDonald and Mitchell (1975a,b) found that Type I cells outnumber Type II cells by a factor of 3 to 5. The estimation of the proportions of Type I cells and Type II cells is based on counts of cell nuclei identified by electron microscopy for the rat data and on serial reconstruction for the cat. Proportions of Type I cells and Type II cells in the human carotid body determined by light microscopy are similar to electron microscopic values. (The comparison of the proportions of Type I cells and Type II cells in different pathological conditions in the human carotid body will be discussed in the next chapter). The cytoplasm of Type II cells contain the usual organelles; a few mitochondria, endoplasmic reticulum, a poorly developed Golgi

apparatus, centrioles, microtubules and microfilaments. However, Type II cells, unlike Type I cells, do not contain dense-cored vesicles; they do not take up treated monoamines (Fig.1.4 a, and b).

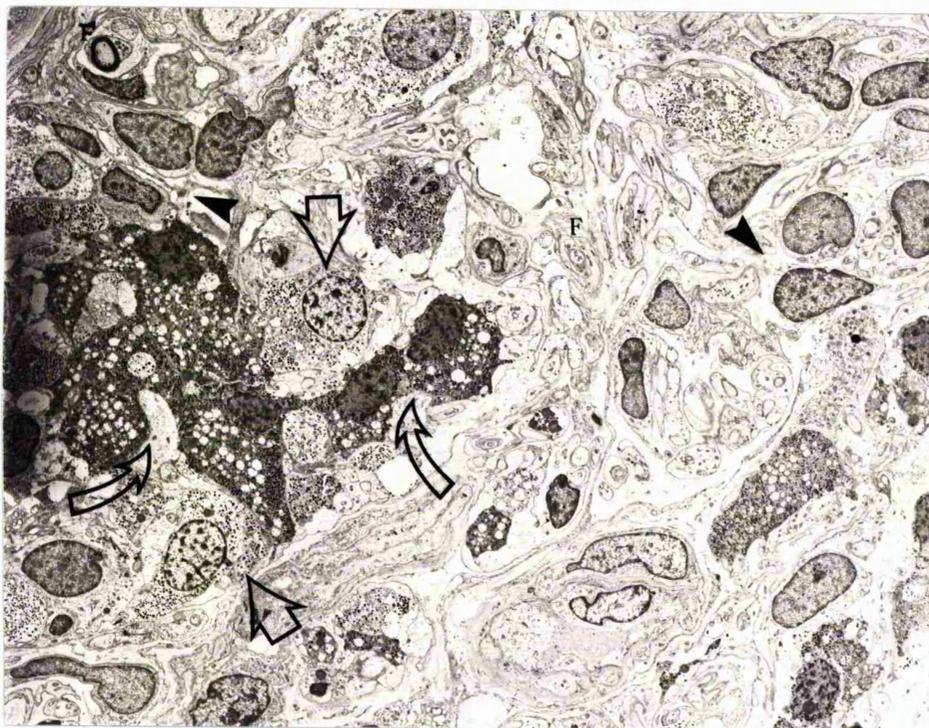
SUBTYPES OF TYPE I CELLS

Since the time of De Castro (1926) several authors have attempted to classify Type I cells on the basis of ultrastructural and/or cytochemical features using a variety of observational and morphometric techniques. Three broad categories have been used in attempts to provide a classification of subtypes of Type I cells;

- 1- Light, Dark and Pyknotic cells,
- 2- Chromaffin and Nonchromaffin Type-I cells,
- 3- Morphometric analysis of Dense-Cored vesicles (Type A and Type B or Large Vesicle Cells and Small Vesicle Cells).

1- Light, Dark and Pyknotic cells:

De Castro (1926) mentioned two types of Type I cells in the human carotid body. One type had a large, pale-staining nucleus, and the other had a small densely staining nucleus. Garner and Duncan (1958) and Lever et al. (1959) have noticed light-dark variations of electron opacity of the Type-I cells in the rabbit, but not in the cat carotid body. Their observation, two types of type-I cells on the basis of the electron density, has been confirmed in later studies for other species such as; horse and dog (Hoglund, 1967), monkey (AL-Lami and Murray, 1968b), monkey and human (Kraus, 1966), man (Grimley and Glenner, 1968), hamster (Chen et al.,1969), cat (Morita et al.,1969); (Al-Lami &Murray, 1968a); (Abbott et al. 1972), camel (Etemadi 1975), and again in rabbit (Verna 1977, 1979). The study of Morita et al. (1969) was the first to introduce morphometric analysis to the carotid body. They have described "Light" and "Dark" Type I cells in the cat carotid body. On the basis of vesicle diameter and density they divided their dark cell into three subtypes. However, they have not given the number of cells and the number of vesicles which have been used to calculate each mean. In the human carotid body a similar situation has been described by Heath et al. (1970), Edwards et al.(1971a/b), Smith et al. (1982). However, they are able to recognize three sub-populations of Type-I cells on the basis of nuclear morphology; these are termed the light (also called as a clear cell, see Pallot et al., 1992), dark and pyknotic cells.



Figures 1.4a, and b. Low power electronmicrographs of human carotid body tissue. Note how clusters of Type I (arrows) and Type II (arrow heads) cells are scattered in a loose connective tissue stroma which contains many nerve fibres (F). Note the cytoplasmic process arising from the cell bodies of Type I cells (curved arrows). (a: x2350, b: x1500)

However, Gosses (1938) observed that there were more dark-coloured nuclei in carotid bodies twenty-four hours after death in human carotid body than in the contralateral organ which was fixed soon after death. The light and/or dark appearance of cytoplasm has been observed in many other tissues such as central nervous system and the adrenal medulla (Cammermeyer, 1962 and Friedrich and Mugnani, 1981). The observation of Mc Donald and Mitchell (1975a,b) on the rat carotid body supports the suggestion of Cammermeyer (1962) on brain and spinal cord that dark cells can be produced by traumatizing tissues before or during fixation. There is great difficulty in this sub-division for it is well known that such apparent differences in electron density are often artefact. Considering all of the available data, Verna (1979), Mc Donald (1981), and Pallot (1983a,b, 1987) considered the density of staining of cytoplasm or nuclei to be unreliable to subclassify the Type I cell. The significance of such observations is doubtful. For many authors it is only a fixation artefact (Benedeczky and Smith (1972); Wacker and Forssmann (1972)). Whilst others consider it to show different physiological states of the cells at the time of fixation (Garner and Ducan, 1958). We will discuss this matter in more detail in the following chapter.

2. Chromaffin and Nonchromaffin Type-I cells:

For many years the chromaffinity or otherwise of the Type I cells has been a matter of controversy. First Kohn (1900) presented his results that the carotid body cells were stained yellow to a variable degree after being treated with potassium dichromate and suggested that the carotid body, along with the adrenal medulla, is a paraganglion which was composed of chromaffin cells. After obtaining different results from different species, Smith (1924) concluded that the number of chromaffin cells in the carotid body varied substantially among different species. They were abundant in the cow, less numerous in the cat and absent in the rat. Kobayashi (1968, 1971a,b) using modern methods reexamined the chromaffin cell issue in the carotid body. He found some chromaffin cells in the dog carotid body but in the rat and mouse he could not find any. However, by using fluorescence histochemistry in all three species (dog, rat and mouse) he demonstrated that all Type I cells store catecholamines or indoleamines. He suggested that the absence of chromaffin reaction in the carotid body did not always mean the non-existence of monoamines in the cells, but it may imply the

presence of small amounts of them. In addition, Bock and Gorgas (1976) showed in an electron microscopic study that electron dense-cored vesicles in Type I cells previously treated with dichromate are selectively stained. They also suggested that the term "chromaffin" should mean the capability to synthesize and store catecholamines or indolamines thus making the controversy redundant. However, Heath and Smith (1992) considered that to the naked eye the human carotid body is undeniably non-chromaffin. In addition they have postulated that from a practical standpoint the human carotid body is non-chromaffin only in that the reaction is too insensitive to label the cells containing biogenic amines which are visible either macroscopically or by light microscopy.

3- Morphometric Analysis of Electron Dense-Cored Vesicles: (Type A or Large Vesicle Cell and Type B or Small Vesicle Cell)

Numerous attempts have been made to differentiate sub-types of Type-I cells on the basis of electron dense-cored vesicle morphology (Kobayashi, 1968; Morita et al., 1969; Mc Donald and Mitchell, 1975a; Hellstrom, 1975; Verna, 1977; Pallot et al., 1985,1986, and Kusakabe et al., 1993). Morita et al. (1969) claimed that they were able to distinguish four types of glomus cells in the cat carotid body. They have given the size of dense cored vesicles in each of the four cell types but no information is given on the number of cells or vesicles evaluated and technique used for measuring vesicles. Without such information it is impossible to evaluate the quality of their data. As mentioned above, Kobayashi (1968,1971a,b) showed that the dog carotid body contained some cells with a positive chromaffin reaction at the light microscope level. These cells contained large electron dense cored vesicles whilst the non-chromaffin cells exhibited smaller vesicles. Because of their morphological and histochemical features he thought that "chromaffin cells" of the dog carotid body might correspond to norepinephrine secreting cells of the adrenal medulla.

The electron dense-cored vesicle (EDCV) diameter and subtypes of the rat Type I cell have been studied by different authors (see table 1.2). Because of either the methodological differences or the lack of methodological information the interpretation of their data is difficult. However, Gronbald and Eranko's study (1978) provides very important evidence upon the effect of the fixation method on

EDCV diameter. Hess (1975), Morgan et al. (1975) and Al Neamy (1983) reported one population of Type I cells on the basis of the diameters of the electron dense-cored vesicles in the rat carotid body.

However, working independently but making similar measurements Hellstrom (1975), and Mc Donald and Mitchell (1975a) came to the conclusion that on the basis of the measurement of the diameter of the electron dense-cored vesicles, two subtypes of Type I cells existed in the rat carotid body. Their results indicate that the mean diameter of electron dense cored vesicles of one cell type (called Type A by Mc Donald and Mitchell, Large vesicle cells "LVC" by Hellstrom) was about 30% larger than that of vesicles of the other cell type (called Type B by Mc Donald and Mitchell, Small vesicle cell "SVC" by Hellstrom). The mean vesicle diameters were calculated as 119 nm for Type A and 90 nm for TypeB by Mc Donald and Mitchell (1975a), whilst Hellstrom measured diameters of 47.4 and 63.0 nm for SVC and LVC respectively. These two types were present in almost equal proportions. It is not clear why the result of their studies are so different despite using the same species. Mc Donald and Mitchell's observations were based upon data taken from a small selection of cells which had already been subjectively classified into two group on the basis of qualitative observations; such a methodology is not conducive to an objective study. It must be emphasized that according to Verna (1979) and Pallot (1983b) most of these studies have suffered to a greater or lesser degree from methodological problems or from incomplete description of the techniques employed. Avoiding such methodological problems (e.g selecting the cells at random and measuring an adequate number of cells and vesicles) is an absolute requirement for valid quantitative studies. Verna (1977) studied the rabbit carotid body. His data showed a great variation in the size of dense-cored vesicle with extreme values of 50 and 250 nm. The mean diameter was calculated for each cell and ranged between 93 and 146 nm. Most cells possessed dense-cored vesicles with a mean diameter of about 115 nm. Such data in isolation could indicate small and large vesicle cells. However, when all of the data from all of the cells is considered together then we see a unimodal distribution of cored vesicle diameter suggesting that such a measure is inadequate to provide concrete evidence for the existence of small and large vesicle cells.

Al-Neamy (1983) studied both the rat and cat carotid bodies. In order to show the importance of random sample technique, he analysed data from the rat in two

ways. First Al-Neamy measured the vesicle diameter of rat Type I cells which were classified on subjective criteria as large and/or small vesicle cells (as done by McDonald and Mitchell 1975a). His results in this study were similar to McDonald and Mitchell's (1975a). Large vesicle cells were 15% larger than the small vesicle cells and the vesicle density in large vesicle cells were about three times higher than small vesicle cells. Secondly he combined all of the data from large and small vesicle cells and found overall mean of 106 nm; furthermore the distribution histogram of the diameters of all of vesicles from all of the cells were unimodally distributed. According to the shape of dense cored vesicles the Type I cell was classified into four types in the rat carotid body exposed to long term (10-12 weeks) hypoxia by Kusakabe et al.1993 (see table 1.2).

Authors	EDCV Diameter	Sub-types of Type I cells
Hess (1975)	60-90 nm	One Type
Morgan et al.(1975)	93-185 nm	One Type
McDonald & Mitchell(1975)	116 nm	Type I A
	90 nm	Type I B
Hellstrom (1975)	71 nm	Large Vesicle Cells "LVC"
	52 nm	Small Vesicle Cells "SVC"
Grandbold & Franko (1978)	110-150 nm	Fixation: with Glutaraldehyde
	90-130 nm	Fixation with Neutral Permanganate
	50-120 nm	Fixation with Acid Permanganate
		(Rechart et al. 1977)
Al-Neamy (1983)	101 nm	One Type
Kusakabe et al. (1993)	50 nm	Small Vesicle Cells "SVC"
	80 nm	Large Vesicle Cells "LVC"
	400-800 nm	Dilated Eccentric Vesicle Cells "EVC"
	Large & Eccentric	Mixed Vesicle Cells "MVC"

Table 1. 2 shows the electron dense-cored vesicle diameter and subtypes of the rat Type I cells studied using identical criteria by different authors

Using morphometric and stereological techniques, Blakeman et al. (1984) and Pallot et al. (1985,1986) could not find any evidence in the rat carotid body for subtypes of Type I cells on the basis of vesicle size and vesicle density but were able to find subtypes in the cat carotid body. With regard to the differences between their results and those of McDonald and Mitchell (1975a) and Hellstrom

(1975) and his group Pallot (1987) suggested that it may have occurred either because of strain differences or as a result of inadequate sampling. On the basis of vesicle diameter in the cat carotid body Pallot et al. (1985) and Pallot (1987) were able to distinguish two cell types. On the significance of different Type I cell types in the carotid body, they suggested that in different species the different Type I cell types may be correlated with catecholamine content of the organ. It will be discussed under the catecholamine headline.

All of the evidence available to date taken together suggest that measurement of vesicles does not permit us to distinguish sub-classes of Type I cells in any species except the cat.

BLOOD VESSELS

The carotid body has a very prominent vasculature. Most of the vessels within the capsule of the organ are typical fenestrated capillaries or thin walled venules but a few are clearly fine branches of the arterial system, being large with thicker walls containing two or more layers of smooth muscle cells. The capillaries account for some 20-25% of total organ volume (Seidl, 1975,1977; McDonald, 1981,1983; Ballard et al. 1982; Pallot, 1987). In keeping with this Vv% for vessels the carotid body has a large blood flow some 2000ml/g wet weight per minute (Daly et al., 1954; Purves, 1970a, and b; Keller and Lubbers, 1972; Lubbers et al., 1977) a comparison of this figure with similar data for brain (60 ml/min per 100g wet weight; Dumke and Schmndt 1943), or with heart muscle (64-150 ml/100g wet weight per min; Gregg 1950) illustrates the enormity of the blood flow.

The structure of the glomic arteries have been studied in a number of different species (e.g. Becker, 1966; in rabbit; Abraham, 1968 in hedgehogs, cats, dogs and man; Edwards and Heath, 1969; Jago et al.,1982, 1984, 1986; Heath et al., 1983 in man; McDonald and Le rue, 1983; McDonald, 1983; McDonald and Haskall, 1984 in rat). In all species studied so far the distribution of the arterial supply of the organ is variable within a given species and it has been reported that the vascularity of the organ increases with age (Murotori, 1943; Adams, 1958; Seidl, 1975; Pallot, 1987). The venous drainage of the organ is also similarly species

dependent and is either to the external and/or internal jugular veins. Abraham (1968) suggested the possibility that baroreceptor nerve endings were located in the walls of the veins draining the carotid body.

The carotid body is supplied by one artery which originates from the carotid bifurcation area most commonly from the external carotid artery but considerable variations occur (the cat carotid body can be supplied by three or more main arteries, Seidl, 1975). After providing branches to the carotid body the glomic arteries or artery leave(s) the organ to supply neighbouring structures such as the carotid sinus and/or the superior cervical ganglion (Chungcharoen et al.1952a,b). The glomic arteries have a very flexible wall made of concentric elastic laminae surrounded by only a few layers of smooth muscle fibres (De Castro and Rubio, 1968; Heath and Edwards, 1971). De Castro suggested that the artery had a flexible wall to permit changes in blood pressure to stimulate baroreceptive nerves in the adventitia. Jago et al., (1984) showed that the glomic artery outside the carotid body had an appearance similar to that of the carotid sinus: with closely packed bundles of elastic fibres separated by tightly packed bundles of collagen fibres, fibrocytes and unmyelinated nerve fibres.

The main glomic artery divides into a number of smaller branches within the organ. The first order branches have a predominant elastic media. Such vessels were found between the lobules of the carotid body. These interlobular arteries gave rise to muscular intralobular arteries which entered the lobules and broke up into capillaries. Between the elastic laminae of the glomic arteries the presence of nerve fibres suggest that the vessels may have a specific function however not all species show this structural arrangement (Pallot, 1987). The capillaries are very thin walled and surrounded by well developed basal laminae. Each capillary is lined by two or more typical endothelial cells of variable thickness (Hodges et al., 1975). Biscoe and Stehbens, (1966) were the first to produce really well fixed material for electron microscopy study and were the first to report that the capillaries were fenestrated (a feature of endocrine glands - an interesting feature which correlates well with the suggestion of Luschka, 1862). Jago et al. (1984) claimed that in human the fenestration feature of the endothelium is inconspicuous; in view of the quality of the fixation of their material this finding is not surprising.

McDonald, (1983) observed that in the rat carotid body the terminal arterioles which supply the specific tissue arise from third or fourth order branches of the glomic artery, apparently some 14% of these terminal arterioles feed directly into veins. Schafer et al., (1973) have described similar structure in the cat and dog carotid bodies. Two kind of arteriovenous anastomoses (bridge anastomoses and spiral anastomoses) have been reported by Schafer et al., (1973) and Seidl, (1975). It appears that the suggestion of De Castro, (1951) that arteriovenous anastomoses are a characteristic feature of the carotid body has been confirmed. In addition the presence of two types of capillaries both with a fenestrated endothelium are reported within the organ (De Castro and Rubio, 1968; Mc Donald, 1983). Type I capillaries are very large vessels (14 to 28 um in diameter) and penetrate clusters of Type I cells whilst Type II capillaries are less numerous, much smaller (6 to 10 um in diameter) and tend to constitute bridges between type I capillaries. Although the type I capillaries have multiple connections with venules, the type II capillaries have only one or two connections and do not enter cell clusters (McDonald, 1983).

The vessels within the carotid body receive an extensive innervation from both sympathetic and parasympathetic nerve fibres. In the rat most of the parasympathetic fibres have their cell bodies within the carotid body while most of the sympathetic fibres have cell bodies in the superior cervical ganglion. Whilst it is assumed that the normal transmitters act at these postganglionic terminals, Lundberg et al., (1979); Wharton et al., (1980); Cuello and Mc Queen, (1980) supplied the evidence that fibres containing VIP (vasoactive intestinal polypeptide) and Substance P end near carotid vessels. From the physiological standpoint, parasympathetic activation leads to vasodilatation (Biscoe et al., 1969; Neil and O'Regan, 1971; O'Regan, 1977) and sympathetic activation leads to vasoconstriction (Daly et al., 1954).

THE INNERVATION OF THE CAROTID BODY

The carotid body is innervated via the carotid sinus nerve which is a branch of the glossopharyngeal nerve (De Castro, 1951). In addition the organ also receives a sympathetic innervation from noradrenergic fibres of the ganglioglomerular nerve which are derived from the superior cervical ganglion (Verna, 1975; Pallot, 1983b) and these innervate the surrounding vasculature. The abundance of nerve fibres is

another typical histological features of the carotid body. I must emphasize that the most outstanding contribution to the study of the carotid body innervation is De Castro's work (1926, 1928, 1940, 1951, & De Castro and Rubio 1968) which first described the innervation and arrangement of nerve fibres and nerve endings of the organ using classical methylene blue and silver stain techniques.

NERVE ENDINGS ON TYPE I CELLS

De Castro, (1926) and others (Al-Lami and Murray, 1968a; Kraus and Martinek, 1970; Verna, 1971; Eyzaguirre et al., 1972; Biscoe and Pallot, 1972; Abbot et al., 1972; Nishi and Stensaas, 1974; Eyzaguirre & Gallego, 1975) reported that the nerve endings on Type I cells have many different shapes and sizes (with great variation from about 1 to 10 μm) and because of either different size or the very complicated shape of many terminals they attempted to classify the nerve terminals based on morphological criteria. The area of individual nerve endings in single sections has been measured in rat and cat carotid body, the results were highly variable. McDonald reported a range of 1-10 μm^2 for the rat. However, Pallot and Blakeman, (1982 cat), and Pallot et al. (1986 rat) measured some smaller endings (for cat; 0.17-7.3 μm^2 , for rat ; 0.18-7.75 μm^2). Later studies have suggested that nerve terminals on Type I cells are very polymorphous therefore, one and the same nerve fibre after giving branches may innervate several Type I cells. Using the electron microscope by serial (ultrathin section analysis) reconstruction studies it has been established that large and small endings are often derived from a common single axon and so that each Type I cell usually receives its innervation from a single nerve fibre (Biscoe and Pallot, 1972; Nishi and Stensaas, 1974; Nishi, 1976; Kondo, 1976 b,c).

Under the electron microscope the nerve endings on Type I cells showed quite different ultrastructure. They contain local accumulations of organelles such as mitochondria, small clear-cored vesicles (synaptic-like microvesicles), neurotubules and neurofilaments in varying amounts. Like other nerve endings anywhere in the nervous system, the nerve endings on Type I cells commonly have some elements of the lysosomal system such as multivesicular bodies and residual bodies. In addition, some electron dense-cored vesicles and glycogen granules have also been reported in some endings (Biscoe, 1971; Verna, 1979; McDonald & Mitchell, 1975a; Pallot & Blakeman, 1982; and Pallot et al. 1986).

MITOCHONDRIA

Mitochondria are plentiful in nerve endings on Type I cells and a more or less regular feature of carotid body nerve endings. They show longitudinally oriented cristae and a very electron dense matrix but are generally smaller than mitochondria of Type I cells (Verna, 1971, 1973; McDonald, 1975a). It has been reported by several authors that in different species (e.g. Bock et al., 1970 in man; Kondo, 1971 in guinea pig; Verna, 1971, 1973 in rabbit; Kobayashi, 1971b in the amphibian; and King et al. 1975 in the avian carotid body), mitochondria sometimes completely fill the neuroplasm. The significance of this observation is that the variability in the mitochondrial content in the carotid body might relate to the high oxygen consumption of the organ. It must be mentioned that Biscoe & Pallot (1972) and Verna (1973) observed that the amount of mitochondria varies greatly from one region of a given nerve ending to another. Hence, the mitochondria accumulation does not allow us to distinguish a special kind of nerve terminal in the carotid body. Using morphometric techniques, Pallot and Blakeman (1982), Pallot et al. (1986) reported ranges of 17%-25% in cat nerve endings and a mean of 18.3% in the rat nerve endings. McDonald (1981) found an average mitochondrial concentration of 1 per 2.2 μm^2 in the rat carotid body. These data indicate that the values of mitochondrial concentration in the rat carotid body nerve endings are little different from the values which are found in other types of nerve endings. Hence, according to these results the variability of mitochondrial concentration within the carotid body nerve endings offers no explanation of the oxygen consumption of the organ.

CLEAR-CORED VESICLES

The presence of small clear-cored (synaptic-like microvesicles or electron-lucent vesicle, Verna, 1979) vesicles has been reported in the nerve endings; however their number and density are highly variable (Biscoe & Pallot, 1972; Nishi & Stensaas, 1974; Mitchell & McDonald, 1975; McDonald, 1981; Pallot et al., 1986).

In the rat carotid body McDonald & Mitchell, (1975 a,b) and Pallot & Blakeman, (1986c) measured the clear-cored vesicles and found similar values in the sinus nerve afferent terminals; as a mean diameter of 61 nm - 60.84 nm respectively. However, for cat carotid body Type I cell nerve endings Pallot et al. (1986) reported much smaller diameters (41 nm) whilst McDonald & Mitchell (1975a) reported similarity with rat (62 nm). Nishi & Stensaas (1974) and McDonald (1981) observed that there is an inverse relationship between nerve ending size and vesicle concentration that is small clear-cored vesicles are more abundant in small sensory nerve endings than in large nerve endings. In addition, Morgan et al., (1979) reported that the density of vesicles within nerve endings is not only variable in different endings but anaesthetic agents also affected this parameter. Nerve endings in animals anaesthetised with pentobarbitone sodium showed much lower vesicle densities than animals anaesthetised with chloralose. According to McDonald (1981), their morphometric studies indicate that the comparatively large size of such vesicles is a distinctive feature of sensory nerve endings and in the carotid body the vesicles are significantly larger than synaptic vesicles of various motor nerve endings in the organ, preganglionic sympathetic nerves ending on Type I cells, preganglionic nerves ending on ganglion cells and postganglionic parasympathetic nerves ending on blood vessels. They suggested that the difference in size of vesicles in sensory and motor nerve endings on Type I cells may reflect differences in the level of activity of the terminals.

ELECTRON DENSE-CORED VESICLES

In addition to small clear-cored vesicles, a few electron dense-cored vesicles (larger and less numerous than small clear-cored vesicle, about 90 to 110 nm in diameter) have been reported in a number of different types of sensory and motor nerve endings (Verna, 1979; McDonald, 1981). McDonald & Mitchell (1981) showed that in the rat carotid body they comprise 8% of the total vesicle population, however in the cat carotid body they constitute only 1% of all vesicles. According to the data of Pallot et al. (1986) electron dense-cored vesicles in the rat carotid body Type I cell nerve endings occur at a mean concentration of 3 vesicles per μm^2 . In the cat carotid body their concentration is also much lower than the rat carotid body. Their data also indicated that their concentration (unlike that of clear-cored vesicle) is not affected by hypoxia.

GLYCOGEN GRANULES

The presence of glycogen granules in the nerve endings shows great variability both within and between species. Large numbers of glycogen granules have been observed by several authors in sensory nerve endings of the cat (Biscoe and Stehbens, 1966; McDonald and Mitchell, 1975a,b; Pallot et al., 1986) amphibians (Kobayashi, 1971b) and rabbit (Verna, 1973). However very few glycogen granule has been observed in the rat carotid body. McDonald & Mitchell (1975 a,b) and Pallot & Blakeman (1986c) reported a similar observation that sensory nerves of the rat contain fewer glycogen granules than those of the cat.

In the central nervous system synapses are characterized by an accumulation of synaptic vesicles near one side of a specialized synaptic junction at which cytoplasmic densities are located asymmetrically on the presynaptic and postsynaptic membranes (Peters et al., 1970,1976). Using such criteria nerve endings on the Type I cells have been classified into three groups by several authors (see Fig 1.5). Upon these criteria, they have described that nerve endings are:

- (1) presynaptic to Type I cells (also called an efferent synaptic complex),
- (2) postsynaptic to Type I cells (also called an afferent synaptic complex) and
- (3) reciprocal (where both pre and post synaptic dense projections are found in the same nerve ending) with Type I cell (McDonald & Mitchell, 1975a,b; Konda 1976a,b; Verna, 1979; Blakeman et al., 1984; Pallot et al., 1986). The frequency of these three synapses varies among species as illustrated in table 1.3.

On the Type I cells of the cat and rat carotid body McDonald & Mitchell (1975a,b), Mitchell & McDonald (1975), Pallot & Blakeman (1982), Pallot et al. (1986) have performed extensive quantitative studies of the different types of nerves endings on the basis of synaptic morphology. Although their data have been obtained in different conditions (for the former considered only sinus nerve endings, whilst the latter, regardless of their origin, obtained from all endings on Type I cells) the results from the two groups were surprisingly similar.

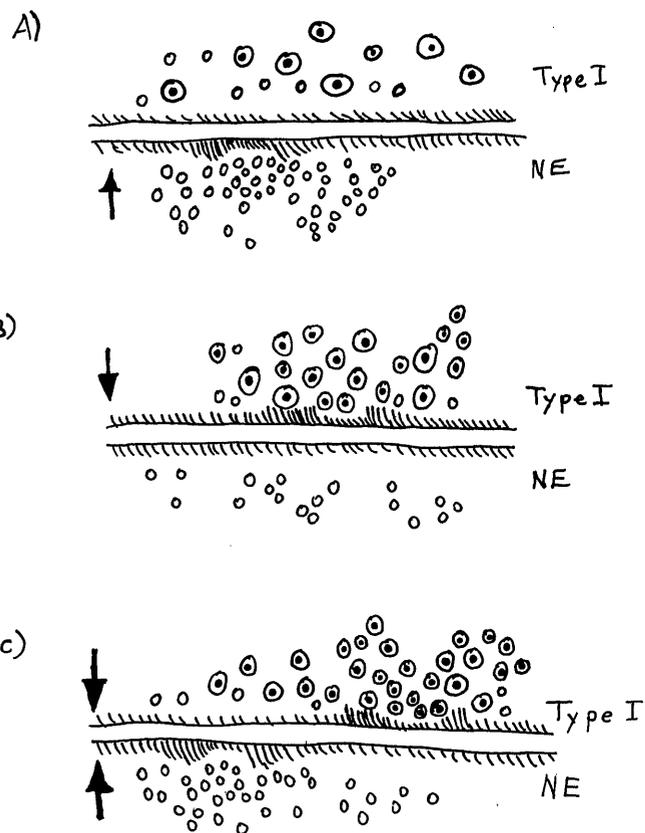


Fig 1.5. shows the synaptic variants on the carotid body Type I cell and nerve endings(NE). A: Presynaptic, B: Postsynaptic, C: Reciprocal.

	Rot Studies		Cot Studies	
Presynaptic	11%	10%	67%	70%
Postsynaptic	78%	84%	24%	25%
Reciprocal	11%	6%	9%	4%

Table 1.3. Percentage occurrence of different varieties of endings, based on data from Blakeman et al., (1984), and McDonald, (1981) respectively.

In the rat, nerve endings which are postsynaptic to Type I cells are about seven times more frequent than presynaptic and/or reciprocal endings whilst in the cat the nerve endings which are presynaptic to Type I cells are three times more numerous than postsynaptic and approximately six times more common than reciprocal endings. Clearly the number of endings which could be classified on the basis of the presence of presynaptic dense projections was very limited. In an attempt to find other criteria to identify and classify the nerve endings McDonald & Mitchell (1975a,b, 1981), Pallot & Blakeman(1982), Pallot et al.(1986) and Blakeman et al. (1984) performed extensive quantitative studies on the area of presynaptic and postsynaptic nerve endings, their vesicle density, diameter and volume of ending occupied by mitochondria. However, they could not find any significant differences based on these criteria.

To determine the source of nerve endings in the carotid body, so far, two methods have been used. One of them is the well known degeneration method (by De Castro, 1926, 1928; Biscoe and Stehbens, 1966; Hess, 1968,1975a,b; Hess and Zapata, 1972; Biscoe et al., 1970 a,b,c; Nishi and Stensaas, 1974; McDonald and Mitchell, 1975a,b); the other is the more recent tracer method (using both autoradiographic techniques and also horseradish peroxidase) which uses the axoplasmic flow (Smith & Mills 1976, 1977; Fidone et al., 1977a,b).

De Castro (1928) showed that 5-12 days after intracranial transection of the glossopharyngeal nerve (that is between the brainstem and the sensory ganglion of the nerve) there was no degeneration of nerve endings in the carotid body. Thus he deduced that the carotid body endings were derived from cell bodies within the petrosal ganglion. According to his previous studies of the ganglion which showed it was composed of typical pseudo-unipolar cells, he reported that the nerve endings were sensory (afferent) terminals. After administration of tritiated amino acids to the petrosal ganglion Smith & Mills (1976,1977) and Fidone et al. (1977a,b) provided additional evidence of the sensory nature of the innervation to the Type I cells. They observed that autoradiographically, most nerve endings on Type I cells were labelled and in the absence of evidence that the petrosal ganglion contained any efferent neurones, they concluded that the nerve endings were sensory. However, as pointed out by Williams et al. (1977) such tracer experiments are, by their very nature, very difficult to quantitate. Hence it may be

that more than one type of ending can exit on Type I cells (Pallot 1987). Horseradish peroxidase (HRP) transport techniques have also been used to find out an answer to this problem. In this case HRP was placed onto the sinus nerve and its transport in a retrograde direction followed to neurons in the petrosal ganglion (Eyzaguirre & Zapata, 1984). Kalie & Davies (1978) reported that only areas containing established sensory neurons were stained in such experiments whilst de Groot (1979), Berger (1980), Davies & Kalie (1981), and Ciriella et al. (1981) observed cells containing the transported enzyme in the rostral part of the nucleus ambiguus and claimed that an efferent component existed in the sinus nerve.

Although there are quite different ultrastructural features on Type I cell nerve endings many authors had assumed that all Type I cell nerve endings were sensory. However, the presence of synaptic vesicles in the Type I cell nerve endings has led to the suggestion that these nerve endings are structurally more like efferent endings rather than sensory (afferent) endings, at the very least a proportion of the endings might be efferent, motor terminals (Biscoe & Stehbens, 1966; Abraham, 1968). De Castro & Rubio (1968) reported that one month after intracranial transection of the glossopharyngeal nerve, central to its sensory ganglion, they were unable to record any changes in the nerve endings. However, Biscoe et al. (1969, 1970 b,c) reperformed the intracranial nerve transection experiment and claimed that some 60% of nerve endings on Type I cells degenerated with a slow time course of several months. During this period they were also able to record the normal chemoreceptor activity. Therefore, they suggested that the Type I cell nerve endings (at least some of them) were efferent. After their claim, this intracranial transection experiment has been repeated by several authors (Hess & Zapata, 1972; Nishi & Stensaas, 1974; McDonald & Mitchell, 1975a,b). Although Biscoe et al. (1970 b,c) specifically remarked on the long time course of degeneration only Hess & Zapata (1972) used such long recovery times (two months). The claim in this paper is that intracranial transection did not alter the number of nerve endings on type I cells. However, as pointed out by Pallot (1987) their illustrations show at least one markedly abnormal ending (their Fig.6) which is claimed as a normal nerve ending. Whether this structure really represented a degenerative nerve ending or was the result of inadequate fixation is not clear.

McAllen & Willshaw (1979) have reported that acute intracranial section of the glossopharyngeal nerve does not abolish the efferent activity which is recorded from the central cut end of the sinus nerve. Thus, they suggested that the cell bodies of the efferent neurons are located peripherally rather than in the brain stem.

The mutant mouse Wobbler has a genetic neurological defect which leads to a spontaneous degeneration of most of its motor nerve fibres. Biscoe & Pallot (1975,1983) and Pallot & Biscoe (1977) have studied chemoreception in this animal. In this animal, the lack of a very large percentage (60 - 90%) of Type I cell nerve endings has been observed by quantitative ultrastructural studies. Physiologically, they reported that the animals increased their minute volume in response to hypoxic stimulus and responded to one or two breaths of 100% O₂ with a brief reduction in minute volume. These effects were eliminated by sinus denervation. They suggested that many of the Type I cell nerve endings are efferent rather than afferent or alternatively that there is massive redundancy with regard to the number of nerve terminals within the mouse carotid body. McDonald & Mitchell (1975a, b) has also reported that in the rat carotid body some (about 50%) Type I cells are not innervated by nerve endings, they have connections only with other Type I cells (by chemical or gap junctions). However, Kondo (1976 b) did not confirm this observation. In the cat Nishi (1976) found 3 noninnervated cells out of 22 Type I cells but structurally, there was no difference between innervated and noninnervated cells.

In summary, as mentioned above, Type I cell are innervated by sensory nerve fibres from the glossopharyngeal nerve, however some efferent autonomic fibres end on Type I cells. The structure of the synapses between Type I cells and nerve endings is similar to the structure of chemical synapses in the nervous system and based upon the location of vesicles: three kinds of synapses can be distinguished on the Type I cell nerve endings. Some Type I cells are not connected to nerve endings but only to other Type I cells.

POSSIBLE CHEMICAL TRANSMITTERS IN THE CAROTID BODY

Carotid body Type I cells produce and release catecholamine, indolamine, neuropeptides and acetylcholine (Fidone & Gonzalez, 1986; Pallot, 1987; Nurse, 1987; Shaw et al., 1989; Eyzaguirre and Koyano, 1965; Eyzaguirre et al., 1990b; Heath & Smith, 1992, and Prabhakar, 1994). Various studies have established the presence of dense -cored chromaffin -like vesicles in the cytoplasm of Type I cells, and these vesicles are known to contain putative neurotransmitters such as the catecholamines dopamine and noradrenaline. The possible role which each of these chemicals play as transmitters of the chemosensory signal will be briefly discussed below.

CATECHOLAMINES

There is no doubt that the carotid bodies of all species, studied so far, contain catecholamines, as shown by the presence of formaldehyde- induced fluorescence and granular vesicles in the Type I cells (e.g. Palkama, 1965; Grimley & Glenner, 1968; Bataglia, 1969; Kobayashi, 1971a; and Kobayashi & Chiba, 1977a). Such cytochemical and ultrastructural observations stimulated numerous authors to determine carotid body catecholamine levels. In most species dopamine and noradrenaline are the predominant amines detected within the carotid body (Chiocchihio et al., 1966, 1981; Dearnaley et al., 1968; Zapata et al., 1969; Hellstrom & Koslow, 1975; Hellstrom et al., 1976; Hellstrom, 1977a,b; Mills et al., 1978; Hanbauer et al., 1981; Mir et al., 1982, 1983, 1984a,b; and Al-Neamy, 1983). However, the amounts of catecholamines in the carotid body vary greatly, even within different reports from the same laboratory (Chiocchihio et al., 1966, 1971a,b, 1981). According to Fidone et al. (1983); Al-Neamy (1983); Pallot (1987) this may be due to several reasons listed below;

- a) physiological status of the animal before carotid body removal,
- b) animal strain, the age and body weight of the animals, methods of sacrificing the animal, different techniques in the dissection procedure and post mortem changes (especially in the human),

c) differences in sensitivity and selectivity of the detection methods employed (Fluorometric, Gas/Mass spectrometry, Radioimmunoassay, High performance liquid chromatography)

d) statistical error in data interpretation, and the fact that some of the methods measured total noradrenaline and adrenaline concentrations together (Dearnaley, 1968; Mills & Slotkin, 1975), and L-dopa and dopa together (Chiocchio et al.1971a,b).

Although there is a degree of controversy as to the way in which catecholamines are stored by the carotid body it has been suggested that noradrenaline and dopamine are stored in different Type I cells since the distribution of dopamine-beta-hydroxylase (DBH) seems to be limited. As mentioned above, the most distinguishing features of Type I cells are the electron dense-cored vesicles within the cytoplasm the distribution, size (2000-20.000 nm) and number of which vary from cell to cell. It is likely that the amines are stored, as in other cells, within these vesicles

Using two highly sensitive methods, namely the technique of High Pressure Liquid Chromatography (HPLC) coupled with electrochemical detection and radioenzymatic assays, Mir et al. (1982) investigated the catecholamine contents of the carotid body of several mammalian species. This data illustrated that whilst all of the species examined stored both dopamine and noradrenaline there were dramatic differences in the concentrations of the two amines and that the absolute levels were not related to the size of the animal. Unilateral superior cervical ganglionectomy or chemical sympathectomy with 6-hydroxydopamine reduced noradrenaline but not dopamine in the rat carotid body. In the rabbit and guinea-pig, ganglionectomy also slightly reduced noradrenaline levels, whilst the same procedure produced no alteration in the catecholamine levels in the cat carotid body. Their observations were consistent with those of Nahorski et al. (1980) who previously performed similar experiments using identical methods.

FUNCTIONS OF THE CATECHOLAMINES AND FACTORS AFFECTING CATECHOLAMINE LEVEL IN THE CAROTID BODY

All of the physiological evidence suggests that dopamine is by far the most important amine with regard to carotid body function. It is well known that the close arterial injection of dopamine leads to a reduction in chemoreceptor activity recorded in the sinus nerve (Zapata, 1975; Lladós & Zapata, 1978; Docherty & McQueen, 1978), and there is also ample evidence to show that dopamine is released from the carotid body Type I cells by hypoxia (Fidone et al., 1982; Pallot, 1987; Shaw et al., 1989).

In the Central nervous system (CNS) dopamine is known to act on specific receptors some of which are positively linked and others negatively linked to adenylate cyclase. Dopamine receptors have been examined in the rabbit carotid body by radioligand binding techniques using labelled spiperone (Dinger et al., 1981). They observed specific binding to carotid body membrane preparations and found that sinus nerve denervation reduced the specific binding by 64%. However, it was not clear in their report what proportion of dopamine and Serotonin (5HT) receptors were being labelled; because spiperone has both dopaminergic and 5HT activity. Mir et al., (1983, 1984a,b) identified dopamine D2-receptors in the rat carotid body using radiolabelled domperidone and suggested that 60% of dopaminergic receptors may be located on the cellular elements of the organ however, the exact location of these cellular D2 receptors is unknown. It is possible that they may be located on Type I cells and are concerned with controlling dopamine release.

The main effect of noradrenaline on the cat and rabbit carotid bodies is a brief (2sec.) inhibition of activity followed by excitation (Sampson, 1972; Folgering et al., 1982). The initial inhibitory effect of noradrenaline can be abolished by dopamine antagonists (Zapata & Lladós, 1977; Folgering et al., 1982). Sampson (1972) and Lladós & Zapata (1978) reported that the delayed excitation can be abolished by the vasopressor compound phenoxybenzamine. Hence the response of the organ to noradrenaline appears to have two components; a dopamine receptor-mediated inhibition and an alpha mediated excitation.

Mills & Slotkin (1975) reported that in the cat carotid body systemic hypoxia (10% O₂ for 60 min.) caused a significant reduction in the combined noradrenaline and adrenaline level. In addition they reported an increase in noradrenaline and adrenaline content of the cat carotid body in hyperoxia (following 40 %O₂ 90 min. ventilation) Mills & Slotkin (1978). However, Hellstrom & Koslow (1975), Hellstrom et al. (1976) Hellstrom (1977a,b) and Hanbauer & Hellstrom (1978) reported that in systemic hypoxia (5% O₂ for 30 min. - hardly a normal physiological stimulus) that there was no change in noradrenaline content while dopamine level was significantly reduced. Since that time it has become accepted that acute hypoxia affects the dopamine concentration specifically (see Pallot 1987). Hanbauer & Hellstrom (1978) found no change in amine levels after hyperoxia (100% O₂ for 30 mins) a finding not supported by the data of Al-Neamy (1983) who observed that hypoxia leads to a reduction in dopamine content of the rat carotid body whilst hyperoxia and hypercapnia increase dopamine level. On the contrary, Chiochio et al. (1981), Pallot & Barer (1982), Barer & Pallot (1984), and Shaw et al. (1989) reported an increase in both noradrenaline and dopamine content in chronic hypoxia.

Chronic hypoxia has the opposite effect of acute hypoxia on the dopamine content of the carotid body. After two-three weeks of 10% O₂ dopamine levels are dramatically increased (up to eight fold) and there is also a considerable increase in the amount of noradrenaline (Chiochio et al., 1981; Pallot & Barer, 1982,1985; Barer & Pallot, 1984). Many animals living at high altitude, and hence under conditions of hypoxia, have a blunted response to acute hypoxia. Wach et al. (1989) showed that after three weeks in an environment of 10% O₂ rats had such a reduced sensitivity to hypoxia and that, this so-called blunting could be abolished by the injection of the D2 blocker domperidone. As the domperidone does not cross the blood brain barrier, then the inhibitory effect must be mediated by dopamine acting in the carotid body.

SEROTONIN (5-Hydroxytryptamine; 5HT)

The presence of this indolamine has been reported in the carotid body (Hamburger et al., 1966; Chiochio et al., 1971; Black et al., 1972; Nishi, 1975; Hellstrom & Koslow, 1975, and Alfes et al. 1977), and it was thought that most of the

compound was found mainly in mast cells and platelets. More recent data has confirmed that Type I cells also store 5HT (McDonald, 1981; Pallot, 1987; Abramovici et al., 1991; Habeck et al., 1994; Oomori et al., 1994). Its localization in Type I cell may have important implications for the control of the organ activity, for in the nucleus accumbens 5-HT at low concentrations causes inhibition of dopamine synthesis, whilst at higher doses it causes increased dopamine release (Abramovici et al., 1991). It has been reported that serotonin concentration is higher in systemic hypertension cases (Steele & Hinterberger 1972).

PEPTIDES

The similarity of the carotid body to other tissues in the APUD (amine precursor uptake and decarboxylation) series led Pearse (1969) to suggest that the carotid body too might produce some low molecular weight hormone which he called glomin. Using similar criteria as Pearse, Koboyashi (1977) included Type I cells in a group of cells termed paraneurons, cells which produce low molecular weight neurotransmitters.

So far, the presence of enkephalins, substance P, vasoactive intestinal polypeptide (VIP), bombesin, cholecystokinin, and neurotensin have been found in the carotid body using radioimmunoassay and immunohistochemical techniques. In the cat carotid body Type I cells Lundberg et al. (1979); Cuello & McQueen (1980), and Wharton et al. (1980) reported the presence of enkephalins (both met- and leu-enkephalins), substance P and VIP-like peptides. The list of peptides which are identified in animal and/or human carotid bodies is discussed by Abramovici et al. (1991); Heath & Smith (1992), and Prabhakar, (1994).

Heath et al., (1988) reported that enkephalins are also present to a much greater concentration in the human carotid body than in other animal species whilst Khan et al. (1990) and Smith et al. (1990) reported that there were no difference in the distribution of enkephalins in human carotid bodies in different pathological conditions that resulted in cellular proliferation and chronic glomerulonephritis (see below). Enkephalins (both met- and leu-) are predominantly observed within Type I cells, however, the intensity of labelling between cases has been observed to vary

considerably. Met- and leu- enkephalins were found only in Type I cells by Abramovici et al. (1991), and Smith et al. (1990) whilst it was reported by Smith et al. (1990) that some nerves within the human carotid body contain met-enkephalin. Smith et al. (1990) claimed that the dark Type I cells showed more intense immunoreactivity whilst most of the light cells have little reaction to enkephalins. In view of the data presented below that show that dark cells are likely to be a result of post mortem artefact this finding is extraordinary. Abramovici et al. (1991) observed that most leu-enkephalin positive cells also showed a positive reaction to met-enkephalin in the cat carotid body. Monti-Bloch & Eyzaguirre (1985) observed that the enkephalins depress the chemosensory discharge in the cat carotid body. Hanson et al. (1986) demonstrated that when rabbits are exposed to hypobaric hypoxia for one hour, the level of the enkephalins is reduced by as much as 40% compared with controls in a normoxic environment. Such data suggest that acute hypoxia stimulates secretion of enkephalins from Type I cells which might act directly on the afferent terminals within the carotid body or possibly through the co-release of dopamine. In this latter respect, Varndell et al. (1982) has demonstrated that met-enkephalin is stored within the periphery of the dense-cored vesicles in the cat carotid body. It is these vesicles that are believed to store dopamine and hence the hypothesis of co-release is at least tenable.

Substance P, predominantly found in a variety of nerve fibres in several organs as well as the CNS, has also been suggested as a sensory neurotransmitter in the carotid body (Polak & Bloom, 1980). The immunocytochemical studies of Lundberg et al. (1979) and Wharton et al. (1980) demonstrated that substance P is located within fine nerve fibres, which are especially adjacent to clusters of Type I cells, throughout the organ. However, Cuello & Mc Queen (1980); Prabhakar et al. (1989), and Smith et al. (1990) reported that some (20% in the cat) Type I cells also had positive substance P immunoreactivity.

Heath et al. (1988) reported that vasoactive intestinal poly-peptide (VIP) was also found in Type I cells in the human carotid body but that there was no immunoreactivity in Type II cells and nerves. However, Lundberg et al. (1979) and Wharton et al. (1980) were not able to demonstrate VIP within the Type I cells of animals but rather found it in varicose nerve fibres, especially those around blood vessels.

A significant concentration of neurotensin (mean concentration 67 pmol/g) has been reported within the human carotid body type I cells by radioimmunoassay (Heath et al., 1988). However the same authors could not demonstrate immunoreactivity within the carotid body (Smith et al., 1990). In human carotid body some Type I cells and myelinated nerves in the stroma were labelled by bombesin which is a 14 amino acid residue peptide originally extracted from the amphibian skin. However, the most striking labelling has been observed in the glomic arteries but not in the glomic veins (Smith et al. 1990). These labelled cells in the glomic arteries are presumably smooth muscle cells since no other cell known to contain peptides has been reported in the media of glomic arteries by electron microscopy (Jago et al., 1982, and Heath & Smith 1992)

ACETYLCHOLINE

Acetylcholine (ACh) has been identified within the cat carotid body, it is believed to be located in Type I cells and has been suggested as a neurotransmitter or modulator of chemosensory activity. (Fidone et al., 1977a,b; Eyzaguirre & Fidone, 1980; Nurse, 1987; Eyzaguirre et al., 1990a,b, and Prabhakar, 1994). Its presence is also reported in the carotid bodies of the rabbit, human (Becker et al., 1967) and rat (Korkala and Waris, 1977) The significance of such reports must be in doubt as the absence of acetylcholinesterase (AChE) has been reported by Biscoe & Silver (1966), and Ballard & Jones (1971).

THE IDENTITY OF THE CHEMOSENSOR

Although more than 70 years passed from the discovery of the chemoreceptive functions of the organ (DeCastro, 1928 and Heymans et al., 1930) neither the mechanism of the process nor the identification of the transducer has yet been established. De Castro (1926, 1928) first suggested that, due to the close association with sensory nerve endings, Type I cells were the receptor cells which tested the blood. Adopting a similar idea as to the site of the receptor, Eyzaguirre and Zapata (1968) suggested that the Type I cells released acetylcholine in response to hypoxia which in turn stimulated afferent nerves and was thus the first to suggest a mechanism of chemotransduction. Since these early experiments, all

of the other elements of the carotid body, with the notable exception of blood vessels, have been postulated as the chemosensor. Much of the experimental data has been reviewed by Pallot (1987) and Heath & Smith (1992) is only touched on here.

A- TYPE I CELLS AS CHEMORECEPTORS:

Two different types of experimental studies have been performed in an attempt to prove that Type I cells are the chemosensor:

- a. the reinnervation of Type I cells with either a foreign, or the carotid sinus, nerve:
- b. attempts to destroy the Type I cells.

De Castro et al., (1940), re-innervated the carotid body with vagal nerve fibres and after a suitable period for growth of the foreign nerve into the organ showed that chemosensitivity had been restored. Essentially similar experiments were performed, using the superior laryngeal nerve by Zapata et al. (1969), and by Dinger et al. (1981,1982a,b,1984) using the lingual nerve. Belmonte & Gonzalez (1983) remarked that these experiments were open to the criticism that the observed reflexes, or chemoreceptor activity, might have originated in chemoreceptor fibres present in the foreign nerve rather than because the fibres had reached some special carotid body element . Another point which is relevant in the evaluation of these studies is that there was little or no histological or ultrastructural quantitative data with regard to connection of nerve endings and Type I cells. In the absence of such quantitative data it could equally well be argued that it was not the presence of Type I cells that mattered but rather that any small nerve fibre in the special environment of the carotid body could act as a chemosensor.

Numerous authors have studied the previously denervated carotid body after the transected carotid sinus nerve has been allowed to grow back into the organ (Zapata et al., 1976,1977; Kienecker & Knoche, 1977; Bingman et al., 1977; Belmonte et al., 1981; Dinger et al., 1981,1984). It has been claimed by some that a direct correlation between the reappearance of nerve endings on Type I cells and the appearance of chemosensory activity in the carotid sinus nerve exists. However

Kieneker & Knoche (1977), and Bingman et al. (1977) reported a different results: in that chemosensory activity appeared long before Type I cells had been re-innervated.

Verna et al. (1975), and Leitner et al. (1981) destroyed the Type I cells by the application of a probe to the carotid body which had been cooled in liquid nitrogen. In the 3-12 months period after this procedure they observed that the chemoreceptor activity in the carotid sinus nerve was reduced or disappeared whilst baroreceptor activity remained normal. It was observed, in some cases where some chemoreceptor activity was found, that some cells remained in the carotid body. Their study suffered from lack of information of technique and data on the quantitative histology of carotid body elements for the method of identifying Type I cells, was by formaldehyde induced fluorescence which would not have identified nerve endings. Indeed, it might be argued that if the cold treatment removed the Type I cells in the carotid body then it must have also removed the nerve fibres and hence it is hardly surprising that there was no chemoreceptor activity. Such experiments require repeating but with the addition of ultrastructural studies of the carotid bodies after the physiological recordings.

Monti Bloch et al. (1981) reported that there was a fall-off in the discharge which could be recorded from the carotid sinus nerve and that this decrease in discharge correlated with increasing damage to the Type I cells during progressive ischaemic periods. He concluded that these results provided enough evidence to show that Type I cells played an essential role as the transducer. The authors do not answer the critical question as to what happens to the nerves during this procedure. If the ischaemia is enough to damage the Type I cells then it is highly likely to have caused even more damage to the nervous elements of the carotid body as nervous tissue is extremely sensitive to hypoxia. If this is correct then it is not surprising that there was a decrease in chemoreceptor activity. Monti-Bloch et al. (1984) also transplanted the cat carotid body to the tenuissimus muscle of the hind leg and tied a branch of the muscle nerve to the carotid body and allowed regeneration for 95-174 days. They recorded activity in the nerve which had been transplanted into the carotid body and observed responses to 100% nitrogen, 100% oxygen, 5% CO₂ and various drugs. It was concluded that "the foreign nerve fibres not only penetrate the lobules but are accepted by the Type II cell and Type I cells of the

graft whose intimate apposition allows them to interact with foreign axons in response to chemosensory stimulation."

Gual & Stensaas (1984) studied the effect of replacing calcium ions with barium ions in the fluid superfusing the *in vitro* carotid body and observed that despite the gross changes in Type I cells after 30 min. of barium treatment, carotid bodies exposed for 3 - 4 hours could still respond to acetylcholine and sodium cyanide and suggested that Type I cell do not act as generators of chemosensory discharge but may serve to modulate chemoreceptor activity. As already mentioned above Biscoe & Pallot (1975,1983) and Pallot & Biscoe (1977) have provided evidence in a neurological mutant mouse that normal chemoreception can exist in the absence of most of the Type I cell nerve endings.

B) NERVE FIBRES AS CHEMOSENSOR:

Biscoe (1971) proposed that small unmyelinated nerve fibres surrounded by Type II cells were the transductive element. There is structural information, from the cat and mouse carotid body, to suggest that unmyelinated nerve fibres enclosed within Type II cell cytoplasm may terminate without contact with other cell types (Pallot 1975). For the nerve fibres as the primary chemoreceptors the most supportive data have been produced from neuroma experiments.

Bingmann et al. (1981a,b,1984), Kienecker et al. (1978a,b), and Tan et al. (1981) all produced evidence that normal chemoreceptor activity could be recorded from neuromas of the carotid sinus nerve. The data of the former authors are particularly impressive in that they found two types of sinus nerve neuromas could be reproduced after implantation of the sinus nerve into the walls of the external carotid artery. The first was highly vascular and from this variety chemoreceptor activity could be recorded. The second neuroma type lacked this vascularity and only pressure sensitive units could be found. Ponte & Sadler (1989) were able to observe small groups of surviving Type I cells and nerve endings in the vicinity of their neuromas and concluded that the chemoreceptor activity which they recorded was due to the association of nerve fibres with these cells.

C) THE TYPE II CELLS AS CHEMORECEPTORS:

The Type II cell has also been suggested as a chemosensor (Paintal 1968; Mills & Jobsis 1972). Heath & Smith (1992) remarked that it should be borne in mind that the Type II cells are well placed to act as oxygen sensors, since they are closer to the blood than either Type I cells or nerves and are also in intimate contact with both. To date little convincing evidence has been produced in their favour.

PATHOLOGY OF THE CAROTID BODY

The carotid body is a peripheral chemoreceptor that monitors arterial blood gas tensions and pH. Its main function is to contribute to the regulation of breathing and there is evidence for a reflex influence on the pulmonary circulation and the kidney. The introductory part of the first chapter of this thesis briefly deals with morphological changes in the carotid body in different pathological conditions, induced either experimentally or by disease. The carotid body has been investigated in diseases such as acute oxygen deficiency, asthma, chemodectoma, chronic hypoxia, anaemia, essential hypertension, sudden infant death syndrome (SIDS), cirrhosis of liver, chronic carotid glomitis and schizophrenia (Geertinger, 1978; Blessing, 1983; Pallot, 1987; Khan et al., 1989; Heath & Khan, 1989; Khan and Heath, 1990; Heath & Smith, 1992; 1994, and Bee & Howard, 1993). It is regrettable with regard to the animal experimental studies on the histopathological change in the carotid body that there are controversial results which are difficult to interpret because of strain and environmental differences. In addition, the responses also may not match human conditions.

It must be mentioned that much of our current knowledge about the histopathology of the human carotid body, particularly chronic hypoxaemia stems from the studies of Heath and his colleagues. In order to improve the understanding of the role of the organ in some diseases, especially in cases of heart and lung diseases, they have included the study of the organ in routine necropsies and suggested that the carotid body should be dissected out routinely, and that failure to do this in the past has led to a peculiar imbalance in our knowledge of the organ (Heath et al., 1970, 1982, 1984, 1985, 1986; Heath & Smith, 1992).

FACTORS AFFECTING THE CAROTID BODY STRUCTURE:

In 1969 Arias-Stella observed that the carotid body of the Quechua Indians living at high altitude were much larger and heavier than those of the Meztizos living at sea level. Subsequent studies by Arias-Stella & Valcarcel (1976) established that these differences were due predominantly to hyperplasia of the Type I cells. Similar observations have been reported in Peru for humans (Saldana et al., 1973; Heath & Williams, 1981, 1989) and for animals (Edwards et al., 1971b, 1972). It has been reported that in the human, chronic obstructive pulmonary disease (COPD), systemic hypertension (Edwards et al., 1971a; Lack, 1978; Heath et al., 1982, 1984), and sudden infant death syndrome (Naeye et al. 1976) cause some structural changes in the human carotid body. Lack et al. (1977, 1985) reported the enlargement of the carotid body and its lobules in young adults with cystic fibrosis and/or cyanotic congenital heart disease. Bronchospasm and the excessive production of viscid mucus in bronchial asthmatic patients bring about alveolar hypoxia and hypoxaemia and therefore hyperplasia of the carotid body might be expected. However, Bencini and Pulera (1991) reported that the carotid bodies from the asthmatic patients were not enlarged. According to their morphometric study there were no increase in the lobules' size but that they were irregular in shape and the proportion of Type II cells and dark variety of Type I cells was increased.

Arias-Stella & Valcarcel (1976) demonstrated that in humans living at high altitude the extent of the carotid body enlargement increased with age and was associated with progressive chemoreceptor insensitivity. Hurst et al. (1985a,b; 1986) also reported that there was a linear increase in the total cross-sectional area of carotid bodies of adult people, which was age-related and due to an increasing amount of connective tissue within the organ. In other words, the mean percentage of glomic tissue or the amount of functioning area of the organs decreased with age due to fibrosis. Excluding chronic hypoxaemia or systemic hypertension cases which might have caused changes in the carotid body structure, they have studied human carotid bodies from patients of widely differing ages and observed four basic different histological patterns within the human carotid body, firstly, in the basic pattern found exclusively in young people (age 16-29) the light variant of Type I cell predominated, in the second group; dark

cell prominence was found which was associated with an increase in the number of pyknotic cells, Type II cell proliferation was seen in the third group whilst evidence of fibrosis was reported in the fourth.

The difficulty of studying human carotid body material is immense as it is well known that the carotid body is very susceptible to anoxia. All of the pathological studies outlined above have been performed using material from routine postmortems and hence there will have been considerable delay between death of the patient and fixation of the material. Hence it must be asked whether some of the features that are being described really represent the structure of the normal human carotid body or rather the result of postmortem artefact.

THE EFFECTS OF CHRONIC HYPOXIA ON THE STRUCTURE OF THE CAROTID BODY

Enlargement of the carotid bodies has been found both in humans and animals exposed to prolonged periods of hypoxemia and/or hypercapnia (Barer et al., 1976,1986,1987; Habeck, 1986; Pallot, 1987; Heath & Smith, 1992). An increase in the size of the carotid body in humans living at high altitudes compared to people of similar ethnic background but living at sea level was reported firstly by Arias-Stella (1969). Subsequent work by Arias-Stella & Valcarcel suggested that this enlargement could be attributed to hyperplasia of parenchymal tissue. Edwards et al. (1971a) provided evidence that the enlargement of the carotid body occurred in patients with chronic lung disease (such as chronic bronchitis and emphysema). They also showed that the carotid bodies of animals living at high altitudes were larger than those living at sea level and observed some changes in the Type I cell ultrastructure of guinea pigs that were born and lived at high altitude (Edwards et al., 1971b,1972 but note that guinea pigs were originally high altitude dwellers). Laidler & Kay (1975,1976) studied rats kept at a simulated altitude of 4300 m for 27-35 days in a hypobaric chamber and demonstrated an enlargement of the carotid body due to dilation of the vasculature and hypertrophy of Type I cells. Some authors report that the increase in the amount of glomic tissue is caused predominantly by a proliferation of Type I cells (Arias-Stella & Valcarcel, 1976; Janzer & Schneider, 1977), whereas others found enlarged

carotid bodies accompanied by a proliferation of Type II cells and/or Schwann cells (Heath et al., 1982; Smith et al., 1982; Habeck, 1986).

The chronic stimulation of the carotid body by placing an animal in an environment where the inspired oxygen levels are artificially maintained at low levels, results in enlargement of the organ whether the low oxygen environment is produced by simulated high altitude or by providing a lowered oxygen content in a normobaric chamber. However, there is considerable controversy on the processes of the enlargement and the associated increase in catecholamine levels. Some authors suggest that it is a result of hypertrophy of the cellular and vascular components (Hellstrom, 1977a,b; Hellstrom and Hanbauer, 1981) whilst others (Barer & Pallot, 1984; Barer et al., 1986; Pallot et al., 1990) report that there is also hyperplasia of at least some tissue elements within carotid body in addition to hypertrophy. A great increase in the vasculature and an increase in the size of Type I cells in chronic hypoxia are accepted as a general aspect of enlargement. Compared with normal rats, chronically hypoxic rats have more Type I cell nuclei (two or three fold), more connective tissue, more endothelial cells and greater blood vessel surface area (Barer et al. 1972, 1976; Pequignot & Hellstrom 1983a,b, 1984; Dhillon et al. 1984). The data on the number of Type I cell nuclei within the carotid body were those which led to the suggestion that there might be hyperplasia of the Type I cells. Many authors regarded this idea with suspicion as the Type I cells are probably derived from neural crest cells and hence as neuronal derivatives would not be expected to divide.

In 1986, Bee and her colleagues reported unequivocal evidence for cell division in chronically hypoxic rats. Animals were exposed to 10% O₂ for from 1- 4 days and, four hours before sacrifice received an injection of vincristine sulphate (an inhibitor of mitosis). After the four hour period, the animals were anaesthetised, fixed by perfusion and the carotid bodies were prepared for light and electronmicroscopical examination. These workers showed that mitotic figures were found in the carotid body on the first four days of exposure to hypoxia. It was apparent that the Type I cells, connective tissue elements and also the endothelial cells were capable of hypoxia-induced division. In subsequent experiments Bee et al. (1989) performed similar experiments but used ³H-thymidine to label cells entering the mitotic cycle and provided additional, and almost irrefutable, evidence that hyperplasia was an early part of the response to

hypoxia. The fact that cell division occurs so early after exposure, and before the organ has reached its maximum size, might argue that hyperplasia precedes hypertrophy in the carotid body response to hypoxia. It is also significant that Bee et al. (1986, 1989) have been unable to find evidence for Type II cell division (see also below).

In the animal experiments referred to so far, we have only considered the effects of hypoxia. In human disease states associated with hypoxia there is almost invariably hypercapnia as well as hypoxia. The data of Dhillon et al. (1984) suggests that combining hypoxia and hypercapnia does not further increase the increase in size of the carotid body seen with hypoxia alone, indeed to date no worker has been able to demonstrate hyperplasia in response to hypercapnia alone.

The dopamine and noradrenaline contents of animal carotid bodies are substantially increased after 2-3 weeks exposure to chronic hypoxia. After chronic hypercapnia only dopamine levels are raised (Pallot & Barer, 1982; Barer et al., 1986). There is dispute between the groups of Barer and Hellstrom, over the role of the innervation of the carotid body in the processes of enlargement and the increase in catecholamine level. Hellstrom and Hanbouer (1981) recorded greater increase in catecholamine levels during exposure to chronic hypoxia after sympathectomy whereas Barer & Pallot (1984) showed that sympathectomy attenuated the increase in amine levels during chronic hypoxia while the increase in size remained unchanged. Barer et al. (1976), and Pallot et al. (1990) remarked that the presence of large amounts of dopamine, which is known to depress carotid body activity, suggests the interesting possibility that the depression in hypoxic sensitivity (as a response of chronically hypoxic animals to hypoxia) might be due to inhibition of chemoreceptor afferents by dopamine. Such a possibility has been confirmed by the work of Wach et al. (1986, 1987, 1989).

There is a difficulty with regard to combining the animal and human data on the effects of hypoxia described above which concerns the fact that Heath and his colleagues report hyperplasia of the Type II cells as the most impressive finding in their hypoxic cases. Two possibilities exist for this apparent discrepancy. Firstly most, if not all, of the Heath cases will have been hypercapnic as well as hypoxic. This is an inadequate explanation as hypercapnia in experimental animals does not lead, in the short term at least, to proliferation of Type II cells. Pallot (1987)

suggested that the response of the human carotid body to hypoxia is a two stage phenomenon. Initially the response is a proliferation and hypertrophy of the Type I cells, continued exposure to the hypoxic stimulus lead to a gradual diminution in the number of Type I cells and their replacement by elongated cells which probably represent a mixture of Type II cells and Schwann cells. This possibility is examined in Chapters 4 and 5 which examine the effects of chronic hypoxia in humans on carotid body structure.

SYSTEMIC HYPERTENSION

Heath et al. (1970) found a clear relation between left ventricular weight and the weight of the carotid body. Edwards et al. (1972) reported that not only were the carotid bodies enlarged in patients with systemic hypertension, but the histological changes in the hypertensive human carotid bodies were similar to those in chronic hypoxia with a concentric proliferation of Type II cells around compressed groups of Type I cells (Smith et al. ,1982, 1984; Heath et al.,1982,1985). However, Kluge (1985), and Habeck (1986) could not confirm this histological picture and found an increase in carotid body volume associated with proliferation of Type I cells (particularly in renal hypertension) and also an obliterating atherosclerosis of glomic vessels. Habeck (1986) observed that patients with severe lung diseases together with essential hypertension showed the biggest carotid bodies in his study and younger patients with essential hypertension and patients with renal hypertension, who were relatively young, did not show carotid body enlargement whilst vascular damage in the organ was spectacular in the latter group. Such findings suggest that size increase may not be related to tissue anoxia.

A number of groups have studied the carotid bodies in animals with either genetically or experimentally induced hypertension. Pfeiffer et al. (1984) showed that the carotid bodies in spontaneously hypertensive rats of the Wistar Okamoto strain had enlarged carotid bodies. However, these authors used normal Wistar rats as controls, as the genetic make up of the animals is quite different this is clearly unacceptable. Barer et al. (1986) and Barer & Pallot (1985) and Pallot et al. (1986) studied the New Zealand strain of genetically hypertensive rat in which normotensives from the same colony are available. Their data showed that there was no relationship between carotid body size and mean arterial blood pressure.

Bee et al. (1989) also observed a reduction in the number of Type I cell nuclei and the vascular lumen and an increase of vascular wall volume as a percentage of total carotid body volume in her study of hypertensive rats suggesting that hyperplasia of the carotid body was not a feature. In experimental models of renal hypertension there is no increase in carotid body size (Habeck et al. 1987 in rat, Angell-James et al. 1985 in rabbit)

There have been a number of studies of chemoreceptor activity in animal and human hypertensives. Przybylski (1978) reported a resting hyperventilation and respiratory alkalosis in young SHR rats (using Wistar rats as controls) and subsequently (Przybylski, 1981) produced a hypothesis suggesting that an over active chemoreflex might be the trigger factor in setting the elevated blood pressure in the early labile phase of hypertension. Trzebski et al. (1982) and Quies et al. (1983) reported that the chemoreceptor drive to ventilation is increased in systemic hypertension in humans. In the New Zealand strain of hypertensive rats, where adequate controls were available, this changed chemosensitivity could not be found (Bee et al., 1989).

We thus appear to have a difference in the response of the animal and human carotid body to hypertension (see Chapter 5). Habeck (1991) discussed this point recently in his comparative review on human and animal experimental studies involving all forms of hypertension in the carotid body. He indicated that the structural and biochemical alterations of the organ in hypertension seem to be secondary and are not sufficient to explain the overactivity of chemoreceptors. He suggested that further systematic studies are necessary to clarify the interactions between arterial hypertension and peripheral arterial chemoreceptors.

CHEMODECTOMA

The carotid body tumour is called chemodectoma. They are true neoplasms and have a monomorphic population of Type I cells which have lost their normal relation to nerve fibrils, and are separated by a vascular stroma. The characteristic appearance of large balls of Type I cells has led to their other name of potato tumour. Saldana et al. (1973) reported that there is a greater frequency of the

tumour in high altitude and cattle and dogs are apparently particularly susceptible to developing chemodectomas.

Chemodectomas may occur at any age, most patients seek advice in the third or fourth decade of life. The youngest known patient with the chemodectoma was a three months old child (Akkary, 1964) while the oldest patient was a 89 year old male (Besznyak & Pinter, 1959). Although there is no convincing evidence to support sex linked transmission of the chemodectoma, there are some reports that show familial incidence (Sprong & Kirby, 1949; Katz, 1964; Glenner & Grimley, 1974; Zak & Lawson, 1982; Shedd et al., 1990; Ophir, 1991; Borowy et al., 1992; Ridge et al., 1993).

The tumour may be limited to the area of the carotid bifurcation. However, Conley (1965) observed three types of tumour extension :

- 1) encircling the common carotid artery and extending to the base of the skull,
- 2) growing around the external carotid artery and extending to the palate and pharynx,
- 3) proliferation along the course of the internal carotid artery.

Shamblin et al. (1971) described three groups of patients with chemodectoma. In the first group the tumours are very small, the patients are asymptomatic and removal is relatively straightforward. In the second group, patients have larger tumours which are beginning to produce symptoms and to become adherent to surrounding vessels and nerves. The last group, patients have several symptoms and metastases to many structure in the region of the carotid bifurcation. Compression of the common carotid artery or its internal carotid branch can cause diminished cerebral blood supply. In addition; interference with the function of different nerves including the vagus can produce a variety of symptoms (Brown & Fryer, 1952; Wilson 1964; Capella and Solica, 1971; McSwain & Spencer, 1974; Gupta et al., 1976; Zak & Lawson, 1982; Ophir, 1991; Schild et al., 1992; Mertens et al., 1993).

The histological appearances of the chemodectoma are not uniform and show considerable variation (Pallot, 1987 and Heath & Smith, 1992). The most common appearance is that of nests or islands of cells (Zellballen: representing the Type I cells) which maintain a close association with numerous blood vessels. Grimley & Glenner (1967) described spindle-shaped cells (resembling Type II cells) at the

periphery of the Zellballen. Nerve fibres and ganglion cells may occasionally be found within the stromal regions of the tumour but the normal relation of nerve fibres to Type I cells is normally absent in the chemodectoma. Grimley & Glenner (1967), and Crowell et al. (1982) reported the presence of neurosecretory granules in these tumours which provides morphological support for the biochemical evidence of catecholamine secretion in carotid body tumours. The fibrous tissue within the chemodectoma may undergo hyaline and myoedematous degeneration and even calcification or metaplastic bone formation (Lack et al., 1977,1979). Although the size of the tumour at presentation depends to a large extent on the number of years it has been growing; tumours as large as 10 to 20 cm (Brown & Fryer 1952) and over 110 g in weight (190 g Reid, 1920; 120 g; Javid et al., 1976) have been reported (see also Browse 1982). Krupski et al. (1983) suggested that because of the proposed role of the innervation in the release of stored catecholamines in the normal carotid body, a lack of innervation to the Type I cells may explain the rarity of amine secreting tumours.

The treatment of chemodectoma, due to the rich vascularity of the neoplasm, is hazardous. The postoperative morbidity or complications are high. Many surgeons believe that it is best to leave these tumours well alone. However, carotid body tumours have 6% risk of malignancy comprising 3% local invasion and 3% distant metastases. Because of this, some authors recommend a surgical approach (Javid et al., 1976; Shedd et al., 1990; Bernard, 1992). According to Browse (1982) no surgeon should attempt to remove a carotid body tumour unless he is experienced in the surgical technique used for carotid artery surgery. Recently Wax and Briant (1992) reported that they have utilized a new method for resection of these highly vascular tumors that results in less blood loss and increased ease of removal. In addition the successful result of radiotherapy treatment of chemodectomas has also been reported by Schild et al.(1992).

SUDDEN INFANT DEATH SYNDROME (SIDS)

In Britain, as in many other industrialized countries, sudden infant death syndromes, also commonly called cot deaths, are the most common cause of mortality in infants in the first year of life. The death is unexpected and unexplained by any clinical or routine post mortem findings. There is no single

cause of SIDS yet identified and it is now generally accepted that several factors come together to result in death. Notwithstanding this, abnormalities in the respiratory system have come to be regarded as important in the aetiology of SIDS.

Steinschneider (1972) reported prolonged apnoeic periods during sleep in two SIDS victims and Naeye (1977) considered sleep apnoea as a central problem in SIDS. Apnoea results in a decrease in oxygen and an increase in carbon dioxide in the blood. If apnoea is further prolonged, the brain is finally starved of oxygen, leading to an infant death. The assertions concerning apnoea and SIDS have led to a number of groups examining the carotid bodies in this condition (see Dapena 1983).

Naeye (1973,1974, 1983) and Naeye et al. (1976) described post mortem evidence of hypoxaemia in some SIDS victims. Naeye et al. (1976) measured the total volume of glomic tissue in the carotid bodies of subjects dying with a diagnosis of SIDS at an age between 1 and 10 months. They found that the ratio of glomic volume to body weight was significantly smaller in the non-infected SIDS victims than in controls and 63% of cases had a subnormal volumes of glomic cells, while 23% had an enlarged volume of such cells. However they did not describe the histological appearances of the carotid bodies. In 1977, Dinsdale et al. found no increase in size of the carotid bodies of SIDS victims except in some children dying at the age of 1 year or older. They suggested that the degree of hypoxia likely to occur in SIDS would be insufficient to produce carotid body hypertrophy. Cole et al. (1979) reported a reduction in the number and size of type I cells and absence of dense-cored vesicles in Type I cells of SIDS victims' carotid bodies. They claimed that some of the Type I cells in the carotid bodies in cases of SIDS were immature. Heath et al. (1990) supported their claim and suggested that the immature cells described by Cole et al. (1979) may correspond to their early progenitor cells (see Chapter 2 & 3 which discuss whether progenitor cells exist).

Using Chalkey's (1943) point counting technique to estimate the area occupied by glomus tissue Pearson & Brandeis (1983) measured the mean volumes of carotid body and found no abnormality in SIDS cases. Becker (1983) observed no qualitative or quantitative differences in the cytoplasmic granularity of the carotid bodies of SIDS cases. Perrin et al. (1984a,b) and Lack et al. (1985,1986) reported that there is no difference in the ultrastructure of carotid bodies of SIDS cases

compared to the age-matched controls. There is a major difficulty in all of the studies referred to with the exception of that of Dinsdale et al. (1977) which is the lack of adequate, age-matched and sex-matched controls. In addition to this, the delay between death and fixation of the tissue is usually great so that the morphology of the carotid body is poor and frequently the studies are performed on very small numbers of cases. It would seem, taking these factors into account, that no convincing evidence has yet been found for structural abnormalities of the carotid body.

Perrin et al.(1984b) found that catecholamine levels were significantly higher in the SIDS cases than in the controls. Again this study suffered from the difficulty of adequate controls. Vaughan (1992) studied the catecholamine levels in the carotid bodies of all children under the age of one year coming to postmortem over a period of eighteen months, the cases were analysed blind. Many of the carotid bodies analysed had incredibly low (or undetectable) dopamine levels and this was interpreted as being related to the delay between death and obtaining and processing the tissue. However there were a number of cases (~33%) in which very high dopamine levels were recorded. With one exception, these cases were from the SIDS group, that which came from the non SIDS patients had a history of lung disease. Hence there may be some evidence to suggest that at least a proportion of SIDS patients experience hypoxaemia sufficient to cause an increase in amine levels.

To date workers have concerned themselves with looking for abnormalities in respiratory control in children at risk for SIDS. What if the respiratory control problem that arises is not an abnormality but rather a part of normal development? The recent work of Bee et al. (1994) has highlighted exactly this possibility. Bee et al. (1994) studied the development of the chemoreflex in neonatal rats and rabbits. Both species showed a brisk response to hypoxia on the day of birth which gradually increased with increasing age. However at about 15 days in the rat and about 6 weeks in the rabbit, the response to hypoxia was abolished. This situation existed for a few days in the rat and about one week in the rabbit and then hypoxic sensitivity was gradually restored. If a similar developmental process takes place in the human infant then clearly, at the time of depressed hypoxic sensitivity the baby would be at great risk during an apnoeic spell.

THE CAROTID BODY AND ANAEMIA

As mentioned earlier, Pearse (1969) classified the Type I cells as being part of the APUD system for which a hormonal function might be predicted. He proposed the name glomin for this hypothetical hormone but did not predict a function for it. The fact that the carotid body senses oxygen in the blood has led a number of authors to postulate that, in addition to being a chemoreceptor, the carotid body is an endocrine organ secreting erythropoietin which is concerned with the control of erythropoiesis (Tramezzani et al. 1971; Gillis & Mitchell, 1973; Beynon and Balfour, 1973, and Heath et al., 1973). However, the data of Lugliani et al. (1971), Sorensen et al. (1973), and Winson & Heath (1973) does not support this suggestion. Lugliani et al. (1971) studied a series of patients who, as part of the treatment of chronic obstructive pulmonary disease (COPD), were undergoing bilateral resection of the carotid body, they found no significant change in haematocrit or reticulocyte count from preoperative values when studied for a period of up to three years postoperative. Winson & Heath (1973) remarked that the carotid bodies were found not to be enlarged in anaemia and that this was unlikely to be consistent with recent suggestion that the carotid body secreted erythropoietin. Heinrick & Acker (1977) administered dextran solutions to produce a graded haemodilution in the cat and observed that severe anaemia caused a partial destruction of the cellular architecture of the carotid body, a finding that probably accounted for the impaired chemoreception noted in these experiments. Habeck & Przybylski (1989) reported that neither in normotensive Wistar rats (NCR) nor in spontaneously hypertensive rats (SHR) did chronic anaemia influence the size or histological appearance of the carotid bodies. In addition, functional studies have shown that haemoglobin concentration has but a negligible effect on the activity of afferent carotid body chemoreceptor fibres (Hatcher et al., 1978; Davies et al., 1981). Whilst the suggestion of Pearse concerning an endocrine function has not been disproven it seems unlikely that the carotid body is a major source of erythropoietin.

CHAPTER TWO

GENERAL HISTOLOGY AND ULTRASTRUCTURE OF THE NORMAL HUMAN CAROTID BODY

INTRODUCTION

The mammalian carotid body consists of groups of specific cells set in a vascular connective tissue stroma containing numerous nerve fibres. The parenchymal cells consist of two varieties, namely the Type I and Type II cells. In all experimental animals studied to date a variable number of polygonal Type I cells are surrounded by Type II cells, the latter being elongated cells with long fine, cytoplasmic processes (Biscoe, 1971; McDonald, 1981; Pallot, 1987). In the human carotid body a similar arrangement has been described by Heath and his colleagues (Heath et al., 1970; Smith et al., 1982, and Heath et al. 1984) except that they are able to recognize three subpopulations of Type I cells on the basis of nuclear morphology, these are called the light, dark and pyknotic cells. The details of these subvarieties of Type I cells have been described in Chapter 1.

As mentioned above the general histological features of the mammalian carotid body have been described in Chapter 1, here I describe the histology of the human carotid body as seen in our postmortem samples using light and electron microscopy. The observations reported here are derived from carotid bodies which were obtained from patients below the age of 50 years whose postmortem examination ruled out the presence of cardiovascular disease, diabetes and chronic lung disease.

METHODS

Light microscopy

Carotid bodies collected at routine necropsies were fixed in either ethanol or Zamboni's fluid and processed for embedding in paraffin wax (see also

appendix 2.1.). Sections stained with haematoxylin and eosin were studied with regard to general architecture using a Zeiss microscope (Axiophot). In addition some sections stained with antibodies to Neurofilament protein and Von Willibrand factor (F8) were also used to display general morphology (see Chapter 5).

Electron Microscopy

Seven carotid bodies were obtained from routine necropsies (2 hours, 3 cases, and one case each for 4, 18, 20, and 42 hours after death). All specimens were fixed in 2.5% glutaraldehyde in phosphate buffer (PO_4 , pH 7.2) for 3-24 hours at room temperature and post-fixed in 1% osmium tetroxide (in PO_4 buffer pH 7.2) for 2-3 hours. Following dehydration in graded ethanol solutions and treatment with propylene oxide they were embedded in resin (Agar 100). Semithin sections stained with 1% toluidine blue were used for identification of carotid body tissue under the light microscopy, the blocks were then trimmed and ultrathin sections for electron microscopy prepared. Three sets of sections separated by 20 μm were prepared from each block and stained with uranyl acetate and lead citrate (see appendix 2.2). The sections were examined using a Philips CM10 transmission electron microscope.

RESULTS

Light microscopy observations:

The carotid body is an encapsulated organ which essentially consists of clusters of cells embedded in a highly vascular connective tissue stroma (Figs 2.1. & 2.2), as in many organs the stromal connective tissue is derived from extensions of the capsule into the substance of the carotid body. Connective tissue of the carotid body may be divided into interlobular (between lobules) and intralobular (between cell clusters) connective tissue.

The cell clusters consist of two types of cells. The majority of cells have plump spheroidal or oval nuclei (Fig 2.3). These are the Type I cells. The chromatin

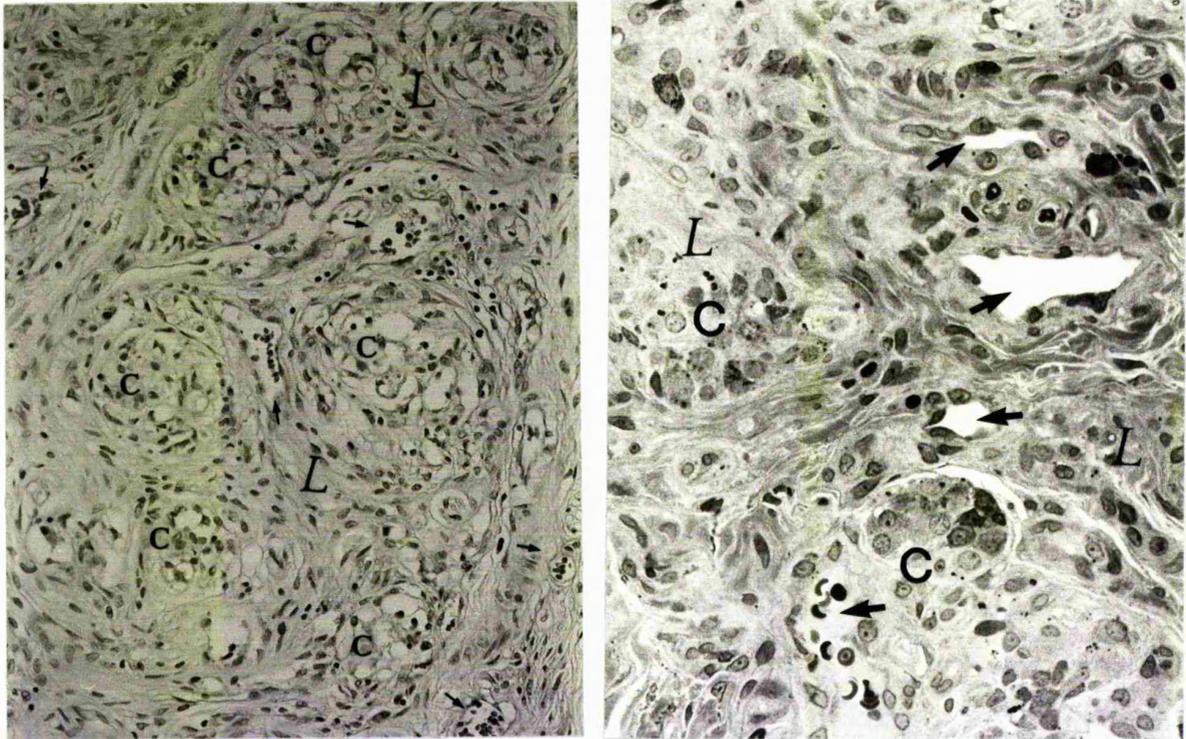


Figure 2.1 shows the classic human carotid body structure which consists of cell clusters (C) within lobules (L) embedded in a highly vascular connective tissue stroma (x20) and Fig. 2.2 shows similar pattern in a higher magnification (x40). Arrows indicate blood vessels.

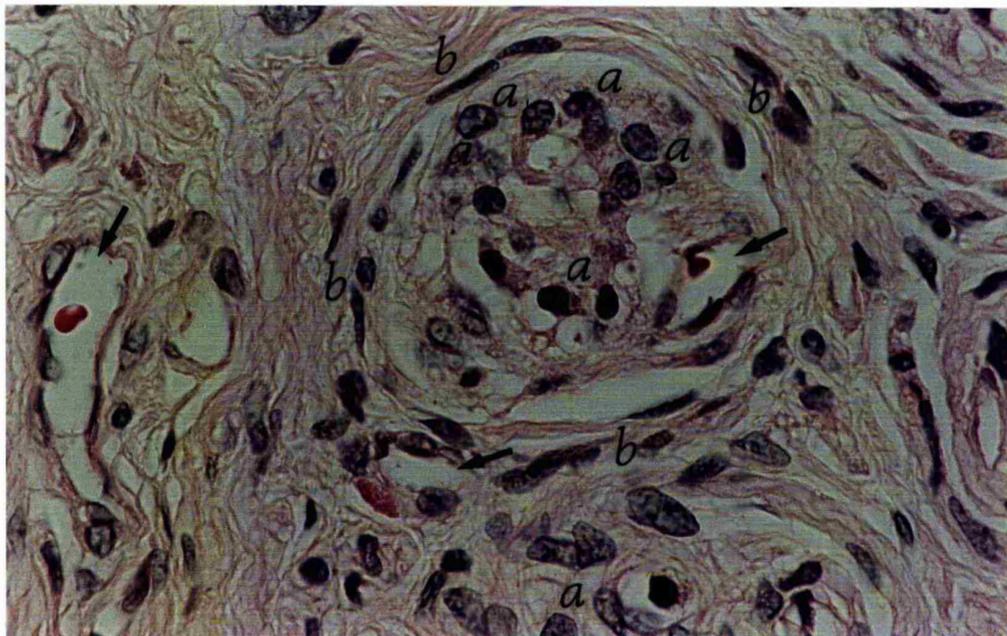
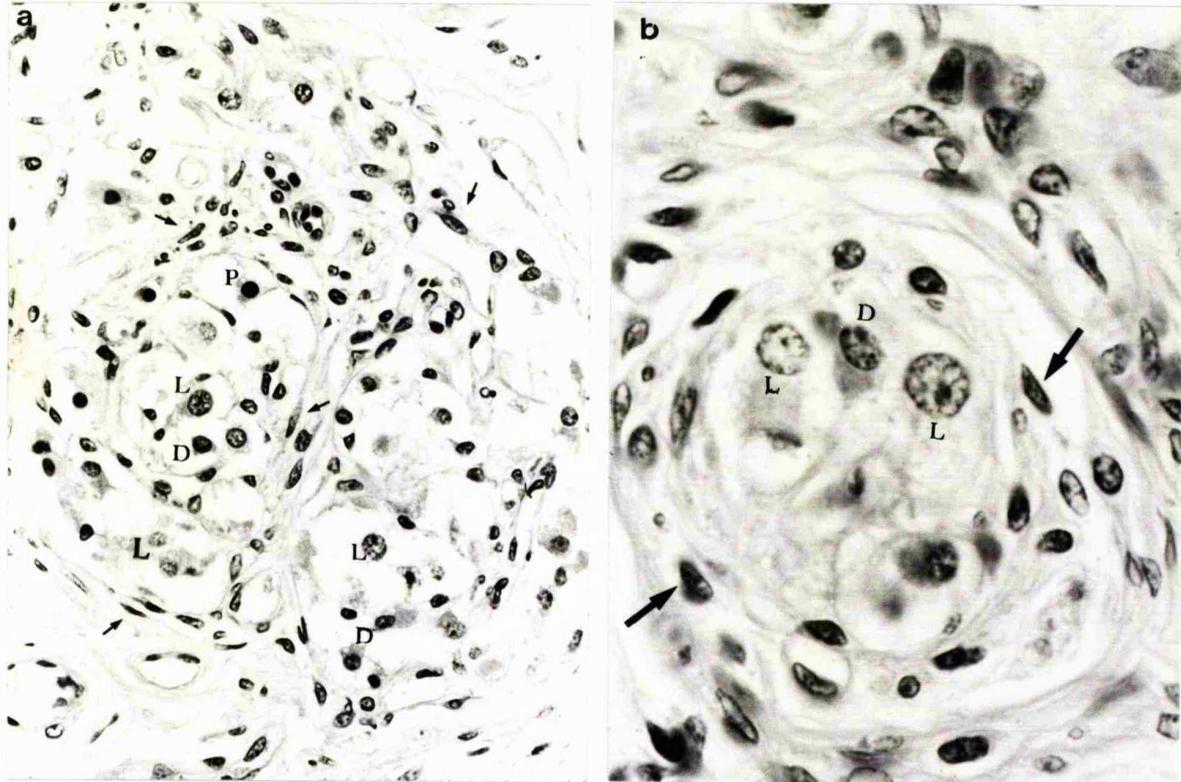
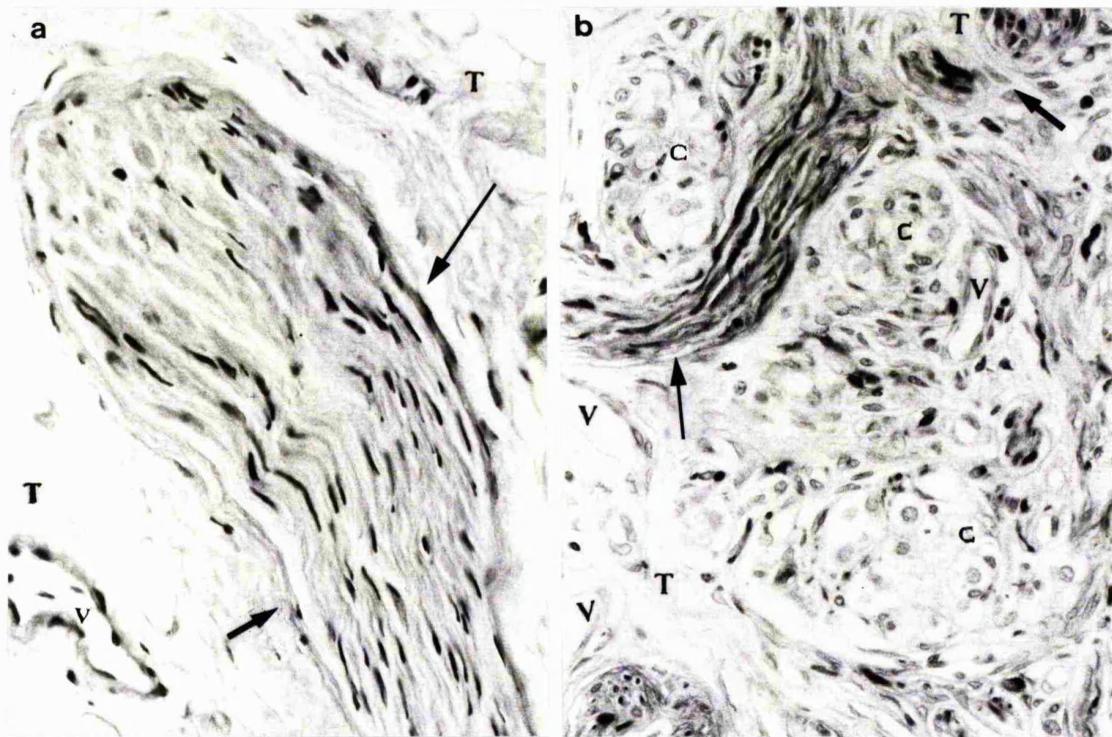


Figure 2.3. shows a micrograph of a human carotid body cell cluster. Note the two types of cells, namely; Type I cells (a) which have plump spheroidal or oval nuclei and Type II cells (b) which have elongated nuclei with condensed chromatin. The connective tissue stroma surrounds the cluster and contains a number of blood vessels (arrows) (x63).



Figures 2.4a, and b show cell clusters which consist of number of Type I cells enclosed by Type II cells (arrows). The three types of Type I cells; Light cells (L), Dark (D) and Pyknotic (P) cell may be seen including their intermediate form. (x40 & x100)



Figures 2.5a, and b show two photomicrographs of human carotid body tissues stained using the immunohistochemistry technique for neurofilament protein. Nerve bundles (arrows) around and within cell clusters can be seen (x100, and x40 respectively, C: cell cluster, V: blood vessels, T: connective tissue).

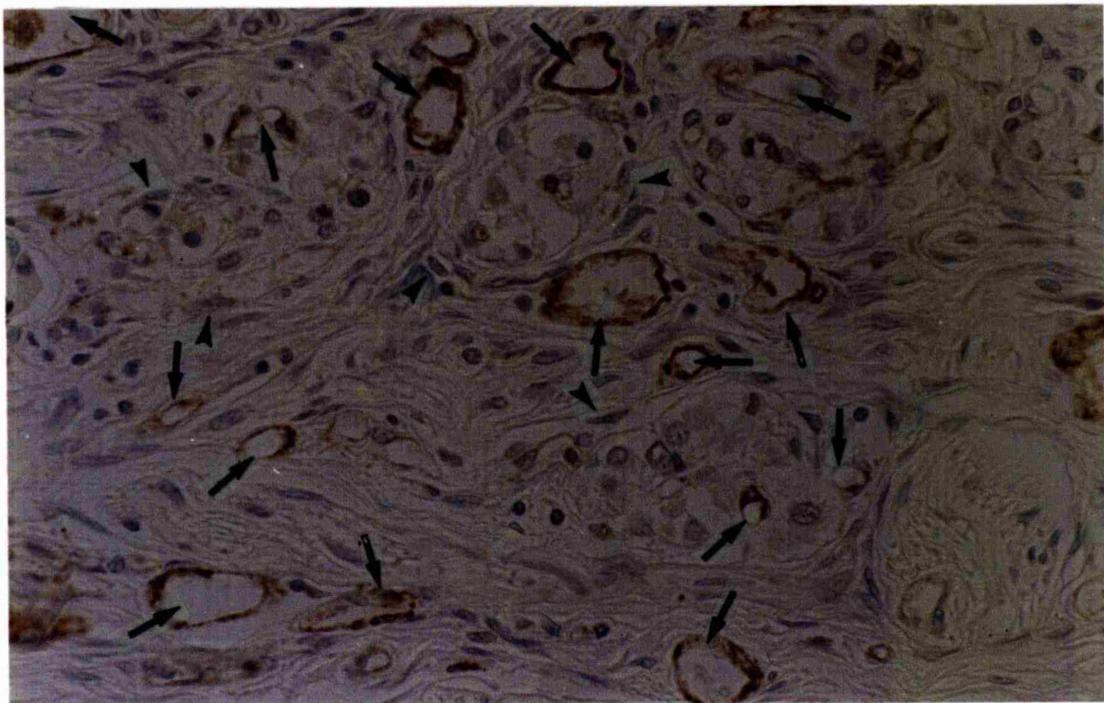


Figure 2.6. Micrograph of human carotid body tissue stained with antibody to Von Willebrand Factor. Numerous blood vessels (arrows), clusters of Type I cells (I), and Type II cells (arrow heads) may be observed (x40).

pattern in the nuclei varies from a light staining pattern to an intensely dark staining pattern which is almost pyknotic in appearance (see Fig 2.3, 2.4a, & b this feature is discussed in detail in Chapter 3). In addition to the normal Type I cells some cells with the general characteristics of Type I cells are found save that their nuclei are much larger, the significance of these is unclear (see Fig 2.4a,b & 2.6).

The second cell type is characterised by elongated nuclei with condensed chromatin (Fig 2.3, 2.4a,b). These are the Type II cells. The Type I cells are arranged in clusters containing various numbers of cells (Figs 2.1 & 2.3). At the periphery of each cell cluster a variable number of Type II cell nuclei are found. The cytoplasm of the Type II cell is attenuated and at the light microscope level is difficult to discern (Fig 2.3). They appear similar to Schwann cells and enclose unmyelinated nerve fibres. Type II cells are identified by their position and/or their elongated basophilic nuclei.

Closer examination of the connective tissue stroma shows that it contains bundles of nerve fibres (Fig 2.5a) which can often be seen entering the clusters of cells (Fig 2.5b) in addition to the arterial vessels supplying the carotid body tissue and the venous elements draining it (Fig 2.6). The capsule also contains many blood vessels which cause the organ having a reddish appearance *in situ*.

The carotid body is a vascular organ. Blood vessels are found within the connective tissue (interlobular stroma) and also within the cell clusters (intra-lobular stroma). Within the cell clusters the vessels are mainly capillaries and small venules and within the connective tissue stroma larger veins and small arterial vessels are found (Figs 2.3 & 2.6).

Electron microscopy observations:

Low power electron micrographs of the organ show a highly vascular structure with clusters of cells containing the two specific cell types: the Type I and Type II cells mentioned above (Fig 2.7a,b). A variety of nerve fibres and nerve endings were seen within the connective stroma and within the lobules (Fig 2.7b).

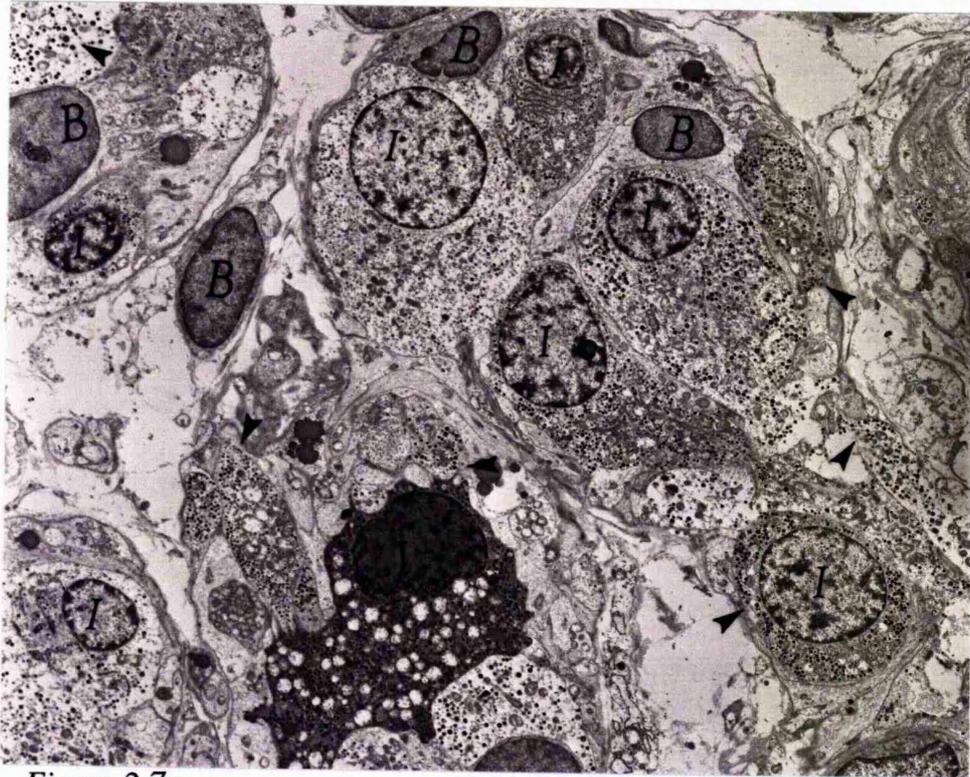


Figure 2.7a

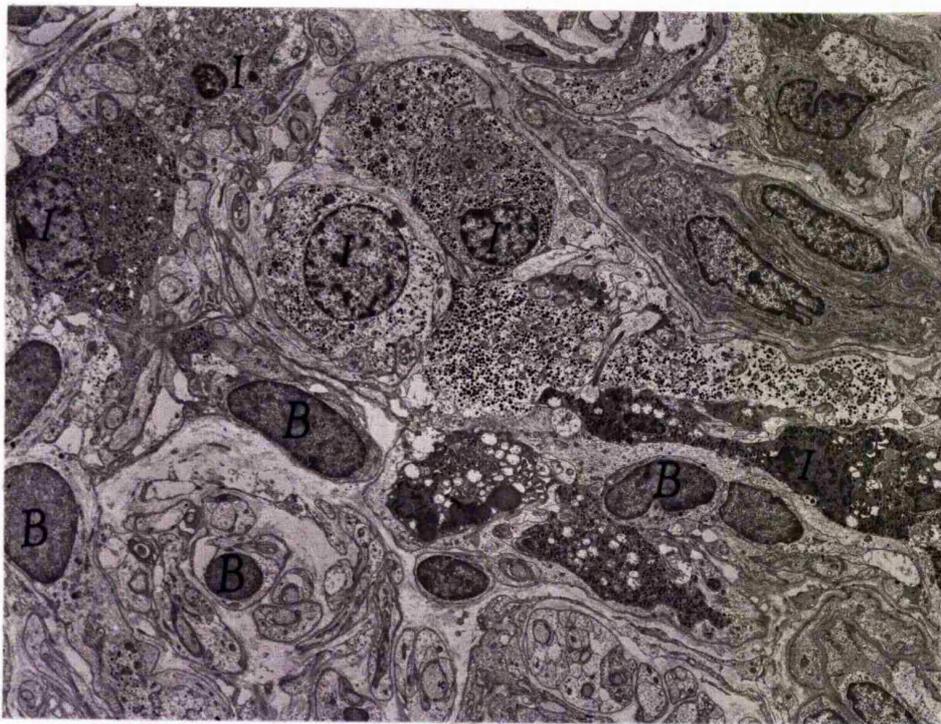


Figure 2.7b

Figure 2.7a, b, c, and d. The presence and distribution of electron dense-cored vesicles (arrow heads) within the cytoplasm of the Type I cells (I) and the complex shape of the Type I cells. Figure 2.8b provides evidence that Type II cells (B) may also be seen in round shape. Note the nerve fibres around and within the cell clusters (arrows) (a: x2600, b: x1900, c: x3500, d: x4700)

The Type I cells were seen sometimes to have cytoplasmic processes which were mostly found in contact with neighbouring Type II cells (Figs 2.7a,b). The Type I cells groups were partially surrounded by the processes of Type II cells as in other species.

The presence of electron dense-cored vesicles within the cytoplasm is the most distinguishing feature of the Type I cells (Fig 2.7a,b & c). Their distribution, size and number were found to vary from cell to cell, and from area to area within the cytoplasm of the same cell. The accumulation of the dense-cored vesicles adjacent to the cell membrane was also observed.

The Type I cells exhibited nuclei of quite differing densities and also cytoplasmic variability. As mentioned above light cells had large complex shaped cell processes and round pale nuclei. The electron dense-cored vesicles in the light cells were distributed throughout the cytoplasm (Fig 2.7a,b, c).

The dark cell nucleus is slightly darker and smaller than the light cell nucleus as seen in light microscopy. The density of the dense-cored vesicles appears greater than in light cells (Fig 2.8a). In addition, some cells could also be seen in an intermediate form between light cells and dark cells (see Fig 2.7a, b).

Pyknotic cells had small, dark staining, heterochromatic nuclei (Fig. 2.7a, b). In addition to this, the cytoplasm was densely stained and characterised by many vacuoles. The Pyknotic cell nucleus was often elongated in shape and characterised by a crenated surface rather than the round or ovoid nuclei of light and dark cells (Fig 2.7a,b, c). As in the light microscopy, cells with a morphology between that of dark cells and pyknotic cells were regularly seen.

Vacuolation of the cytoplasm of dark and pyknotic cells was a common feature but the number of vacuoles in pyknotic cells was much greater (Figs 2.8b). In light cells vacuoles were much less common. Examination of these vacuoles shows that many of them represent ruptured mitochondria (Fig 2.8b).

Electron microscopic studies have shown that the Type II cells partially enclosed the groups of Type I cells. Mostly, they sent out numerous fine cytoplasmic extension which enclosed the Type I cells (Fig 2.7a, & 2.9b). However, they were also found between or within Type I cells group and, as in other species they enclosed small unmyelinated axons (Fig 2.7d, & 2.9b).

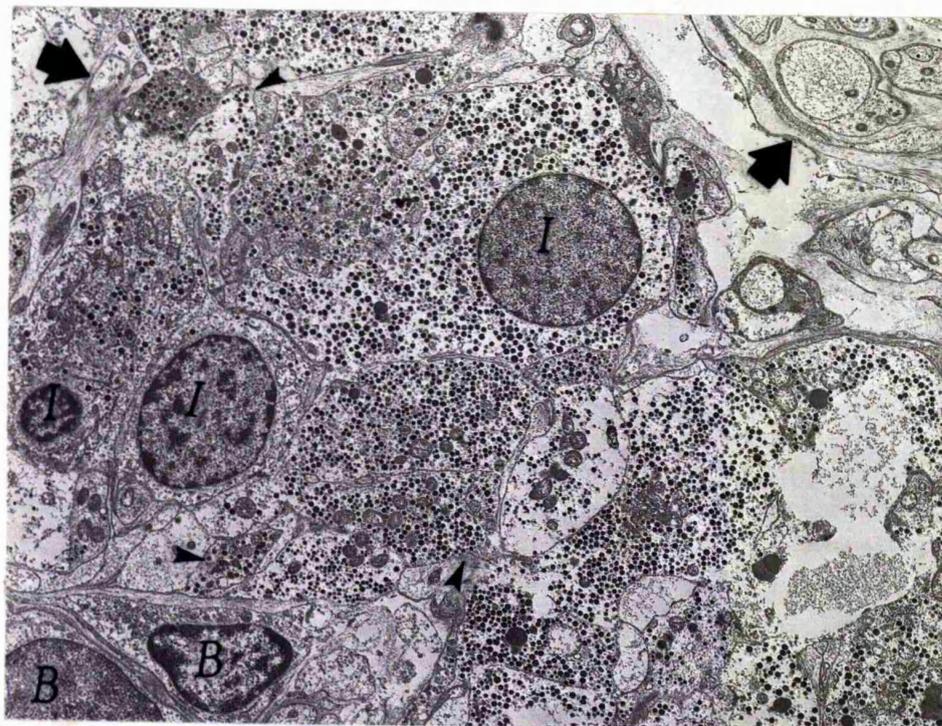


Fig 2.7c

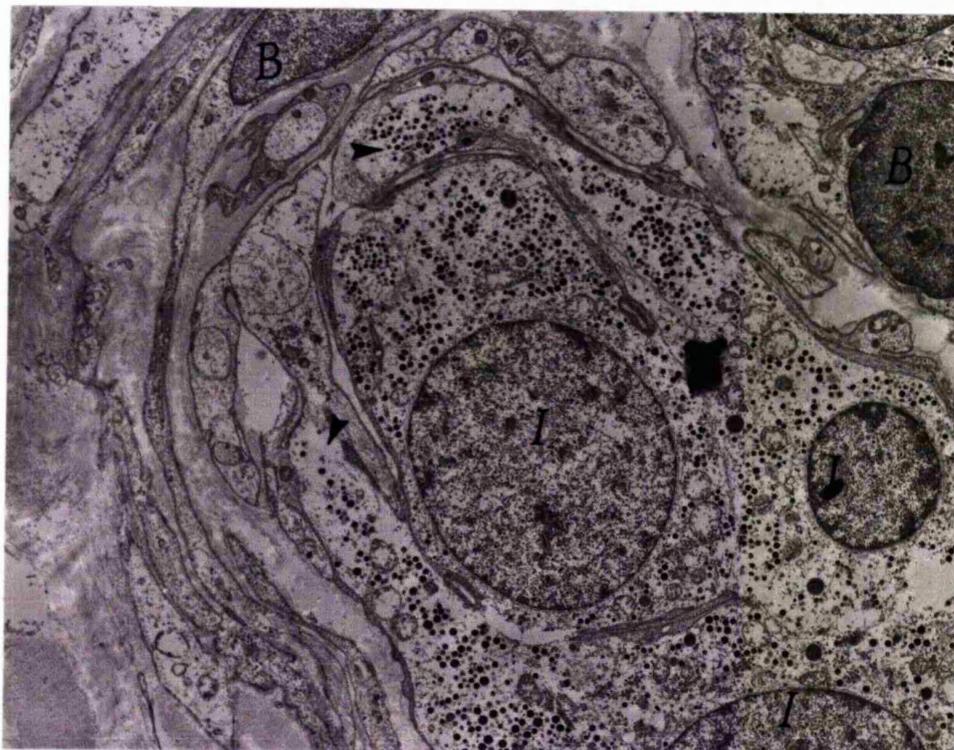


Fig 2.7d

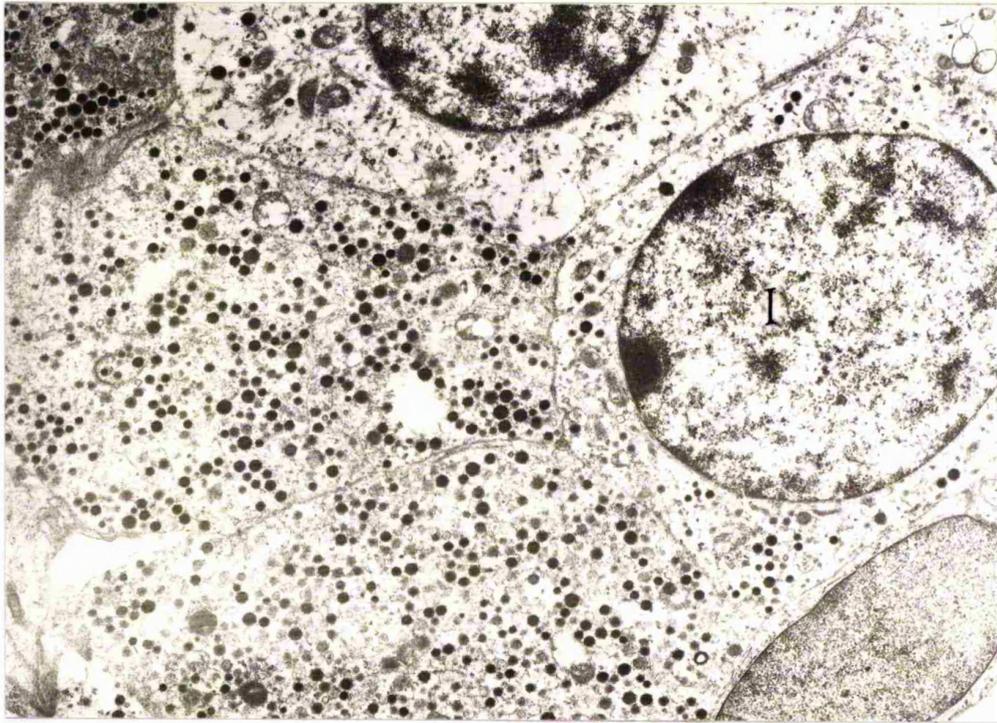


Figure 2.8a

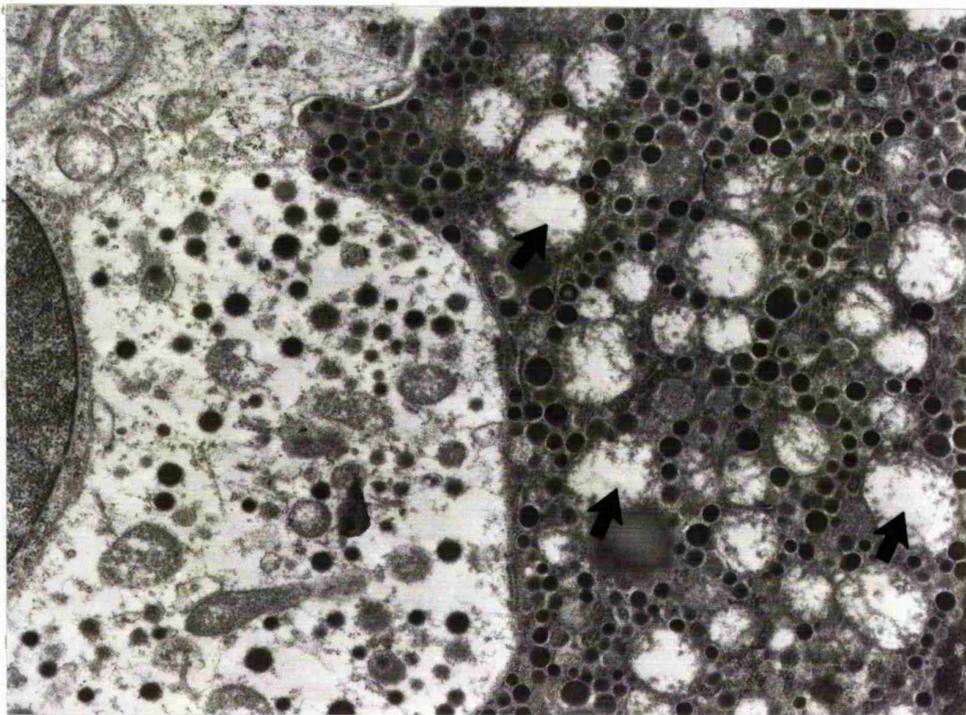


Figure 2.8b

Figure 2.8 a,b,c, and d show the distribution of dense-cored vesicles within the cytoplasm of Type I cells of the nerve endings (N) in Fig 2.8c & d. The variety of dense-cored vesicle shape, size and distribution may be seen. The great amount of vacuolation of the cytoplasm (arrows) of dark and pyknotic cells is clearly seen (Fig.2.8b & c). (a:x 4000, b, c, & d: x14250).

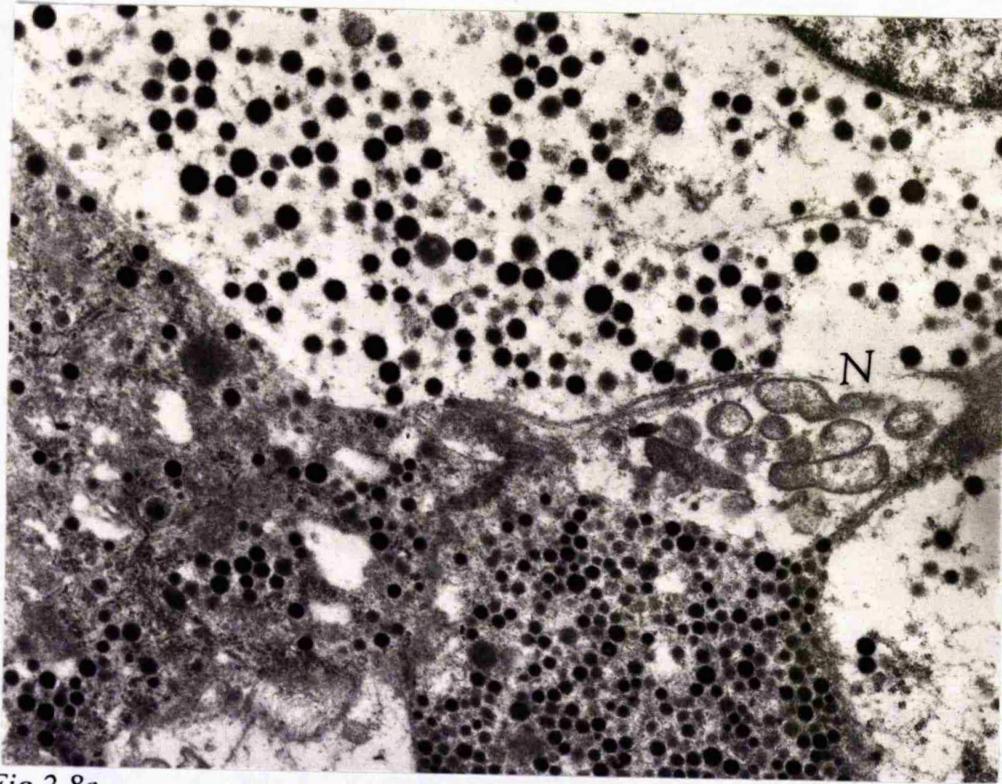


Fig 2.8c

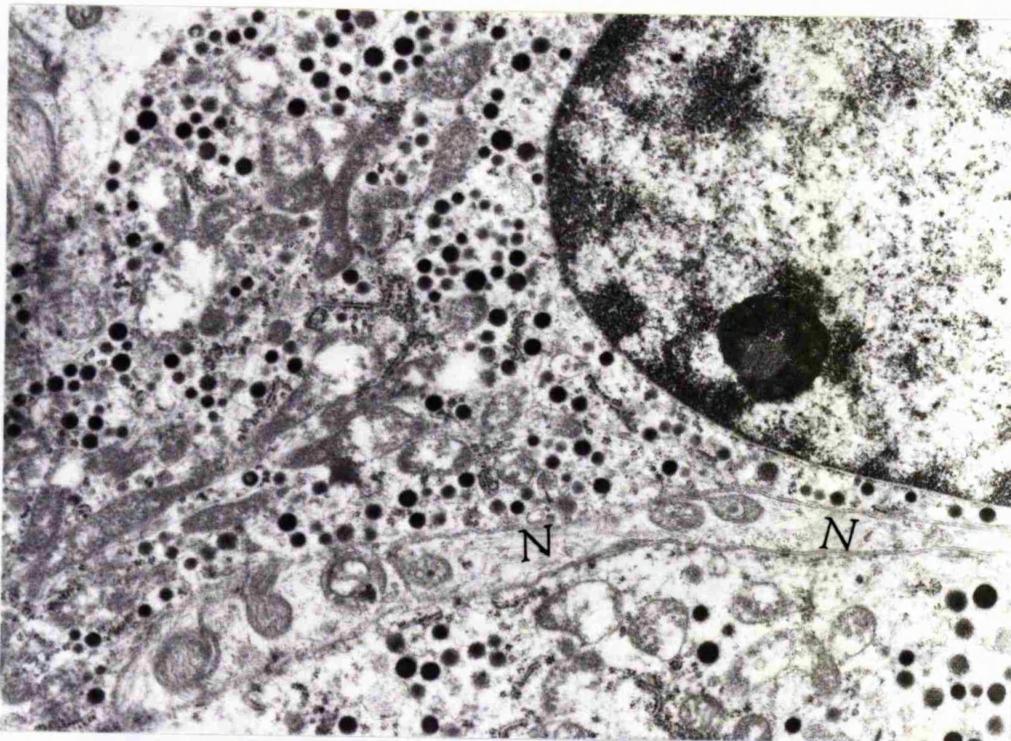


Fig 2.8d

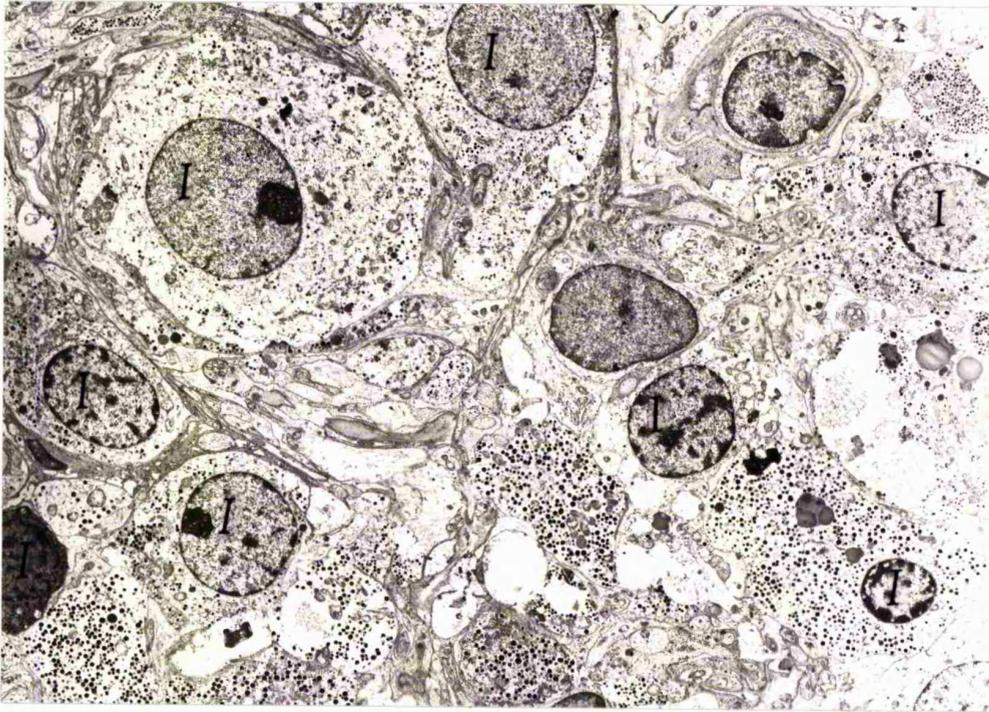


Figure 2.9a

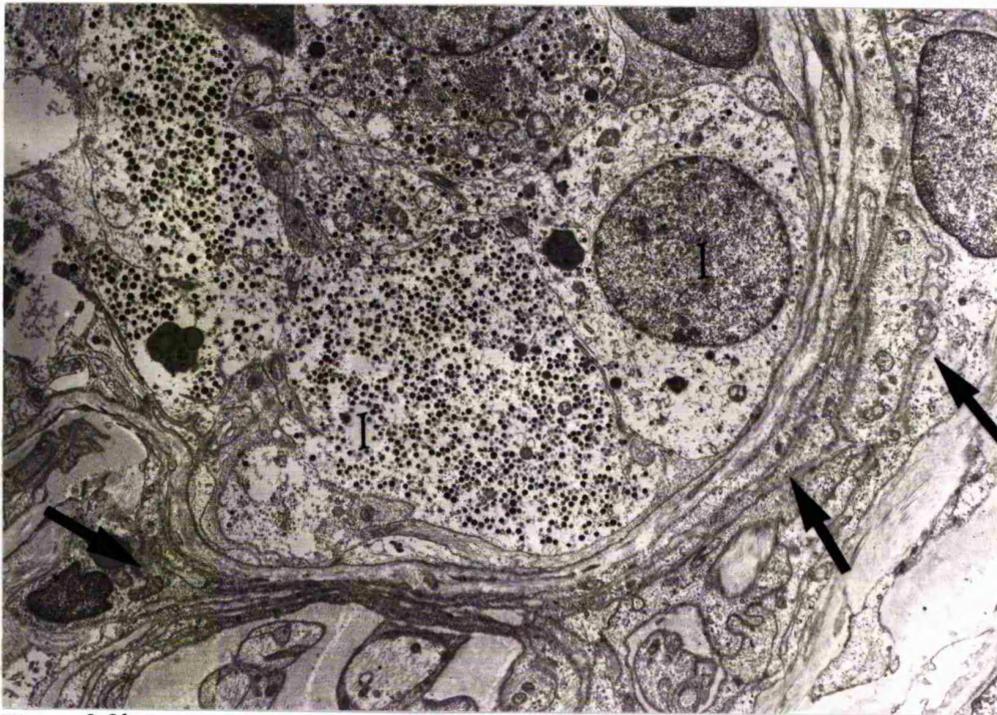


Figure 2.9b

Figure 2.9a, shows the great variety of Type I cell (I) nuclear size. Figure 9b, an electron micrograph of human carotid body showing the complex shape of Type II cell cytoplasm (arrows) and its cytoplasmic extension which enclose the Type I cells cytoplasm (a: x2600, b: x4700).



Figure 2.10a

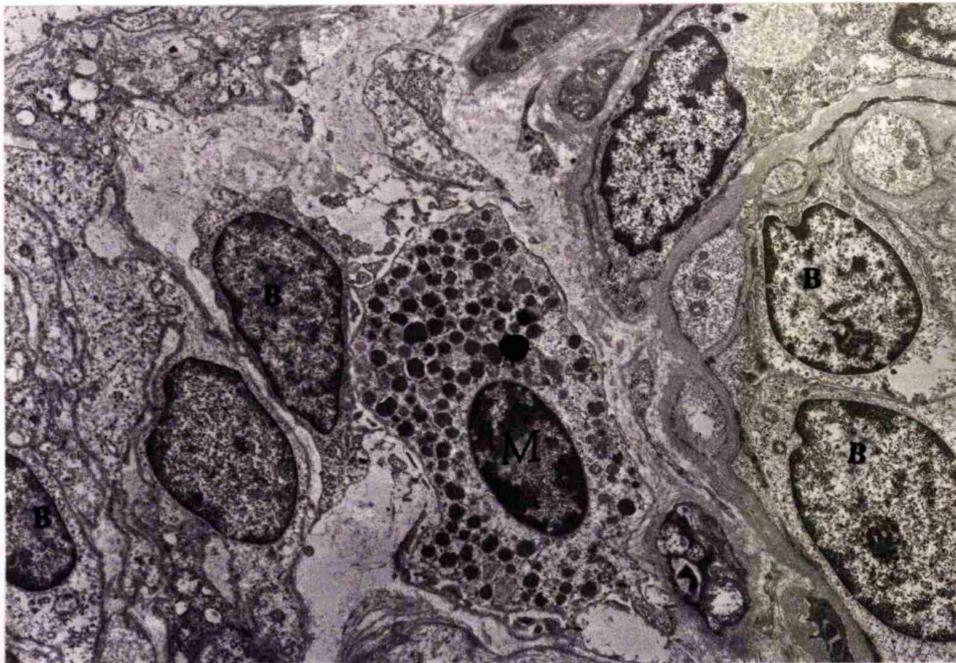


Figure 2.10b

Figure 2.10.a,b Two electron micrographs showing the mast cells (M) which are very close to Type I (A) and Type II cells(B). The mast cells show the with characteristic cytoplasmic granules and an irregular cytoplasmic shape (a:x3100, b:x4700).

It has been observed that when Type II cells have been sectioned transversely the nucleus appears round in shape (Fig 2.7b). In Chapter 5 the use of various immunological markers in the carotid body is described and one of these markers confirms the peripheral distribution of the Type II cells.

Mast cells were found with characteristic cytoplasmic granules and irregular borders in close proximity to interstitial vessels and the cell clusters (Figs 2.10a & b).

A new finding was the presence of isolated groups of cells with the characteristics of epitheloid cells (Fig 2.11a,b, & c). These cell groups were enclosed by elongated cells with a structure similar to that of Type II cells and were associated with nerves, they were found close to the usual cell clusters (Fig 2.11a). The cytoplasm of the epithelial-like cells contained a normal complement of organelles but lacked electron dense-cored vesicles (Fig 2.11c). Like Type I cells they formed junctions with adjacent cells (Fig 2.11c).

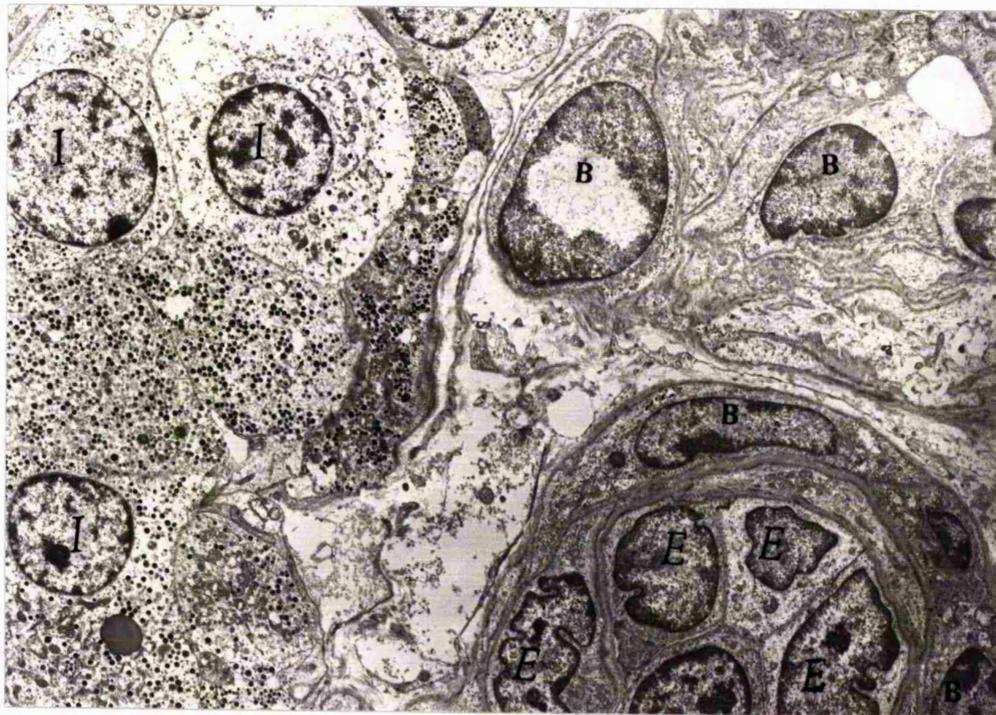


Figure 2.11a

Figures 2.11 a,b, and c shows the presence of isolated groups of cells (E) with the characteristics of epitheloid cells which are enclosed by elongated cells (B) with similar structure to Type II cells. Junctions (arrows) with adjacent cells may be seen (Fig 2.11c). They can be found either close to cell clusters [Type I (a) & Type II cells (b)] (Fig.2.11a & b) or within the intralobular stroma. (a: x 3500, b:x4700, and c:x8300).



Fig 2.11b

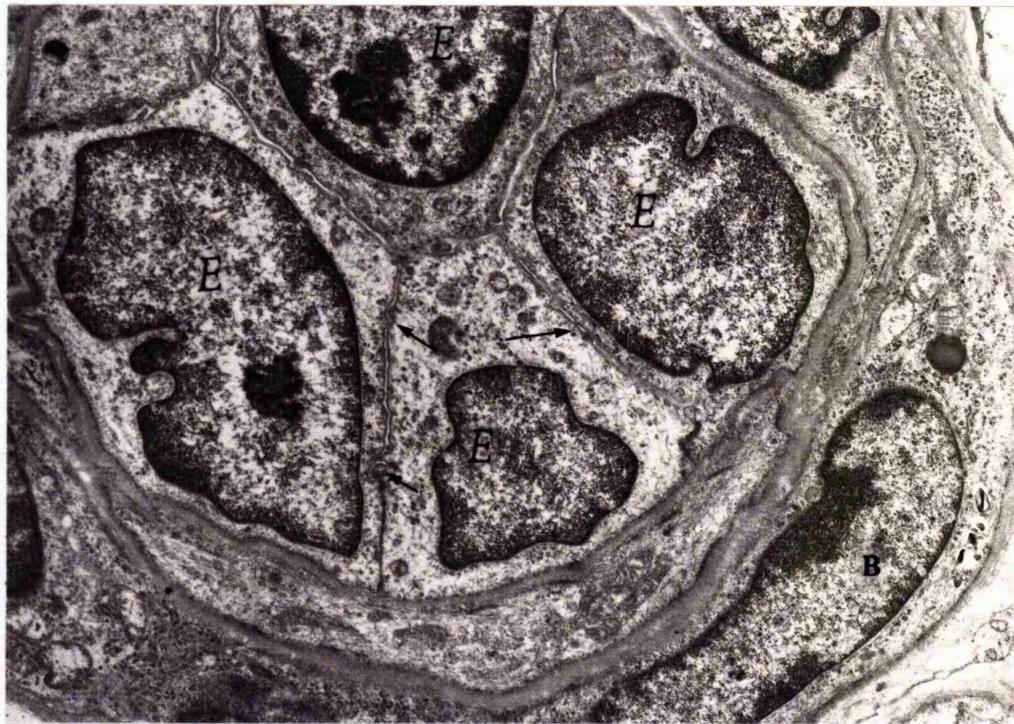


Fig 2.11c

The connective tissue between the cell clusters contained large numbers of unmyelinated nerve fibres and also the odd myelinated nerve fibre (Figs 2.7b). Very few nerve endings were found on Type I cells (Fig 2.8c,d) in this study presumably because of the speed with which nerve endings autolyse.

DISCUSSION

These observations confirm those of other workers that the human carotid body has a structure which is essentially similar to that reported in other mammals (see Chapter 1).

One difference between the human carotid body and that of other mammals, reported extensively by Heath and his colleagues (Heath and Smith 1985, Heath et al., 1990; Heath & Smith, 1992), concerns the existence of different staining patterns within the nuclei of Type I cells. Do they represent different physiological states of Type I cells with quite different functions, or they are merely the result of postmortem change ?

All of the papers of Heath and colleagues suggest that the three cell varieties which are present in the human carotid body represent a true finding and they have suggested that these different varieties may have different functions. The data reported here throws some doubt on this interpretation.

At the level of the light microscope, it appears that there is a decrease in size of the cell as well as increasing heterochromicity of the nucleus as we pass from light, to dark to pyknotic cell. Such a picture would fit in with the idea of postmortem change. The electron microscopic study has added additional support for this view.

Apart from the light, dark and pyknotic cells, the data here show that there are cells with an intermediate appearance between light / dark and dark / pyknotic cells. According to Jago et al. (1984), and Heath & Smith (1992) there is no cytoplasmic degeneration in any of the cell varieties.

In this study it is clear that the vacuolation of the cytoplasm which these authors appeared to accept as normal often represents the explosion of mitochondria due to postmortem change. Indeed, although it was not possible to perform quantitative studies, it appears subjectively that vacuolation occurs commonly in dark cells, most commonly in pyknotic cells and much less frequently in light cells. In fact this observation can also be obtained from the examination of their electron microscopic (EM) pictures (see Heath & Smith, 1992. p.146, Figures 17.4 &17.5). It seems, therefore, that there is evidence to support the view that pyknotic and dark cells may be the result of postmortem change (see also Chapter 3).

If the dark and pyknotic cells are some type of postmortem artefact it is interesting to speculate as to why we can find light, dark and pyknotic cells within the same cell cluster which has presumably been exposed to identical conditions of anoxia after death. It may be that indeed different Type I cells exhibit different functional activities within the same cell group. Sometimes one cell is more active than its neighbour. Such a hypothesis could then be extended and it might be postulated that the more active cells undergo more rapid postmortem change.

The present electron microscopic study was unable to provide sufficient numbers of specimen to perform a quantitative EM evaluation of the organ. All of the material studied were from autopsy and despite being able to receive tissues within 2 hours and 4 hours, of death, the degree of postmortem changes in carotid bodies 4 hours after death were too great for adequate fixation. This observation is not in agreement with the suggestion of Heath & Smith (1992) that an interval between death and necropsy of less than 12 hours, provides preservation of cellular detail which is sufficiently good for meaningful EM examination.

Quantitation of moderately well fixed material will provide interesting information concerning the innervation of the human organ. It is merely a subjective impression, but there appear to be many more nerve fibres within the human carotid body than either cat or rat.

The occurrence of epithelial-like cells close to Type I cell clusters is interesting. Their function is unknown and this study has not offered any evidence on the matter. However it is interesting that they are apparently partly enclosed by a Type II like cell and there are apparently nerve fibres associated with them. They may represent a developing population of Type I cells or they may represent a population of Type I cells that are degranulated. This is another area where immunocytochemistry might elucidate a function.

To perform further quantitative studies, it is essential that an adequate number of specimens should be obtained as soon as possible after death (within 3 hours). In addition to that future EM immunocytochemical studies are essential in order to distinguish and finalise the discussion of the subtype of Type I cells (this will be discussed in chapter 5 and 6).

CHAPTER THREE

AN EXPERIMENTAL STUDY ON THE POSTMORTEM CHANGES IN THE RAT CAROTID BODY AND IMPLICATION TO THE HUMAN CAROTID BODY

INTRODUCTION

The results reported in Chapter 2 confirm that the human carotid body has an essentially similar structure to that seen in other mammals. In addition, it highlighted the presence of the three Type I cell types reported by Heath and his colleagues (Heath et al., 1984; Jago et al., 1984, and Heath & Smith 1992). In this chapter, I report experiments to try and decide whether these Type I cell varieties represent postmortem change.

The major difference in the preparation of animal and human carotid bodies for structural examination lies in the delay between death of the animal and fixation of the tissue. Accordingly, one possible reason for the apparent difference in structure of Type I cells may be that there are postmortem changes in human Type I cells which account for the appearance of dark and pyknotic cells. Such an explanation is suggested by the electron microscopy studies reported in Chapter 2. This chapter describes experiments in rat and human carotid bodies which examine the distribution of subvarieties of Type I cells and correlates the cell types observed with the delay between death and fixation.

MATERIALS AND METHODS

Rat Experiments

The experiments were performed using adult Wistar rats of approximately 4 months of age. The animals, obtained from the Unit of Biomedical Services, University of Leicester or alternatively, the Animal House of the University of the U.A.E., were killed by an overdose of pentobarbitone sodium (75-100 mg/kg, May & Baker, Ltd.) intraperitoneally, and treated as follows;

- 1- The carotid bodies were removed immediately at the time of death (0 hour): these tissues acted as controls.

2- Carotid bodies were removed at different times after death (2, 4, 8, 16 and 24 hours) from animals in which the carcass had been kept at room temperature for 2 hour and thereafter at 4°C

3- Carotid bodies were removed at 4 and 16 hours postmortem delay from animals in which the carcass had been kept at room temperature.

A total of 30 animals were studied. The carotid bodies were fixed overnight in neutral buffered formalin, processed by routine techniques for embedding in paraffin wax and stained either with Harris haematoxylin and eosin or by the periodic acid-Schiff (PAS) method (for fixation, embedding, and staining methodology see Appendix : 2.1).

The number of Type I cells with either light, dark or pyknotic nuclei was counted in random sections of carotid bodies using a 40x objective on a Zeiss microscope equipped with a camera lucida, the average of at least four or more such fields were counted in each carotid body.

Human Experiments

There is considerable difficulty in obtaining large numbers of carotid bodies in Western Europe. All of the material studied in this thesis was collected by Dr. J-O. Habeck from the State Hospital in Chemnitz. A total of 75 carotid bodies were removed at routine necropsy and fixed in ethanol or Zamboni's fluid, the time interval between the death of the patient and fixation of the organ was recorded. The organs were obtained in patients where the primary cause of death was known from previous case history and/or postmortem findings and the presence or otherwise of other chronic diseases was known. After routine processing for embedding in paraffin wax, serial sections were prepared and stained with Haematoxylin & Eosin (see Appendix 2.1). Sections, separated by a minimum gap of 250 um, were examined and random fields using a 40x magnification were used to classify all of Type I cells within the field as either light, dark, or pyknotic cell variants. In two cases the carotid body was removed and each carotid body was divided into two pieces: one of these pieces was fixed immediately whilst the other was kept in the refrigerator for 17 or 18 hours. Following this additional delay period the tissue was processed as above and analysed as for all other tissue.

Three methods of data analysis were used in the human study. Firstly, all of the cases were ascribed to one of three groups on the basis of the time between death and fixation of the carotid bodies as follows:

- a. up to 8 hours,
- b. over 8 hours and up to 15 hours,

c. more than 15 hours.

Secondly, according to patient's clinical history and the autopsy reports, the carotid bodies were placed initially into seven separate groups:

- a. chronic hypoxia,
- b. essential hypertension,
- c. diabetes mellitus,
- d. thoracic tumors,
- e. nonthoracic tumors,
- f. sepsis and
- g. diseases unrelated to any of these conditions.

The latter cases were considered as "normal" and acted as the control group.

The ages of patients ranged from 19 to 88 years. Thirdly for analysis purposes, the cases were also divided arbitrarily into young (<50 - 13 cases), and old groups (> 50 - 62 cases). There were 35 females, and 40 males. Data on each case analysed in this study is given in Appendix 3.1.

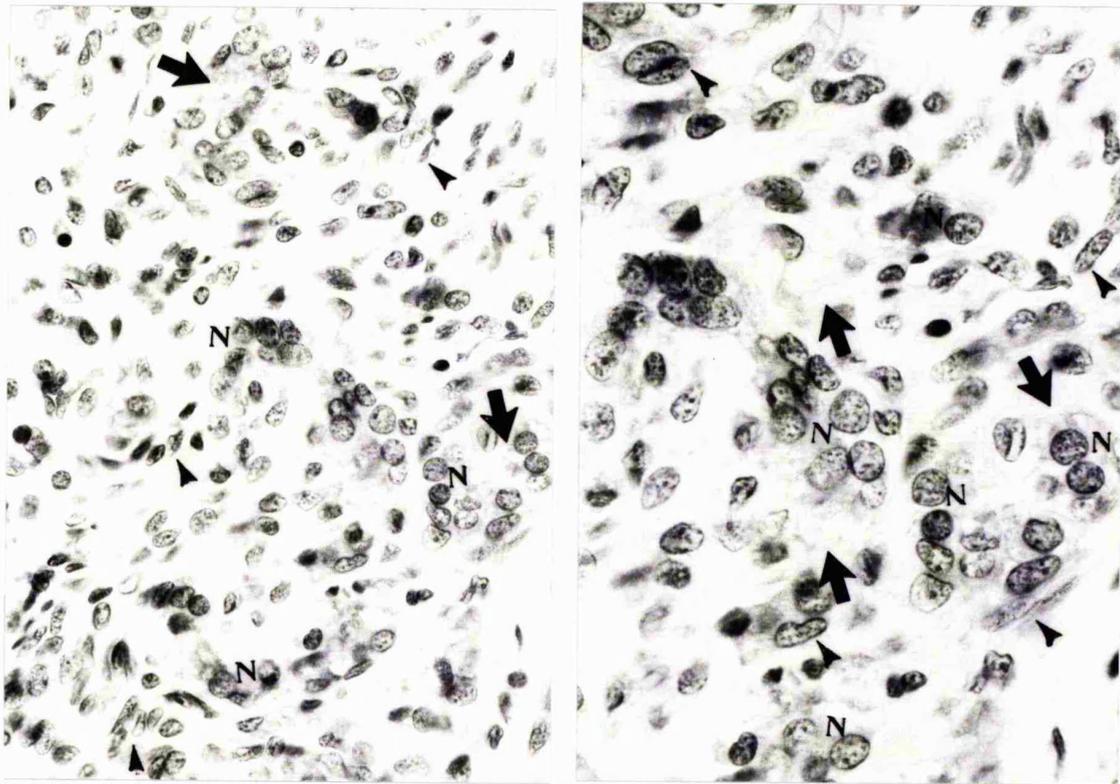
Finally the number of Type I and Type II cell nuclei were counted in all of the cases so that the relative proportion of these two cell types could be assessed.

RESULTS

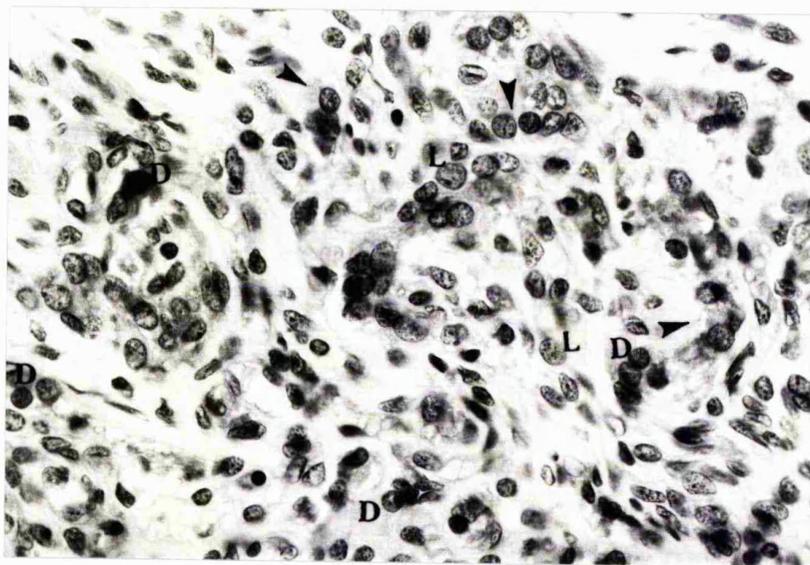
Rat Study

The control carotid bodies (0 hour group) showed the typical clustering of polygonal Type I cells surrounded by elongated Type II cells (Fig.1a and 1b). The Type I cells were of similar size and shape and possessed a light eosinophilic cytoplasm and large round or ovoid nuclei (Fig.3.2). Some Type I cells possessed a slightly hyperchromatic nucleus but lacked the eosinophilic cytoplasm (characteristic of dark cells) at this time. The Type II cells had less definite cell borders than Type I cells and the nuclei were elongated and also hyperchromatic. Few congested capillaries and very few pyknotic cells were seen.

Changes in the morphology of the Type I cells were apparent within 2 hours of death and became prominent after 4 hours at 4°C (Fig.3.3a and 3.3b). There was an increasing vacuolation of some Type I cells and the appearance of more cells with hyperchromatic nuclei, some of these latter cells possessed smaller, eccentrically placed nuclei with a cap of intensely eosinophilic cytoplasm (Fig. 3.4)



Figs 3.1. & 3.2. Micrographs of rat carotid body tissue fixed immediately after death. Note the largely uniform Type I cells nuclei (N) & the lightly stained Type I cell cytoplasm (arrows). Type II cells are also visible at the periphery of Type I cells groups (arrow heads). (Fig 3.1: x63, Fig3.2: x100)



Figs 3.3 Rat carotid body fixed two hours after death. Note many of the Type I cell nuclei are now more hyperchromatic, these are the dark cells (D). There are also intermediate forms between the light cells (L) and the dark cells (arrowheads). (x63)

Such cells are similar to the dark cells described in the normal human carotid body by Smith et al. (1982). A third variant of Type I cell became prominent by 4 hours. This pyknotic variant was characterised by a small, compact, darkly stained nucleus with a narrow peripheral rim of cytoplasm (Fig.3.5).

Similar periods of delay induced different pathological findings depending on whether the carcase of the animal had been kept at room temperature or at 4°C. The degree of change from the normal histological structure was much greater at room temperature where even after 4 hours nuclear vesiculation and loss of cell margins could be seen.

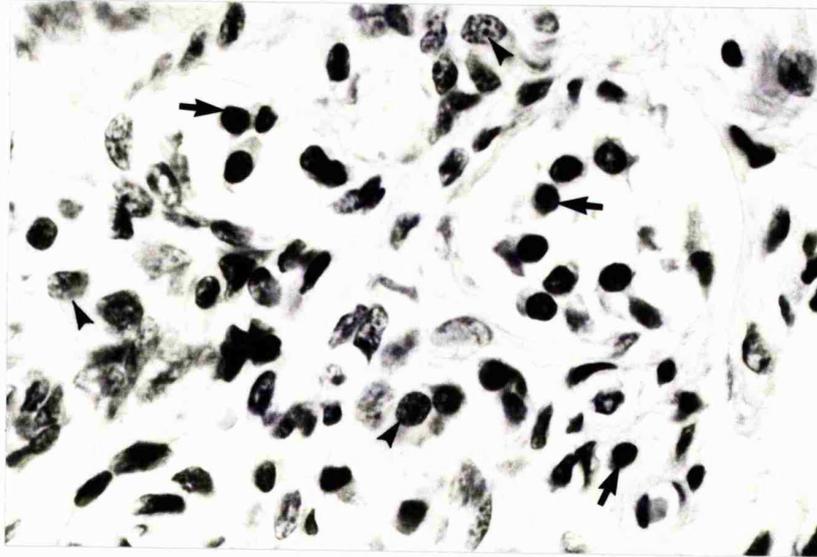
In contrast to Type I cells, the Type II cells appeared resistant to autolytic change. However, after 8 hours, and even more so after 16 hours some Type II cells with pyknotic nuclei were observed.

Marked vascular dilatation and congestion were observed in all specimens studied at postmortem periods greater than 2 hours and the magnitude of the congestion seemed to increase with increasing postmortem delay. No leucocytic infiltration was found in any of the rat carotid bodies examined.

Table 3.1. The percentage occurrence of light, dark and pyknotic cells in the rat carotid body after various postmortem delays in fixation.

Rat study	0 Hour	2 Hours	4 Hours	8 Hours	16 Hours	24 Hours
Light Cells	79.4	42.4	26.8	24.1	23.3	3.5
Dark Cells	18.1	43.9	51.2	51.6	51.5	47.4
Pyknotic cells	2.5	13.7	22	24.3	25.2	49.1

Table 3.1 and Figs 3.6 & 3.7 show quantitative data on the Type I cell variants in the rat carotid body after different fixation delays. The numbers of clear cells decrease up to 8 hours postmortem and this decrease was paralleled by an increase in the number of pyknotic and dark cells.



Figs 3. 5. Rat carotid body tissue fixed 16 hours after death of the animals. Note the cells with small highly stained nuclei and a small cap of eosinophilic cytoplasm (arrows). In this case, no light cells may be seen and only a few dark cells (arrow heads) remain. (x100)

Between 4 and 24 hours fixation delay there is a further fall in the percentage of light cells and rise in the number of pyknotic cells while there is no change in the percentage of dark cells (Fig 3.6). Fig 3.7 shows a plot of light and pyknotic cell variants against time. The linear correlations were -0.92 for the clear cells and $+0.92$ for the pyknotic cells; in both cases $p < 0.001$.

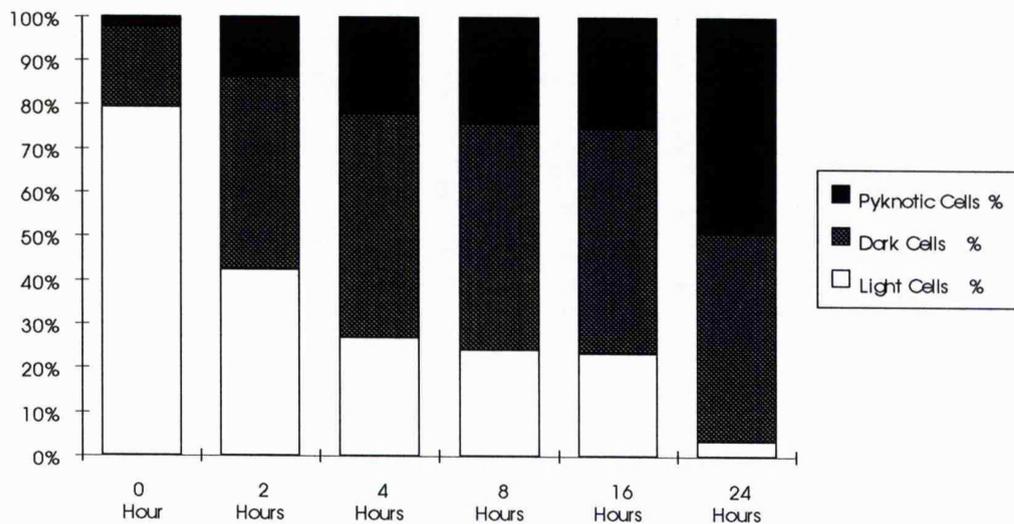


Fig. 3.6 The percentage occurrence of light, dark and pyknotic cells in the rat carotid body after various delays in fixation (Data from table 2.1.).

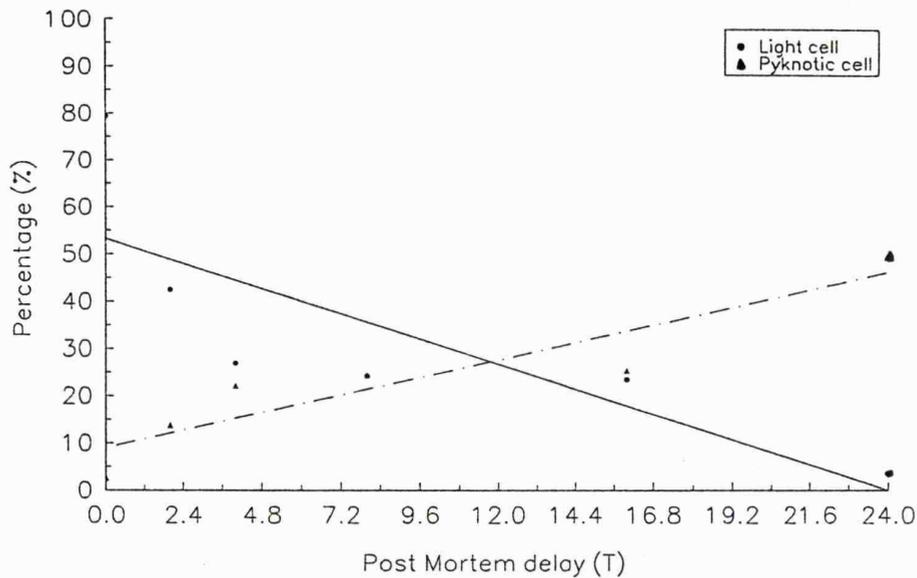
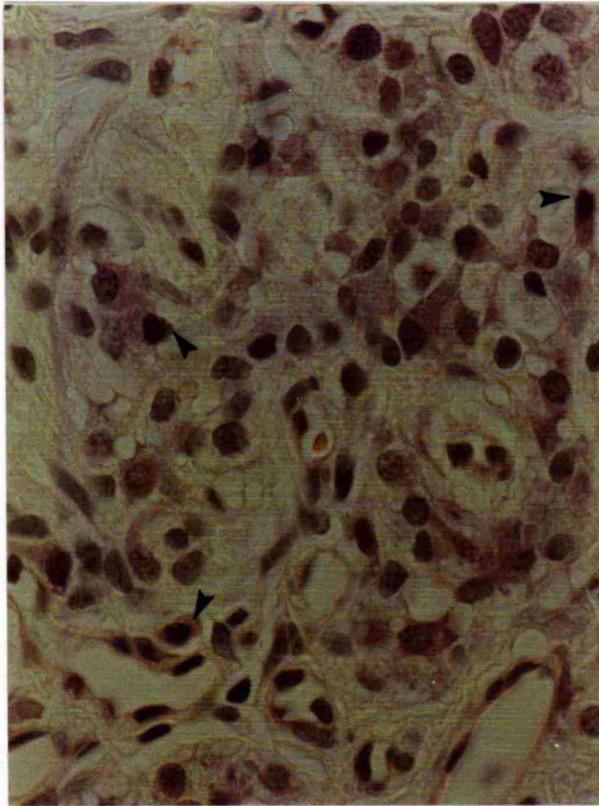


Fig 3.7. A plot of the percentage occurrence of light and pyknotic cells in the rat carotid body against time, the line is that of best fit.

Human Study

Figs 3.8 & 3.9 shows photomicrographs of carotid body tissue which was fixed 1.5 and 3.5 hours after the death of the patients. Initially most of the Type I cells are of the light variety with plump, lightly stained nuclei and only mild eosinophilia of the cytoplasm. By 3.5 hours three varieties of Type I cells, light, dark and pyknotic, are clearly seen, it is also possible to observe cells that seem to be intermediate in appearance and form. Apart from increasing staining within the nuclei, the nuclei become smaller and there is increasing eosinophilia of the cytoplasm. The end result of this process is a cell with a small densely stained nucleus in which no detail of the distribution of the chromatin can be seen, in these cells the cytoplasm is intensely eosinophilic and often only amounts to a small cap placed on one pole of the nucleus.

Fig 3.10 illustrates the distribution of light, dark and pyknotic cells in all of the carotid bodies studied. For convenience the data is arbitrarily divided into three



Figs 3.8 & 3.9. Human carotid body tissue fixed 1.5 hours (Fig 3.8: x63) and 3.5 hours (Fig 3.9: x100) after death. After two hours delay most of the Type I cells are of the light variety (arrows) but already some nuclei show increased chromatin density (arrowheads). At 3.5 hours postmortem the three sub-types of Type I cell may be seen (light: L, dark: D, pyknotic: P). Note also the cells which appear intermediate in form between light and dark cells (I).

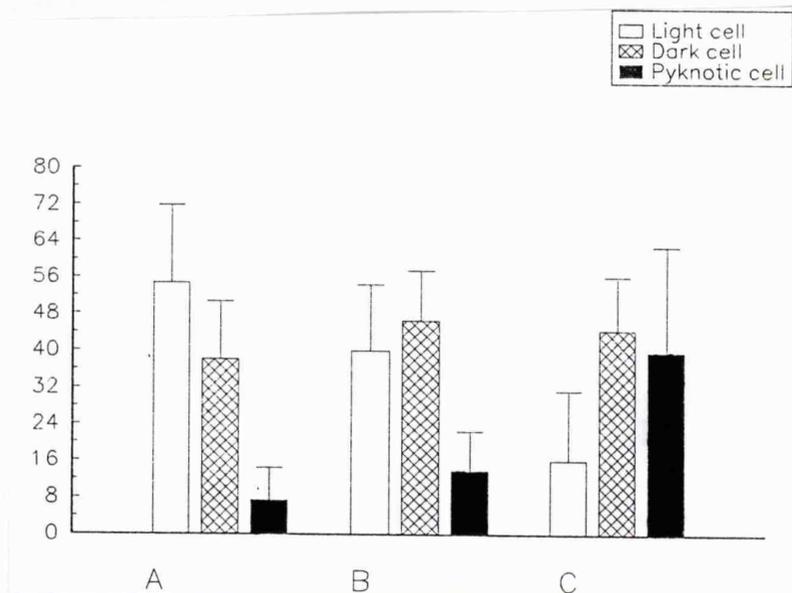


Fig 3.10. Distribution of light, dark and pyknotic cells in all of the carotid bodies studied. A: fixed between 2 and 8 hours postmortem (PM), B: fixed between 8 and 15 hours PM, C: more than 15 hours PM. Error bars represent standard deviations.

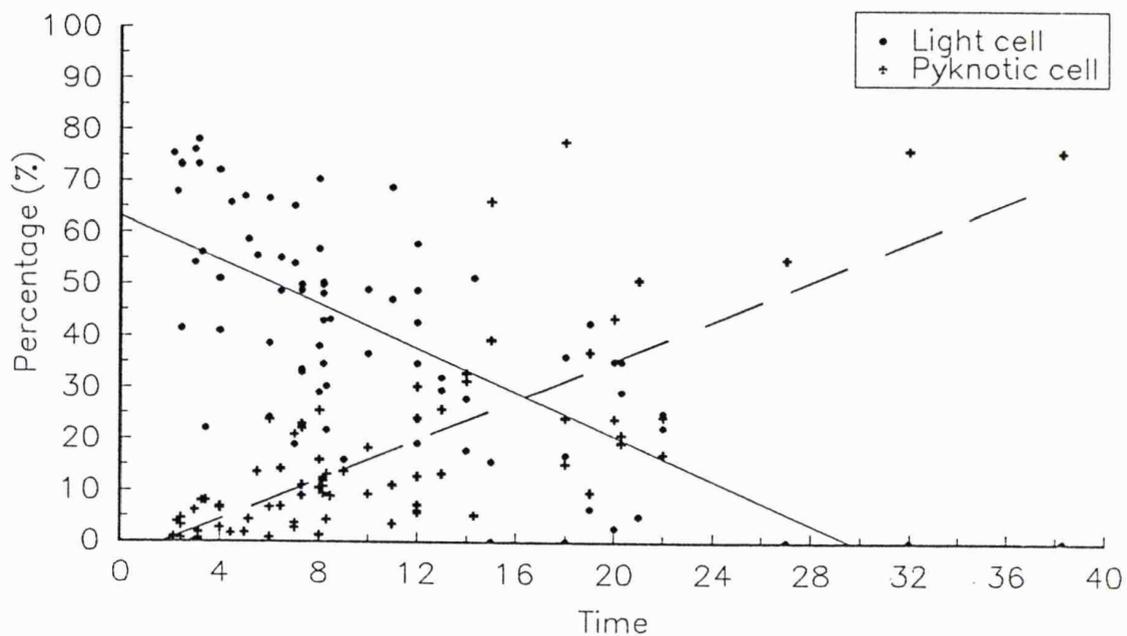


Fig 3.11. Plot of light and pyknotic cells against time (i.e. postmortem delay). The lines are those of best fit.

time delay groups. Clearly increasing postmortem delay led to a reduction in the number of light cells and an increase in the number of pyknotic cells within the human carotid body.

The correlation between delay and the disappearance of light cells and the increase in pyknotic cells is shown in Fig 3.11 where the percentage of light and pyknotic cells in each carotid body is plotted against time of delay. A linear correlation for the appearance of pyknotic cells (correlation coefficient 0.71; $p < 0.001$), and an inverse linear correlation for the disappearance of light cells (correlation coefficient -0.77; $p < 0.001$) is seen.

Fig 3.12 illustrates data from carotid bodies that were fixed in two parts. The data clearly show that the number of light cells decreased during the period of storage whilst the number of pyknotic cells increased.

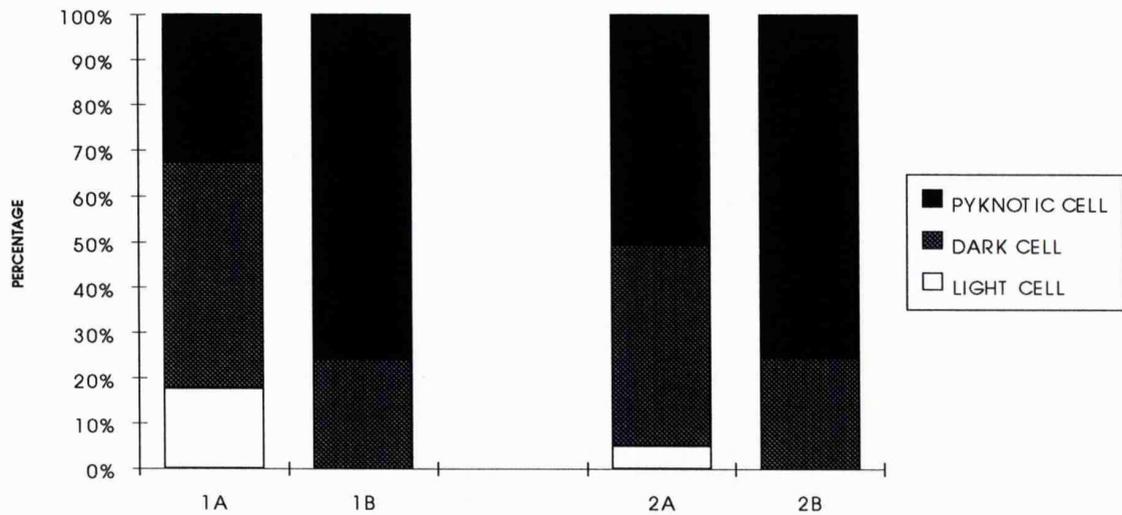


Fig. 3.12 . Type I cell variants in two carotid bodies fixed at 14 hours (1A) and 32 hours (1B) and 21 hours (2A) and 38 hours (2B) after death.

In the first carotid body the number of light cells decreased by 18% whilst the number of pyknotic cells increased by 44%; there was also a decrease in the number of dark cells of 25%. In the second organ, light cells decreased by 5% whilst dark cells decreased by 20% and pyknotic cells increased by 25 %.

Table 3.2 shows the data separated into the disease classifications. Whilst there is variation between the individual groups which in the indicated cases reaches significance, the general observation that light cells decreased in number with increasing delay whilst pyknotic cells increased is confirmed. It is interesting to note that in essential hypertension there were significantly more light cells at all postmortem delays than in the control group and that the number of pyknotic cells was significantly reduced at all delays. We have no explanation of this phenomenon.

However, it was a fact that in all of the cases studied where there was a variation between the disease class and control, that the both classes contained carotid bodies that were clustered towards the lower end of the range of postmortem delay.

DISEASES	HOURS	Number of Cases	LIGHT CELL	DARK CELL	PYKNOTIC CELL
			% - ST.DEV.	% - ST.DEV.	% - ST.DEV.
UNRELATED	2-8	4	46.25 ± 16.62	39.9 ± 7.73	13.85 ± 9.95
	8-14	3	37.96 ± 4.29	50.06 ± 7.16	11.96 ± 5.67
	15-->	3	5.2 ± 9.0	37.63 ± 13.2	57.13 ± 19.3
CHRONIC HYPOXIA	2-8	5	56.66 ± 13.56	37.68 ± 11.6	5.64 ± 3.11
	8-14	6	29.15 ± 8.56 ***	53.68 ± 11.66	17.16 ± 10.9
	15-->	2	4.5 ± 2.68	55.4 ± 1.97 ***	40.1 ± 4.66
Ess.HYPERTENSION	2-8	10	67.5 ± 8.76 ***	29.76 ± 7.61 ***	2.73 ± 2.09 ***
	8-14	12	48.93 ± 13.29 ***	41.95 ± 8.47 ***	9.08 ± 5.89
	15-->	5	34.66 ± 6.22 ***	47.66 ± 6.59	17.26 ± 5.44 ***
D.MELLITUS	2-8	5	55.7 ± 15.27	41.12 ± 14.25	3.16 ± 2.89 ***
	8-14	2	47.25 ± 13.22	39.6 ± 17.11	13.1 ± 3.81
	15-->	2	14.02 ± 13.75	43.52 ± 13.39	42.42 ± 26.08
THORACIC CANCER	2-8	2	35.25 ± 18.73	53.65 ± 23.12	11.05 ± 4.31
	8-14	2	53.2 ± 4.80 ***	32.75 ± 7.42 ***	13.95 ± 2.61
	15-->	1	16.8 ±	59.2 ±	24 ±
NONTHORACIC CANCER	2-8	5	47.34 ± 19.8	41.04 ± 15.7	11.24 ± 6.41
	8-14	5	36.62 ± 15.7	43.3 ± 4.77	20.08 ± 11.3
	15-->	6	11.5 ± 16.1	36.45 ± 11.09	51.86 ± 25.3
SEPSIS	2-8	5	49.18 ± 17.08	40.6 ± 13.7	9.84 ± 9.14
	8-14	3	23.13 ± 9.98 ***	58.4 ± 10.67	18.46 ± 10.11

Table 3.2. The % occurrence of light, dark and pyknotic cells in the human carotid body after various delays in fixation. The data is subdivided according to disease classification. *** in this table and all subsequent figures indicates statistically different from unrelated (normal) at $p < 0.05$ level (t -test).

Table 3.3 provides data that indicate that there were no significant differences in the relative occurrence of Type I cell variants on the basis of age.

Table 3.3. The distribution of light, dark and pyknotic cells in all of carotid bodies studied and in those carotid bodies from patients < 50 and older than 50 years of age.

GROUP	Hours	Number of Cases	LIGHT CELL	DARK CELL	PYKNOTIC CELL
			% - ST.DEV.	% - ST.DEV.	% - ST.DEV.
TOTAL	2-8	30	54.64 ± 17.12	38.02 ± 12.60	7.25 ± 7.04
Human Carotid Bodies	8-14	28	39.80 ± 14.49	46.47 ± 10.94	13.7 ± 8.68
	15-->	17	15.92 ± 15.36	44.31 ± 11.83	39.61 ± 23.23
OLD PATIENTS	2-8	28	55.26 ± 16.54	37.86 ± 12.63	6.79 ± 6.52
Human Carotid Bodies	8-14	22	41.45 ± 13.87	46.70 ± 10.25	11.80 ± 7.77
	15-->	12	16.33 ± 15.26	44.32 ± 12.03	39.16 ± 23.22
YOUNG PATIENTS	2-8	2	45.95 ± 30.90	40.3 ± 16.82	13.75 ± 14.07
Human Carotid Bodies	8-14	6	33.73 ± 16.41	45.61 ± 14.31	20.63 ± 8.96
	15-->	5	14.96 ± 17.35	44.3 ± 12.72	40.7 ± 25.94

The relative numbers of Type I & Type II cells were also determined in all of the specimens. The data is shown in Tables 3.4, and 3.5. It can be seen that the carotid bodies of older patients fixed up to 15 hours postmortem appear to have a lower percentage of Type I cells than the young patients, the reverse is true of Type II cells (Table 3.4). There were no differences in relative occurrence of Type I & Type II cells in any of the disease classifications with the exception of essential hypertension. In the latter case the relative numbers of Type II cells were significantly elevated and is well illustrated in Figs 3.13 & 3.14 where whorls of Type II cells surrounding the groups of Type I cells may be seen; this issue will be returned to in Chapter 5.

Table 3.4. The proportion of Type I & Type II cell nuclei in the human carotid body on the basis of age.

GROUP	Hours	Number of Cases	TYPE I CELL	TYPE II CELL
			% - ST.DEV.	% - ST.DEV.
TOTAL	2-8	30	54.34 ± 9.02	45.33 ± 8.68
Human Carotid Bodies	8-14	28	55.57 ± 8.22	44.35 ± 8.23
	15-->	17	54.20 ± 7.56	45.71 ± 7.47
OLD PATIENTS	2-8	28	53.93 ± 9.17 ***	45.72 ± 8.83 ***
Human Carotid Bodies	8-14	22	54.07 ± 8.39 ***	45.84 ± 8.42 ***
	15-->	12	53.57 ± 7.51	46.41 ± 7.52
YOUNG PATIENTS	2-8	2	60.05 ± 4.17 ***	39.95 ± 4.17 ***
Human Carotid Bodies	8-14	6	61.08 ± 4.78 ***	38.9 ± 4.75 ***
	15-->	5	55.72 ± 8.33	44.04 ± 7.94

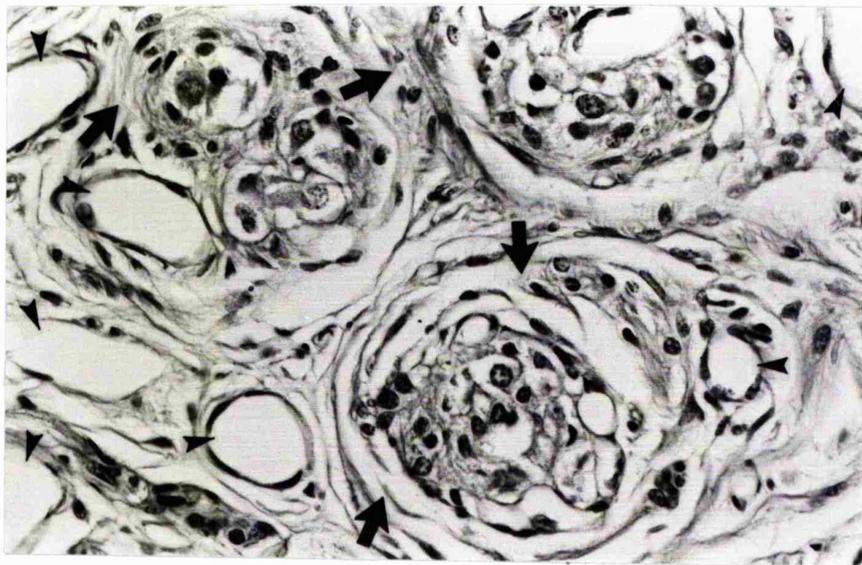


Figure 3.13

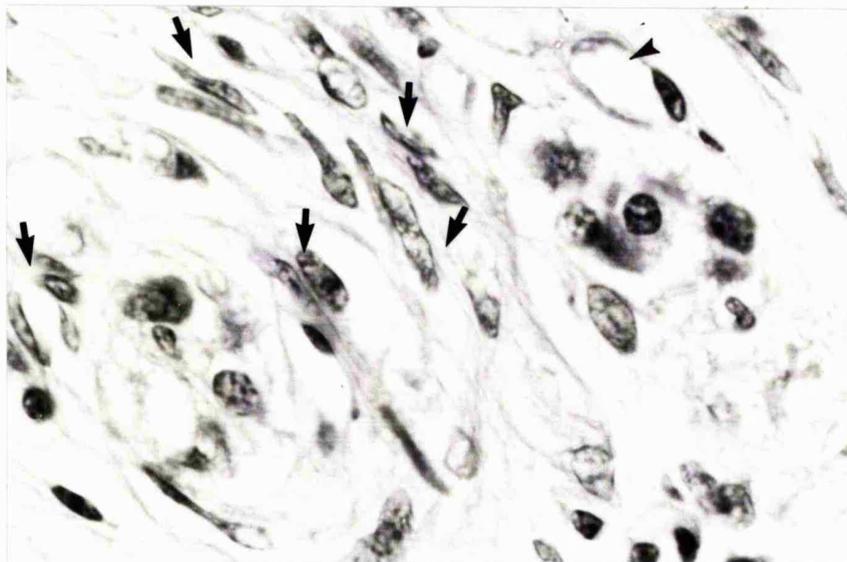


Figure 3.14

Figs 3.13 & 3.14 Photomicrographs of two different hypertension cases. Type II cell (or Schwann cells) proliferation can be seen clearly (arrows). Note also the large dilated vessels (arrow heads) in fig 3.13. (fig 3.13: x40, fig 3.14: x100)

Table 3.5. The relative proportion of Type I cell & Type II nuclei in different pathological conditions. An increase is observed in the percentage of Type II cell in essential hypertension patients.*** indicates statistically different from normal at $p < 0.005$ level (t-test).

DISEASES	HOURS	Number of Cases	TYPE I CELL		TYPE II CELL	
			% - ST.DEV.	T.TEST	% - ST.DEV.	T.TEST
UNRELATED	2-8	4	58.45 ± 6.10		41.55 ± 6.10	
	8-14	3	61.36 ± 2.49		38.36 ± 2.17	
	15->	3	59.23 ± 3.55		40.73 ± 3.5	
CHRONIC HYPOXIA	2-8	5	58.02 ± 4.74		41.98 ± 4.74	
	8-14	6	58.36 ± 5.07		41.63 ± 5.07	
	15->	2	55.75 ± 4.59		44.25 ± 4.59	
Ess.HYPERTENSION	2-8	10	43.91 ± 4.20	***	55.13 ± 4.44	***
	8-14	12	48.05 ± 5.25	***	51.85 ± 5.37	***
	15->	5	44.88 ± 4.04	***	54.92 ± 3.89	***
D. MELLITUS	2-8	5	60.14 ± 7.71		39.86 ± 7.71	
	8-14	2	59.3 ± 0.28		40.7 ± 0.28	
	15->	2	58.06 ± 7.14		41.92 ± 7.13	
THORACIC CA	2-8	2	62.25 ± 1.34		37.75 ± 1.34	
	8-14	2	60.6 ± 1.55		39.4 ± 1.55	
	15->	1	62.1 ±		37.8 ±	
NONTHORACIC CA	2-8	5	53.76 ± 9.50		46.22 ± 9.47	
	8-14	5	59.02 ± 12.09		40.98 ± 12.09	
	15->	6	54.29 ± 8.42		45.68 ± 8.42	
SEPSIS	2-8	5	59.5 ± 7.41		40.5 ± 7.41	
	8-14	3	58.16 ± 3.07		41.83 ± 3.07	

DISCUSSION

The present observations from experimental animal clearly indicate that the three variants of Type I cells, the light, dark, and pyknotic cells, do not represent a stable population but rather that their numbers fluctuate in relation to delay the between death of the animal and fixation of the tissue.

It is interesting that isolated autolytic changes were noted in the rat tissues where the fixation delay was of the order of 15 minutes (the time to complete the

dissection). This situation is not surprising as it is well known that other rat organs, for example

the liver or the thymus, may undergo autolytic changes within minutes after death (Duwall and Wyllie, 1986; Rubin and Farber, 1988). The fact that previous morphological studies of rat, cat and rabbit carotid bodies failed to demonstrate dark and/or pyknotic cells may be explained by the fact that all of these studies used vascular perfusion of the fixative (McDonald & Mitchell, 1975; Verna, 1979; Pallot et al., 1986). The design of these experiments was adopted in an attempt to mimic the situation that might exist in routine autopsy protocols. Because of this reason there was a delay of 2 hours before refrigeration of the carcass and permeation of the fixative rather than perfusion.

The human data, whilst not controlled in the manner of that from the experimental study, is essentially similar. Thus increasing delay in fixation leads to:

- a. a decrease in the number of light cells,
- b. an increase, at least initially, in the number of dark cells
- c. an increase in the number of pyknotic cells.

Confirmatory evidence of this view is provided by the data from the two carotid bodies that were divided and fixed at different times. It is inconceivable that cell types can change except as a result of postmortem processes.

These experiments provide no information on the events that lead to the appearance of dark and pyknotic cells within the carotid body. Based upon what is known to occur elsewhere during the development of irreversible anoxic cell injury (Rubin & Farber 1988) it may well be that the light cells undergo hydropic degeneration as indicated by their vacuolated appearance, indeed it is interesting to note that a number of authors have reported such a vacuolation as a regular feature of normal Type I cells (e.g. Hurst et al., 1985). An increase in the eosinophilic affinity of the cytoplasm, together with changes in the chromatin array, would lead to the formation of dark cells which can be regarded as an intermediate step in the process of autolysis. During the subsequent hours after death the cytolytic process will continue and the nucleus becomes heavily hyperchromatic leading to the formation of the so-called pyknotic cells. If this is a true interpretation of events in the carotid body then it could be argued that the light cell is the precursor of the dark cell which in turn is the precursor of the pyknotic cell. The electron microscopic study (Chapter 2) provides some evidence for this view because of the occurrence of intermediate forms of light, dark and pyknotic cells. If this is correct then it would

be predicted that the number of light cells would decrease with increasing delay whilst the number of pyknotic cells would increase. This seems to be the case. The number of dark cells would increase with time initially as they are formed from the light cells and then decrease as more and more of them are converted to pyknotic cells being produced at times when most of the light cells have been converted. Some evidence for this view is provided by the experiments where two pieces of the same carotid body were fixed at different times post - mortem.

As mentioned before, there is considerable controversy in the carotid body literature regarding the origin and nature of the dark and pyknotic cells. The possibility that these cells increased in number with increasing postmortem delay has been suggested by a number of authors (Gosses, 1938; De Castro, 1951), whilst others presumed them to be normal constituents of the carotid body (Smith, 1924; White, 1935; Smith et al., 1982). Recently, it has been suggested that the pyknotic cells in the carotid bodies of children represent "precursors" of the dark and clear cell variants (Heath et al., 1990). The fact that their numbers are so low in carotid bodies fixed rapidly after death makes this idea unattractive. This is the more so as the carotid body of the one day old rat fixed at death contains virtually no pyknotic cells (Pallot personal communication).

The changes in the relative numbers of Type II cells are interesting. There is an increase in the relative number of Type II cell nuclei with age suggesting that Type I cells may degenerate with age. The situation is complex, however, in that with delays in fixation of >15 hours there is also a decrease in the percentage occurrence of Type I cells. Perhaps with these delays the degree of postmortem change is so great that the Type I cells are no longer identifiable. The different percentage occurrence of Type I and Type II cells in disease will be discussed in Chapter 5.

The experimental study of the rat carotid body provides compelling evidence that delay in fixation after death of the animal leads to a progressive decrease in the light Type I cells and increase in the number of pyknotic cells. The finding that the percentage of light cells within the human carotid body is inversely related to the delay between death and fixation and that the percentage of pyknotic cells is directly related to this delay, when coupled to the animal findings suggests that dark and pyknotic cells are merely an artefact due to autolytic change.

CHAPTER FOUR

QUANTITATIVE STUDIES OF THE HUMAN CAROTID BODY USING MORPHOMETRIC AND STEREOLOGICAL TECHNIQUES

INTRODUCTION

Stereological techniques provide a mechanism for obtaining three dimensional information about a structure from two dimensional sections of that structure. Stereology deals with quantitative aspects such as shape, size, number and orientation. The techniques of stereology were first used in geology and metallurgy and were subsequently applied to biomorphological problems. Morphometry is the measurement of shape and it overlaps with stereology; indeed many anatomists use the terms interchangeably. A great variety of techniques have been devised to answer specific morphological questions together with a number of statistical methods for analysing the significance of the data obtained; good reviews of the range of stereological and morphological applications in biology are provided by Williams (1977), Aherne & Dunnill (1982), Gundersen et al.(1988a,b), and Royet (1991).

As mentioned above (Chapter 1), it is well documented that the carotid body, in both experimental animals and humans responds to abnormal physiological stimuli. Chronic hypoxaemia and systemic hypertension (Habeck, 1986; Pallot, 1987; Heath and Smith, 1992; and Bee & Howard, 1993) have been the most extensively studied and there is evidence that in both of these conditions there is an increase in the size of the carotid body (Heath et al.,1970, 1982; Dhillon et al., 1984; Barer et al.,1987).

In the case of chronic hypoxaemia it has been reported that this increased size is brought about by a combination of hypertrophy, the enlargement or overgrowth of an organ or partly due to an increase in size of its constituent cells, and hyperplasia, the increase in the number of normal cells in normal arrangement in a tissue (see Pallot 1987). In the carotid bodies of experimental

animals the data of Dhillon et al. (1984) and Bee et al. (1986) undoubtedly confirm that there is both hypertrophy and hyperplasia upon exposure to 10% oxygen for three weeks. In this situation the carotid body shows increased numbers of Type I and possibly Type II cells and a greatly enlarged vasculature (Dhillon et al., 1984; Bee et al., 1986; Barer et al., 1987) and the latter authors have provided clear evidence of increased mitotic activity as a result of hypoxic exposure. In the human clear evidence of hyperplasia has not been obtained. In human the carotid body is certainly bigger in patients suffering from diseases associated with chronic hypoxaemia; i.e. there is hypertrophy. It is not established whether any cell division, or hyperplasia occurs (see discussion). Furthermore the reason for the hypertrophy is apparently different in that the human hypoxic carotid body is characterised by a dramatic proliferation of Type II cells and a reduction in the number of Type I cells (Heath et al., 1970, 1982).

The situation in the cases of the effects of hypertension is more complex, the effects of hypertension are examined in some detail in Chapter 1, and are only reviewed briefly here. Heath et al. (1970) showed a relationship between carotid body and left ventricular weights suggesting that hypertension might be associated with hypertrophy of the carotid body. Edwards et al. (1972) reported that the structure of the carotid body from hypertensive patients was identical to that of the organ obtained from patients with chronic hypoxaemia, an observation that has been repeatedly confirmed by the Liverpool group (e.g. Smith et al. 1982, 1984; Heath et al., 1982, 1985). Despite this, other workers (Kluge, 1985; Habeck, 1986) found an increased volume of the carotid body to be associated with Type I cell rather than Type II cell proliferation.

The search for an animal model of the hypertensive carotid body has been fraught with methodological problems; the arguments and counter arguments have been reviewed in Chapter 1.

In this study I have examined three aspects of the human carotid body structure, namely, cell cluster size, lobule size and the amount of intralobular connective tissue within the lobules using morphometric and stereological data from "normal", hypoxic, hypertensive and diabetic patients. In addition a number of patients with tumours and infective conditions have also been studied.

MATERIALS AND METHODS

The same material as used for the studies described in Chapter 2 was used here. A total of 66 carotid bodies were removed at routine necropsy, fixed in either ethanol or Zamboni's fluid; processing techniques were as described in Chapter 2 (see Appendix 2.1). Sections stained with H&E and separated by a minimum distance of 250 μm , were examined and random fields using a 10x objective on a Zeiss microscope equipped with a camera lucida were used to obtain the quantitative data as below.

Single sections were viewed and the area of carotid body tissue, the outline of each lobule and the outlines of all of the cell clusters within each lobule within the field were drawn using a camera lucida. The areas of these drawings was determined using a Videoplan analysis system (Kontron Ltd., Germany). An example of a field is illustrated in Figure 4.1a,b. The areas of each lobule and each cell cluster within the lobule was measured directly in arbitrary units. Subtraction of the combined cell cluster areas in each lobule from the area of the lobule gave a measure of the non-cell cluster content (intralobular stroma/connective tissue) of that lobule. A cumulative mean technique was used to ascertain that a sufficient sample had been analysed. In practice the average of at least four or more such sections were needed to obtain an adequate cumulative mean criteria of no more than 5% deviation of the cumulative mean from the final mean.

The cases were divided into seven categories according to the clinical history and the autopsy report of each patient as described in Chapter Three. The records of some patients indicated the presence of a number of conditions and that these might have contributed to the cause of death. In such cases the primary cause of death as provided at autopsy was used to place the case in the appropriate category. Any patients where a primary cause of death was not established was not included in the study.

The exception to this general rule was those patients with a history of diabetes. These patients were divided into two groups based on the presence or absence of systemic hypertension. In addition those patients with severe infections were examined separately as well as within the group of major cause of death.

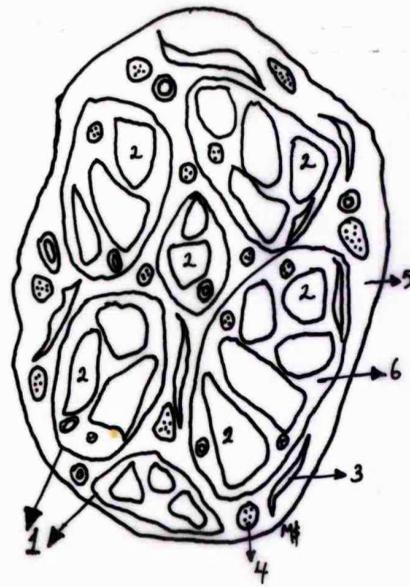


Fig 4.1A. Diagram illustrating a carotid body lobule and its component parts. 1: Lobule, 2: Cell cluster, 3: Blood vessels, 4: Nerve fibres, 5: Interlobular connective tissue, 6: Intralobular connective tissue.

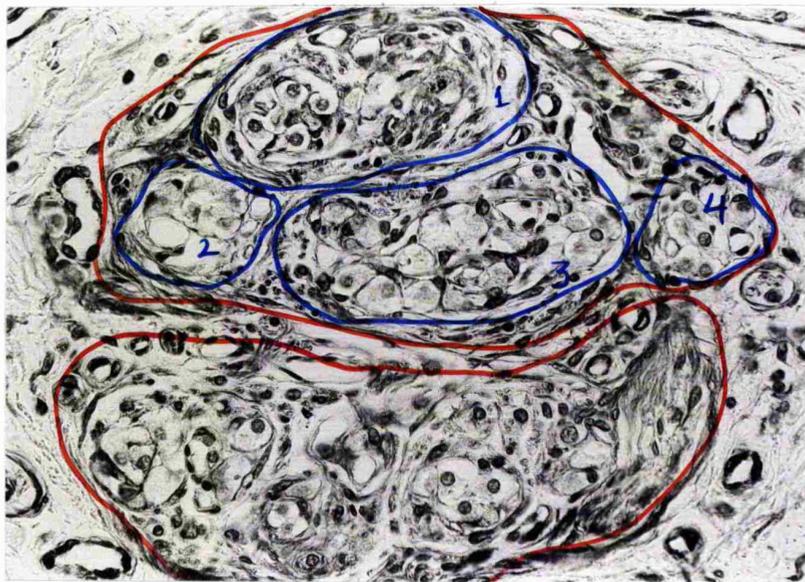


Fig.4.1B. Photomicrograph showing two carotid body lobules. Each lobule is outlined in red and the cell clusters in blue. Subtraction of the total cell cluster area (i.e. 1+2+3+4) from the lobule area provides a measure of connective tissue in the lobule. (x40)

It must be emphasised that these studies report a comparison between the various disease classifications and a group of cases which have been termed unrelated. This term was used to indicate that the group contained only patients who had no history of any of the other disease classifications. Because of this it is important to realise that the term control cannot be used in its true meaning as it was impossible to obtain, for obvious reasons, tissue that could unequivocally be stated to be normal.

The ages of the patients ranged from 2 to 88 years; for analysis purposes they were also divided arbitrarily into young (<50; 14 cases) and old groups (>50; 52 cases) and comparisons were attempted on the basis of age as well as the whole disease group. There were 29 females and 37 males. Data on each case analysed in this study is listed in Appendix. 4.1.

RESULTS

Fig 4.2a,b,c,d,e, and f show photomicrographs of representative areas of carotid body tissue from unrelated, chronic hypoxia, essential hypertension and diabetes mellitus associated with hypertension. Subjectively it would appear that the area of the lobules might be greater in the disease classes when compared to the unrelated.

Table 4.1 illustrates all of the data on lobule area, cell cluster area and the area within each lobule occupied by connective tissue which is here called intralobular stroma. It can be seen that chronic hypoxia, hypertension, diabetes mellitus with hypertension and chronic infection are all associated with an increase in the mean lobule area within the carotid body. In chronic hypoxia and hypertension there is an increase in the area of the cell clusters and the amount of connective tissue within the organ whilst in the diabetes group only the connective tissue element is apparently increased. Diabetes in the absence of hypertension and non thoracic carcinomas apparently have no effect on the size of these parameters in the carotid body.

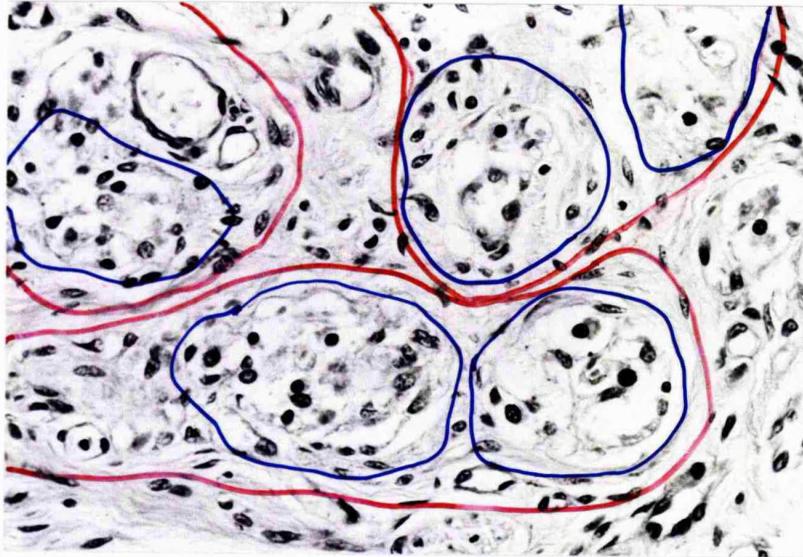


Fig 4.2A

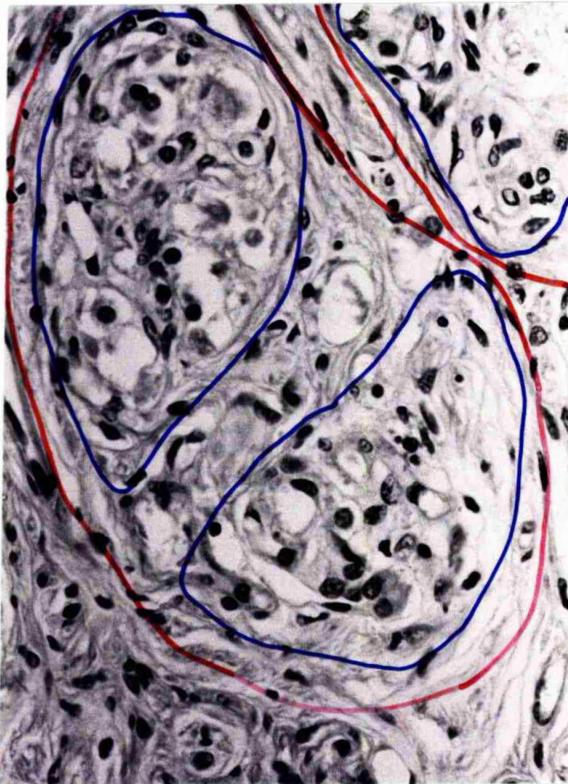


Fig 4.2B

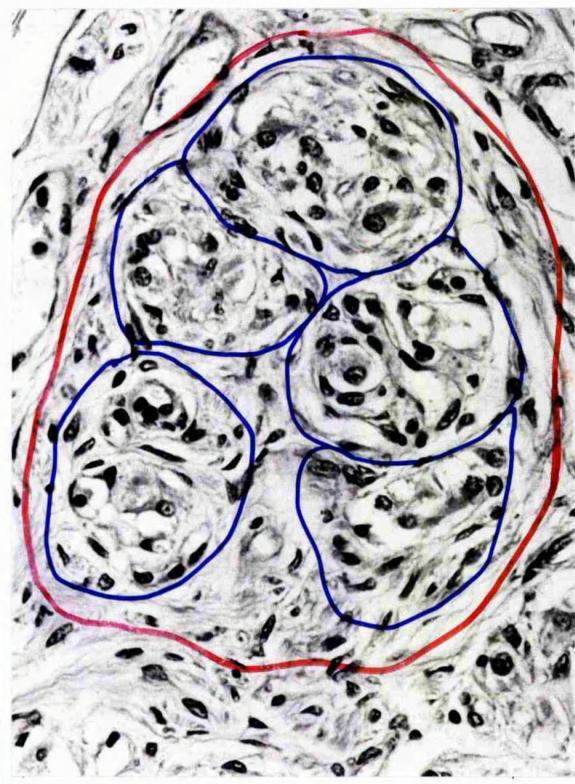


Fig 4.2C

Figures 4.2a,b,c,d,e, and f. Photomicrographs of an unrelated (A), two different COPD (B & C) and hypertension (D & E) cases and a diabetic case (F). Photomicrographs provide supportive evidence consistent with the data obtained from cases by morphometric analysis (x40). Note that the size of lobules in different pathological conditions as compared to unrelated.

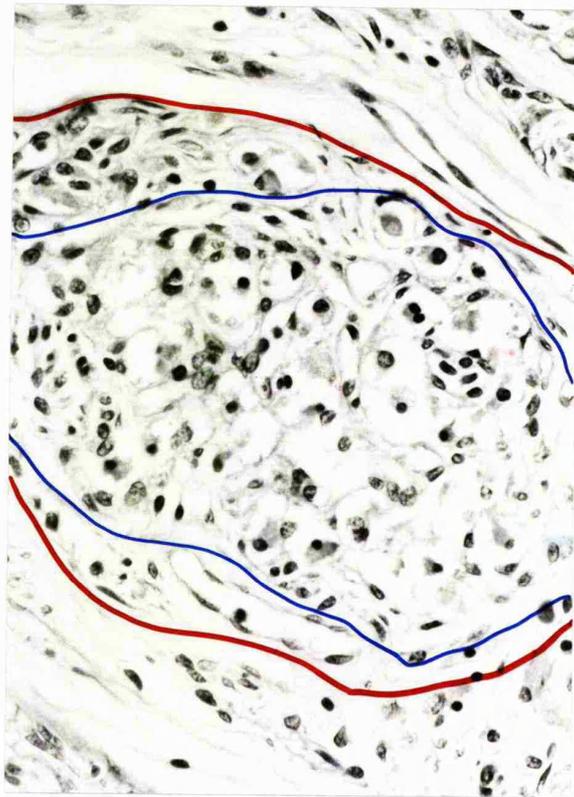


Fig 4.2D



Fig 4.2E

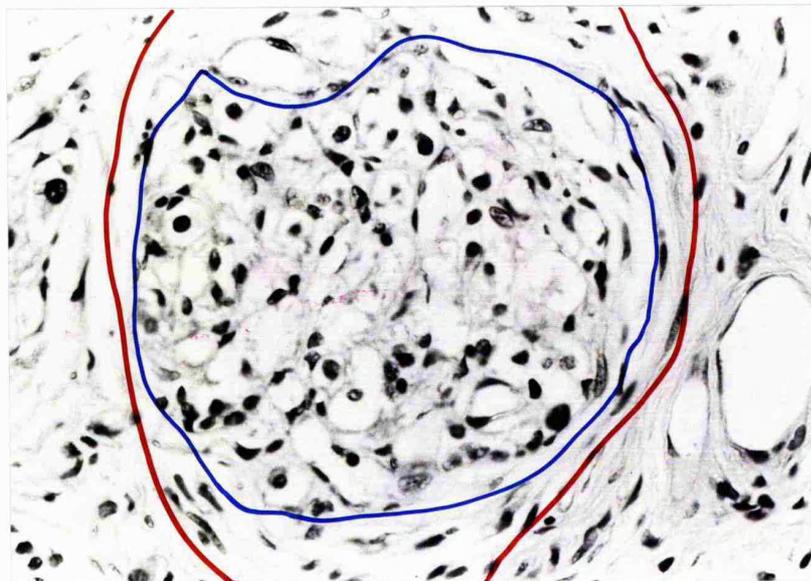


Fig 4.2F

The existence of the larger lobules, cell clusters and increased connective tissue provides only limited information about the microstructure of the carotid body. Additional information was obtained by calculating the volume fraction of each lobule occupied by cell clusters and connective tissue. The data is shown in Table 4.2. From this data it is apparent that the volume fraction of cell clusters and connective tissue in chronic hypoxia is not altered i.e. the larger lobules contain the same proportion of cellular and connective tissue elements. In hypertension there is a reduction in the fraction of the carotid body occupied by cells clusters and an increase in that occupied by connective tissue.

DISEASE	NUMBER	LOBULE	CELL CLUSTER	CONNECTIVE TISSUE
		AREA	AREA	AREA
UNRELATED	8	0.094 ± 0.023	0.0392 ± 0.010	0.0547 ± 0.013
CHRONIC HYPOXIA	13	0.165 ± 0.062 **	0.0644 ± 0.022 **	0.1000 ± 0.042 **
E.HYPERTENSION	25	0.133 ± 0.031 **	0.0470 ± 0.011 **	0.0859 ± 0.023 **
D.MELLITUS	9	0.107 ± 0.05 *	0.037 ± 0.014 *	0.0692 ± 0.038 *
D.MELLITUS* (+E.H.)	19*	0.121 ± 0.042 **	0.0417 ± 0.013 *	0.0793 ± 0.032 **
NONTHORACIC CA	13	0.106 ± 0.035 *	0.0413 ± 0.014 *	0.0649 ± 0.026 *
SEPSIS	7	0.143 ± 0.075 **	0.0565 ± 0.028 *	0.0859 ± 0.052 *

Table 4.1 provides data of area measurement of lobules, cell clusters and connective tissues in different pathological conditions. All data are means and standard deviations. * not significantly different from unrelated, ** $P < 0.05$ as compared to unrelated (*t* test).

Diabetes mellitus cases lacking hypertension had similar lobule areas to unrelated cases whilst those cases with the additional complication of hypertension had increased lobule sizes (Table 4.1/4.2). When the volume fractions are calculated it is seen that in both cases there were significant reductions in the fraction for cell clusters and an increase in the amount of connective tissue.

Nonthoracic carcinomas had parameters which were similar to those in the unrelated cases.

The sepsis group data shows an increase in lobule size, however, the volume fraction of cell clusters and connective tissue were unchanged and similar to unrelated cases and nonthoracic carcinomas.

DISEASE	NUMBER	LOBULE	CELL CLUSTER	CONNECTIVE TISSUE
		AREA	AREA FRACTION	AREA FRACTION
UNRELATED	8	0.094 ± 0.023	41.8 ± 4.1	58.2 ± 4.2
CHRONIC HYPOXIA	13	0.165 ± 0.062 **	39.9 ± 5.7*	60.1 ± 5.7 *
E.HYPERTENSION	25	0.133 ± 0.031 **	35.7 ± 5.5 **	64.3 ± 5.5 **
D.MELLITUS	9	0.107 ± 0.05 *	36.0 ± 6.6 **	63.9 ± 6.6 **
D.MELLITUS* (+E.H.)	19*	0.121 ± 0.042 **	35.1 ± 6.1 **	64.9 ± 6.1 **
NONTHORACIC CA	13	0.106 ± 0.035 *	39.3 ± 9.5 *	60.7 ± 9.5 *
SEPSIS	7	0.143 ± 0.075 **	40.8 ± 11.2 *	59.1 ± 11.1 *

Table 4.2 provides data of area fractions of lobules occupied by cell clusters and connective tissues in various pathological conditions. All data are means and standard deviations. * not significantly different from unrelated, ** $P < 0.05$ as compared to unrelated (t test).

Carotid bodies were obtained from four patients who died as the consequence of thoracic tumours. The data is found in Table 4.3, individual values for each case are presented as well as the mean of lobules and the mean and the volume fraction of cell clusters and connective tissues for the whole group because of the variability and the small number of cases.

Case number	Age	M/F	Lobule	Cell Cluster	Connective tissue	Main Diseases
Thoracic tumour						
			Area			
16k-91	2	M	0.2024	0.1092 (54 %)	0.0932 (46 %)	Mediastinal CA
810	47	M	0.1794	0.0547 (30.5%)	0.1247 (69 %)	Bronchial CA
90.91	49	M	0.1828	0.0943 (52 %)	0.0884 (48 %)	Bronchial CA
763	65	M	1.2719	0.3462 (27 %)	0.9257 (73 %)	Lung CA & thrombembolism
Unrelated			0.0939	0.0392 (41.8%)	0.0547 (58.2%)	

Table 4.3 provides individual data of 4 cases from patients with thoracic tumours. The average value of unrelated cases is also included for comparison. The area fractions of individual thoracic carcinoma cases included into the data are in brackets.

Because of insufficient numbers of cases, the data concerning thoracic carcinomas is impossible to describe as it is so variable. Thus in two cases of carcinoma of the bronchus one showed marked fibrosis of the carotid body (810.90) whilst the other showed a dramatic increase in the volume fraction occupied by cell clusters (90.91). In addition to that, one common feature in three of the four cases of thoracic carcinoma concerned changes in the architecture of the carotid body. The lobules appeared extremely large whilst cell clusters appeared irregular (Fig 4.3.a,b,c).

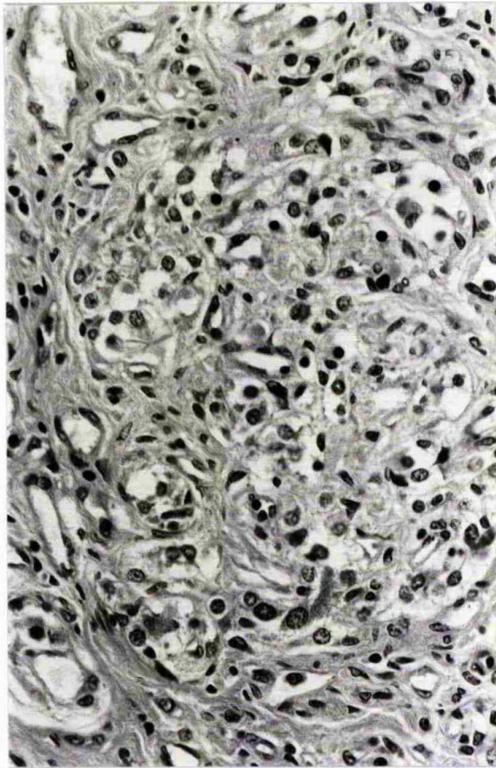


Fig 4.3A

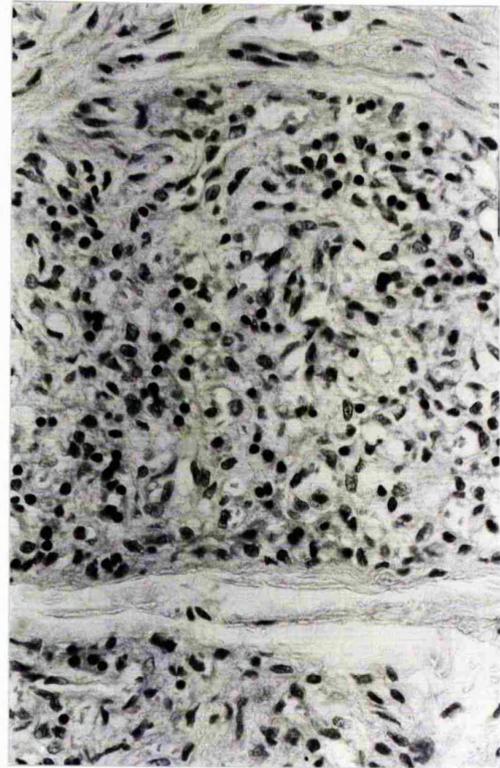


Fig 4.3B

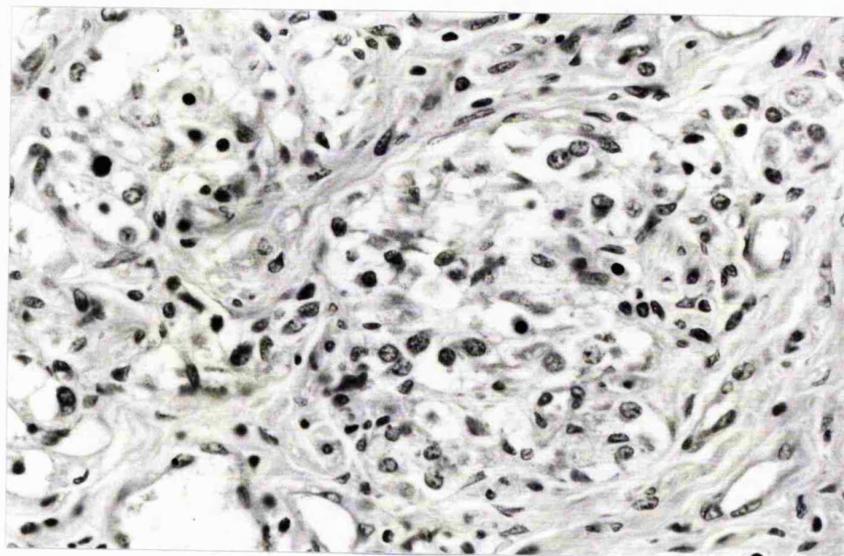


Fig 4.3C

Figure 4.3A, B, and C. Photomicrographs of three thoracic carcinoma cases. It seems that the normal structure of lobules and cell cluster is absent. Note the large lobules and irregular cell clusters (x40).

abnormal histological appearance of the carotid body tissue from three different thoracic carcinoma cases. There remains the possibility that the data in Table 4.3 might be influenced by age.

Table 4.4. contains the data for all of the cases on the basis of group regarding to age, and means of lobule area and volume fraction of cell cluster and connective tissues for young and old cases (young < 50, old > 50). Whilst, due to the lack of young cases this data is very difficult to interpret, there is clearly no consistent effect of age on any of the parameters measured.

DISEASE	Number of Cases	LOBULE AREA	CELL CLUSTER A.Fraction ± st.dev	CONN. TISSUE A.Fraction ± st.dev
UNREALATED	8			
YOUNG	3	0.089 ± 0.029	%42.5 ± 6.4	%57.4 ± 6.4
OLD	5	0.097 ± 0.021	%41.3 ± 3.0	%58.6 ± 3.0
CHRONIC HYPOXIA	13			
YOUNG	1	0.1739	%37.1 ±	%62.8 ±
OLD	12	0.164 ± 0.064 **	%40.1 ± 5.9	%59.8 ± 5.8
E.HYPERTENSION	25			
YOUNG	2	0.1726 ± 0.0358*/**	%30.2 ± 2.7**/*	%69.8 ± 2.7**/*
OLD	23	0.1297 ± 0.0286*/**	%36.1 ± 5.4**/*	%63.8 ± 5.4**/*
D.MELLITUS	9			
YOUNG	1	0.0952	%37.7 ±	%62.2 ±
OLD	8	0.108 ± 0.054	%35.8 ± 7.1**	%64.1 ± 7.1**
D.MELLITUS (+E.H)	19			
YOUNG	3	0.141 ± 0.042	%33.5 ± 3.7**	%66.5 ± 3.8**
OLD	16	0.117 ± 0.042	%35.4 ± 6.5**	%64.8 ± 6.5**
NONTHORACIC tumor	13			
YOUNG	4	0.112 ± 0.016	%45.9 ± 7.7*	%54.1 ± 7.7*
OLD	9	0.104 ± 0.041	%36.3 ± 9.0 *	%63.6 ± 9.0*
SEPSIS	7			
YOUNG	1	0.0874	%34.3 ±	%65.6 ±
OLD	6	0.152 ± 0.077 **	41.9 ± 11.8	%58.1 ± 11.8
* Significantly different between young to old with same pathological condition				
** Significantly different to unrelated in same age group				

Table 4.4 shows the data on the basis of age for various cases. It seems that there is no significant influence of age on the carotid body of the different pathological conditions except in hypertension. * & ** indicates statistically differences compared to their groups at $p < 0.05$ level (t-test).

DISCUSSION

The data reported in this chapter confirms previous suggestions that hypoxia and hypertension lead to an increase in size of carotid body lobules. In addition it shows that diabetes, accompanied by hypertension and chronic infection also alter carotid body structure.

The human carotid body is a lobulated structure set in a connective tissue stroma. One of the features of the organ is its rich vasculature and innervation with numerous blood vessels and bundles of nerve fibres traversing the connective tissue to reach the cell clusters within the lobules. (see Chapter 5. for additional data). Within any lobule in an unrelated group some 58% of carotid body tissue was composed of connective tissue stroma. It must be emphasised that no attempt has been made to analyse the contents of this connective tissue stroma into its component parts of blood vessels, nerves and cellular and fibrous elements. Cell clusters (Type I and Type II cells) together with their vasculature and innervation made up 42% of the normal carotid body. Again we have not differentiated between Type I and Type II cells in this part of the study. Although in Chapter 5. there is a description of a method that might be used to distinguish between Type I and Type II cells and blood vessels and nerves.

Data reported by Heath and various colleagues suggested that chronic hypoxia and hypertension produced similar changes in the human carotid body. In essence the major change was an increase in the number of Type II cells and thus an apparent decrease in the number of Type I cells. No attempt was made in the study to count relative numbers of specific cells, but the pictures frequently shown by the Liverpool group were very different from the pictures in our study. This is discussed further in Chapter 5.

When the stereological data for chronic hypoxia and hypertension are considered an interesting new finding is apparent. Whilst there is no doubt that the size of both lobules and cell clusters are increased it is only in hypertension that there is any change in basic morphology of the carotid body. That is in the case of chronic hypoxia, the area fractions of cell clusters and connective tissue are no different from those seen in the unrelated group. Therefore, the carotid bodies in chronic hypoxaemia have bigger lobules and bigger cell clusters but the essential architecture, at least with regard to these features, remains unchanged. In the case of hypertensive patients the lobules

and cell clusters are also bigger but the area fraction of cell clusters is significantly reduced from normal whilst that of the connective tissue element is increased. In hypertension, therefore, there appears to be a fibrosis of the carotid body and decrease in the relative amount of specific cellular material.

50% of the cases of diabetes mellitus that were studied also had hypertension as a complication of the diabetes. It was thus possible that any of the changes produced in the carotid body of such patients was due to the effects of hypertension rather than the diabetes. In fact when diabetic carotid bodies were compared with carotid bodies from patients with diabetes and also hypertension it became apparent that even in the absence of hypertension diabetes had led to a significant change in both the cell clusters and connective tissue area fractions. Addition of hypertension to the diabetes had little additional effect. This finding is interesting as hypertension, on its own, was always thought to be the more potent stimulus to changes in carotid body architecture. In order to examine the effects of diabetes on the carotid body I have started an examination of the carotid body in diabetic rats.

In summary, as mentioned above all of the stereological data from different pathological conditions taken together confirm that chronic obstructive pulmonary disease (COPD) and essential hypertension alter human carotid body structure by hypertrophy and/or hyperplasia. In addition the present study reports that similar changes can be observed in diabetes mellitus, thoracic carcinoma and chronic infection.

CHAPTER FIVE

THE ANALYSIS OF CELL CONSTITUENTS OF HUMAN CAROTID BODY USING IMMUNOCYTOCHEMISTRY TECHNIQUE

INTRODUCTION

Immunocytochemistry and immunohistochemistry are methods of locating tissue components by the use of specific antibodies to those components. These methods are very sensitive and have found extensive use in histopathological analysis and diagnosis for they can be used to label populations of cell types within compound tissues.

The technique relies upon the fact that the injection of a foreign protein (antigen) results in production of antibodies to that protein. The antibody can be purified and placed on a tissue section, if the antigen is present in that section then the antibody will bind to it. With suitable detection systems it is thus possible to localise the presence of the antigen within the tissue.

There are many methods which can be used to localize antigens such as, direct method, indirect method, avidin biotin complex method (ABC), alkaline phosphatase/anti-alkaline phosphatase complex (APAAP) and peroxidase/anti-peroxidase complex (PAP). There are also numerous reagents and kits available. The choice is based on the individual need of each laboratory, such as the type of specimen being investigated, the degree of sensitivity required, and the processing time and cost requirements. Details of the various methodologies are given in the series edited by Bullock, and Petrusz (1988). In this study the PAP technique was used and this is described below.

The PAP method which utilizes a peroxidase anti-peroxidase (PAP) complex is a commonly used technique for demonstrating tissue and cell antigens by light

microscopy. The PAP complex consists of three molecules of peroxidase enzyme. This method uses a primary antibody, a secondary "link" antibody and an antibody that is produced against and linked with peroxidase enzyme (PAP complex). The secondary antibody is produced in a species different from the primary and PAP complex, and the primary antibody and PAP complex are produced from the same species. The secondary antibody therefore acts as a bridge or link antibody. The technique is illustrated diagrammatically in Fig 5.1. (See also Sternberger, 1979; Polak & Noorden, 1983, 1988, and Boenisch, 1989).

A number of immunocytochemical studies of animal carotid body have been performed and these have confirmed the presence of a number of biogenic amines and neuropeptides. Data from such studies is summarized in Table 5.1. However, very few studies have been performed on the human carotid bodies so far (Heath & Smith, 1992; Habeck et al., 1994), and these are reviewed in Chapter 1.

The aim of the present chapter is to investigate the pattern of distribution of Neurofilament 200 (NF), S100-protein (S100), Synaptophysin (SYN), Von Willebrand factor (VWF and also called Factor VIII; F8), and Vimentin (VIM) in the normal human carotid body and to compare such patterns in different pathological conditions.

The neurofilaments are one of the five major groups of intermediate filaments and are found predominantly in cells or tissues of neuronal origin, therefore NF antibodies stain nervous components of the tissue. The S100 protein is widely distributed in both neural, and non-neural tissues. Its staining demonstrates glial and ependymal cells in the brain and Schwann cells of the peripheral nervous system.

Synaptic vesicles play an important role in neurotransmission. At the synapse, neurotransmitters are stored in synaptic vesicles or mediated by proteins in the synaptic vesicle membranes that are being released by synaptic vesicle exocytosis. Synaptophysin (SYN) is one of several major protein components that are present in synaptic vesicles possibly responsible for neuronal transmission. Therefore, neuromuscular junctions and synapses can be marked by SYN. Von Willebrand factor (VWF or F8) is present in endothelial cells. Therefore it outlines the blood vessels of tissues.

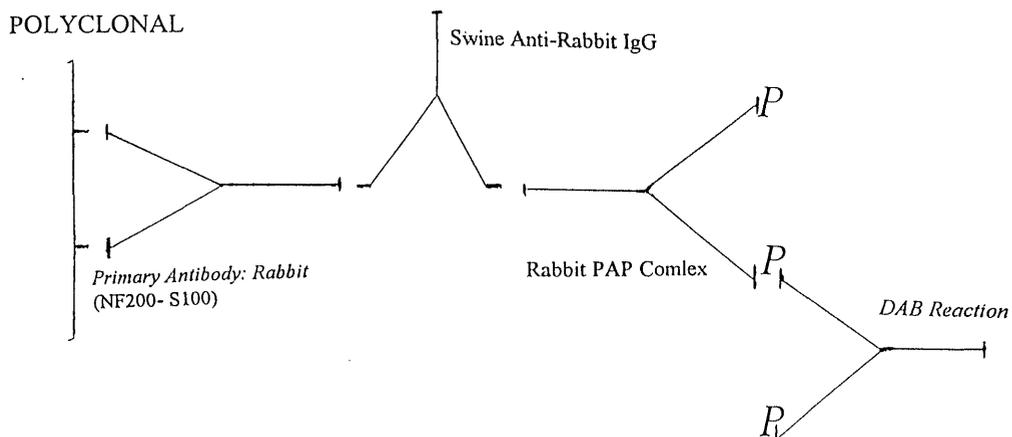
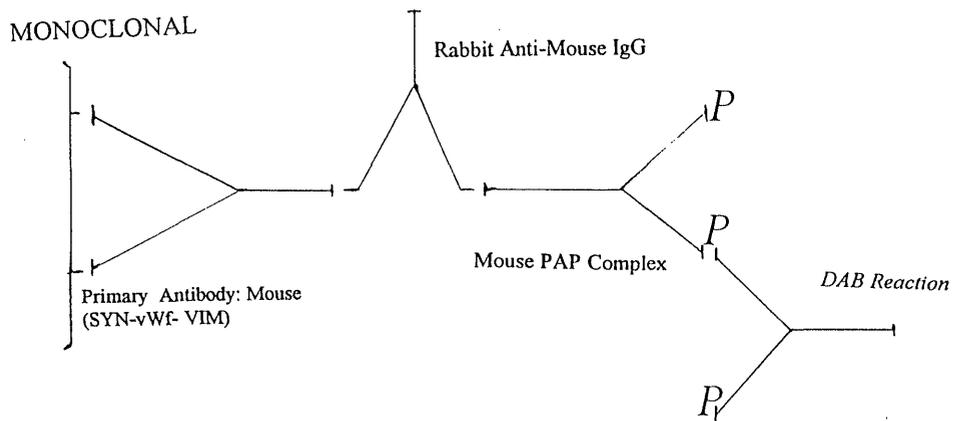


Figure 5.1 Schematic representation of the PAP immunocytochemistry method used in the present study.

Vimentin is present in cells of mesenchymal origin, hence a wide variety of cells, because of their mesenchymal origin, (e.g. endothelial cells, lymphoid cells, fibroblasts and smooth muscle cells) can be labelled by the vimentin antibody.

REFERENCES	SPECIES & TECHNIQUES	ANTIBODIES
Lundberg et al. (1979)	CAT	MENK/ LENK/ SP/ VIP
Cuello & McQueen (1980)	CAT	SP
Wharton et al. (1980)	CAT	MENK/ LENK/ SP/ VIP
Kondo (1982)	RAT	NSE/ S100
Kobayashi et al. (1983)	DOG	MENK
Hanson et al. (1986)	RABBIT	MENK/ SP
Kameda (1989)	CHICKEN	SP/ CGRP/ VIP/ Somastatin
Prabhakar et al. (1989)	CAT	SP/ Neurokinin A
Maxwell et al. (1990)	Human (16)	VIP/ SP
Happola et al. (1990)	RAT	Peptide YY/ Peptide YY-Like
Khan et al. (1990)	Human (9)	MENK/ LENK
Smith et al. (1990)	Human (26)	MENK/ LENK/ SP/ VIP/ Neurotensin/ Bombesin
Kummer W. (1990)	Guinea Pig	TH/ NPY-LI/ VIP-LI
Kummer & Habeck (1991)	Human/ Double lab.(LM & EM)	SP/ CGRP-LI
Abramovici et al. (1991)	CAT / PAP	MENK/ LENK/ 5HT/ NSE/ S100/ GFAP/ NF/ TUB
Wang et al. (1991a,b)	CAT/ APAAP & PAP Double lab.	ANP-LI/ TH/ DA/NE
Abramovici et al. (1991)	Human (10) / PAP	ANP
Achilles et al. (1991)	Human	S100/ GFAP
Pequignot et al. (1991)	RAT	SP/ NPY-LI
Scraggs et al. (1992)	Human fetuses(15) / ABC	MENK/ LENK/ SP/ Bombesin
Kummer & Habeck (1992)	Human (9)/ FITC* & ABC(for EM)	TH/ NF/ SP/ AADC/ CGRP/ DBH
Habeck et al. (1994)	Human (4)/ PAP & FITC*	5HT/ SYN/ PGP
Oomori et al. (1994)	Mouse (20)/ ABC	TH/ DBH / PNMT/ 5HT/ GAD/ GABA

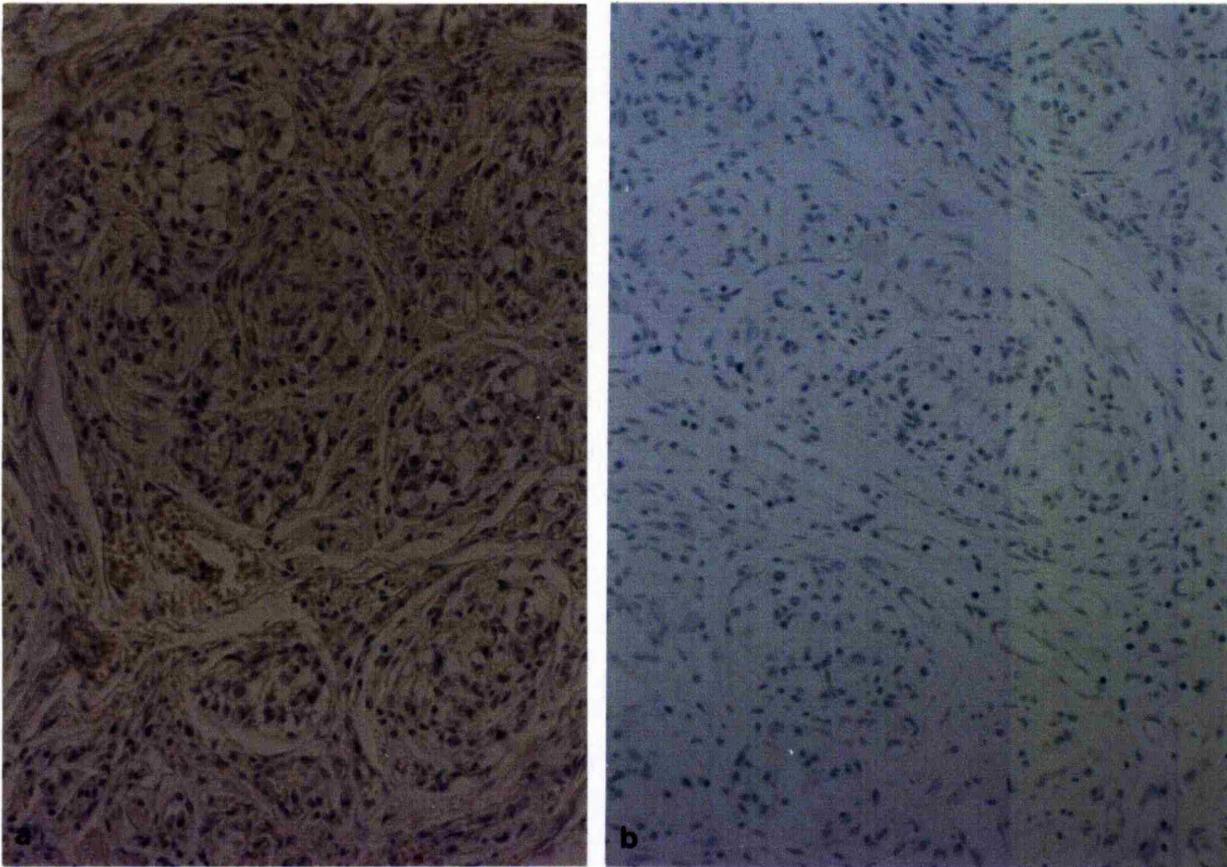
Table 5.1 shows immunocytochemistry studies on the carotid body so far. (Abbreviations for antibodies: MENK: Methionine enkephalin, LENK: leucine enkephalin, SP: Substance P, VIP: Vasoactive Intestinal Polypeptide, NSE: Neuronal specific enolase, CGRP: Calcitonin Gene-Related Peptide, 5-HT: 5-Hydroxytryptamine/Serotonin, ANP: Atrial Natriuretic Peptide, TH: Tyrosine hydroxylase, SYN: Synaptophysin, DA: Dopamine, NE: Norepinephrine, NF: Neurofilament protein, TUB: Tubulin, DBH: Dopamine Beta-hydroxylase, GFAP: Glial fibrillary acidic protein, NPY-LI: Neuropeptide Y-like, AADC: Aromatic-L-amino-acid-decarboxylase, PNMT: Phenylethanolamine N-methyltransferase, GAD: Glutamate decarboxylase, GABA: Gamma-aminobutyric acid.)

MATERIAL AND METHODS

Carotid bodies were obtained from same source for the studies described in chapter 3. A total of 77 carotid bodies were removed at routine necropsy, fixed in either Ethanol or Zamboni's fluid, the techniques of processing were as described in Chapter 3. Data on each case analysed in this study including age, postmortem delay and pathological conditions are listed in Appendix 5.1. The cases were divided into six categories according to their clinical history and the autopsy report of each patient as described in Chapters 2 and 3, except for nonthoracic tumours which have been included with unrelated cases. Processing temperatures were adjusted 55 °C to avoid excess heat that might destroy the antigens and cellular morphology. Five micrometer sections were mounted on slides coated with either Poly L-Lysine or Gelatine Chrome Alum. At least two slides were stained for each antibody and for each case, the average was four slides per case.

The sections of carotid bodies were labelled by the peroxidase anti-peroxidase (immunoperoxidase, PAP) technique. The specification of antibodies used in our study are shown in table 5.2. The primary antisera were diluted with 10% normal serum in either Tris buffer saline (TBS) or Phosphate buffer saline (PBS) and kept in a deepfreezer at -40°C. The working dilutions of primary antibodies were selected to produce a visible immunoreactivity in the sections (see table 5.2 for optimal dilutions).

Sections were dewaxed in xylene, rehydrated in descending alcohol concentrations and incubated in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Following washing in TRIS or PBS, the sections were incubated with Trypsin, a proteolytic enzyme (if required), then following washing with three changes of buffered saline incubated for 30 minutes at room temperature in diluted normal serum of the animal used to raise the link antibody (in order to reduce nonspecific staining). According to antibody specification two antisera either polyclonal or monoclonal were used (see table 5.2 and appendix 5.2). The sections were incubated overnight in primary antibody at 4°C and then washed three times in buffered saline. The link antibodies were added and incubated for 30 minutes at room temperature. Following washing with three changes of buffer, the sections were incubated for 45 minutes at room temperature with either rabbit PAP (for polyclonal antibodies) or mouse PAP (for monoclonal antibodies).



Figs 5.2a, and b. As negative controls, in these sections incubation with the primary antiserum was omitted. Note the absence of brown reaction product (x20).

After three washes in phosphate buffered saline the immunoreaction was visualized by incubating for 5 minutes in 3,3-diaminobenzidine tetrahydrochloride solution (DAB) which served as chromogen and produced a brown colour reaction. In order to stop the DAB reaction the slides were washed in running tap water for 30 minutes, all preparations were then counterstained lightly with either Harris haematoxylin or Methyl green. Following this treatment sections were washed well in running tap water for 30 minutes and dehydrated through ethanol followed by xylene and mounted in DPX (see also Appendix 5.1 & 5.2).

Negative controls consisted of either serially diluting the primary antibody until all immunoreactivity was undetectable and/or omitting the primary antibody altogether and replacing it with TBS or PBS. Substances stained with the specific antisera but unstained in the negative controls were considered to be specifically stained according to the PAP technique (Sternberger, 1979; Polak & Noorden, 1988, see Figs 5.2a & b).

All structures within the sections of human carotid body were examined for the presence of immunoreactivity. Particular attention was paid to Type I cells (for SYN and NF), bundles of nerve fibres (NF-200), Type II cell and Schwann cells (S100) and blood vessels (VWf and VIM) using a Zeiss Axiophat microscope. Each of these structures was subjectively marked as being non immunoreactivity (IR) or negative (- - -), weakly IR (+ - -), positive IR (+ + -) and strongly positive IR (+ + +) as summarized in table 5.3.

In order to compare the result of our immunostaining the Enhanced Polymer One-Step Staining (EPOS/HRP which has recently been launched by Dako) technique was also applied for Vimentin and S100 reactions. This technique is based on a single incubation step and provides very strong immunoreactions with the added advantage of less background (see appendix 5.3).

RESULTS

The results of the examination of antibody immunoreactivities is summarized in table 5.4 using a subjective marking system. The intensity of immunoreactivity for all antibodies was variable both within all pathological groups and within the individual cases.

Antibody Against	Donor species	The working dilutions	Source and Code No:
Neurofilament (NF200)	Rabbit (Polyclonal)	1:35	Sigma, USA, N-4142
S100 Protein	Rabbit (Polyclonal)	1:100	Dako, Denmark, Z-311&U-029* Sigma, USA, S-2644
Synaptophysin (SYN)	Mouse (Monoclonal)	1:50	Sigma, USA, S-5768
Von Willebrand factor (vWf, F8)	Mouse (Monoclonal)	1:20	Dako, Denmark, M-616
Vimentin V9 (VIM)	Mouse (Monoclonal)	1:15	Dako, Denmark, M-725, U-7034*

Table 5.2. shows the specification of the antibodies used in present chapter.

Antibodies	Type I Cell	Type II & Schwann cell	Nerve Fibers	Blood Vessels
Neurofilament (NF200)	+ - -	- - -	+ + +	- - -
S100 Protein	- - -	+ + +	+ + +	- - -
Synaptophysin (SYN)	+ + +	- - -	- - -	- - -
Von Willebrand factor (vWf, F8)	- - -	- - -	- - -	+ + +
Vimentin V9 (VIM)	- - -	+ + -	+ + -	+ + +

Table 5.3 shows the immunoreactivity of the antibodies in the carotid body recorded as negative (- - -), weakly IR (+ - -), positive IR (+ + -), and strongly positive IR (+ + +).

ANTIBODIES	Unrelated	COPD	E.Hypertensio	Thoracic CA	D.Mellitus	Sepsis
Neurofilament	+ - -	+ + +	+ + +	+ + +	+ + -	+ - -
S-100	+ + -	+ + +	+ + +	+ + +	+ + -	+ - -
Synaptophysin	+ + -	+ + +	+ + +	+ + +	+ + -	+ + -
Vwf (F8)	+ + -	+ + +	+ + +	+ + +	+ + +	+ + -
Vimentin	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Table 5.4 illustrates the intensity of the immunoreactivity in different pathological conditions in the human carotid body

Some weak or negative immunoreactivity including heavy background staining appeared to develop after 8-10 hours postmortem delay. This was intense for Neurofilament, Synaptophysin and Vimentin antisera. Nonspecific background was also found in some sections whilst other sections from the same case, stained at the same time with the same antisera were not affected by this artefact. I have no explanation for this problem.

Neurofilament (NF-200)

a. Normal cases

As would be expected, NF stained the nervous elements of the normal carotid body. Fig 5.3a & b illustrate the distribution of neurofilament protein within the carotid body. The technique shows the presence of axons within interlobular connective tissue and within the cell clusters.

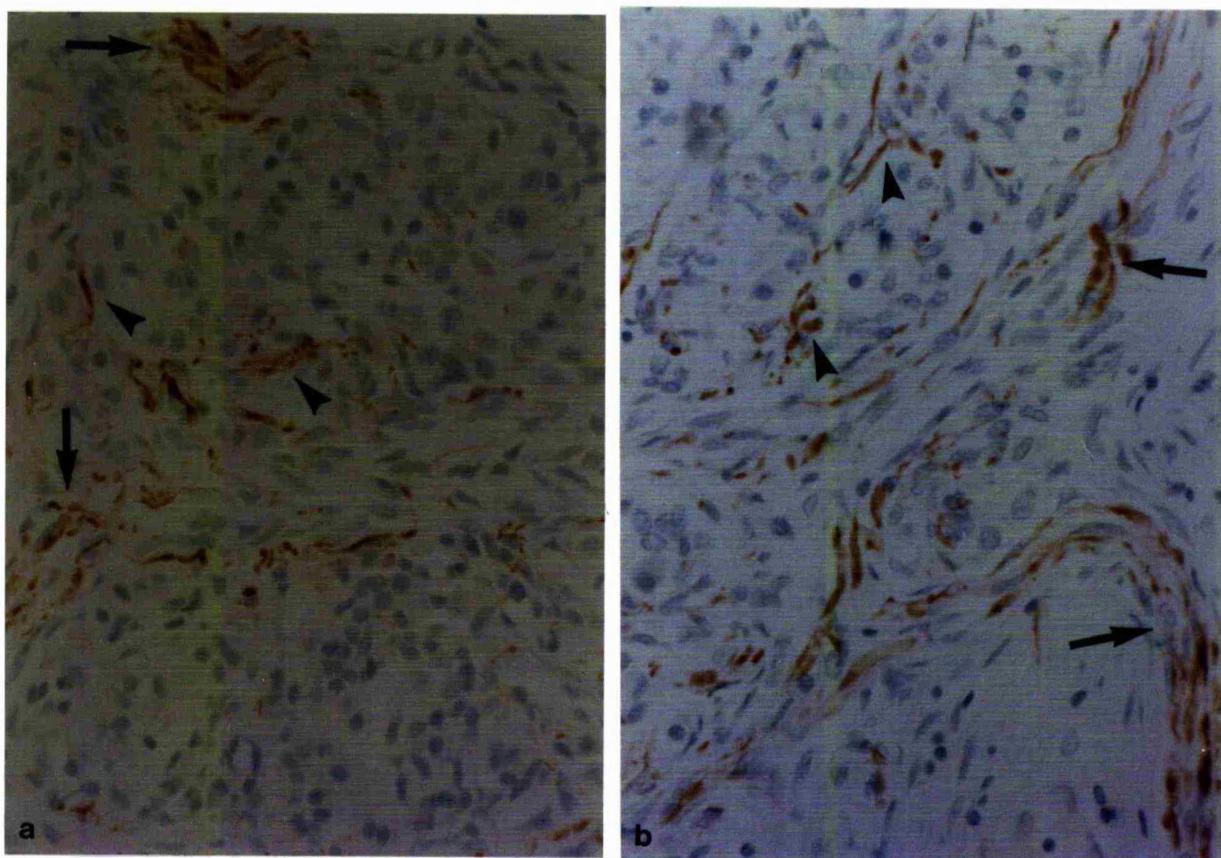
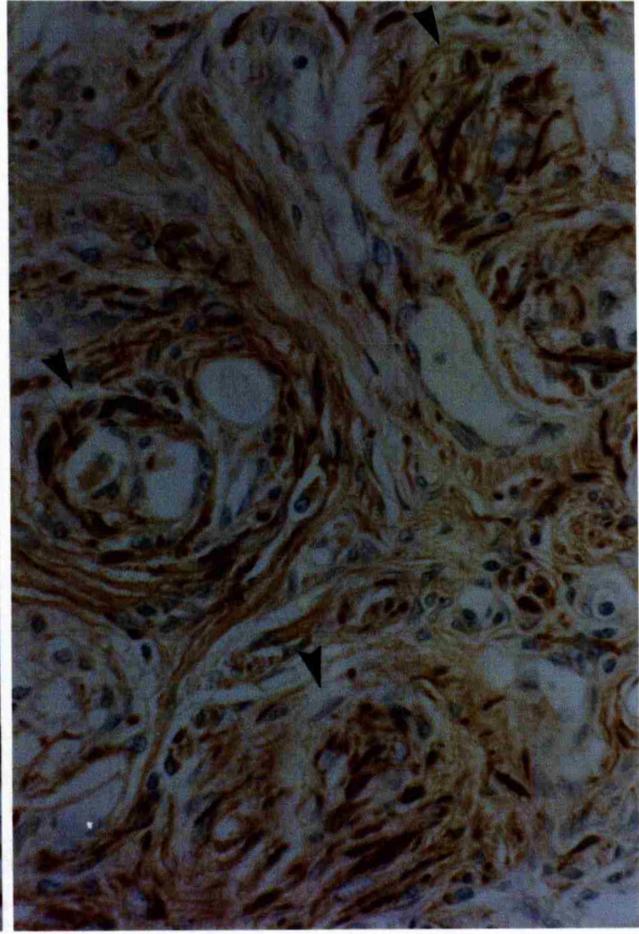
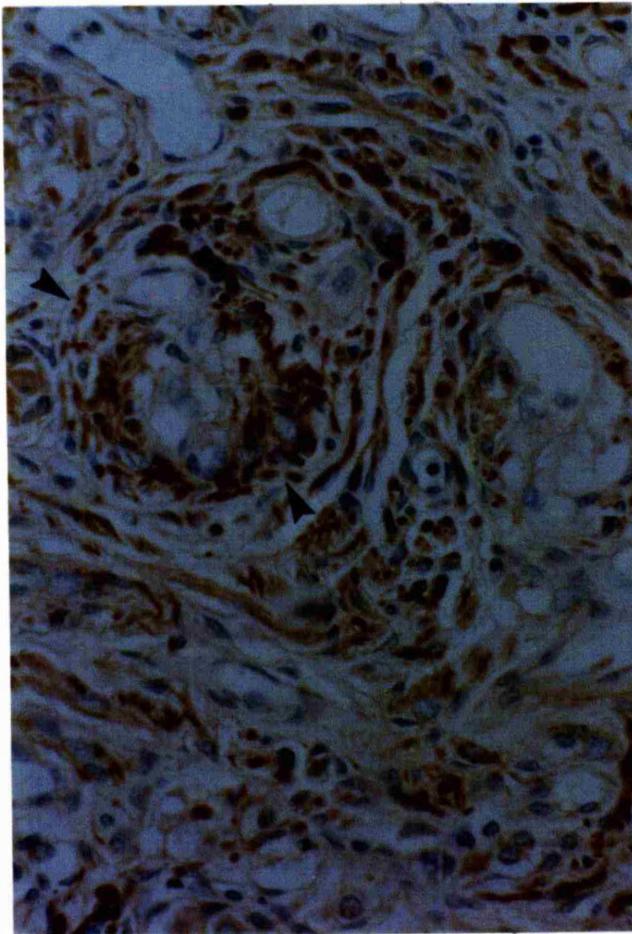
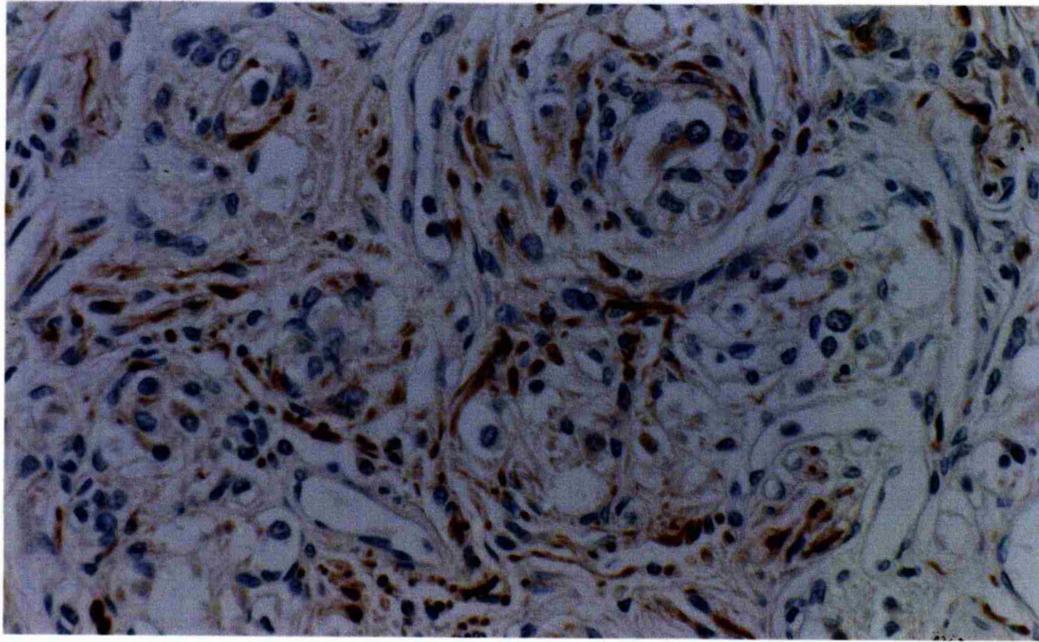


Fig 5.3a, and b show micrographs from two unrelated carotid body cases stained for neurofilament. Note the axons within the connective tissue elements (arrows) and also within cell clusters (arrow heads). (x40)



Figs 5.4, 5.5, and 5.6 show strong immunoreactivity of neurofilament in three different COPD cases. Note cell clusters (arrow heads) surrounded by neural material stained brown. These three figures illustrate how in some cases of COPD the amount of nerve is near to normal (5.4) whilst 5.5. & 5.6 show grossly increased amount of nerve.(x40)

b. Pathological Conditions.

It was possible to obtain an immunoreaction in all of the pathological conditions studied as shown in table 5.4.

In chronic hypoxia the amount of neuronal material present within the carotid body was extremely variable. Figs 5.4, 5.5 & 5.6 illustrate this phenomenon. In Figure 5.4 the amount of neurofilament positive tissue was essentially similar to that seen in the control cases with bundles of fibres seen in the connective tissue stroma and individual axons visible within the cell clusters. In other cases there appeared to be whorls of axons surrounding the clusters of Type I cells and in this situation it seemed that the number of Type I cells were reduced (Fig 5.5). In extreme cases of this apparent hypertrophy of neural material the axons appeared to have invaded the cell clusters and only isolated Type I cells were visible within them (Fig 5.6). In addition, in chronic hypoxia some of the Type I cells were also neurofilament positive (Figs 5.4, 5.5). Such an observation was rare in normal cases. When this staining pattern was observed the number of Type I cells that were stained was variable, the presence or absence of Type I cell staining did not appear to be related to age, cause of death of the patient or the postmortem delay.

In essential hypertension the pattern of distribution of nerve fibres was similar to that seen in normal cases as illustrated in Fig 5.7. except that it seemed that some of the axons were larger.

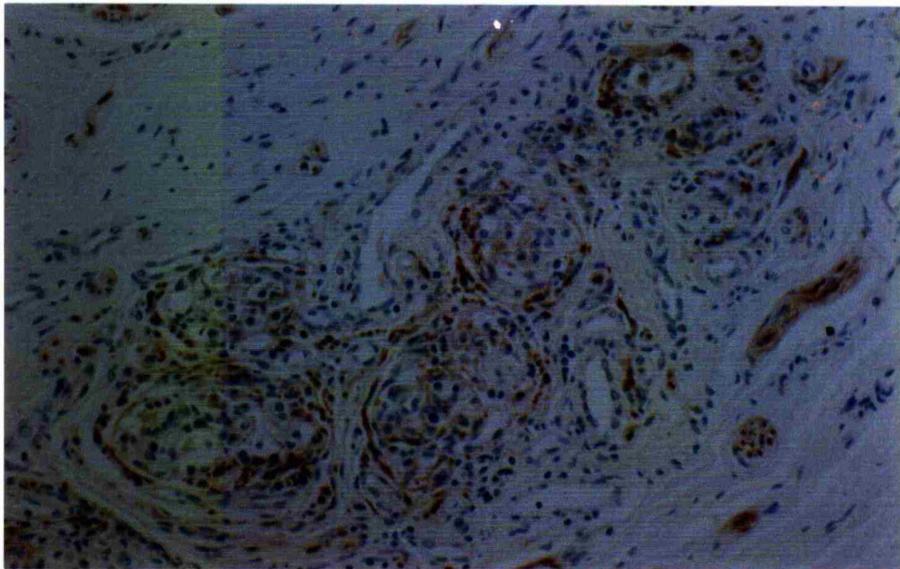


Fig 5.7. Photomicrograph of an essential hypertension case showing the immunoreactivity of neurofilament antibody (x20).

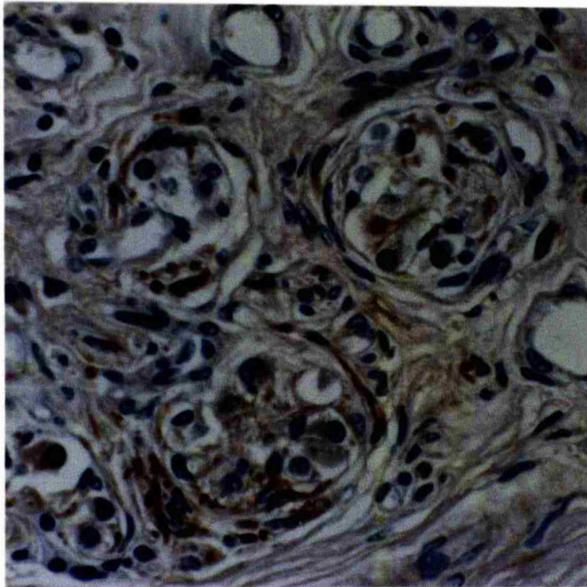


Figure 5.8

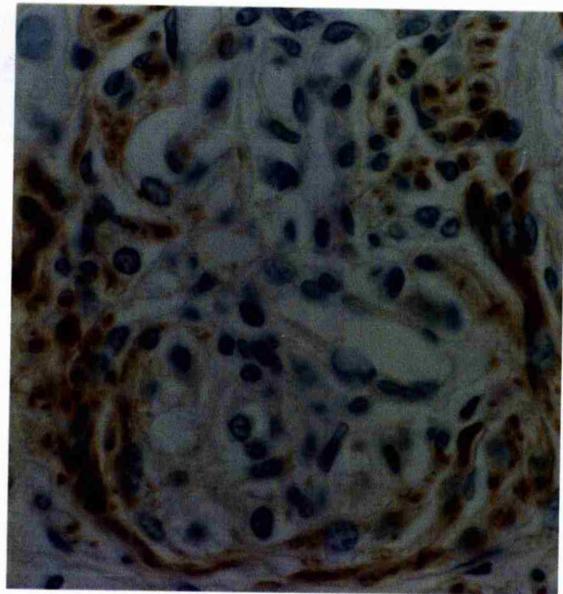


Figure 5.9

Fig 5.8 shows neurofilament IR in the carotid body of a diabetes case (x40). Fig 5.9. showing immunoreactivity of neurofilament in the hypertension case carotid body (x63)

In diabetes mellitus the distribution of nerve fibres was again similar to that seen in the control specimens. The distribution of axons was not altered by the presence of hypertension as a complicating factor in the diabetes (Figs 5.8 & 5.9).

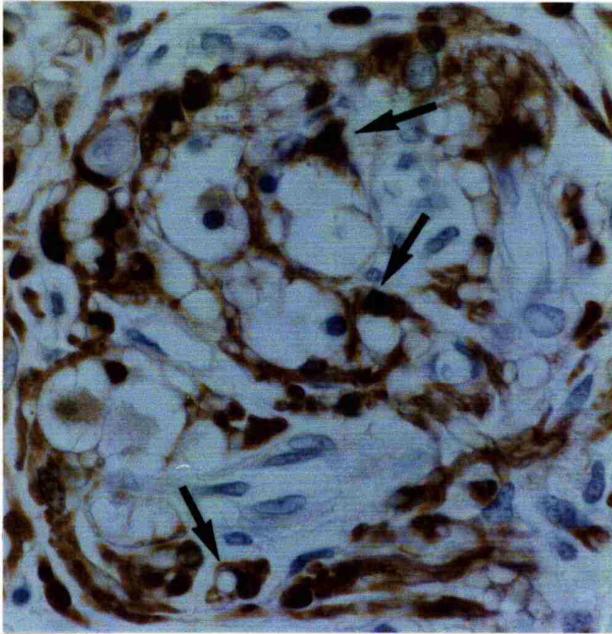


Figure 5.10

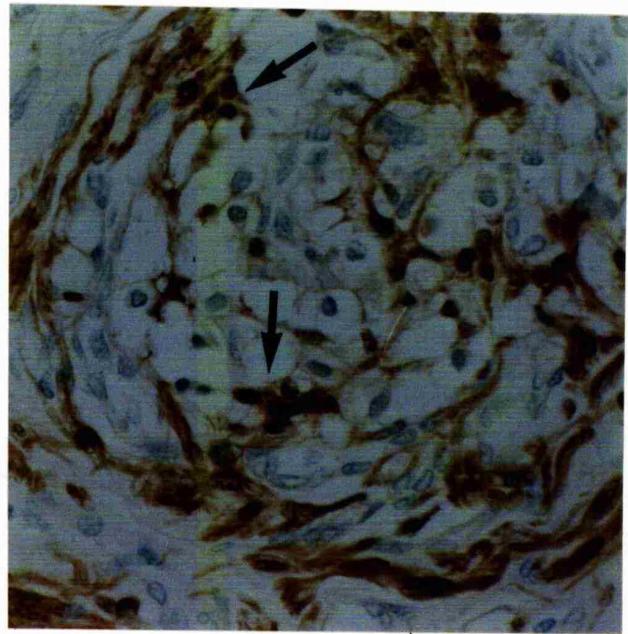


Figure 5.11

Figs 5.10 and 5.11 show S100 antibody immunoreactivity in unrelated cases. Note how some cells with circular nuclei (arrows) are also positive to S100 protein (Fig 5.10). (x63, x40 respectively)

S-100 Protein

a. Normal Cases

Sections of normal human carotid body incubated with antibodies to S100 showed intense deposits of reaction product in the bundles of nerve fibres, at the periphery of the cell clusters and also between the Type I cells (Figs 5.10 & 5.11); qualitatively the distribution of staining pattern was similar to that seen with neurofilament (see below). Typically the positively stained cells possessed elongated nuclei, occasionally some cells with circular nuclei were also stained (Fig 5.11). The cytoplasmic extension of Type II cells between the Type I cells within the cluster was a regular feature (Fig 5.10 & 5.11). It is also interesting to note the presence of many cells at the periphery of the cell clusters which possessed elongated nuclei (and would therefore be called Type II cells) but which lacked any immunoreactivity to S100 protein.

b. Pathological Conditions.

In chronic hypoxaemia the staining pattern seen after reaction with anti S100 was extremely variable as illustrated by Figs 5.12, 13, 14, & 15. In some cases it was

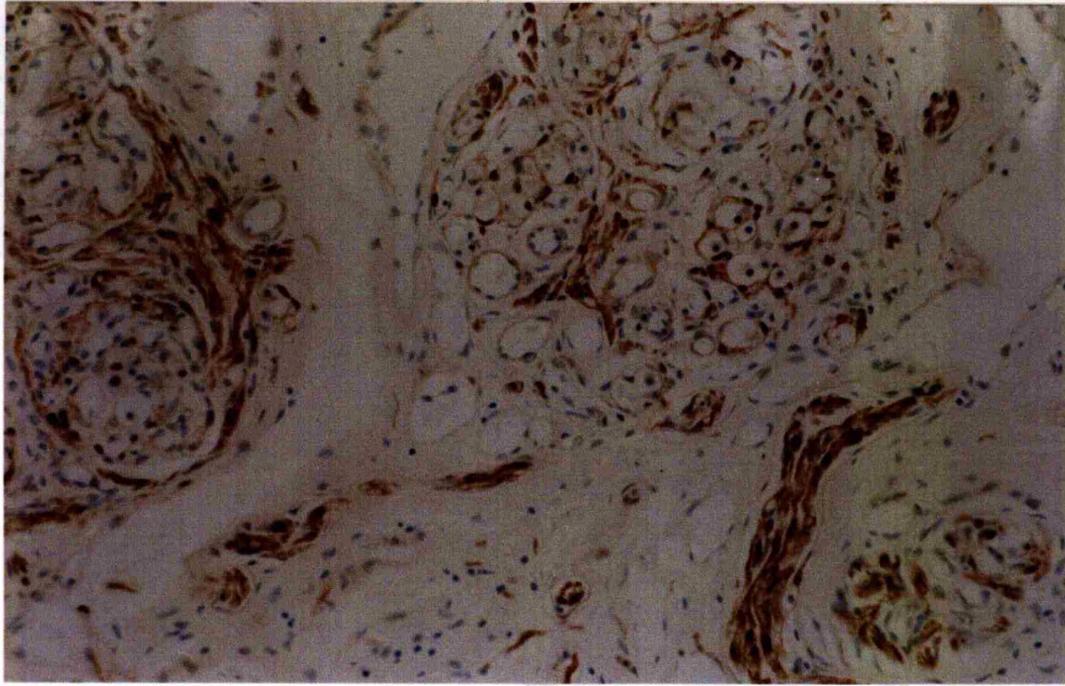


Fig 5.12

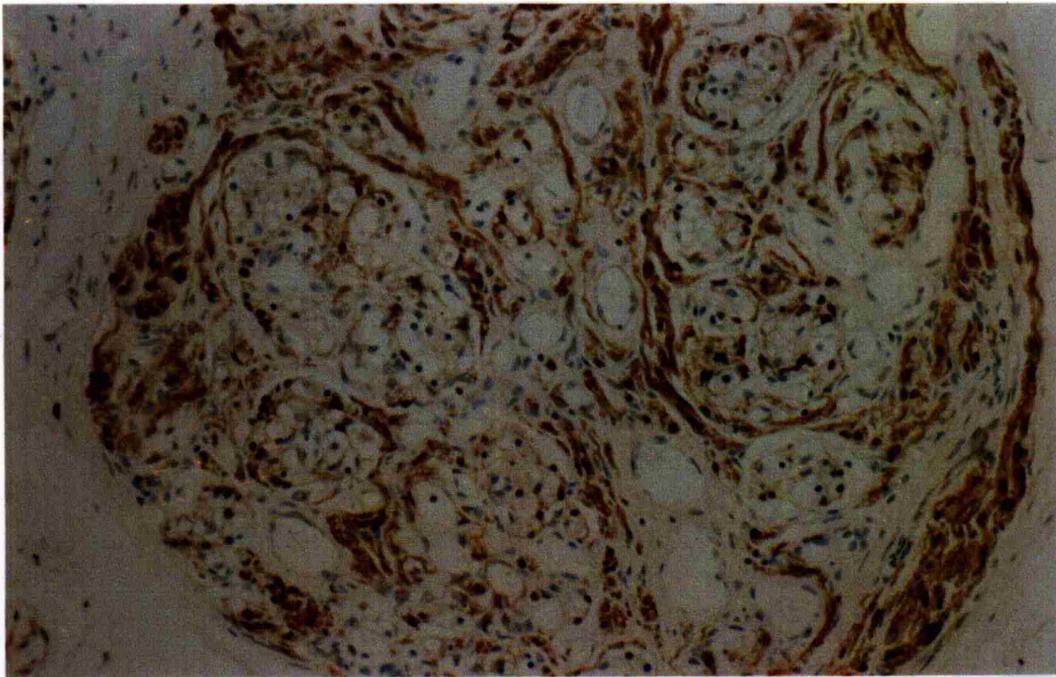


Fig 5.13

Fig 5.12 and 5.13 Two photomicrographs showing S100 immunoreactivity in carotid body of COPD cases. Note that the reaction is similar to unrelated cases. (x20)

impossible to predict the existence of hypoxaemia from the reaction (Fig 5.12) as the amount and distribution of immunoreactivity was indistinguishable from that seen in the control cases. In other cases the effect of the disease had produced a dramatic effect on the amount of reactivity. Thus in some cases there were whorls of positively stained cells at the periphery of the cell clusters. In others the periphery of the clusters appeared normal but there was a massive amount of S100 positive material between the Type I cells (Figs 5.14 & 5.15). In those cases where there was hypertrophy/hyperplasia of S100 positive cells there were still areas within the carotid body which appeared relatively normal (see Fig 5.14).

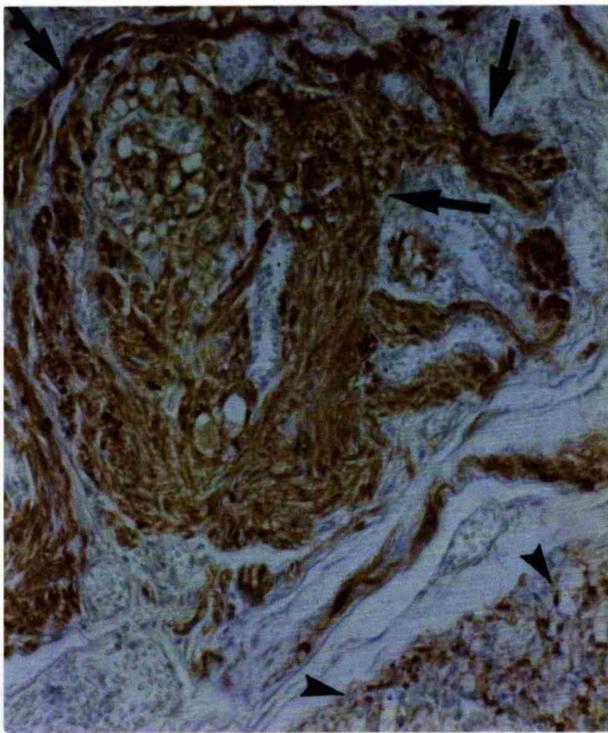


Fig.5.14

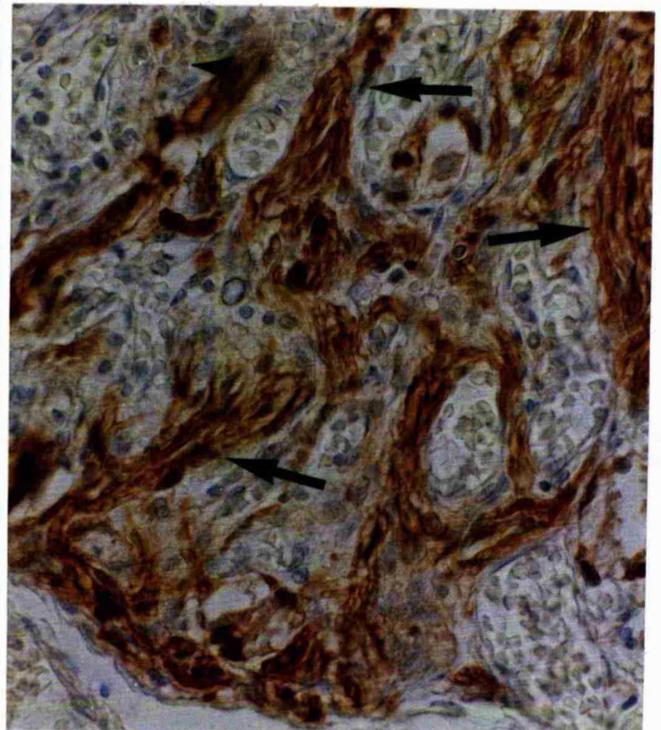


Fig 5.15

Figs 5.14, and 5.15. Two photomicrographs of COPD patients carotid bodies showing the intense immunoreactivity of S100 in some areas (arrows) whilst there were still areas (arrow heads) within the carotid body that seems to be normal. (x20, x40 respectively)

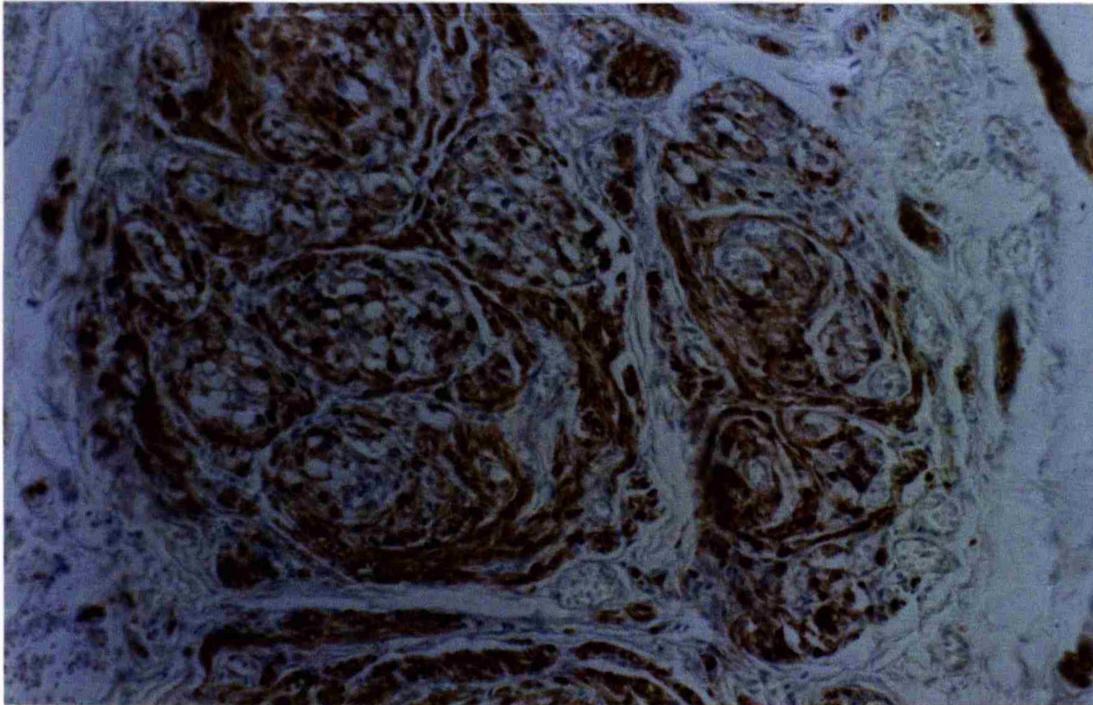


Fig 5.16a

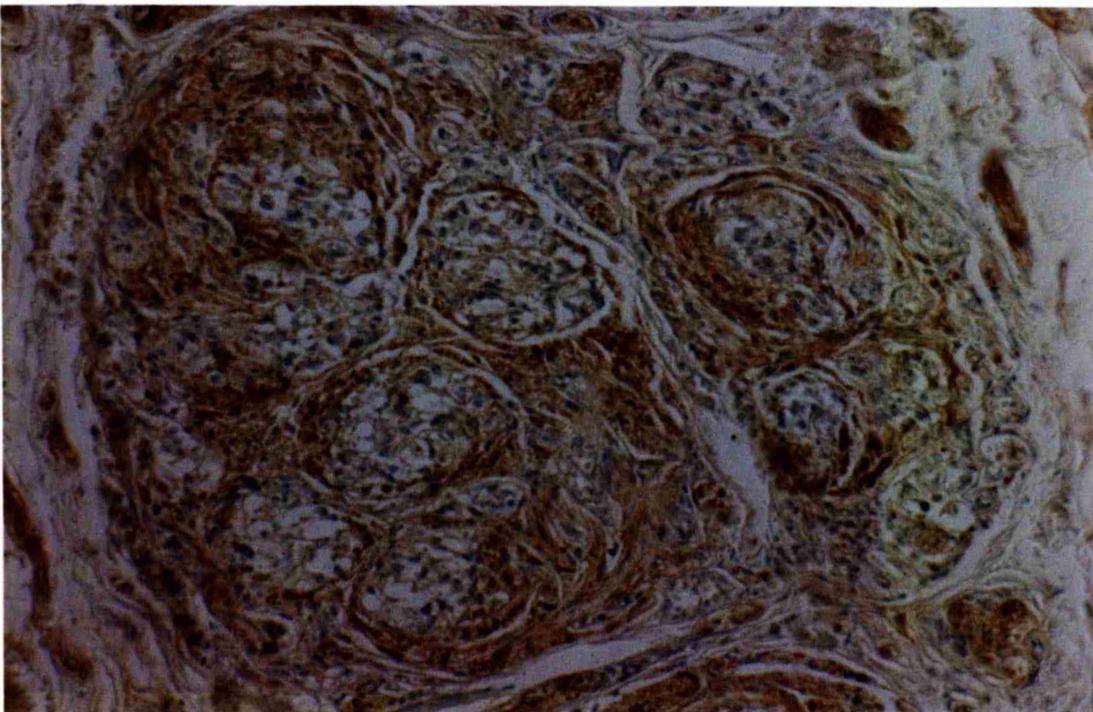


Fig 5.16b

Figs 5.16a, and b. Two photomicrographs showing similar areas from the same case stained with S100 (fig 5.16a) and neurofilament (fig 5.16b) antibodies. Note the similarity in the distribution of immunoreactivities. (x20)

The cases of diabetes mellitus and essential hypertension all produced satisfactory staining with anti S100; it was not possible to find any qualitative differences in either the distribution or amount of immunoreactivity in these cases.

The similarity in the distribution of reaction product after incubation with anti-neurofilament and S100 is well illustrated in Figs 5.16a & b which illustrates similar areas from a case of chronic hypoxaemia.

Synaptophysin

a. Normal Cases

As would be expected immunoreactivity for synaptophysin occurred within the cytoplasm of the Type I cells (Figs 5.17a & b). In many cases the shape of the Type I cell could be delineated after immunoreaction with synaptophysin but in some cases the reaction product was patchy and sometimes located only in the perinuclear part of the cytoplasm. No immunolabelling for synaptophysin was seen in the Type II cells, bundles of nerve fibres, or around the glomic vasculature.

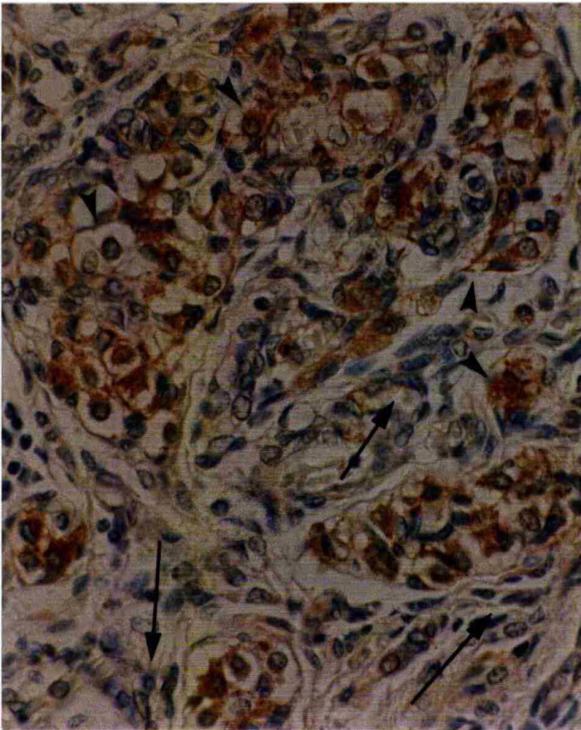


Fig 5.17a

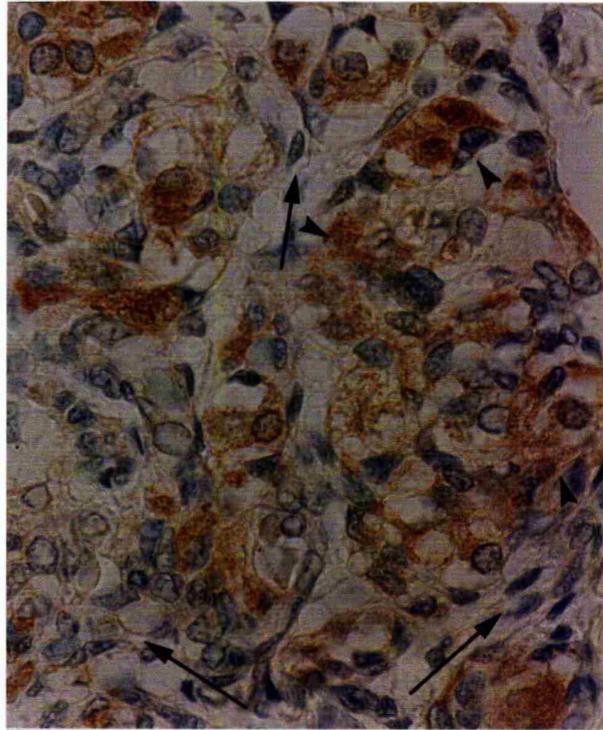


Fig 5.17b

Figs 5.17a, and b. Two micrographs showing the immunoreactivity of synaptophysin in unrelated cases. Note irregular reaction in Type I cells (arrow heads) and no reaction can be seen for Type II cells (arrows). (Fig 5.17a: x40, and b:x63)

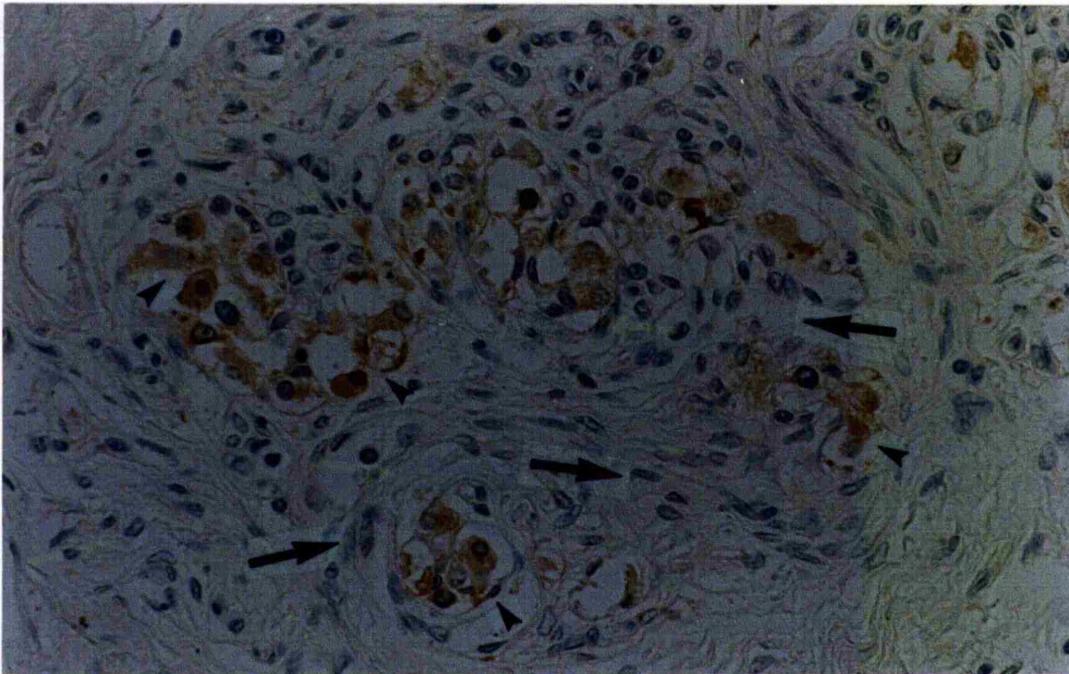


Fig 5.18a

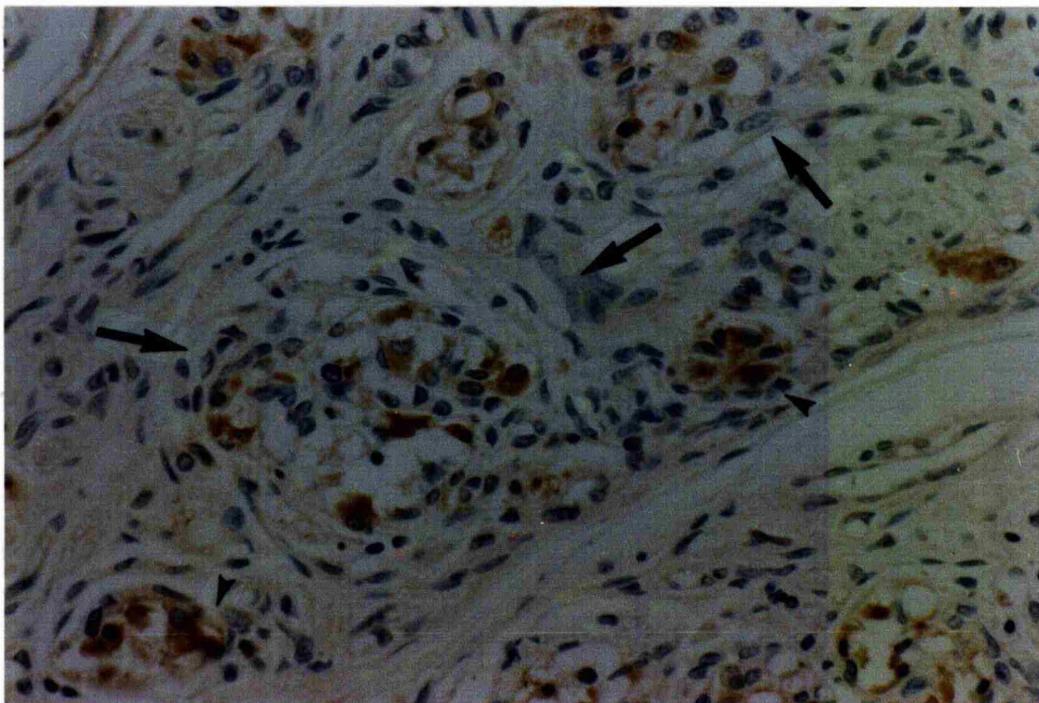


Fig 5.18b

Fig 5.18a, and b. Photomicrographs of COPD(a) and hypertension(b) showing the variety of the reaction of synaptophysin. Note the extension of cytoplasm of Type I cells (arrow heads) and no reaction in Type II cells (arrows). (x40)

b. Pathological Conditions.

In all pathological conditions studied it was possible to obtain a positive immunoreaction to synaptophysin. Without quantitative techniques it is not possible to comment on changes in the distribution or number of positive cells. However, in cases of hypoxaemia and essential hypertension it appeared that the numbers of Type I cells within different cell clusters was much more variable than in the control cases. This variation is illustrated in Figs 5.18 a & b.

In all cases of hypoxaemia, diabetes (with and without hypertension) and essential hypertension a variable number of isolated synaptophysin positive cells were found (figs 5.19)

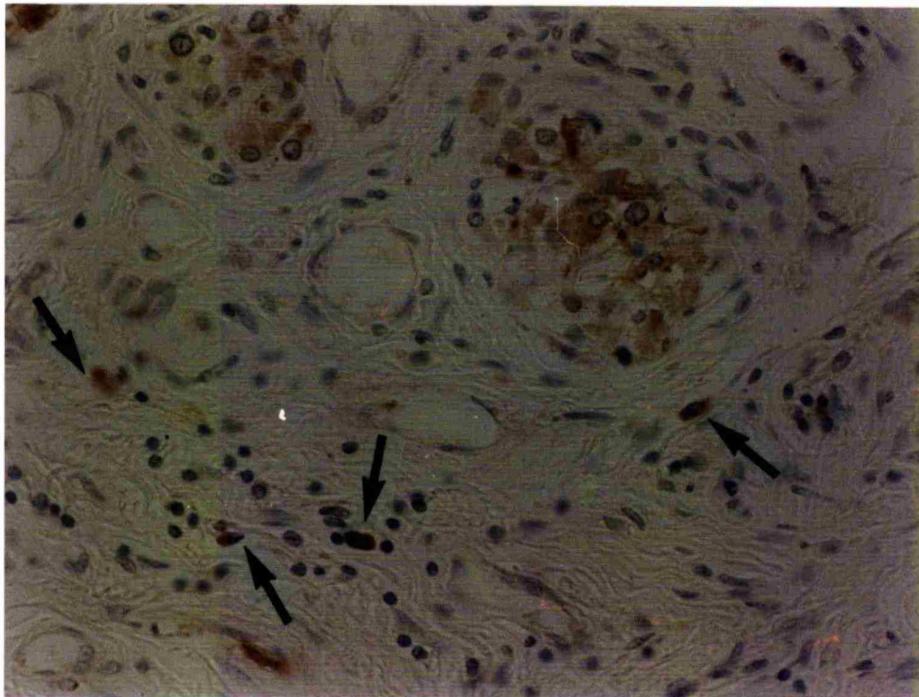
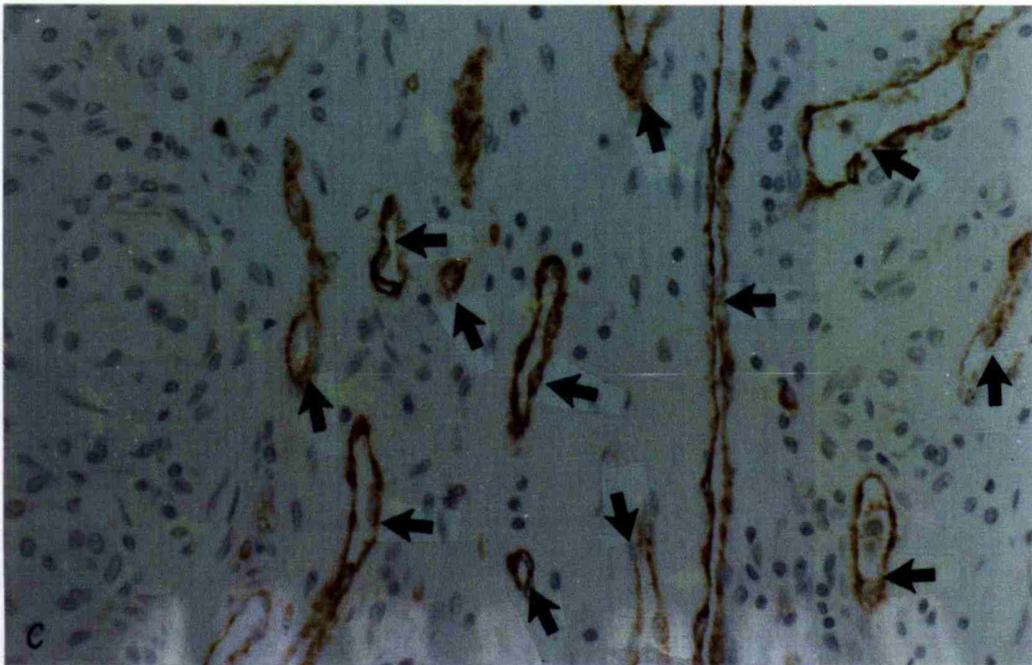
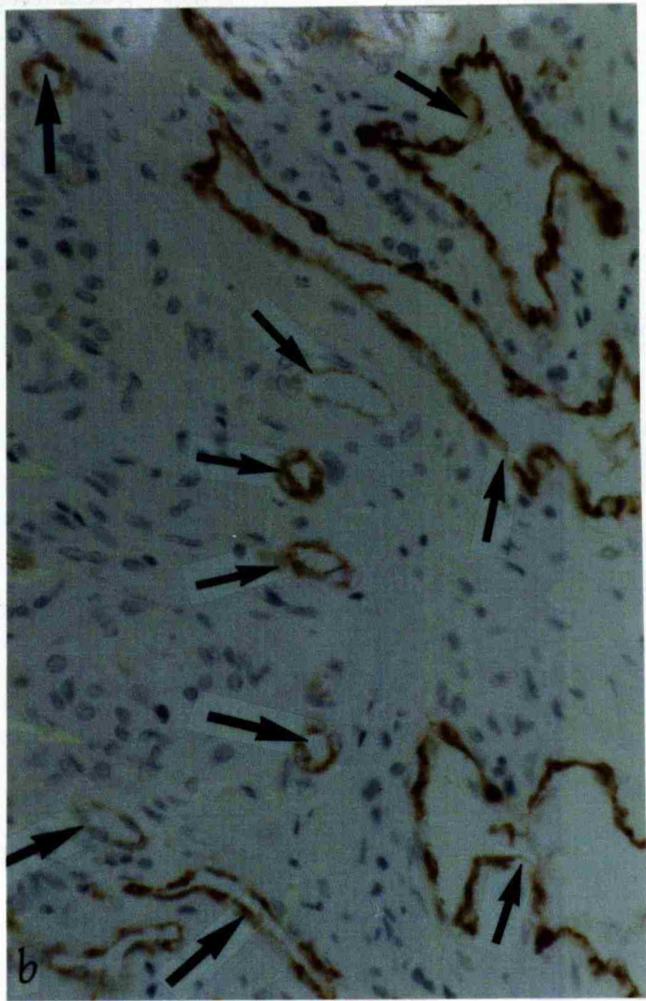
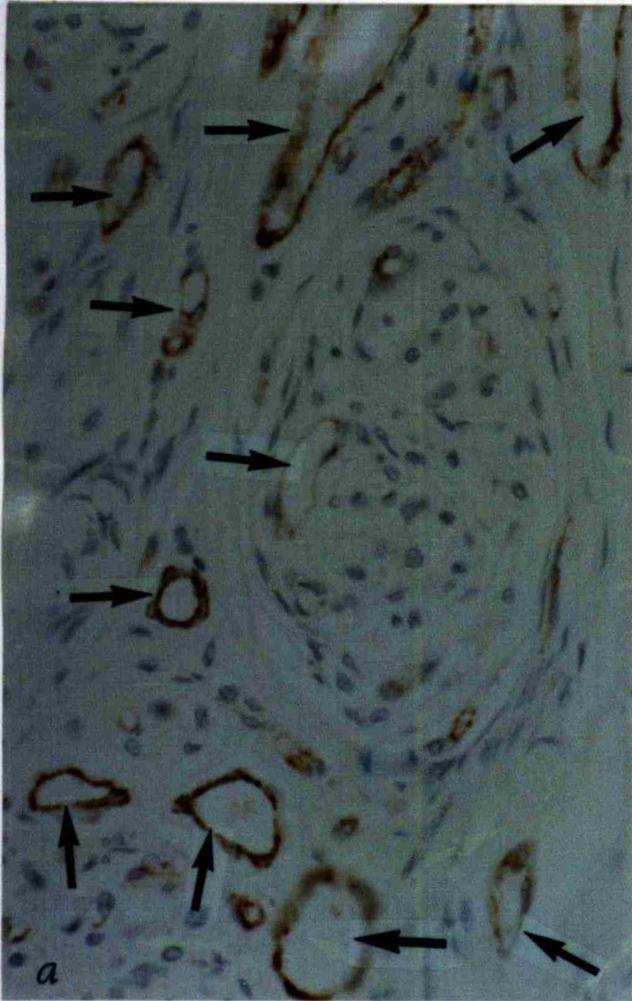


Fig.5.19 shows a number of isolated synaptophysin positive cells (arrows) around the cell clusters.



Figs 5.21a, b, and c show three different pathological cases in which blood vessels (arrows) showed vWf (F8) immunoreactivity. Note that comparing to the unrelated, the pathological cases have large blood vessels (dilatation) and more in numbers. A: COPD, B: Hypertension, C: Diabetes (x40)

Von Willebrand Factor (vWf, Factor VIII: F8)

a. Normal Cases

Incubation of the normal human carotid body with antibodies to Von Willebrand's factor led to intense staining of the endothelial cells of the blood vessels within the carotid body. (Fig. 5.20). Note how the vessels are not confined to the connective tissue stroma but are also found within the cell groups.

b. Pathological Conditions

Chronic hypoxaemia, essential hypertension and diabetes all produced essentially similar changes in the vasculature of the carotid body. (Fig 5.21 a, b, & c). These changes consisted of an increase in the number of vessels and also dilatation of the vessels.

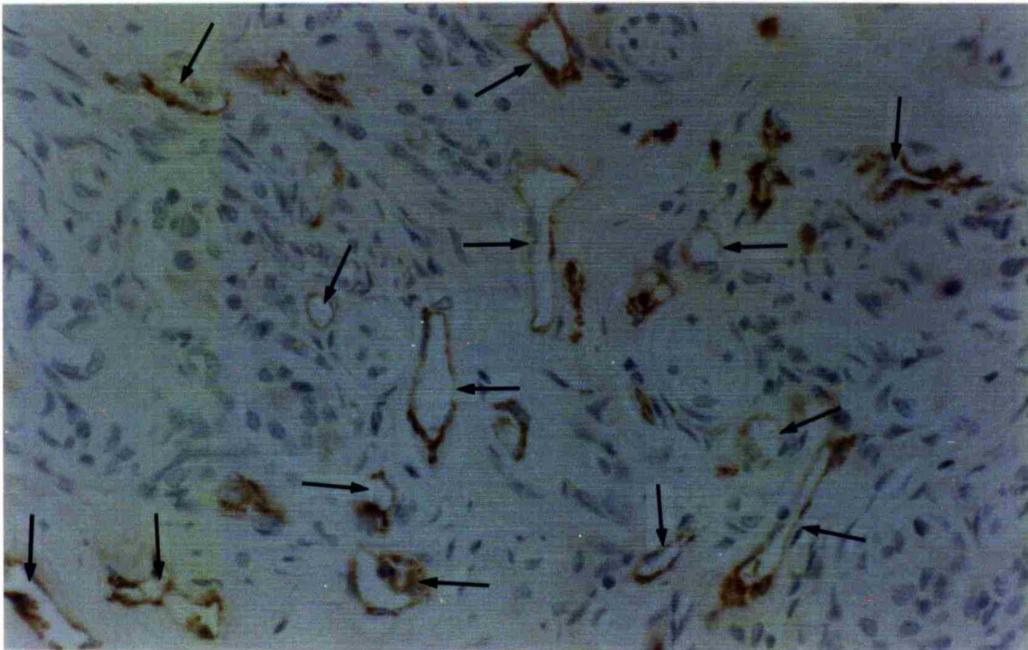


Fig.5.20 shows photomicrograph of a carotid body (unrelated case) with vWf immunoreaction in the wall of blood vessels (arrows). (x40)

Vimentin

All constituents of human carotid body except Type I cells reacted positively to vimentin antibody incubation. As such, the staining pattern was of little use in defining changes in the different pathological groups (see Fig 5. 22).

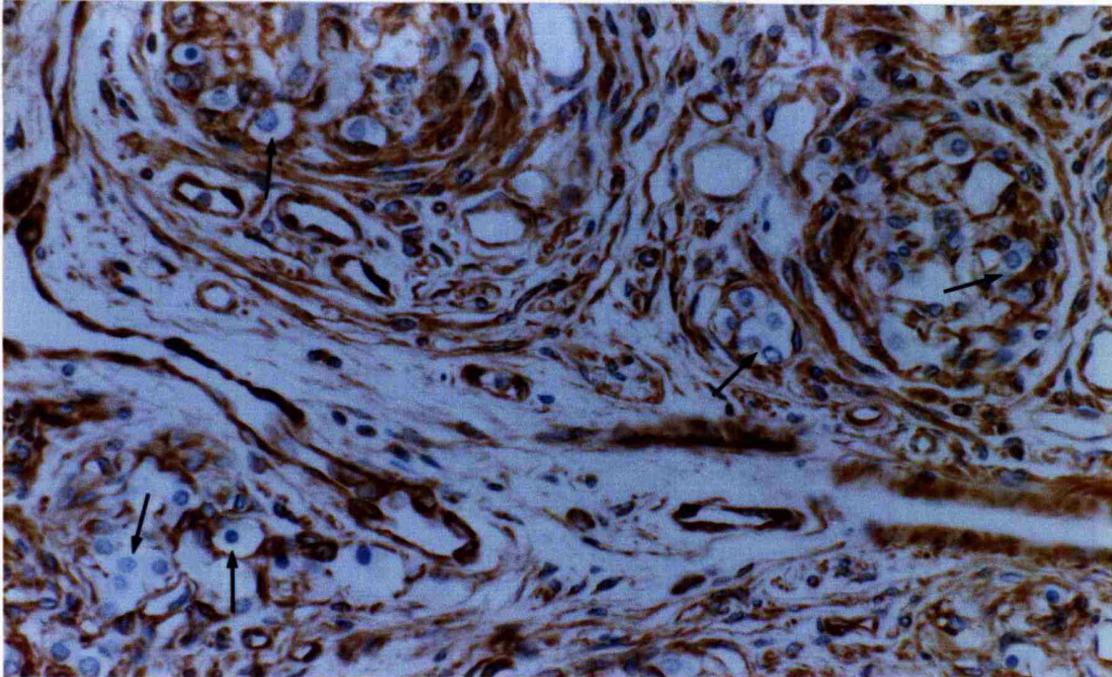


Fig 5.22 illustrates an example of vimentin immunoreactivity in the human carotid body. Note that only Type I cells (arrows) were not positive to Vimentin.

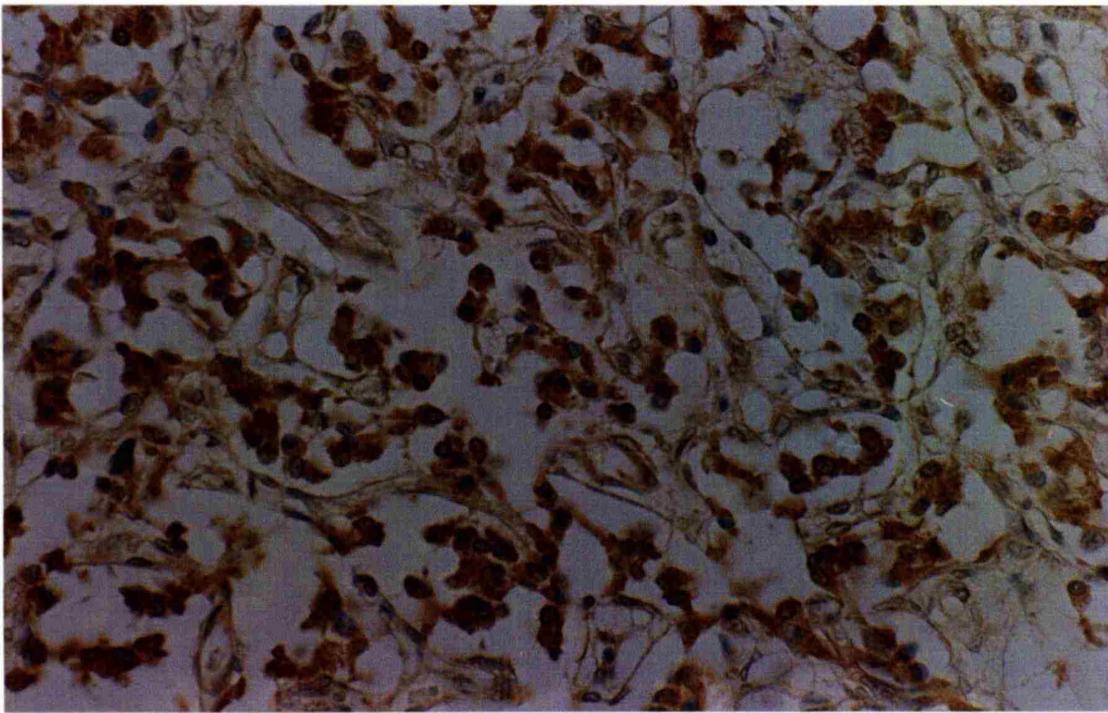


Fig 5.23c. A chemodectoma case reacted with anti-synaptophysin immunoreactivity in the numerous Type I cells of the carotid body. Note how all of the tumour cells are synaptophysin positive and their cytoplasmic connections to each other. (x40)

A Single Case of Chemodectoma

In one specimen, from a patient with essential hypertension, a large part of the carotid body was occupied by a chemodectoma. The immunoreaction of the tumour cells with anti synaptophysin, S100 and neurofilament is shown in Fig 5.23a, b, & c). Synaptophysin immunoreactivity was strongly positive in all of the cells within the tumour. There were few S100 positive cells save for a few forming a capsule like structure around the periphery of the tumour and a few at the surface. In the case of neurofilament we were unable to detect any nerve fibres within the tumour. Notwithstanding these findings, the areas of normal carotid body tissue outside of the tumour had essentially normal distributions of these compounds (e.g. S100 in Fig 5. 23a).

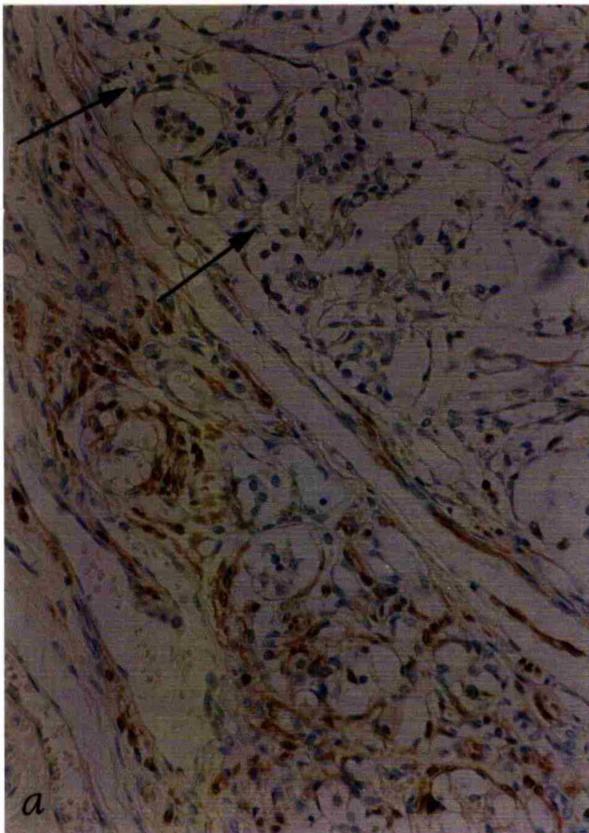


Fig 5.23a

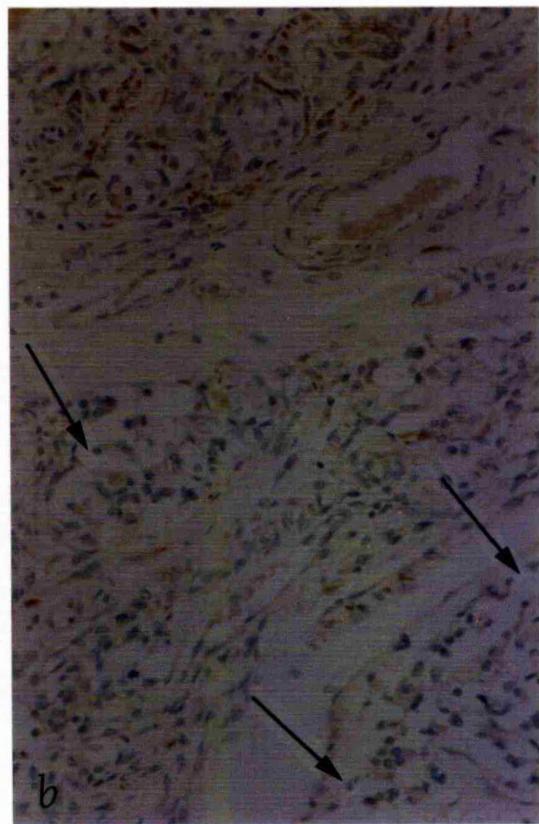


Fig 5.23b

Figs 5.23 a, and b. Showing immunoreactivity of S100 (Fig 5.23a), NF(Fig 5.23b), SYN (Fig 5.23c) antibodies in a chemodectoma case. Note that no immunoreactivity for S100 and NF is found within the chemodectoma (arrows) whilst there is reaction in the normal parts of the organ.(Fig 5.23a, and b:x20)

DISCUSSION

The present study confirms that the different components of the human carotid body can be distinguished using appropriate immunocytochemical markers as suggested by Abramovici et al. (1991). As in the cat, the nervous constituents of the organ can be delineated by antibodies to neurofilament protein and the Type II cells with antibodies to S100. In addition to this Type I cells contain synaptophysin, and blood vessels react positively with antibodies against Factor VIII.

Synaptophysin has been reported as a good marker for the identification of Type I cells of human carotid body (Habeck & Kummer 1993; Habeck et al. 1994). The result of the present study confirms their reports. However not all Type I cells were stained even within a single cluster of cells. It is possible that this may reflect the fact that even with very short postmortem delays some Type I cells are severely damaged (see EM section in Chapter 2) or a different functional state of the cells. Alternatively we have found some cells by EM which have the nuclear characteristics of Type I cells but which lack any electron dense-cored vesicles within their cytoplasm. As synaptophysin is associated with the vesicles in neuronal tissue such cells would be expected to show a negative staining reaction.

S100 antibodies mark the cells in three locations within the carotid body. Those cells associated with bundles of nerve fibres are certainly Schwann cells whilst those cells with elongated nuclei at the periphery of the cell clusters are probably Type II cells. The cells within the clusters which are plump and round and possess the nuclear characteristics of Type I cells which are apparently S100 positive cannot be identified positively. The EM study reported in Chapter 2 confirms the presence of round cells lacking electron dense-cored vesicles. Whether they represent a variant of Type I cells or the nuclei of the Type II cell which have been sectioned transversely is not clear. EM immunocytochemistry may provide an answer to this dilemma.

One other interesting feature of the staining reaction with S100 was the fact that some cells with elongated nuclei at the periphery of the cell clusters were S100 negative. Cells with the morphology and position so described would undoubtedly be recorded as Type II cells in sections stained with H&E. Whether there is a

population of Type II cells which lacks S100 or whether they represent a different cell type is unclear. It is clear, however that in any other tissue such cells might be identified as fibroblasts and it is thus possible that, particularly in hypertension and hypoxaemia, there can be proliferation of both Type II cells and connective tissue cells.

Heavy background and/or very weak immunoreactivity for some antibodies (namely, NF, SYN, VIM) were found in some cases in which the postmortem delay was greater than 8-10 hours. In part, this artefact is probably the result of autolysis due to postmortem delay. Why it only affected some cases is not clear. Similar findings have been reported for serotonin immunoreactivity in Type I cells of human carotid body where more serotonin immunoreactivity has been observed in sections with postmortem delays below 4 hours (Habeck et al., 1994).

The data reported here in cases of COPD and hypertension are different from those classically reported by Heath and his colleagues (Smith et al., 1982; Heath et al., 1984, 1985; Jago et al., 1984; Khan et al., 1990; Heath, 1991; Heath and Smith, 1992). To date, it had always been assumed that all cases of COPD and hypertension were associated with hyperplasia of Type II cells and a diminution in the number of Type I cells. Such a description is totally different from the situation seen in the animal model of chronic hypoxaemia where there is marked hypertrophy and hyperplasia of Type I cells (Dhillon et al., 1984; Barer & Pallot, 1984, and Bee et al., 1986, 1989). In the hypoxaemic and hypertensive cases reported here all of the carotid bodies had areas where the architecture was apparently normal. Examination of large numbers of microscope fields, however, showed that in most organs there were areas where there were indeed Type II cell hyperplasia and an apparent reduction in the number of Type I cells within the cell clusters associated with the hypertrophied Type II cells. In areas where this Type II cell hypertrophy occurred there was also an increase in the amount of axonal material visualised by antibodies to neurofilament protein. It is interesting to speculate on the apparent difference between these results and those previously reported.

All of the cases reported here came from the former East Germany. The standard of care available for the chronically ill was less developed than in the West and many of the patients only sought medical help towards the end of their disease.

Thus the intervention that in the West leads to increasing longevity in cases of COPD and hypertension was lacking in many of these patients. It is hence possible that the full blown picture of Type II cell hyperplasia described previously has had insufficient time to develop. The data here reported gives some support to this view as in all of the cases studied there were areas of Type II cell hyperplasia in COPD and hypertension which were localised. It might be argued, therefore, that Type II cell hyperplasia is a late result of these two conditions rather than the primary response of the carotid body.

Why should there be a proliferation of Type II cells in the carotid body in cases of hypertension and chronic hypoxaemia? The data from animal studies of hypoxic carotid body are unequivocal. There is hypertrophy and hyperplasia of Type I cells (Bee et al.1986) leading to increased size of the Type I cell nests. The data presented in Chapter 4 shows that the cell clusters are increased in size in both hypoxaemia and hypertension in the absence of overall Type II cell proliferation; therefore it is likely that the increased cell cluster size is due to the Type I cell component as was found in animal studies. It would seem that some factor then leads to the development of the Heath picture of Type II cell proliferation and reduction in the number of Type I cells. It may be that repeated stimulation of the Type I cells by low arterial oxygen partial pressure leads to the death of Type I cells. Perhaps then the axons, deprived of their target cells undergo sprouting, winding around the cell clusters and producing the characteristic picture of chronic hypoxia. The increased amount of axonal material detected by antibodies to NF reported above is at least consistent with this idea as is the reduced number of Type I cells detected by SYN reactivity in the proliferative areas. Direct experimental evidence of such a mechanism is lacking and as such this idea is purely hypothetical (see also Habeck, 1994).

The reactions with Factor VIII antibody provide a simple method for comparing the amount of vasculature in the different cases. From these purely descriptive studies it is clear that the amount of vasculature is increased in cases of hypertension and hypoxaemia. In animal studies of hypoxia a similar finding has been reported (Dhillon et al. 1984).

The finding of the chemodectoma was fortuitous. However, it was useful to be able to demonstrate that the tumour consisted exclusively of modified Type I cells

which could be marked with synaptophysin and that there was an absence of nerve fibres and Type II cells within the tumour. The strong immunoreactivity of synaptophysin suggests that this reaction may be useful in the diagnosis of metastases of chemodectomas.

It must be emphasised that all of the observations reported here are based on observation. The methods used here, however, would provide a simple technique for performing quantitative studies on the human carotid body in various situations. Thus by using antibodies to synaptophysin, neurofilament protein, Factor VIII and S100 it would be possible to quantify the relative contributions of Type I cells, Schwann & Type II cells and blood vessels to the total volume of carotid body tissue. I have tested this possibility and found it to be feasible but was prevented from performing such a study due to the non-availability of equipment.

CHAPTER SIX

FINAL DISCUSSION

The general histological and ultrastructural features of the human carotid body are essentially similar to those seen in other mammals. The carotid body consists of groups of specific cells set in a highly vascular connective tissue stroma containing numerous nerve fibres. The functional unit of the carotid body, the cell clusters or glomoids, consist of two different types of cells, namely the Type I and Type II cells. A variable number of polygonal Type I cells are surrounded by Type II cells.

As mentioned earlier, Heath and his colleagues (Heath et al., 1970; Smith et al., 1982; Heath and Smith, 1992) described a similar structure for human carotid body except that they were able to recognize three subtypes of Type I cells (light, dark, and pyknotic cells) mainly on the basis of the nuclear morphology of the Type I cells. The data reported here throws some doubt on their interpretation. Like Heath and his colleagues, I also observed three varieties of Type I cells in the human and rat carotid bodies which were obtained some time after the death of the subject. However, the quantitative studies of the distribution of Type I cell variants in both the rat and human carotid bodies at different times after death provide data that they do not represent the *in vivo* situation, but rather the effect of delays in fixation (autolytic changes) on the Type I cell. The inverse relationship between percentage of light cells and pyknotic cells, the increase in occurrence of pyknotic cell with time together with decrease in the number of light cells, provide very good evidence that pyknotic cells are a postmortem artefact. Additional, and confirmatory evidence of this view, has been obtained from the data from the carotid bodies that were divided into two pieces and fixed at different times postmortem where the number of light cells decreased with time whilst the number of pyknotic cells increased.

In addition, the ultrastructural study of human carotid body provides supportive evidence that dark and pyknotic cells are postmortem artefacts in that the vacuolation of the cytoplasm was found commonly in dark cells and most commonly in pyknotic cells and much less frequently in light cells.

The presence of epithelial-like cells close to Type I cell and their relationship both with each other and Type I cells is interesting. Electron microscopic and immunocytochemical studies are necessary to obtain adequate information about their structure and function. The nervous elements in the human carotid body appear to consist of many more nerve fibres within the humans organ than either cat or rat carotid body.

To obtain adequate quantitative data about the human carotid body ultrastructure, performance of further EM studies, with adequate numbers of specimens is necessary; they were not available during the course of this study.

As reviewed in Chapter 1, the effect of various pathological conditions has been reported in experimental animal and human carotid body. There is evidence that in chronic hypoxaemia and systemic hypertension there is an increase in the size of the organ (Heath et al., 1970, 1982; Dhillon et al., 1984; Habeck, 1986; Barer et al., 1987; Pallot, 1987; Heath and Smith, 1992; and Bee and Horward, 1993). The present study confirms previous studies that chronic hypoxaemia and hypertension lead to an increase in size of the carotid body. In addition, it provides data that thoracic carcinoma and diabetes, as well as chronic infection also alter carotid body structure. However, the data concerning thoracic carcinomas needs further study, due to insufficient number of cases (only four cases and very variable data obtained). Although, 50% of the cases of diabetes mellitus that were studied also had hypertension as a complication of the diabetes, even in the absence of this complication, diabetes led to a significant change in both the cell clusters and connective tissue area fraction. In order to clarify the effects of diabetes on the carotid body structure, an experimental study in diabetic rats has been started recently.

The stereological studies provided some new data. Hence, the carotid bodies in chronic hypoxia have bigger lobules and bigger cell clusters but the main structure, at least with regard to these features, remains unchanged. In the hypertension group the lobules and cell clusters are also bigger; however, the area fraction of cell clusters is significantly reduced from normal whilst that of

the tissue interlobular connective tissue element is increased. As a result of hypertension, there appears to be a fibrosis of the carotid body and decrease in the relative amount of specific cellular material.

It must be mentioned that in the human study good control material group is very difficult to obtain. It is also very difficult to say only one disease as a cause of death. Especially, in older cases so many parameters may have been involved as a cause of death which may themselves have altered the carotid body structure.

Using immunocytochemistry techniques the analysis of cell constituents of the human carotid body confirms that the different components of the human carotid body can be distinguished using appropriate immunocytochemical marker as suggested by Abramovici et al. (1991). In human carotid body the nervous constituents of the organ were demonstrated by antibodies to neurofilament protein and the Type II cells (and/or Schwann cells) with antibodies to S100 protein.

The data reported in Chapter 5, in cases of COPD and hypertension are different from those classically reported by Heath and his colleagues (Smith et al., 1982; Heath et al., 1984, 1985; Jago et al., 1984; Khan et al., 1990; Heath, 1991; Heath and Smith, 1992). To date, it had always been assumed that all cases of COPD and hypertension were associated with hyperplasia of Type II cells and a diminution in the number of Type I cells. Such a description is totally different from the situation seen in the animal model of chronic hypoxaemia where there is marked hypertrophy and hyperplasia of Type I cells (Dhillon et al., 1984; Barer & Pallot, 1984, and Bee et al., 1986, 1989). In the hypoxaemic and hypertensive cases reported here all of the carotid bodies had areas where the architecture was apparently normal; this is not in accord with previous reports. Examination of large numbers of microscope fields, however, showed that in most organs there were areas where there were indeed Type II cell hyperplasia and an apparent reduction in the number of Type I cells within the cell clusters associated with the hypertrophied Type II cells. In areas where this Type II cells hypertrophy occurred there was also an increase in the amount of axonal material visualised by antibodies to neurofilament protein. The arguments outlined in Chapter 5, to explain the discrepancy of data suggests a long term study of hypoxia in animals might be interesting.

The stereological studies of hypertension suggested that fibrosis of the carotid body was a feature of this condition. A study using double labelling technique for fibronectin antibodies (Dako- A245, P246 or Sigma F 3648) as a marker of fibroblast and S100 protein as a marker of Type II cells would provide useful data on the relative proliferation of the Type II / Schwann cells and fibroblasts.

The endothelial cells of the wall of the blood vessels react positively with antibodies against Von Willebrand factor (vWf, Factor VIII: F8). The positive immunoreactivity for vWf provides a simple method to compare the amount of vasculature in the different pathological conditions. It is clear that subjectively, the light microscopic observation of all the cases provides data that there is an increase in the amount of vasculature of hypertensive and hypoxic cases. This observation is consistent with early reports in animal studies (Dhillon et al., 1984) and could be tested quantitatively in the human.

As a result of the present work, some future studies are needed to clarify the structure and ultrastructure of human carotid body using an adequate number of specimen which should be obtained as soon as possible after death. In addition to that, future EM immunocytochemical studies are necessary in order to finalise the discussion of epithelial-like cells. In particular, an adequate numbers of specimens are also necessary to finalise the level of structural changes in human carotid body in cases of thoracic carcinoma. In order to examine the effects of diabetes mellitus on the carotid body I have already started an examination of the carotid body in diabetic patients and experimental animals.

The apparent differences between the effects of chronic hypoxia in animals and humans require further study. As outlined in chapter 5, it is necessary to study much longer periods of hypoxia on experimental animals in order to see whether these longer exposures result ultimately in Type II cell hyperplasia.

Finally, the immunocytochemical data offers the chance to obtain quantitative data of the various pathological conditions on the human carotid body (see chapter 5).

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APPENDICES

Appendix 2.1*

ETHANOL FIXATIVE

Keep tissue in absolute or 95 % alcohol for 12- 24 hours with three changes at room temperature.

ZAMBONI'S FIXATIVE

(4% Paraformaldehyde - 0.2% Picric Acid)

1-Phosphate Buffer (0.2M):

Sodium Phosphate, Dibasic (Na_2HPO_4 , Molecular Weight 141.96).....	22.8g
Potassium Phosphate, Monobasic(KH_2PO_4 , M.W.136.09).....	5.4g
Distilled Water.....	1litre

Mix and adjust pH to 7.4

2-Paraformaldehyde:

On a thermomixer in a fumehood, heat and dissolve 80g paraformaldehyde in 696ml distilled water. Add a few drops of 10N NaOH until the solution clears. Cool and filter.

3-Add in order

0.2M Phosphate Buffer, pH 7.4.....	1000ml
Saturated Picric Acid in distilled water.....	300ml
Paraformaldehyde.....	696ml

Check pH, and adjust if necessary to pH 7.2-7.4

Tissue Processing

- 1- Rinse tissue briefly in running water,
- 2- Hold in 70% or 80% alcohol,
- 3- Wash x3 times in 95% alcohol; 60 minutes each,
- 4- Wash in absolute alcohol, x3 times; 60 minutes each,
- 5- Equal parts absolute alcohol and xylene; 60 minutes,
- 6- Xylene, 2 changes; 60 minutes each,
- 7- Paraffin, 3 changes, 60 minutes each,
- 8- Paraffin, under vacuum, 60 minutes,
- 9- Embed

**Appendices are numbered in relation to chapters to which they refer.*

H&E Staining Procedure

- 1-Label slides with pencil
- 2-Deparaffinize and Rehydrate slides (2 or 3 minutes per step) through :
 - Xylene x2,
 - Absolute ethanol x2,
 - 90% Ethanol,
 - 70% Ethanol,
 - 50% Ethanol,
 - Distilled water,
- 3-Stain in Haematoxylin solution for 15 minutes
- 4-Wash in running tap water for 15 minutes
- 5-Place in distilled water
- 6-Place in 80% ethanol for 2 minutes
- 7-Counterstain in eosin solution for 2 minutes.
- 8-Dehydrate and clear through two minutes and two changes each of
 - 70 % Ethanol,
 - 90 % Ethanol,
 - 100% Ethanol,
 - Xylene
- 9-Mount with DPX.

Solutions for Haematoxylin and Eosin Staining

Harris' Haematoxylin

Haematoxylin crystals	5g
Absolute ethanol	50ml
Potassium or Ammonium Alum	100g
Mercuric oxide red	2.5g
Distilled water	1000ml

1. Dissolve Potassium Alum in Distilled water on a thermomixer
2. Dissolve Haematoxylin in Alcohol in a small flask
3. Slowly combine two solutions, return the combined solution to the heat and bring to boiling point as rapidly as possible (within 1 minute).
4. Remove from heat and slowly add Mercuric oxide and return to the heat until it becomes a dark purple colour.
5. Remove from the heat and plunge into cold water to cool

6. Add 20ml of Glacial Acetic Acid and filter before use.

Eosin Stock (1%)

1g Eosin (water soluble) dissolve in 20 ml water and add 80 ml of 95% ethanol

Working solution

Stock eosin.....	1 part
80% Alcohol.....	3 part

Add 0.5ml Glacial acetic acid to each 100ml of solution, (just before use).

Appendix 2.2

Transmission Electron Microscopy Processing Schedule

Fixation

3 hours in 2.5% Glutareldehyde in Cacodylate buffer (0.1M-pH 7.4) or in Karnowsky's fixative at room temperature.

Trim the tissues into small pieces(2-3mm) and wash 5 times in Cacodylate buffer.

Leave overnight in fridge.

Postfixation

- 1- Osmium tetroxide for 1 or 3 hours
- 2- Wash in Cacodylate buffer eight times for 15 minutes
- 3- Dehydrate through 50% Ethanol (2 changes of 15 minutes), 70% (2 changes of 15 minutes), 95% (2 changes of 15 minutes), 100% (5 changes of 15 minutes), and Propylene oxide (2 changes of 15 minutes).
- 4- Equal parts Propylene oxide and Resin (Agar 100) for 1hour
- 5- One part of Propylene oxide and two parts of Resin for 1 hour
- 6- One part of Propylene oxide and three parts of Resin for 1 hour
- 7- Resin overnight
- 8- Embed and Polymerize at 60°C in oven for 24 hours.

Cacodylate Buffer (0.2M), pH. 7.4)

Sodium Cacodylate (M.Wt.214.03).....	42.8g
Distilled water	1000ml

Mix them properly and adjust the pH to 7.4

Phosphate Buffer (0.2M, pH 7.4)

KH ₂ PO ₄ M.Wt.136.09)	5.4g
Di Sodium Hydrogen Orthophosphate, Na ₂ HPO ₄ (M.Wt. 141.96).....	22.8g

Mix to 1000 ml of Distilled water and check the pH
Dilute 1:1 with Distilled water before use

Postfixation and Embedding

1- Working phosphate buffer, 3 changes.....	15 minutes each
2- Osmium tetroxide 1.0%, phosphate buffered.....	1 hour
3- Distilled water, 4 changes.....	15 minutes each
4- Uranyl acetate, 1% aqueous.....	1 hour
5- 50% ethyl alcohol.....	15 minutes
6- 75%ethyl alcohol.....	15 minutes
7- 95% ethyl alcohol.....	15 minutes
8- 100% (absolute) ethyl alcohol, 4 changes.....	15minutes each
9- Equal parts 100% ethyl alcohol and propylene oxide.....	15 minutes
10- Propylene oxide, 4 changes.....	15 minutes each
11- Equal parts propylene oxide and epoxy resin.....	1 hour
12- Epoxy resin, 3 changes.....	1 hour each
13- Epoxy resin.....	2 hours
14- Embed	

Semi-thin Section Staining (2% Toluidine Blue Staining)

Solutions

1% Sodium Borate	
Sodium borate.....	1g
Distilled water.....	100ml
2% Toluidine Blue O in Sodium Borate	
Toluidine blue O.....	2g
Sodium borate 1%,.....	100ml

Staining Procedure

- 1- Flood sections with the toluidine blue solution.
- 2- Place slides on a hot plate(80°-90°C) for 1minute. Edges of the stain will have a sheen.
- 3- Wash in distilled water.
- 4- Air dry completely
- 5- Coverslip with DPX.

Thin Sectioning Procedure :

The block selected for thin section is placed in the block holder and with the aid of the stereoscope is trimmed smaller for thin sections. The trapezoid shape is retained and the unwanted areas eliminated.

- 1- Place trimmed block in the ultramicrotome
- 2- Using a diamond or glass knife, cut the thin sections at the desired thickness.
- 3- Collect the thin sections in a trough.
- 4- Examine the thin sections using the stereoscope binoculars. Only those sections with a silver or pale gold colour (50-60nm) are collected
- 5- Pick up sections by inserting into the trough, a 200mesh grid held with forceps. The grid is brought under the sections and then raised.
- 6- Drain the grid on filter paper.
- 7- Store grids in petri dishes, on filter paper labelled with the identification numbers

Manual Grid Staining

Solutions

70% Ethanol
Ethanol..... 35ml
Sterile water..... 65ml

4% Uranyl Acetate in Ethanol
Uranyl acetate..... 4g
Ethanol, 70%..... 100ml

Sodium hydroxide
1%-1g Sodium hydroxide in 100 ml of Distilled water

Lead Citrate
Lead nitrate..... 1.33g
Sodium citrate..... 1.76g
Sterile water..... 30.0ml

Shake the above ingredients in a 50 ml flask for 20 minutes. Add 8 ml of 1N sodium hydroxide. Dilute to 50 ml with sterile water. Mix by inversion. The solution is ready for use when completely clear.

Procedure:

- 1- Place 5 ml of uranyl acetate solution and 5 ml of lead citrate solution in separate screw top vials, Falcon tubes 13x100 mm, then centrifuge each at 1500 rpm for 5 minutes.
- 2- Prepare two petri dishes, lining each with a square of dental wax. (Label petri dishes e.g. *1 and *2)
- 3- Place several drops of centrifuged uranyl acetate on wax in petri dish *1, one drop of uranyl acetate for each grid to be stained. Keep dishes covered before and during staining.
- 4- Place sodium hydroxide pellets under wax in petri dishes*2.
- 5- Place several drops of lead citrate on wax one drop for each grid to be stained.
- 6- Transfer grids, specimen side down, with the aid of fine forceps, onto the uranyl acetate drops in petri dishes*1. Stain in covered dishes for 10 minutes.
- 7- Remove grids with fine forceps.
- 8- Wash well with sterile water using a syringe.
- 9- Dry grids on clean filter paper or lens paper.
- 10-Stain in lead citrate, Petri dish *2, for 5 minutes.
- 11-Remove grids with fine forceps.
- 12-Wash well with sterile water using a syringe.
- 13-Dry grids on clean filter paper or lens paper.

Appendix 3.1. Table showing age, sex PM time and cause of death of patients whose carotid bodies were used in this chapter.

	Group	Case No	Age	F/M	P.M.time	Diagnosis or Main Disease
1	1st	570	76	F	2.15	M.I. + D.M.
2		498	46	M	2.3	Pneumonia/Control
3		748	76	F	2.45	Thrombembolism -Pneumonia / COPD
4		729	59	M	2.45	Pancreatitis + D.M. /Sepsis
5		720	72	M	2.45	Carcinoma of the Stomach /E.H.
6		493	76	F	3	Pneumonia-Stroke + D.M. /E.H.
7		610	77	M	3	M.I.-Obi.coronary sclerosis + D.M.
8		719	58	M	3.15	M.I.-Thromb.of the lung /E.H.
9		507	76	M	3.15	E.Hypert. + D.M.
10		806	78	M	3.3	Bronchopneumonia/MI /COPD
11		763	65	M	3.45	Lung CA-Thrombembolism /TCA
12		39/91	79	F	4	MI-Ac.coronary Insuf.-obi.cor. sclerosis /E.H.
13		901	75	F	4	Leukemia + D.M.
14		344/91	80	F	4	M.I. /Control
15		710	64	M	4.45	P.hypert-obliter.pleuritis /COPD
16		780	61	M	5	Thrombembolism of the lung + D.M.
17		477	85	F	5.15	M.I.-coronary obli.+ D.M. /E.H.
18		13/91	69	F	5.15	M.I.+ Icterus + Liver CA + D.M. /Sepsis
19		38/91	82	F	6	Interstitial lung fibr. E.Hyp. Ac.Insuf.R.heart /COPD
20		843	21	F	6	Toxic shock(Pregn.)Streptococci /Sepsis
21		48/91	81	F	6	E.Hypertension
22		419/91	83	F	6.45	E.Hypertension
23		399/91	60	M	6.45	Lung carcinoma/TCA
24		44/91	68	F	7	Acute pyelonephritis + D.M. /E.H.
25		45/91	74	F	7	Amp.of a Leg-Thrombemb.of lung+ D.M. /E.H.
26		789	57	M	7	Leukemia-lymphatic
27		917	62	F	7.3	Bronchopneumonia-P.hypert. /COPD
28		265/91	55	M	7.3	Cirrhosis of the liver /Control
29		66-91	59	F	7.3	Cirrhosis of the liver/In. cereb. bleeding/Control
30		317/91	65	F	7.3	Carcinoma of the thyroid *
31	2nd	725	75	F	8	M.I.-Obi.coronary sclerosis + D.M./E.H.
32		760	56	M	8	M.I. - atherosclerosis + D.M.
33		810	47	M	8	Bronchial CA + D.M. /TCA
34		286/91	55	F	8	Pleisomocytom
35		305/91	51	M	8	Bronchial carcinoma /TCA
36		329/91	48	M	8.15	M.I. /E.H.
37		60/91	55	M	8.15	Intra cerebral bleeding+ E.H.
38		848	88	F	8.15	B.pneumonia-Emphysema /COPD. Sepsis
39		19/91	84	F	8.15	Anevrysm of the aorta-rupture /Control
40		266/91	60	M	8.3	Insufficiency of the coronary arter /E.H.
41		499	61	M	8.3	P.hypert-antroacilicosis lung /COPD
42		313/91	72	F	8.45	M.I. /E.H.
43		642	30	F	9	Sepsis-Endomyometritis
44		648	35	M	10	M.I. /Control
45		397/91	84	F	10	E.H./Insufficiency of the coronaria

	Group	Case No	Age	F/M	P.M.time	Diagnosis or Main Disease
46		252/91	68	M	11	Rectal CA / E.H.
47		809/90	53	M	11	M.I. / E.H.
48		31-91	56	F	12	Cervic.CA + E.Hypertension
49		395/91	54	M	12	Thrombosis of coronary artery / E.H.
50		804	61	M	12	M.I. - Thromb. of the coronary arter / E.H.
51		638	62	M	12	Thrombembol lung/Pleuritis / COPD, Sepsis
52		831	72	M	12	COPD
53		332/91	85	M	12	Cirrhosis of the Liver / Control
54		772	77	M	13	Chr.bronchitis / P.hypert. / COPD
55		347/91	84	F	13	Breast Carcinoma
56		625	46	M	14	Pulmonary hypert./Lung resection / COPD
57		247/1	47	F	14	Tumor of the brain
58		30/91	68	M	14.3	E.Hypertension + D.M.
59	3rd	53/91	19	F	15	Thrombosis of cerebral veins / Control
60		269/91	82	F	15	Breast carcinoma *
61		33/91	51	M	18	Coronary a. a. sclerosis / E.H.
62		262/91	57	M	18	Cirrhosis of the Liver / Control
63		90/91	49	M	18	Bronchial CA / TCA
64		29/91	46	M	19	Diabetic coma + D.M. / E.H.
65		24/91	63	M	19	Thrombembol lung / P.hypert. / COPD
66		17/91	59	F	20	COPD
67		287/91	61	M	20	Colon CA / E.H. / D.M.
68		280/91	78	F	20.3	Mesenteric embolism / E.H.
69		294/91	75	F	20.3	Ovary CA / D.M.
70		248/1	68	M	21	Carcinoma of the thyroid / D.M.
71		300/91	52	F	22	Insufficiency coronaria / E.H.
72		295/91	62	F	22	Insufficiency of the coronaria / D.M.
73		284/91	28	M	27	Chronic Pancreatitis / Control
74		247/2	47	F	32	Tumor of the brain
75		248/2	68	M	38.3	Thyroid CA / D.M.

Abbreviations for terms used describe the diagnosis and main disease in the appendices are:

Control: Unrelated (accepted as a normal), COPD: Chronic Obstructive Pulmonary Disease, E.H.: Essential Hypertension, D.M.: Diabetes Mellitus, TCA: Thoracic Carcinoma, MI: Myocardial Infarction.

Appendix 4.1. Table showing age, sex, PM time and cause of death of patients whose carotid bodies were used in this chapter.

	Case number	Age	M/F	Lobule	Conn-tissue	Cell Cluster	Diagnosis or Main Diseases
1	16k-91	2	M	0.2024	0.0932	0.1092	Mediastinal Tumor/(T.CA)
2	18K-91	12	F	0.0982	0.0425	0.0556	Spinal tumor
3	53.91	19	F	0.1205	0.0679	0.0526	thrombosis of cerebral veins/Control
4	69.91	26	F	0.0625	0.0323	0.0302	Pneumonia+thromboemb. of the lung/Control
5	642	30	F	0.0874	0.0573	0.03	Sepsis-Endomyometritis
6	648	35	M	0.0829	0.0534	0.0296	MI/Control
7	32.91	41	M	0.1081	0.0653	0.0428	Nasopharyngeal CA
8	29.91	46	M	0.1473	0.0999	0.0474	Diabetic coma+ D.M. /(E.H.)
9	625	46	M	0.1739	0.1092	0.0646	Pulmonar hyper. Lung resection / COPD
10	247.1	47	F	0.1356	0.0728	0.0627	Tumor of the brain
11	810	47	M	0.1794	0.1247	0.0547	Bronchial CA + D.M. / (T.CA)
12	247.2	47	F	0.1051	0.0619	0.0431	Brain CA
13	329.91	48	M	0.1979	0.1419	0.056	MI / (E.H.)
14	90.91	49	M	0.1828	0.0884	0.0943	Bronchial CA / (T.CA)
15	15.91	50	F	0.0952	0.0592	0.0359	Hepatic coma/after liver transpl. + D.M
16	33.91	51	M	0.179	0.1198	0.0592	Coronary oblit.sclerosis / (E.H.)
17	64.91	51	F	0.0804	0.05	0.0304	Thromboembolism of the lung+ D.M *
18	300.91	52	F	0.1276	0.0823	0.0452	Insufficiency coronaria / (E.H.)
19	809	53	M	0.1219	0.0765	0.0453	MI / (E.H.)
20	395.91	54	M	0.1615	0.1024	0.0591	Thrombosis of coronary artery / (E.H.)
21	60.91	55	M	0.0921	0.0524	0.0396	Intra cerebral bleeding / (E.H.)
22	265.91	55	M	0.0869	0.0528	0.034	Cirrhosis of the liver/ Control
23	31.91	56	F	0.0818	0.0555	0.0262	Cervic CA
24	760	56	M	0.1431	0.0957	0.0473	MI- atherosclerosis+ D.M
25	262.91	57	M	0.13	0.0736	0.0564	Cirrhosis of the liver /Control
26	789	57	M	0.1057	0.0489	0.0568	Leukemialymphatic
27	719	58	M	0.1484	0.0888	0.0596	MI-Thromb. of the lung / (E.H.)
28	17.91	59	F	0.1819	0.1069	0.075	COPD
29	66.91	59	F	0.0757	0.0451	0.0305	Cirrhosis of the liver-Pneumonia /Control
30	729	59	M	0.2184	0.1583	0.06	Pancreatitis + D.M. /Sepsis
31	266.91	60	M	0.1198	0.0749	0.0449	Insufficiency of the coronary arter / (E.H.)
32	287.91	61	M	0.1585	0.1193	0.0391	Colon CA / (E.H.)+ D.M.
33	499	61	M	0.1405	0.0693	0.0712	P.hypert-arteriosclerosis lungs / COPD
34	780	61	M	0.1133	0.0765	0.0367	Thromboembolism of the lung+ D.M.
35	804	61	M	0.1428	0.1083	0.0345	MI/Thromb. of the coronary arter / (E.H.)
36	638	62	M	0.1129	0.0627	0.0502	Thromboembol lung Pleuritis / COPD, Sepsis
37	917	62	F	0.1362	0.0827	0.0535	Bronchopneumonia-P. hypert / COPD
38	24.91	63	M	0.1188	0.0756	0.0431	Thromboembol lung-P.hypert / COPD
39	710	64	M	0.2574	0.1814	0.076	P.hypert-obliter. pleuritis / COPD
40	763	65	M	1.2719	0.9257	0.3462	Lung CA - thromboembolism / (T.CA)
41	30.91	68	M	0.1469	0.0968	0.05	F.Hyp+ D.M
42	44.91	68	F	0.0941	0.0621	0.032	Acute pyelonephritis + D.M. / (E.H.), Sepsis
43	248.1	68	M	0.0522	0.033	0.0191	Carcinoma of the Thyroid + D.M.
44	252.91	68	M	0.1414	0.0925	0.0489	Rectal CA / (E.H.)
45	248.2	68	M	0.0592	0.0427	0.0165	Thyroid CA + D.M
46	13.91	69	F	0.0887	0.0548	0.0339	MI+ Icterus + Liver CA /Sepsis
47	313.91	72	F	0.1632	0.0965	0.0666	MI / (E.H.)
48	720	72	M	0.1611	0.1083	0.0527	Stomach CA / (E.H.)
49	831	72	M	0.2463	0.1441	0.1021	COPD
50	45.91	74	F	0.0977	0.0674	0.0302	Amput. of a Leg/Thromboembol lung+ D.M. / (E.H.)
51	294.91	75	F	0.0866	0.0464	0.0402	Ovary CA+ D.M
52	725	75	F	0.1298	0.0706	0.0591	MI-Obt. coronary sclerosis+ D.M. / (E.H.)
53	493	76	F	0.0925	0.0626	0.0299	Pneumonia-Stroke + (E.H.)+ D.M.
54	507	76	M	0.1488	0.0893	0.0595	E.H. + D.M.
55	570	76	F	0.1102	0.0608	0.0494	MI + D.M.
56	748	76	F	0.1588	0.0937	0.0651	Thromboembolism-Pneumonia / COPD
57	772	77	M	0.1444	0.1	0.0444	Chr. bronchitis-P.hypert. / COPD
58	280.91	78	F	0.0927	0.0537	0.0389	Mesenteric embolism / (E.H.)
59	806	78	M	0.2769	0.1675	0.1093	Bronchopneumonia-MI / COPD, Sepsis
60	39.91	79	F	0.1353	0.0797	0.0555	MI-Acute coron. Insuff-obl. cor. sclerosis / (E.H.)
61	38.91	82	F	0.0708	0.0394	0.0314	Interstitial lung fibro. Es.-ac. Insuf. R. Heart / COPD
62	419.91	83	F	0.098	0.0573	0.0407	E.H.
63	19.91	84	F	0.0905	0.0493	0.0412	Aneurysm of the aorta-rupture /Control
64	332.91	85	M	0.1025	0.0632	0.0393	Cirrhosis of the Liver /Control
65	477	85	F	0.1471	0.091	0.056	MI- coronary oblit. + (E.H.)+ D.M.
66	848	88	F	0.1194	0.0678	0.0516	B. pneumonia-Embrysema/ COPD, Sepsis

Appendix 5.1. Table showing age, sex, PM time and cause of death of patients whose carotid bodies were used in this chapter.

	Case no	Age	F/M	P.M.D	Main Diseases & Diagnosis
1	374/90	83	F	3.3	Acute coronary insuf. / E.H.
2	431/90	67	M	6	Acute pancreatitis/insuf.of the heart/Sepsis
3	921/90	82	M	4.3	M.I. / E.H.+ D.M.
4	498/90	46	M	2	Pneumonia
5	499/90	61	M	8.3	P.hyper-duodenal ulcer/ COPD, Sepsis
6	638/90	62	M	12	Thrombemboli lung-Pleuritis / COPD
7	642/90	30	F	9	Endomymetritis, Sepsis
8	648/90	35	M	10	MI / Control
9	710/90	64	M	4.45	P.hyprt-obliter,pleuritis / COPD
10	719/90	58	M	3.15	MI-Obli.coronary sclerosis / E.H.
11	720/90	72	M	2.45	Carcinoma of the stomach / E.H.
12	725/90	75	F	8	MI-Obl.coronary sclerosis / E.H.+ D.M.
13	729/90	59	M	2.45	Ac.pancreatitis / Sepsis, + D.M.
14	748/90	76	F	2.45	Thrombembolism-Pneumonia / COPD
15	760/90	56	M	8	MI-atherosclerosis + D.M.
16	763/90	65	M	3.45	Lung CA-Thombembolism of lungs/TCA
17	772/90	77	M	13	Chr.bronchitis-P.hypert. / COPD
18	780/90	61	M	5	Trombembolism of the lung + D.M.
19	789/90	57	M	7	Leukamia-lymphatic / Control
20	804/90	61	M	12	MI-Thromb.of the coronary arter / E.H.
21	806/90	78	M	3.3	Bronchopneumonia-MI / COPD, Sepsis
22	809/90	53	M	11	MI / E.H.
23	810/90	47	M	8	Bronchial CA/ TCA +D.M
24	831/90	72	M	12	COPD
25	843/90	21	F	6	Toxic shock (Pregnancy) streptococal/ Sepsis
26	848/90	88	F	8.15	B.pneumonia-Emphysema/ COPD, Sepsis
27	901/90	75	F	4	Leukaemia / E.H. + D.M.
28	917/90	62	F	7.3	B.pneumonia-P.hypert. / COPD
29	13/91	69	F	5.15	Myocardial insuf.-icterus-Liver CA, Sepsis
30	16k/91	2	M	18	Mediastinal tumor
31	17/91	59	F	20	COPD
32	18k/91	12	F	16	Spinal tumor / Control
33	38/91	82	F	6	COPD
34	39/91	79	F	4	MI-Ac.coronary insuf.,obl.cor.sclerosis/ E.H.
35	44/91	68	F	7	Acute pyelonephritis, Sepsis, E.H.+D.M.
36	45/91	74	F	7	Amput.of a leg-Thr.embolism lung/ E.H.+D.M
37	53/91	19	F	15	Thrombosis of cerebral veins / Control
38	60/91	55	M	8.15	Intracerebral bleeding / E.H.
39	64/91	51	F	10	Thrombembolism of the lung + D.M.
40	66/91	59	F	7.3	Pneumonia,I.cerebral bleeding/ Control
41	69/91	26	F	20	Pneumonia,thrombemb. of the lung/ Control
42	90/91	49	M	18	Bronchial CA / TCA
43	252/91	68	M	11	Rectal CA / E.H.
44	262/91	57	F	18	Cirrhosis of the liver / Control
45	265/91	55	M	7.3	Cirrhosis of the liver / Control

	Case no	Age	F/M	P.M.D	Major Diseases & Diagnosis
46	266/91	70	F	8.3	Insuf.of the coronary arter / E.H.
47	269/91	82	F	15	Colon CA / Control
48	280/91	78	F	20.3	Mesenteric embolism / E.H.
49	284/91	28	M	27	Chronic Pancreatitis / Control
50	286/91	55	F	8	Plasmocytom / Control
51	287/91	61	M	20	Colon CA / E.H.+ D.M.
52	294/91	75	F	20.3	Ovary CA + D.M.
53	295/91	62	F	22	Insuf.of the coronaria + D.M
54	300/91	52	M	22	Insuf.of the coronaria / E.H.
55	305/91	51	M	8	Bronchial CA / TCA
56	313/91	72	F	8.45	MI / E.H.
57	317/91	65	F	7	Thyroid CA / Control
58	329/91	48	M	8.15	MI / E.H.
59	332/91	85	M	12	Cirrhosis of the liver / Control
60	344/91	80	F	4	MI / Control
61	347/91	84	F	13	Breast CA / Control
62	395/91	54	F	12	Thrombosis of coronary artery / E.H.
63	397/91	84	F	10	E.hyp.Insuf.of the coronaria / E.H.
64	398/91	84	F	6	Bleeding within Pons/Rheumatic SAV/Unrelated
65	399/91	60	M	6	Lung CA / TCA
66	419/91	83	F	6.45	M.I. / E.H.
67	52k/91	21w	M	17	Normal / Control
68	59k/91	37w	M	11	Normal / Control
69	245/91	64	M	20	Emphysema of the lungs / COPD
70	278/91	77	F	24	Cerebral embolism / E.H.+ D.M.
71	287/91	61	M	20	Colon CA / E.H. + D.M.
72	289/91	47	F	15.3	M.I. / E.H. +D.M
73	296/91	85	M	19	Cor kyphoscolioticum / Control
74	326/91	40	M	5.3	yolk sac CA of the retroperitoneum / Control
75	417/91	63	F	7.3	Chronic hypoxia / COPD
76	418/91	55	M	7.3	Normal / Control
77	A.L.	21	M	1.3	Traffic accident / Control

Appendix 5.2

Immunocytochemistry PAP Technique

- 1- Label slides with pencil
- 2- Rehydrate (2-3min. per step) through Xylene, Xylene, Absolute ethanol, Absolute ethanol, 90% Ethanol, 80% Ethanol, 70% Ethanol.
- 3- Block endogenous peroxidase activity by soaking sections in 5% H₂O₂ in Methanol for 30 minutes at room temperature (should be made fresh).
- 4- Distilled water bath 5min.
- 5- Wash in TBS x3 (0.05M, pH7.3,) or PBS (0.02m - pH7.4) with very gentle agitation for 5minutes.
- 6- Draw a circle around tissue sections using immunocytochemistry pen (Dako-S2002)
- 7- Incubate with a proteolytic enzyme (e.g. Trypsin Dako-S2012) at room temperature for 20-30minutes using freshly prepared solution (Optional step : for proteolytic demasking of antigens in formalin- fixed, paraffin embedded tissues; If required; such as FVIII, VIM).
- 8- Wash x3 in TBS or PBS 5min.
- 9- Dry off excess TBS or PBS with a soft tissue paper (around the tissue should be as dry as possible).
- 10- Add the non immune serum with a pipette, leave in a humidity chamber for 30 minutes at room temperature (to reduce the unspecific binding). For monoclonal antisera Normal Rabbit Serum (1:25 dilution); For Polyclonal antisera Normal Swine Serum (1:1-1:5 dilution).
- 11- Pour off the Non Immune Serum.
- 12- Add the primary antisera at optimum working dilution and incubate overnight at 4°C in a humidity chamber. Monoclonal; SYN:1:50, FVIII:1:20, VIM: 1:15, Polyclonal; NF200; 1:50, S100;1:100 dilution in TBS or PBS.
- 13- Wash x3 in TBS or PBS 5min.
- 14- Add the secondary antisera leave for 30min. at room temperature (for monoclonal antisera; Rabbit anti-mouse IgG; 1:40, for polyclonal antisera; Swine anti-rabbit IgG; 1:30)
- 15- Wash x3 in TBS or PBS 5min.
- 16- Add the PAP solution and leave 30 min. at room temperature (Dilution must not contain Sodium azide Na N₃).
- 17- Wash x3 in TBS or PBS 5 min.

- 18- Add 1 tablet of DAB* (3,3'-diaminobenzidine tetrahydrochloride; Sigma-D5905, Dako-S3000; 10mg) to 18ml of Tris or PBS once dissolved filter and add 0.3 ml of H₂O₂ (30% H₂O₂) to 10ml of filtered DAB (Dilution must not contain Sodium azide Na N₃).
- 19- React the slides with the DAB solution 5-7min (under observation; light brown colour should appear).
- 20- Wash the slides immediately in tap water 30 min. to stop the DAB reaction.
- 21- Counterstain lightly by Haematoxylin (30-45second) or Methyl green
- 22- Wash in the running tap water for 20 min.
- 23- Dehydrate and clear through 70%, 95%, Absolute ethanol and Xylene (two changes each) for 2-3 min.
- 24- Coverslip with DPX.

The Procedure of EPOS Immunoperoxidase Staining

- 1- Follow same procedure steps until the normal serum step (10th) of the PAP technique,
- 2- Incubate with EPOS/HRP antibodies for 45 minutes at room temperature **
- 3- Wash x3 in TBS or PBS 5min.
- 4- Add 1 tablet of DAB* (3,3'-diaminobenzidine tetrahydrochloride; Sigma-D5905, Dako-S3000; 10mg) to 18ml of Tris or PBS once dissolved filter and add 0.3ml of H₂O₂ (30% H₂O₂) to 10ml of filtered DAB (Dilution must not contain Sodium azide).
- 5- React the slides with the DAB solution 5-7min (under observation; light brown colour should be appeared).
- 6- Wash the slides immediately in tap water 30 min. to stop the DAB reaction.
- 7- Counterstain lightly by Haematoxylin (30-45second) or Methyl green
- 8- Wash in the running tap water for 20 min.
- 9- Dehydrate and clear through 70%, 95%, Absolute ethanol and Xylene (two changes per each) for 2-3 min.
- 10- Coverslip with DPX.

* After use DAB solution should be disposed by rinsing with a domestic bleach (sodium hypochlorite) to oxidize the used and unused DAB solution.

** Dako-EPOS reagents are ready to use and need no dilution.

TBS (Tris - Buffered Saline 0.05M - pH 7.2 or 7.4)

Trizma Base.....	4.00g
Trizma HCl.....	34.25g
Sodium Chloride.....	44.50g

Dissolve these in 5litres of Distilled water and adjust to pH 7.2-7.4 by adding HCl or NaOH.

PBS (Phosphate - Buffered Saline 0.02M - pH 7.2 or 7.4)

Sodium dihydrogen Orthophosphate (NaH_2PO_4).....	6.24g
DiSodium Hydrogen Orthophosphate (Na_2HPO_4).....	11.36g
Sodium Chloride (NaCl).....	44.50g

Dissolve these in 5litres of Distilled water and adjust to pH 7.2-7.4 by adding HCl or NaOH.

Bovine Serum Albumin (BSA)1% in TBS or PBS

Albumin from Bovine Serum.....	0.50g
Sodium Azide (Na N_3).....	0.05g

Slowly dissolve the above ingredients in TBS (0.05M- pH: 7.2-7.4) or PBS (0.02M-pH: 7.2-7.4) 50ml and keep the solution at 4°C.

Harris' Haematoxylin and 1%Methyl Green Procedure

Harris' Haematoxylin

As mentioned in chapter 2. (see appendix 2.1)

1%Methyl Green

Dissolve 1g Methyl green in 100ml distilled water and filter before use.

Trypsin Solution

Trypsin (Dako S2012).....	0.1g
TBS or PBS solution	100ml

Prepare just before use

Slide Adhesive

Gelatine Chrome Alum (G.C.A.) :

Cold Distilled water.....	75ml
Boiling Distilled water.....	425ml
Gelatine.....	2.5 g
Chromium Potassium Sulfate, CrK(SO ₄).12H ₂ O.....	0.25g
Thymol.....	few grains

- 1- Mix gelatin with cold distilled water in a 1litre beaker and let stand until soft
 - 2- Boil and measure 425 ml distilled water
 - 3- Add to gelatin
 - 4- Mix and let dissolve completely
 - 5- Add chromium potassium sulphate
 - 6- Add a pinch of thymol and filter into a clean bottle
- Store at 4°C

Method:

To prepare the slides, place racks of clean slides (clean them with acid alcohol; 0.5% HCl in 95% ethanol) and a lint-free cloth if necessary for 20 minutes in a dish of G.C.A. which has been warmed (run hot tap H₂O) in a tray and warm the bottle of G.C.A. in it. Dry vertically overnight at room temperature or 1 hour at 60°C. For best results use the slides within 2 weeks. Use 30 ml in the waterbath for paraffin sections.

Poly - L - Lysine (P.L.L.)

Solution:

Poly - L - Lysine Solution (Sigma; P6893).....	50 ml
Deionized water	450ml

Method

- 1-Place clean slides, a rack at a time in diluted poly - L - lysine solution for 5 minutes.
- 2-Drain slides and dry in 60°C oven for 1 hour or at room temperature overnight.
- 3-Filter the P.L.L. solution into a plastic bottle and refrigerate.
- 4-Be sure to allow the solution to come to room temperature before using again
- 5-Discard if turbidity or bacterial growth occurs
- 6-The diluted solution should be stable several months
- 7-The slides are most stable when fresh (maximum life of 2 weeks)

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PUBLICATIONS

During the period of Ph.D. course the following papers have been published:

1- Pallot, D.J., Seker, M., and Abramovici, A. (1992) Postmortem changes in the rat carotid body: possible implications for human histopathology. *Virchows Archiv A. Pathol. Anat. & Histopathology*. 420: 31-35.

2- Seker, M., Pallot, D.J., Habeck, J.O, and Abramovici, A. (1994) Postmortem changes in the human carotid body. In:"Arterial Chemoreceptors, Cell to System." (eds.) O'Regan, R.G. et al. *Advances in Experimental Medicine and Biology*. Plenum Press, New York, London. Vol:360: 349-351.

3- Seker, M., Pallot, D.J., Habeck, J.O, and Abramovici, A. (1994) Effect of various diseases upon the structure of the human carotid body. In:"Arterial Chemoreceptors, Cell to System." (eds.) O'Regan, R.G. et al. *Advances in Experimental Medicine and Biology*. Plenum Press, New York, London. Vol: 360: 353-355.

In addition four papers on the morphometric and immunocytochemical analysis of the human carotid body in normal and different pathological condition are in preparation.

Post-mortem changes in the normal rat carotid body: possible implications for human histopathology

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Summary. The carotid bodies in experimental animals contain only one variety of type I cells whilst in the human organ three varieties of this cell type have been described. We have examined the effects of post-mortem change on the structure of the type I cell of the rat carotid body. When the organ is examined immediately after death of the animal all of the type I cells exhibit similar morphology. With increasing delay in fixation of the tissue the type I cells undergo autolytic changes. Within 2 h of death the nuclei become hyperchromatic and the cytoplasm exhibits an increasing eosinophilia. In carotid bodies fixed 4 h post-mortem a further type I cell variant is seen in which the nucleus lacks a chromatin pattern and becomes pyknotic. We believe that previous descriptions of three varieties of type I cells in the human carotid body are based upon a description of post-mortem change. Furthermore, in any study of this highly oxygen dependent tissue it is essential that due account be taken of the delay between death and fixation.

Introduction

The mammalian carotid body consists of groups of specific cells set in a vascular connective tissue stroma containing numerous nerve fibres. The parenchymal cells consists of two varieties, the type I and type II cells. In all experimental animals studied to date a variable number of polygonal type I cells are surrounded by type II cells, the latter being elongated cells with long fine, cytoplasmic processes (Biscoe 1971; Pallot 1987). In the human carotid body a similar arrangement has been described by Smith and his colleagues (Smith et al. 1982) except that they are able to recognise three sub-popula-

tions of type I cells on the basis of nuclear morphology: these are termed the light, dark and pyknotic cells.

A number of workers have attempted to identify type I cell sub-types in the rat, rabbit and cat carotid body using a variety of morphometric techniques (McDonald and Mitchell 1975; Verna 1979; Pallot et al. 1986): to date no convincing evidence for sub-classes of type I cells has been produced in these species (Pallot 1987). We were intrigued by this apparent difference in the structure of the human carotid body and that of other mammalian species. As studies of the human carotid body must involve the examination of post-mortem material we examine here the effects of post-mortem changes on the structure of the rat carotid body.

Materials and methods

The experiments were performed using adult Wistar rats of approximately 4 months of age. The animals, obtained from the Unit of Biomedical Services, University of Leicester, were killed by an overdose of pentobarbitone sodium and treated as follows.

1. The carotid bodies were removed at the time of death (0 h; these tissues acted as controls).
2. Carotid bodies were removed at different times after death (2, 4, 8, 16 and 24 h) from animals in which the carcass had been kept at room temperature for 2 h and thereafter at 4° C.
3. Carotid bodies were removed at 4 and 16 h post-mortem from animals in which the carcass had been kept at room temperature. A total of 24 animals were studied.

The carotid bodies were fixed overnight in 4% neutral buffered formalin, processed by routine techniques for embedding in paraffin wax and stained either with Harris haematoxylin or by the periodic acid-Schiff (PAS) method.

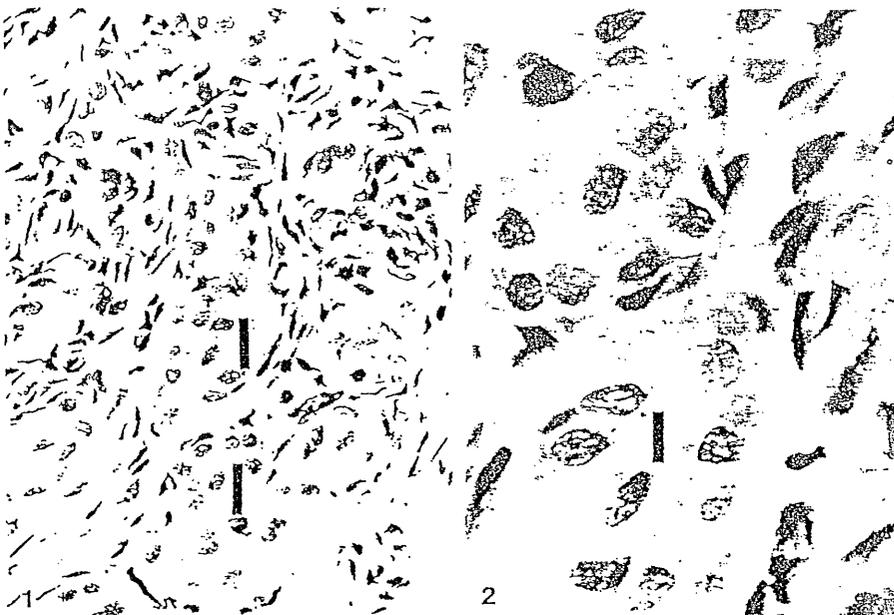
The number of type I cells with either light, dark or pyknotic nuclei was counted in random sections of carotid bodies with a 40x objective; averages of at least four such fields were counted for the quantitative studies.

Results

The control carotid bodies (0 h group) showed the typical clustering of polygonal type I cells surrounded by

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Figs. 1, 2. Low and high power views of a carotid body removed from a rat immediately after the overdose of anaesthetic. Note how all of the type I cells (*I*) have a similar nuclear morphology and that their cytoplasm lacks eosinophilia

elongated type II cells (Fig. 1). The type I cells were of similar size and shape and possessed a light eosinophilic cytoplasm and large round or ovoid nuclei (Fig. 2). Some type I cells possessed a slightly hyperchromatic nucleus but lacked the eosinophilic cytoplasm at this time. The type II cells had less definite cell borders and the nuclei were elongated and hyperchromatic (Figs. 1, 2). Few congested capillaries and very few pyknotic cells were seen.

Changes in type I cell morphology were more frequent within 2 h of death and became prominent after 4 h at 4° C (Fig. 3). There was an increasing vacuolation of some type I cells and the appearance of more cells with hyperchromatic nuclei; some of these latter cells possessed smaller, eccentrically placed nuclei with a cap of intensely eosinophilic cytoplasm (Fig. 4); these cells are similar to the dark cells described in the normal human carotid body by Smith et al. (1982). A third variant of type I cell became prominent by 4 h. This pyknotic variant was characterised by a small, compact, darkly stained nucleus with a narrow peripheral rim of cytoplasm (Fig. 5). Similar periods of delay induced different pathological findings depending on whether the carcass had been kept at room temperature or at 4° C. The degree of change from the normal structure was much greater at room temperature where even after 4 h nuclear vesiculation and loss of cell margins could be seen.

In contrast to type I cells, the type II cells appeared

resistant to autolytic change. However, by 16 h some type II cells had also developed pyknotic nuclei.

Marked vascular dilatation and congestion was observed in all specimens studied at post-mortem periods greater than 2 h and the magnitude of the congestion seemed to increase with increasing post-mortem delay. No leucocytic infiltration was found in any of the carotid bodies examined.

Figure 6 illustrates quantitative data on type I cell variants. The number of light cells decrease almost linearly up to 4 h post-mortem and this decrease is paralleled by an increase in the number of pyknotic and dark cells. Between 4 and 24 h fixation delay there is a further fall in the percentage of clear cells and rise in the number of pyknotic cells. It is also noteworthy that when large numbers of cells are examined, in sections from different levels of the carotid body, that there are a small number of pyknotic cells can be found at time zero.

Discussion

The present observations clearly indicate that three variants of type I cells, the clear, dark and pyknotic cells, do not represent a stable population but rather that their numbers fluctuate in relation to the delay between death of the animal and fixation of the tissue.

It is noteworthy that isolated autolytic changes were

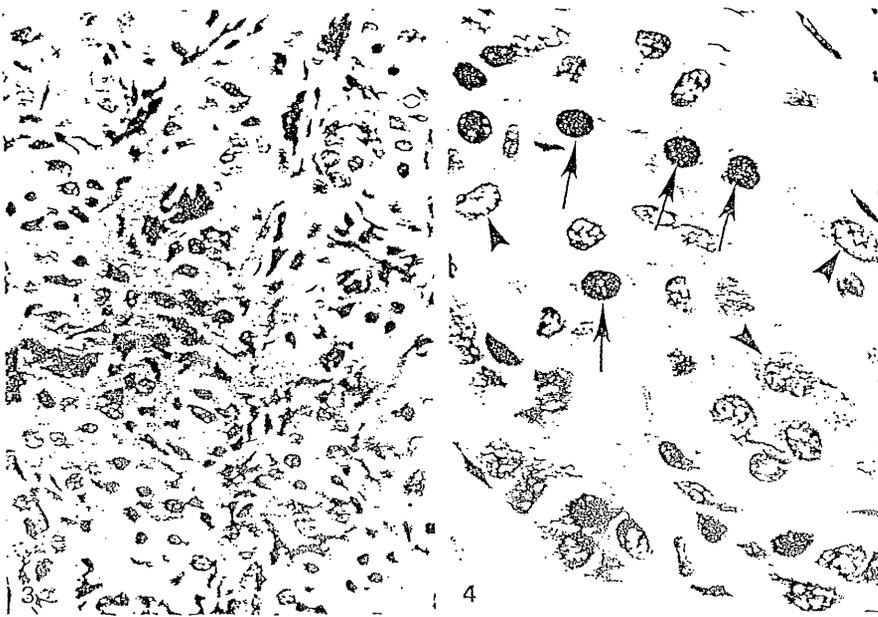


Fig. 3, 4. Low and high power views of carotid body tissue fixed 4 h after death of the animal. Some type I cell nuclei have densely staining chromatin patterns (*arrows*) and that these cells show a markedly eosinophilic cytoplasm (*arrows* in Fig. 4). Other cells (*arrowheads* in Fig. 4) have a similar chromatin pattern to that seen in Figs. 1, 2 but increased vacuolisation of the cytoplasm

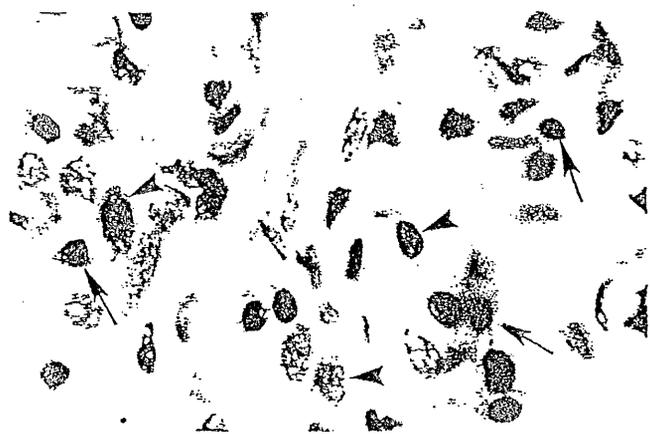


Fig. 5. High power micrograph of carotid body tissue fixed 16 h after death. Some cells lack any visible chromatin pattern in their nuclei (*arrows*) whilst others have dark nuclei as in Figs. 3, 4 (*arrowheads*)

noted in tissues were the fixation delay was of the order of 15 min (the time taken to complete the dissection). This situation is not surprising as it is well known that other rat organs, for example the liver, may undergo autolytic changes within minutes after death. The fact

that previous morphological studies of rat, cat and rabbit carotid bodies failed to demonstrate dark or pyknotic cells may be explained by the fact that all of these studies used vascular perfusion of the fixative (McDonald and Mitchell 1975; Verna 1979; Pallot et al. 1986). The de-

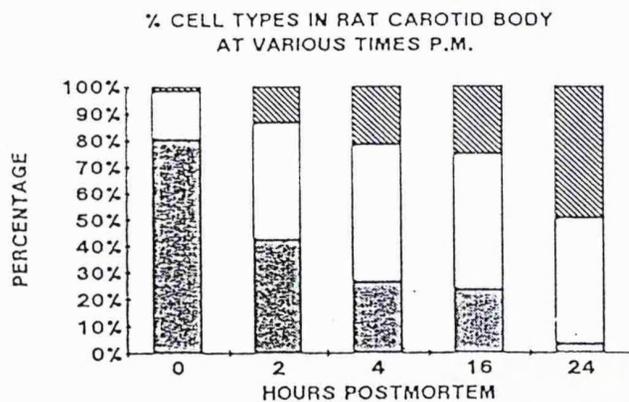


Fig. 6. Stacked bar histogram illustrating the percentage of clear, dark and pyknotic cells in the rat carotid body after various fixation delays. (▨ = clear; □ = dark; ▨ = pyknotic)

sign of these experiments was adopted in an attempt to mimic the situation that might exist in routine autopsy protocols. Thus there was a delay of 2 h before refrigeration of the carcass and permeation of the fixative rather than perfusion.

We have as yet no direct information on the precise events that lead to the appearance of dark and pyknotic cells within the carotid body. However, based upon what is known to occur elsewhere during the initiation of irreversible anoxic cell injury (Rubin and Farber 1988) and on our histopathological findings let us assume that a similar sequence of events could take place in the carotid body. According to such an assumption the clear type I cells rapidly undergo hydropic degeneration, as indicated by their vacuolated appearance, followed by an increase in the eosinophilic affinity of the cytoplasm and changes in the nuclear chromatin array. These transformations represent an intermediate step in the process of autolysis, the dark cell variant. During the following hours after death (4–24 h) the cytolytic process will continue and the nucleus becomes heavily hyperchromatic, representing the so-called pyknotic cell.

The existence of a sequential and dynamic transformation of the type I cell population as a function of the autolytic process is well illustrated in our data by the reciprocal interrelationship between the decreasing number of clear cells and increasing number of pyknotic cells.

There is considerable controversy in the carotid body literature regarding the origin and nature of the dark and pyknotic cells. The possibility that these cells increased in number with increasing post-mortem delay was suggested by a number of authors (Gosses 1938; de Castro 1951), whilst others presumed them to be normal constituents of the carotid body (Smith 1924; White 1935; Smith et al. 1982). Recently it has even been suggested that the pyknotic cells in the carotid bodies of children represent "precursors" of the dark and clear cell variants (Heath et al. 1990). The confusion is compounded by the fact that to date no clear function has been ascribed to the dark and pyknotic variants.

Our studies have also revealed an apparent resistance of the type II, or sustentacular, cells to autolytic change as well as the absence of any intermediate variant between the normal and pyknotic type II cell. The existence of different patterns in response to anoxic injury in the type I and type II cells might be explained by their differing autogenetic origins and functions.

The type I cells are known to originate from the neural crest (Le Douarin et al. 1972) and show similar cell markers to neuronal cells such as neuron specific enolase and neurofilament protein (Abramovici and Pallot 1990) as well as acting as a storage site of various neuropeptides (Wharton et al. 1980; Abramovici and Pallot 1990; Oomori et al. 1991) and catecholamines (Pallot 1987). Since type I cells undoubtedly respond to changes in oxygen tension it seems plausible that a sudden and dramatic drop in oxygen supply, even for a few minutes, might result in irreversible changes in the cell.

The exact origin and function of the type II cell is yet to be elucidated and it is difficult to distinguish them from the Schwann cells (McDonald 1981; Jago et al. 1984). There is some immunohistochemical evidence (they store S100 and glial fibrillary acidic protein) suggesting that they are similar to Schwann cells and oligodendroglia (Abramovici and Pallot 1990). The possibility exists that their resistance to anoxia might be related to a lower oxygen requirement than the type I cells.

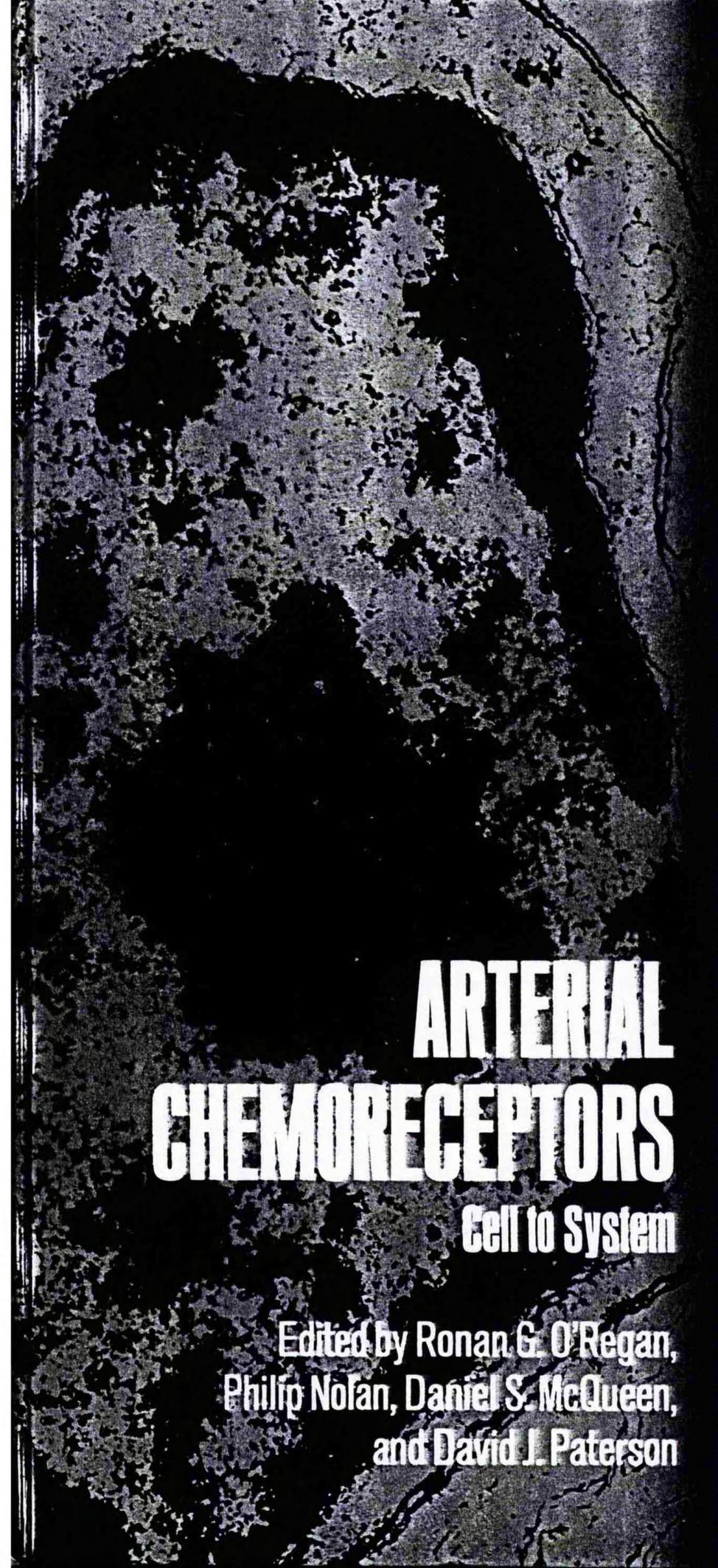
The existence of vasodilatation and congestion in the longer post-mortem delay animals (4–24 h) are further post-mortem changes affecting carotid body morphology. The intensity of both the vasodilatation and the degree of congestion were related to the post-mortem delay and were further increased if the head of the animal was placed below the level of the heart suggesting that the phenomenon was entirely passive.

The data reported here would council caution in the interpretation of carotid body type I cell differential counts as it may be that the existence of substantial numbers of dark and pyknotic cells is a post-mortem artefact. This suggestion is substantiated by our recent observations of human carotid bodies fixed at various times after death where there was a positive correlation between the post-mortem delay in fixation and the number of pyknotic cells and a negative correlation between delay and the number of clear cells (Seker et al., unpublished).

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POSTMORTEM CHANGES IN THE HUMAN CAROTID BODY

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INTRODUCTION

Heath and his colleagues have described three varieties of Type I cells based upon differences in nuclear morphology (Heath et al., 1970); this is at odds with the situation in experimental animals. Pallot et al. (1992) examined rat carotid bodies fixed at various times after death of the animal. Their data showed that, with increasing delay in fixation of the carotid body, first Type I cells with dark nuclei became apparent and with further delay cells with pyknotic nuclei appeared. Here we correlate the percentage occurrence of clear, dark and pyknotic nucleated cells in the human carotid body with the delay between death of the patient and fixation of the tissue.

METHODS

A total of 75 carotid bodies were fixed in Zamboni's fluid at postmortem of and after routine processing sections were stained with H&E. Sections separated by a minimum gap of 250µm were examined and random fields using a x40 objective used to classify the Type I cells as either clear, dark or pyknotic.

RESULTS

Fig 1 shows a photomicrograph of carotid body tissue which had been fixed 3.5 hours after death of the patient. The three varieties of Type I cell, clear, dark and pyknotic are clearly seen.

All of the cases were ascribed to one of three groups on the basis of the time between death and fixation of the carotid body. The percentage occurrence of the three varieties of type I cells are illustrated in Fig 2. It can be seen that, with increasing delay, the number of clear cells decreased whilst the number of pyknotic cells increased. We also removed two carotid bodies and divided them into two parts; the first was fixed on removal whilst the second was stored at 4°C for 18 hours before fixation. After storage the number of pyknotic cells increased and the number of clear cells decreased (see Fig 2 D1,D2 & E1,E2).

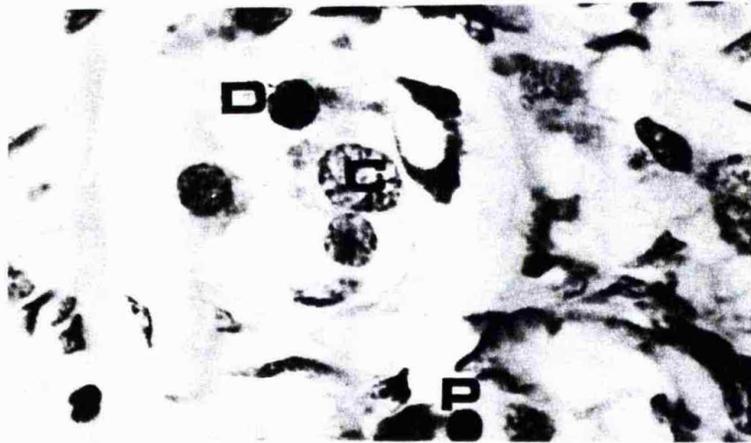


Figure 1. Photomicrograph of the human carotid body showing clear (C), dark (D) and pyknotic (P) cells.

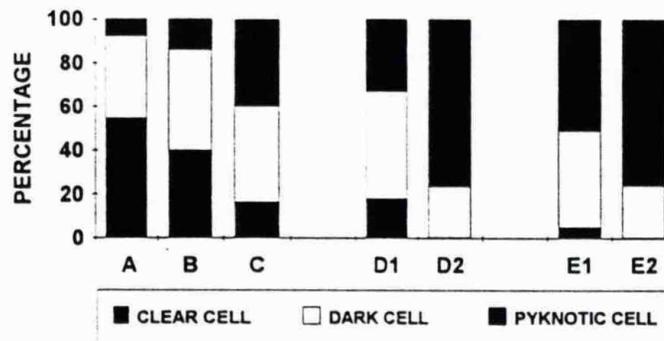


Figure 2. Distribution histograms of the percentage occurrence of the three cell varieties in the human carotid body at various times after death. A = 2-7, B = 7-15, C = >15 hours. D & E show similar data for two carotid bodies divided into two parts and fixed at 14 & 32 hours (D1, D2) and 21 & 38 hours (E1, E2) after death.

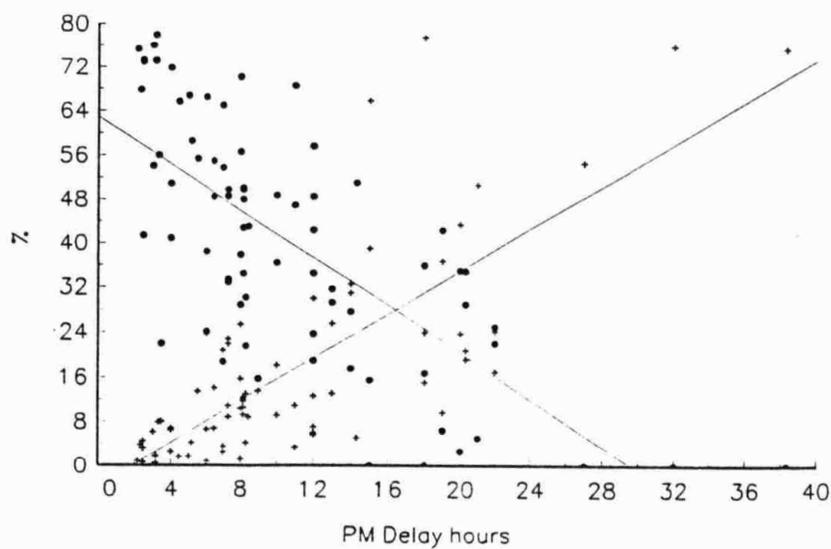


Figure 3. Plot of the percentage number of clear (dots) and pyknotic (crosses) cells against time.

Fig 3 illustrates a plot of the percentage occurrence of clear cells and pyknotic cells as a function of time together with the line of best fit. The linear correlation coefficient for clear cells against time was -0.77 ($p > 0.001$) whilst for pyknotic cells it was 0.71 ($p > 0.001$).

All of the above data indicate that there is a relationship between the number of the different varieties of Type I cells and the time that elapses between death and fixation of the tissue.

DISCUSSION

Like Heath and his colleagues we found three varieties of Type I cells in the human carotid body obtained postmortem (Heath et al., 1970); these were similar to those reported in the rat carotid body (Pallot et al., 1992). In essence the clear Type I cells possessed a lightly eosinophilic cytoplasm and pale nuclei with dispersed chromatin. In dark cells there was increased eosinophilia and a condensation of the nuclear chromatin. Pyknotic cells had only a small rim of cytoplasm around a dense nucleus. In both the rat and human carotid bodies there were increases in the number of pyknotic cells and decreases in the number of clear cells with increasing delay between death and fixation of the tissue. The evidence from the experiments on the rat carotid body (Pallot et al., 1992.) indicate that the dark and pyknotic variants of Type I cells represent postmortem changes, for when fixed very rapidly the carotid body consisted exclusively of clear Type I cells; delay resulted in the appearance of dark cells and then pyknotic cells. Our data show a reduction in the number of clear Type I cells and an increase in the number of pyknotic cells with increasing fixation delay.

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EFFECTS OF VARIOUS DISEASES UPON THE STRUCTURE OF THE HUMAN CAROTID BODY

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INTRODUCTION

It is well documented that the carotid body responds to a number of abnormal physiological stimuli such as chronic hypoxaemia and hypertension (Heath & Smith, 1992; Pallot, 1987). In both of these conditions there is an increase in the size of the carotid body and, in experimental animals, there is evidence that this increased size is brought about by a combination of hypertrophy and hyperplasia (Bee et al., 1986); in the human evidence of hyperplasia has not been obtained.

In this study we have examined human carotid bodies obtained at routine necropsy and examined three aspects of the carotid body structure, cell cluster size, lobule size and the amount of connective tissue within the lobules.

METHODS

Carotid bodies were removed at routine necropsy, fixed in Zamboni's fluid, processed for embedding in paraffin wax, serially sectioned at 5µm and stained with H&E. Sections separated by a minimum distance of 250µm were then examined as below. The cases were divided into the following categories; chronic hypoxia, essential hypertension, diabetes mellitus, sepsis and non thoracic carcinoma; in addition there were a group of patients who lacked and evidence of these conditions in neither their medical history nor postmortem results, these were used as controls and referred to as unrelated cases.

Single sections were viewed with a ×10 objective and the area of carotid body tissue, the outline of each lobule and the outline of all of the cell clusters within the field drawn using a camera lucida; the areas of these drawings was determined using a Videoplan. By simple subtraction the area occupied by connective tissue was calculated from the field area minus the lobule plus cell cluster area. A cumulative mean technique was used to ascertain that a sufficient sample had been analysed.

RESULTS

Table 1 illustrates all of the data relating area of the lobules, cell clusters and connective tissue. From this table it may be seen that hypoxia and hypertension result in an increase in size of all three parameters; what is surprising however is that sepsis and diabetes mellitus both increase the area of lobules. Diabetes mellitus also increases the amount of connective tissue, an effect not seen in patients with sepsis.

Table 1. The area of lobules and cell clusters and the area of connective tissue within our various cases. All data are means and standard deviations. * not significantly different from unrelated, ** $p < 0.05$ as compared to unrelated (t test)

DISEASE	NUMBER	LOBULE AREA	CELL CLUSTER AREA	CONN. TISSUE AREA
UNRELATED	8	0.094 ± 0.023	0.0392 ± 0.010	0.0547 ± 0.013
COPD	13	0.165 ± 0.062**	0.0644 ± 0.022**	0.1000 ± 0.042**
HYPERTENSION	25	0.133 ± 0.031**	0.0470 ± 0.011**	0.0859 ± 0.023**
D. MELLITUS	19	0.121 ± 0.042**	0.0417 ± 0.013*	0.0793 ± 0.032**
CARCINOMA	13	0.106 ± 0.035*	0.0413 ± 0.014*	0.0649 ± 0.026*
SEPSIS	7	0.143 ± 0.075**	0.0565 ± 0.028*	0.0859 ± 0.052*

We divided the chronic hypoxia cases into those with pneumonia and chronic hypoxia due to chronic obstructive pulmonary disease (COPD) and those who lacked the infection; the data showed little difference in the two situations.

We also calculated the area fraction of each lobule occupied cell clusters and connective tissue (see Table 2). The table shows how there is no change in the area fractions of the carotid body lobule occupied by cell clusters and connective tissue in cases of COPD, carcinoma and sepsis, but that the area fractions of the lobule occupied cells in both hypertension and diabetes is decreased whilst there is an increase in the area occupied by connective tissue.

Table 2. Area fraction of lobules occupied by connective tissue and cell clusters in our various cases plus standard deviations. * not significantly different from unrelated, ** $p < 0.05$ when compared to unrelated (t test)

DISEASE	NUMBER	LOBULE AREA	CELL CLUSTER AREA FRACTION	CONN. TISSUE AREA FRACTION
UNRELATED	8	0.094 ± 0.023	41.8 ± 4.1	58.2 ± 4.2
COPD	13	0.165 ± 0.062**	39.9 ± 5.6*	60.1 ± 5.6*
HYPERTENSION	25	0.133 ± 0.031**	35.7 ± 5.5**	64.3 ± 5.5**
D. MELLITUS	19	0.121 ± 0.042**	35.1 ± 6.1**	64.9 ± 6.1**
CARCINOMA	13	0.106 ± 0.035*	39.3 ± 9.5*	60.7 ± 9.5*
SEPSIS	7	0.143 ± 0.075**	40.8 ± 11.1*	59.1 ± 11.1*

DISCUSSION

Our data show, as previously reported, that chronic hypoxia and hypertension result in hypertrophy of the lobules and cell clusters of the human carotid body (see review by Heath & Smith, 1992); in addition to this there is an increase in the amount of connective tissue within the lobule. The new data relates to diabetes mellitus which also increases the lobule

area and amount of connective tissue; cell cluster size is also increased in diabetes mellitus. Interestingly the area fraction of each lobule occupied by cell clusters and connective tissue remain unchanged indicating that the hypertrophy and/or hyperplasia occurs uniformly in both elements in the case of COPD whilst in diabetes and hypertension there is a reduction in the proportion of the lobule occupied by cell clusters and a consequent increase in the area of connective tissue. We have no data at the moment to address the issue of hypertrophy versus hyperplasia; counts are in progress to determine the numbers of cells in normal and hypoxaemic carotid body lobules.

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