Telomere Length and Cellular Senescence in Atherosclerosis

Thesis submitted for the degree of
Doctor of Philosophy
At the University of Leicester

By

Rachel Louise Boultby BSc (Hons)
Department of Medicine
University of Leicester

March 2003

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To: **Mum and Dad**

"Do, or do not. There is no 'try'"

- Yoda 'The Empire Strikes Back'

Statement of Originality

The accompanying thesis, submitted for the degree of Doctor of Philosophy, entitled 'Telomere Length and Cellular Senescence in Atherosclerosis', is based on work undertaken solely by the author in the Department of Medicine at the University of Leicester during the period from September 1997 to September 2000. The work presented in this thesis is original unless otherwise acknowledged in the text or references. None of the work has been submitted for any other degree in this or any other university.

Rachel Boultby March 2003

Acknowledgements

Firstly I would like to thank Professor David de Bono who's initial ideas formed the basis of this project. Also, I would like to express my greatest gratitude to Dr. Alison Goodall and Professor Nilesh Samani for their supervision during all of the work carried out for this thesis. Special thanks go to Dr. Alison Goodall for her enthusiasm, encouragement and constructive criticism during the preparation of this thesis.

I would also like to thank:-

The British Heart Foundation and the Medical Research Council for proving funds, in grant form, for all aspects of the work carried out.

Simon Frantz, Dr Weidong Yang and Dr. Nicola Royle for their technical guidance which was invaluble and tireless throughout.

Amanda Stephens-Lloyd, Louise Reeve, Neil Swanson, Kai Hoegrefe and Ravi Singh for their help during my time in the laboratory. Sharon Neill, Christine Carnegie, Claire McCall, Hedley Knight and Dr Brian Morris for their constant encouragement during the writing up process.

Finally, to my long-suffering husband Andrew, who has cooked and cleaned for the last two years, without any thanks. I couldn't have done it without you!

Rachel Louise Boultby
University of Leicester

Doctor of Philosophy
March 2003

Telomere Length and Cellular Senescence in Atherosclerosis

Abstract

Telomeres are found at the extreme ends of all eukaryotic chromosomes. Due to the nature of DNA replication, a small amount of telomere is lost with each round of mitosis. This telomere shortening has been associated with many disease processes and also the induction of cellular senescence, a feature of the endothelial cell lining in atherosclerotic plaques.

This study sought to investigate possible links between coronary artery disease (CAD) and telomere length by measuring telomere restriction fragments (TRF) in peripheral white blood cells (WBC) from subjects with CAD and also in the cells of atherosclerotic plaques. Also, an *in vitro* cultured endothelial cell model was designed to determine the effects of oxidative stress (via hydrogen peroxide exposure), a known risk factor for atherosclerosis, on the TRF of this cell type, which is known to play a vital role in the progression of CAD.

Adjusting for age and sex, WBC from subjects with CAD had mean TRF lengths of 303bp (SD±90bp) shorter than those of controls ($p=0.002$) which is equivalent in size to individuals with no clinically overt CAD who are 8.6 years older. Plaque cells had significantly shorter TRF lengths, 6.30Kb (SD±0.75Kb) compared with the corresponding WBC, 7.46Kb (SD±0.90Kb) ($P=0.001$).

The *in vitro* cell study produced results consistent with other groups in that following exposure to hydrogen peroxide, endothelial cells demonstrated senescent-like behaviour in a dose dependent manner. Telomere attrition was also tentatively linked to this exposure.

In conclusion, the results from the white cell study suggests that differences seen in telomere length may be genetically mediated or due to a 'catch up' period of growth, making telomere length a possible primary factor in the disease process. Furthermore, there appears to be additional site specific telomere attrition in the cells of the plaque.

Publications arising from this thesis

Samani NJ, Boultby RL, Butler R, Thompson JR, Goodall AH. Telomere shortening in atherosclerosis. *Lancet* 2001; **358**: 472-473

Boultby RL, Butler R, Hayes PD, Thompson JR, Goodall AH, Samani NJ. Telomere shortening in atherosclerosis. *Heart* 2001; **85**: P23 Supplement I

Boultby RL, Yang W, de Bono DP. Oxidative Stress-Mediated Telomere Loss: A potential mechanism for the accelerated senescence of endothelial cells in atherosclerosis. *Circulation* 1999; **100** (18): 1353

Contents

Chapter 1: Introduction 1

1.1 Cell cycle and senescence 2

1.2 Telomeres 7

1.2.1 Telomere Function 8

1.2.2 Telomere structure and sequence 10

1.2.3 Telomere length 14

1.2.4 Telomerase 15

1.2.5 Telomere Shortening 17

1.2.5.1 *In vivo* 17

1.2.5.2 *In vitro* 19

1.2.6 Telomere measurement 20

1.2.7 Senescence and Immortality 20

1.2.8 Telomeres and Disease 24

1.3 Atherosclerosis 27

1.3.1 The Endothelial Cell 27

1.3.2 Endothelial Cells and Atherogenesis 29

1.3.3 Atherosclerotic risk factors 31

1.3.3.1 *Lipids* 32

1.3.3.2 *Homocysteine* 33

1.3.3.3 *Oxidative stress* 34

1.3.4 Endothelial Cell Replication *in vivo* 34

1.3.5 Endothelial Cell Culture 35

1.4 Oxidative stress 37

1.4.1 Effects of ROS on the Cell 38

1.4.2 Oxidative stress and Atherosclerosis 39

1.5 Aims of the study 41

Chapter 2: Materials & Methods **44**

2.1 Human tissue 44

2.1.1 Peripheral Blood 44

2.1.2 Atherosclerotic plaques 45

2.2 Bovine Aortic Endothelial Cells 45

2.2.1 Tissue Culture Materials 45

2.2.2 Primary Culture of Bovine Aortic Endothelium 46

2.2.3 Cloning of Bovine Aortic Endothelial Cells 47

2.2.4 Bovine Aortic Endothelial Cell Sub-culturing 49

2.2.5 Liquid Nitrogen Storage of Cells 49

2.2.6 Recovery of Cells from Liquid Nitrogen Storage 50

2.2.7 Endothelial Cell Identification 50

2.2.7.1 *Morphological Criteria* 50

2.2.7.2 *Immunofluorescence staining of endothelial cells for vWF* 51

2.2.8 Treatment of BAEC's with Hydrogen Peroxide 53

2.2.9 Apoptosis Assay 55

2.3 Isolating Genomic DNA 57

2.3.1 Phenol-Chloroform Extraction of DNA from Plaque samples 57

2.3.2 Phenol-Chloroform Extraction of DNA from BAEC 58

2.3.3 PureGene® Extraction of DNA from Whole Blood Samples 59

2.3.4 Analysis of DNA Samples 60

2.3.4.1 *Spectroscopy Method for Quantitation* 60

2.3.4.2 *Electrophoresis Method for Measuring DNA Integrity* 61

2.4 Analysis of Telomere Lengths 61

2.4.1 Electrophoresis 62

2.4.2 Membrane hybridisation 64

2.4.2.1 *Random primed labelling* 65

2.4.2.2 *TdT labelling* 65

2.4.3 Image Quant Analysis of TRF 67

Chapter 3: Assay Optimisation **68**

3.1 Introduction 68

3.2 Telomere Assay Optimisation 69

3.3 Inter-assay variation 79

3.4 Validation of the telomere assay 87

3.4.1 Experimental Protocol 87

3.4.2 Preliminary Study Results 88

Chapter 4: Comparison of Telomere Length in Peripheral White Blood Cells **91**

4.1 Introduction 91

4.2 Experimental Protocol 93

4.3 Results 94

4.4 Discussion 100

Chapter 5: Comparison of Telomere Length in Atherosclerotic Plaque Cells **106**

5.1 Introduction 106

5.2 Experimental Protocol 106

5.3 Results 107

5.4 Discussion 113

Chapter 6: Oxidative Stress, Telomeres and Endothelial Cells **118**

6.1 Introduction 118

6.2 Experimental Protocol 119

6.2.1 Bovine Aortic Endothelial Cells 119

6.2.2 Endothelial Cell Identification 120

6.2.3 Effects of long term culture on BAEC 120

6.2.4 Hydrogen Peroxide Treatment 122

6.2.5 Annexin Staining 125

6.3 Results 125

6.3.1 Experiment 1 125

6.3.2 Experiment 2 133

6.4 Discussion 145

Chapter 7: Discussion **149**

7.1 Atherosclerosis and senescence 151

7.2 Atherosclerosis and telomere inheritance 155

7.3 Atherosclerosis and oxidative stress 157

7.4 Improvements & Future work 159

7.4.1 Telomere assay 159

7.4.2 Specimen selection 161

7.4.3 Alternative oxidative exposure 163

7.5 Summary 165

References **166**

List of Suppliers **182**

List of Figures

Chapter 1: Introduction

Figure 1.1 *The Cell Cycle* 5

Figure 1.2 *The End Replication Problem* 11

Figure1.3 *A model of telomere length regulation* 16

Figure 1.4 *Telomere hypothesis of cellular ageing and immortalisation* 22

Chapter 3: Assay Optimisation

Figure 3.1 *Digestion of peripheral blood DNA using RsaI/HinfI or RsaI/HaeIII* 73

Figure 3.2 *Separation of telomeric DNA using two agarose concentrations* 74

Figure 3.3 *Transfer of telomeric DNA from agarose gel to Hybond-N using classic blotting or stringent vacuum techniques* 75

Figure 3.4 *Telomeric DNA from human white blood cells after Southern hybridisation using conditions of choice* 76

Figure 3.5 *Separation of telomeric DNA which had not been quantitated after the digestion process* 78

Figure 3.6 *Separation of resultant PCR products using a 1% HGT agarose gel* 81

Figure 3.7 *Separation of purified PCR products using a 1% HGT agarose gel* 82

Figure 3.8 *Southern Analysis of PCR products* 84

Figure 3.9 *Southern analysis of S21 telomeric DNA* 86

Figure 3.10 *A representative autoradiogram for the Preliminary Study* 90

Figure 3.11 *A bar chart to show the mean TRF results obtained in the Preliminary Study* 91

Chapter 4: Comparison of Telomere Length in Peripheral White Blood Cells

Figure 4.1 *Representative autoradiogram for the first cohort in the white blood cell study.* 97

Figure 4.2 *Representative autoradiogram for the second cohort in the white blood cell study* 98

Figure 4.3 *Plot of mean terminal restriction fragments (TRF) lengths of white blood cells from subjects with severe coronary artery disease and normal coronary arteries.* 99

Chapter 5: Comparison of Telomere Length in Atherosclerotic Plaque Cells

Figure 5.1 *Plaque Study Autoradiogram 1* 108

Figure 5.2 *Plaque Study Autoradiogram 2* 109

Figure 5.3 *Plaque study plot of telomere analysis results obtained from white blood cells vs. plaque cells* 111

Figure 5.4 *Plaque study graph plotting the Mean TRF of the plaque and blood specimens for each subject.* 112

Chapter 6: Oxidative Stress, Telomeres and Endothelial Cells

Figure 6.1 *Identification of Bovine Aortic Endothelial Cells* 121

Figure 6.2 *Replicative senescence of BAEC following long term passage.* 123

Figure 6.3 *Experiment 1: Control BAEC* 127

Figure 6.4 *Experiment 1: BAEC treated with 50mM hydrogen peroxide* 128

Figure 6.5 *Experiment 1: BAEC treated with 100mM hydrogen peroxide* 129

Figure 6.6 *Representative autoradiogram of telomere assay using DNA harvested from BAEC (Experiment 1).* 131

Figure 6.7 *Mean TRF for hydrogen peroxide treated BAEC (Experiment 1)* 132

Figure 6.8 *Experiment 2: Control BAEC* 135

Figure 6.9 *Experiment 2: BAEC treated with 25mM hydrogen peroxide* 136

Figure 6.10 *Experiment 2: BAEC treated with 50mM hydrogen peroxide* 137

Figure 6.11 *Experiment 2: BAEC treated with 100mM hydrogen peroxide* 138

Figure 6.12 *Annexin staining of BAEC for identification of apoptosis* 140

Figure 6.13 *Representative autoradiogram of telomere assay using DNA harvested from BAEC (Experiment 2).* 141

Figure 6.14 *Mean TRF for hydrogen peroxide treated BAEC (Experiment 2)* 142

Figure 6.15 *Cumulative population doublings of hydrogen peroxide treated BAEC (Experiment 2)* 144

List of Tables

Chapter 1: Introduction

Table 1.1 *Telomere repeat sequences in a variety of organisms* 13

Chapter 3: Assay Optimisation

Table 3.1 *A table to show all eight results which were obtained after the S2 DNA had been analysed using Southern hybridisation.* 85

Chapter 4: Comparison of Telomere Length in Peripheral White Blood Cells

Table 4.1 *Characteristics of cases and controls in the white blood cell study* 96.

Abbreviations

APC	Anaphase-promoting complex
BAEC	Bovine aorta endothelial cells
BMI	Body mass index
bp	Base pair
BSA	Bovine serum albumin
CAD	Coronary artery disease
CDK	Cyclin-dependent kinase
CRP	c-reactive protein
CV	Coefficient of variation
dATP	Deoxyadenosine triphosphate
DCCT	Diabetic Control and Complications Trial
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dTTP	Deoxythymine triphosphate
eNOS	Endothelial nitric oxide synthase
EtBr	Ethidium bromide
FBS	Foetal bovine serum
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation

H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HDL	High density lipoprotein
hTRT	Human telomerase repeat template
HUVEC	Human umbilical vein endothelial cells
IAA	Isoamyl alcohol
ICAM	Intracellular adhesion molecule
IDDM	Insulin dependent diabetes mellitus
Ig	Immunoglobulin
IHD	Ischaemic heart disease
Kb	Kilobase
LDL	Low density lipoprotein
Mb	Megabase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\cdot}$-	Superoxide anion
OD	Optical density
$OH^{\cdot}$	Hydroxyl radical
OPA	One Phor All
ox	Oxidised
PBS	Phosphate buffered saline, pH 7.3
PCR	polymerase chain reaction

PD	Population doubling
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PFGE	Pulse-field gel electrophoresis
Ph	Philadelphia chromosome
PNACL	Protein and Nucleic Acid Laboratory
PS	Phosphatidyl serine
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SSC	sodium chloride, sodium citrate
SSPE	sodium chloride, sodium phosphate, EDTA
STDEV	standard deviation
TAE	Tris acetate, EDTA
TdT	Terminal deoxynucleotidyl transferase
TE	Tris, EDTA
TNE	Tris, sodium chloride, EDTA
TRF	Telomere restriction fragment
U	Units
UV	Ultraviolet
v/v	Volume:volume

VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
vWF	von Willebrand factor
w/v	Weight:volume

Chapter 1: Introduction

The 'response-to-injury' hypothesis (Ross, 1993) has long been suggested as one of the prominent mechanisms involved in the pathogenesis of atherosclerosis. Many processes are involved in this hypothesis and will be discussed in greater detail later, but one which is of particular interest is that of augmented cell turnover at the site of the atherosclerotic plaque. This was initially suggested on theoretical grounds, and has since been supported experimentally by numerous groups. All of these groups reported that cells isolated from these plaque regions were similar in behaviour to senescent cells which had been cultured *in vitro* (Moss and Benditt., 1975; Bierman, 1978). The cultured senescent cells were known to have undergone many replications and it was suggested that the isolated plaque cells had experienced a similar replicative history, as both cell types acted in a similar manner. Additionally both the plaque cells and senescent cells were reported to have an age dependent reduction in their replicative potential.

It is possible that the augmented cell turnover in plaque regions and the observed loss of replicative capacity are linked to the atherosclerotic disease process. Also, other conditions may contribute to the cellular changes observed such as oxidative stress. Work in this laboratory confirmed such thoughts during experiments in which bovine aortic endothelial cells were exposed to oxidative stress for a period of time. These cells appeared similar to those isolated from the plaque site in that they too were senescent in behaviour, following the oxidative stress.

Interestingly, both the cultured cells from the oxidative stress experiments (de Bono and Yang, 1995) and those from the plaque region (Tokunaga *et al.*, 1989) shared histological similarities in that they were often multinucleated and much larger than cells which were either from non-plaque regions or had not been exposed to the same oxidative conditions. Finally, the cells exposed to oxidative stress lost their replicative capacity earlier than those cells in the control group.

All of these results suggest that the cells involved in atherosclerosis or induced oxidative culture conditions, respond in an almost identical manner. Therefore could oxidative damage be linked in someway to the atherosclerotic process and if so, is it a causative agent of the cellular changes seen at the site of injury? It is the aim of this thesis to attempt to answer this question and also investigate the mechanism which is involved in this loss of replicative capacity.

1.1 Cell cycle and senescence

A eukaryotic cell cannot divide into two unless two processes alternate:

(i) doubling of its genome in S phase (synthesis phase) of the cell cycle

(ii) halving of that genome during mitosis (M phase)

The period between M and S is called G_1 (growth and preparation of the chromosomes for replication); that between S and M is G_2 (growth and preparation for mitosis).When a cell is in any phase of the cell cycle other than mitosis, it is often said to be in interphase.

The passage of a cell through the cell cycle is controlled by proteins in the cytoplasm. Among the most important involved in animal cells are:

(i) The cyclins (G1, S-phase and M-phase). Their levels in the cell rise and fall with the stages of the cell cycle.

(ii) The cyclin-dependent kinases (G_1 CDKs, S-phase CDKs and M-phase CDKs). Their levels in the cell remain fairly stable, but each must bind the appropriate cyclin (whose levels fluctuate) in order to be activated. They add phosphate groups to a variety of protein substrates that control processes in the cell cycle.

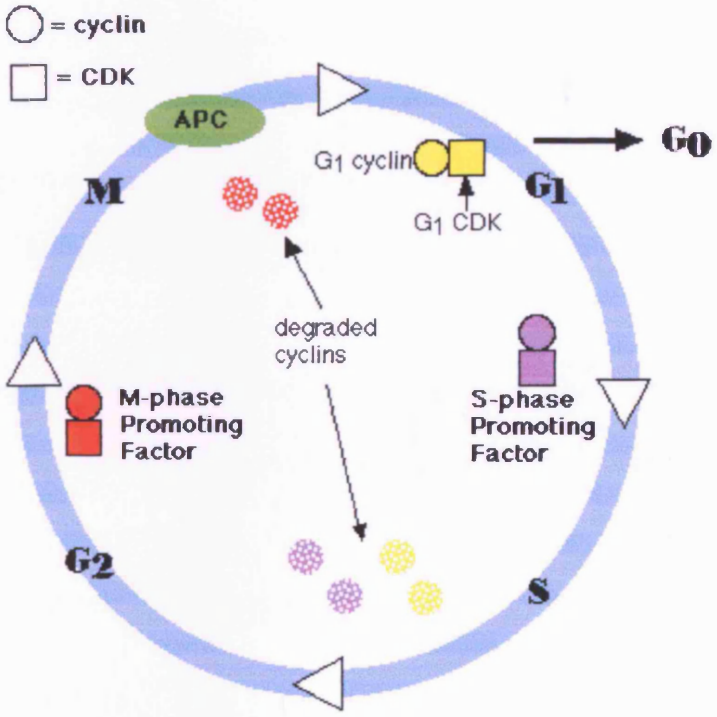
(iii) The anaphase-promoting complex (APC) and other proteolytic enzymes. The APC triggers the events leading to destruction of the cohesins and thus allowing the sister chromatids to separate. It also degrades the mitotic (M-phase) cyclins.

A rising level of G_1 cyclins signals the cell to prepare the chromosomes for replication. A rising level of S-phase promoting factor (SPF) prepares the cell to enter S phase and duplicate its DNA (and its centrioles). As DNA replication continues, one of the cyclins shared by G_1 and S-phase CDKs (cyclin E) is destroyed and the level of mitotic cyclins begins to rise (in G_2). M-phase promoting factor (the complex of mitotic cyclins with M-phase CDK) initiates assembly of the mitotic spindle, breakdown of the nuclear envelope and condensation of the chromosomes. These events take the cell to metaphase of mitosis. At this point, the M-phase promoting factor activates the anaphase promoting complex (APC) which allows the sister chromatids at the metaphase plate to separate and move to the poles (= anaphase), completing mitosis.

The cell has several systems for interrupting the cell cycle if something goes wrong. The first is a check on completion of the S phase. The cell seems to monitor the presence of the Okazaki fragments on the lagging strand during DNA replication. The cell will not proceed in the cell cycle until these have disappeared. Also there are DNA damage checkpoints. These sense DNA damage before the cell enters S phase (a G_1 checkpoint), during S phase, and after DNA replication (a G_2 checkpoint). There are spindle checkpoints which detect any failure of spindle fibres to attach to kinetochores and arrest the cell in metaphase (M checkpoint). Possibly the most important checkpoint is that which triggers apoptosis if the damage is irreparable.

All the checkpoints examined require a complex of proteins. Mutations in the genes encoding some of these have been associated with cancer; that is, they are oncogenes. This should not be surprising since checkpoint failures allow the cell to continue dividing despite damage to its integrity. Examples include the p53 protein which senses DNA damage and can halt progression of the cell cycle in both G_1 and G_2. The p53 protein is also a key player in apoptosis. In some way, p53 seems to evaluate the extent of damage to DNA. p53 triggers arrest of the cell cycle until repairable DNA damage is rectified, or p53 can induce apoptosis if the DNA is irreparable.
A schematic of the cell cycle can be seen in Figure 1.1

Figure 1.1 *The Cell Cycle*



The interactions between the cyclins and cyclin dependent kinases (CDK) during the phases of the cell cycle are demonstrated in this figure.

G_1 – growth and preparation of the chromosome for replication

G_2 – growth and preparation for mitosis

G_0 – resting phase

S – synthesis phase

M – mitosis phase

APC – anaphase promoting complex

CDK – cyclin dependent kinase

Hayflick in the 1960's demonstrated that all normal eukaryotic somatic cells in culture go through a finite number of cell divisions via the cell cycle and then enter a phase of senescence. At this point the cells ceased to divide (Hayflick and Moorhead, 1961), although remained metabolically active (Campisi, 1996). This phase is called the M1 checkpoint (Allsopp *et al.*, 1992). Normally when the cell enters the M1 checkpoint, it permanently exits the cell cycle. However, under certain conditions cells do escape this M1 checkpoint and enter the M2 crisis stage. At this point the cell has an unstable genome and interactions with certain viruses or tumour suppressor genes can result in immortal cells.

Olovnikov, in 1973, developed Hayflick's work further and suggested on theoretical grounds, that this exit from the cell cycle could be triggered by a biological clock residing within each cell. He hypothesised that the event is pre-programmed and purely dependent upon the number of cell divisions and not the chronological age of the cell.

It is now known, or at least strongly suggested, that the biological clock proposed by Olovnikov, resides in the telomeres. These are the extreme ends of chromosomal DNA, made up of large numbers of repeats of a stereotyped and highly conserved, nucleotide sequence. Although this sequence is conserved within a species, differences in base pairs have been reported among species.

Olovnikov realised that although mitosis is a very precise process, due to the way that DNA is replicated, with each round of mitosis a small amount of each of the chromosome ends is lost. Therefore he hypothesised that as a cell undergoes division

the telomere is shortened He referred to this phenomenon as the 'End-Replication Problem' and will be discussed in more detail later.

When the cell has divided to an extent that has rendered the telomere unable to protect the DNA, it is said that the telomere has reached the pre-programmed length (coined the cell's own 'Hayflick limit'), at which point the cell exits the cell cycle and enters senescence. With this in mind and the data on the cells isolated from the plaque region and also those cells which were cultured under oxidative conditions, it is possible that the changes seen in these cells were as a result of telomere shortening.

1.2 Telomeres

Telomeres are the extreme ends of all eukaryotic chromosomes. The concept of the telomere was first raised by pioneering studies, carried out in the 1930s and 1940s by Herman Muller (using *Drosophilla melanogaster*) and Barbara McClintock (using *Zea mays*). Muller found that following X-radiation, terminal deletions and terminal inversion were rare and thus, in order to achieve such chromosome stability, a specialised terminal structure must be present. He hypothesised that this structure must have a special function and he called the region the telomere (Muller, 1938).

McClintock's studies furthered the telomere concept by studying broken chromosome ends. She observed that broken chromosomes ends, which had been induced by X-radiation, as in Muller's experiments, were reactive and often fused with other broken ends, whereas natural chromosome ends were stable. She observed that broken chromosome ends were subject to degradation in the cell, or recombination, often

with deleterious consequences for the chromosome involved, all of which were absent in cells containing intact chromosomes.

1.2.1 Telomere Function

Muller and McClintock both hypothesised that the essential function of the telomere is to provide chromosome stability and protective 'capping' of the chromosome terminus (Muller, 1938; McClintock, 1941).

More recent work has been carried out to test this hypothesis using *Xenopus laevis* eggs (Weber *et al.*, 1993) and *Paramecium* macronuclei (Bourgain and Katinka, 1991). Microinjection of cloned DNA molecules, containing telomeric ends, from different organisms into the macronucleus of *Paramecium primaurelia* resulted in their maintenance throughout vegetative growth. All the injected molecules acquired telomeric repeats at their extremities. This was in contrast to constructs without telomeric ends, which resulted in the macronucleus undergoing intramolecular fusion and degradation (Bourgain and Katinka, 1991).

Work was also carried out on yeast cells, which were constructed so that a single telomere was eliminated from the end of a dispensable chromosome. In the wild type cells, elimination of the telomere repeat caused a RAD9-mediated cell cycle arrest, during which cell cycle progression is prevented due to the presence of damaged DNA. This period of arrest allows time for the DNA to be repaired by delaying the activity of cyclin-cyclin-dependent kinase complexes (Yang *et al.*, 1997). This work indicates that telomeres are a vital structure for the cell to identify intact chromosome ends from broken or damaged chromosomes (Sandell and Zakian, 1993). It also

suggests that in spite of checkpoint and DNA damage repair systems, many chromosomes that lose a telomere are themselves destined for loss.

Telomeres also play an important role in the nuclear organization. In some cell types of some species, chromosomes adopt a "Rabl" orientation where the telomeres become arranged near one another and near to the nuclear membrane, while the centromeres tend to associate on the opposite side of the nucleus (Biessmann *et al.*, 1993). In addition, during leptotene of the first meiotic division for some species, telomeres are clustered together near the nuclear membrane, giving the chromosomes a 'bouquet' formation (Blackburn, 1984). The spatial distribution of mammalian telomeres within the nucleus varies according to cell type and cell cycle stage (Manuelidis and Borden, 1988), but is by no means random. They seem to be involved in establishing the three-dimensional architecture of the interphase nucleus.

Telomeres also participate in mitotic chromosome segregation and telomere defects can impair this process (Kirk *et al.*, 1997). Human telomeres redistribute during the cell cycle, with distal telomeres moving from the interior of the nucleus to the periphery between G1 and G2 phases, and proximal telomeres and centromeres moving in the opposite direction (Vourc'h *et al.*, 1993)

Conventional DNA polymerases can synthesize DNA only in the 5' to 3' direction and cannot begin synthesis *de novo*: they require a primer with a 3'-OH group in order to add nucleotides to a DNA strand; this is usually a short RNA primer. The DNA polymerase extends the RNA primers, generating Okazaki fragments (small DNA fragments, 1000 – 2000 bases long, created by discontinuous synthesis of the

lagging strand). The primers are removed from the lagging strand when synthesis has been carried out, which leaves gaps at the 5' ends of newly replicated strands. Consequently, with each round of DNA replication, there is a loss from the terminus of the chromosome (Olovnikov, 1971; Olovnikov, 1972). Presumably telomeres in some way circumvent this dilemma and prevent the gradual loss of what could be essential genetic information from the ends of chromosomes (Zakian and Pluta, 1989). See Figure 1.2

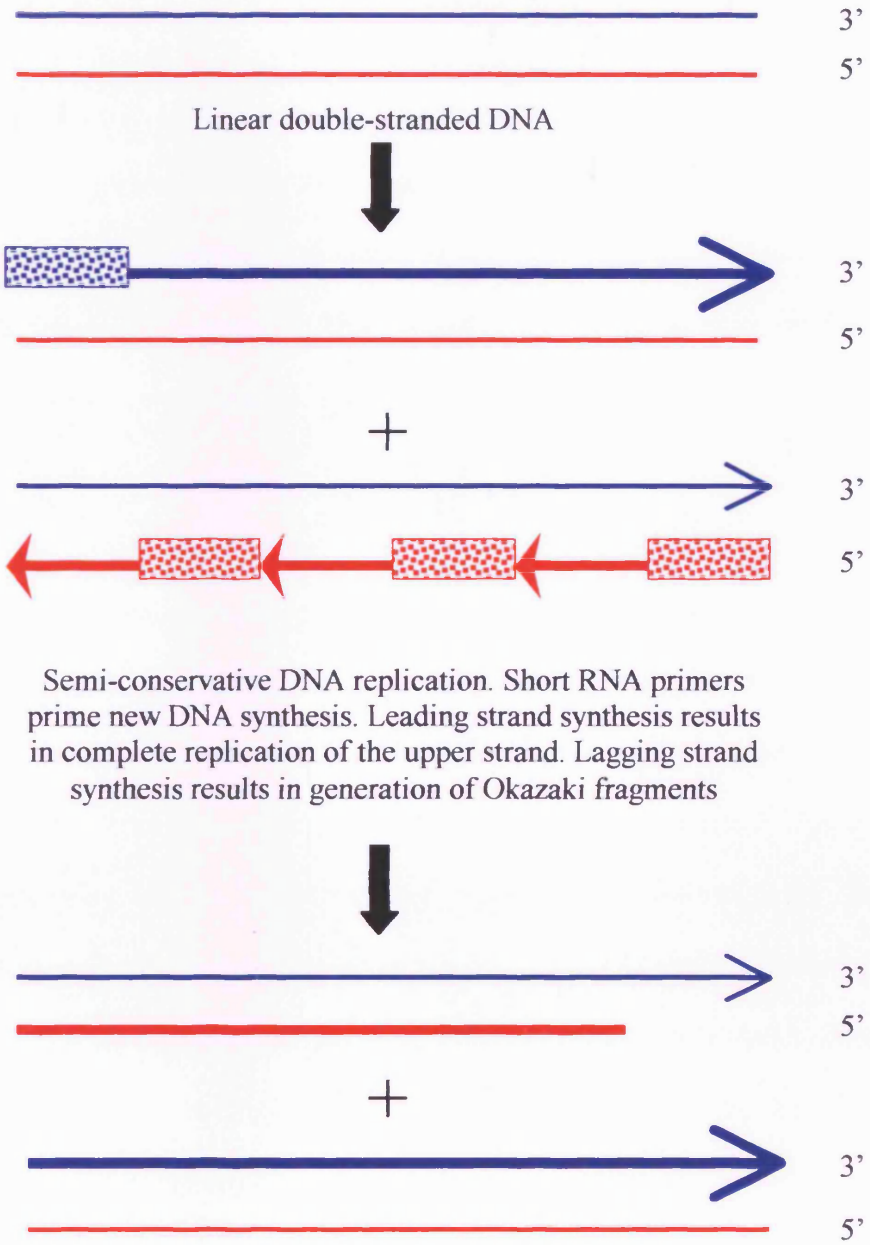
1.2.2 Telomere structure and sequence

Blackburn and Gall in 1978 were the first to study the molecular structure of the telomere. They used the unicellular ciliate *Tetrahymena* and reported a repeating hexa-nucleotide sequence that was present at the chromosome termini.

It was telomeres from *Tetrahymena thermophila* that were also the first to be sequenced. The high copy number (~10^4 copies/macronucleus) and small size (21bp) (Zakian and Pluta, 1989) made studying the telomere much easier than those in the vertebrate cells, which often contain less than 100 chromosome ends per cell (reviewed Greider, 1990). In ciliate macronuclei such as *Tetrahymena*, DNA molecules are linear and require telomeric structure at the termini to be maintained and replicated. New telomeres are generated every time a new macronucleus develops (Prescott, 1994).

In ciliates the G-rich strand is oriented 5' to 3' and the 3' end protrudes beyond the C-rich strand, producing a single-stranded tail (Henderson and Blackburn, 1989). These

Figure 1.2 *The End Replication Problem*



Semi-conservative DNA replication. Short RNA primers prime new DNA synthesis. Leading strand synthesis results in complete replication of the upper strand. Lagging strand synthesis results in generation of Okazaki fragments

Removal of RNA primers and ligation of Okazaki fragments to fill gaps. Lagging strand 5' end cannot be synthesised

G-tails exist during most of the cell cycle and are possibly important for control of telomere length by the telomere maintenance machinery, which requires an exposed 3' terminus (Lingner and Cech, 1996). They may also protect ends from fusion and degradation, and are required for the binding of some telomere-associated proteins.

It was not until 1988 that human telomeres were identified. A highly conserved repetitive sequence, $(TTAGGG)_n$, was isolated from a human recombinant repetitive DNA library. The sequence was found on all of the chromosomes, with major clusters of this repeat at the termini. This sequence was found to have similarities to the functional telomeres found in lower eukaryotes, which suggested that this repeat too, is functional.

The most common structure in human telomeres is the six base pair repeat of TTAGGG. However, there are other types of repeats present at the proximal ends of human chromosomes including TTGGGG and TTTAGGG (Allshire *et al.*, 1989). There seems to be no order to the distribution of these variant repeats, but their presence may be due to the reduced possibility of correction by the telomere maintenance machinery as a result of their extreme proximal positioning (Allshire *et al.*, 1989). Distally, the sequence of the telomere appears to be much more important and variant repeats are less common. This probably reflects the fact that telomere-binding proteins require $(TTAGGG)_n$ for efficient binding.

A summary of telomere repeat sequences in a variety of organisms can be seen in Table 1.1.

Table 1.1 *Telomere repeat sequences in a variety of organisms*

Organism	Repeat	Reference
Single Cell Eukaryotes		
Tetrahymena	TTGGGG	Blackburn and Gall, 1978
Paramecium	TT(G/T)GGG	Forney and Blackburn, 1988
Oxytricha	TTTTGGGG	Klobutcher *et al.*, 1981
Yeasts		
Saccharomyces	$(TG)_{1-3}TG_{2-3}$	Shampay *et al.*, 1984
Mammals		
Homo sapiens	TTAGGG	Moyzis *et al.*, 1988
Mus musculus	TTAGGG	Meyne *et al.*, 1989

Makarov *et al* demonstrated in 1997 that for all human cells tested, >80% of the telomeres have a long G-rich overhang, which averages 130-210 bases in length. This is consistent with the G-rich tails found in ciliates and suggests a similar function is possible.

Therefore, to summarise the structure of the telomere; in most organisms, telomeric DNA consists of a tandem array of very simple sequence DNA (Zakian, 1995). As previously stated, this repeat is TTAGGG in humans (Moyzis *et al.*, 1988), but is also the repeat seen in DNA from chimpanzee (Luke and Verma, 1993), many mammals (de la Sena *et al.*, 1995), other vertebrates, and some invertebrates (Coleman *et al.*, 1993). In all telomeric DNA, regardless of the species, there is a prevalence of tandem G residues (Zakian, 1995).

1.2.3 Telomere length

The average amount of telomeric DNA at a chromosome end varies from organism to organism. For example, *Mus musculus* have as much as 150kb of telomeric DNA per chromosome (Kipling and Cooke, 1990; Starling *et al.*, 1990), whereas telomeres from the ciliate *Oxytricha* macronuclear DNA molecules are only 20bp in length (Klobutcher *et al.*, 1981). In humans, between 5 and 15kb of TTAGGG repeats are found at the ends of all chromosomes (Allshire *et al.*, 1988; Moyzis *et al.*, 1988; de Lange *et al.*, 1990)

1.2.4 Telomerase

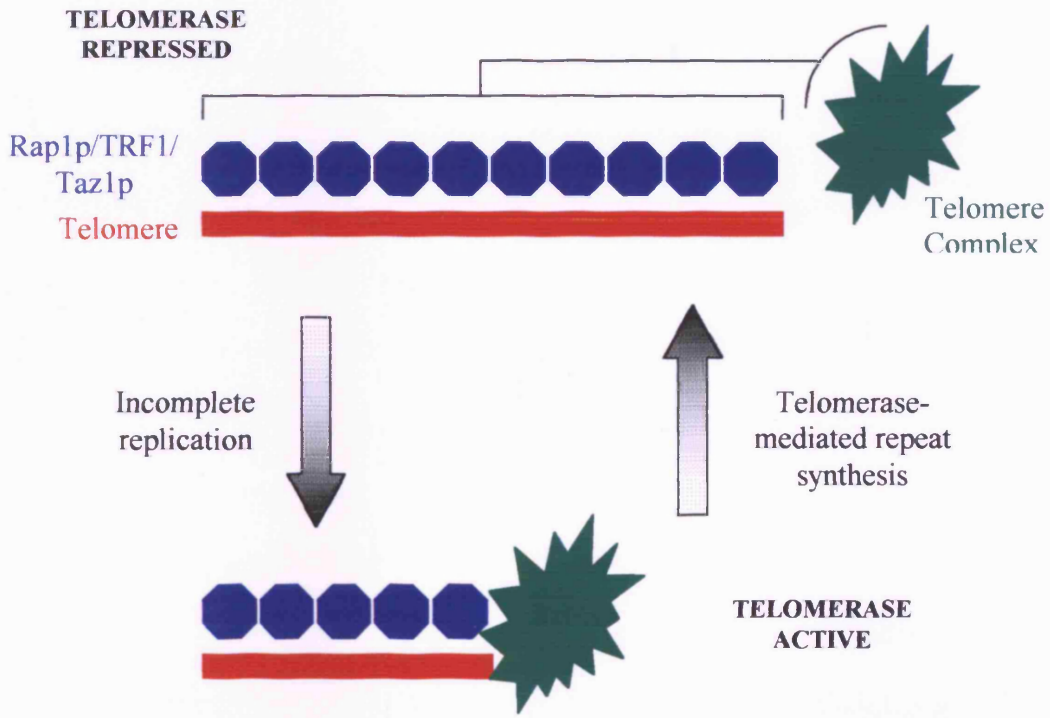
Telomerase, first identified in the ciliated protozoa, *Tetrahymena*, is found in a wide range of eukaryotes from single celled organisms to higher plants and animals, including humans (reviewed by Greider and Blackburn, 1995).

Telomerase is a ribonucleoprotein whose RNA and protein components are both essential for the synthesis of telomeric DNA. Telomerase RNA from different organisms contains an 8 to 30 base segment that serves as a template for the elongation of the 3' overhang that remains after removal of the terminal RNA primer (see Figure 1.2). Telomerase generates tandem repeats of the short sequence encoded by telomerase RNA (See Figure 1.3).

The stabilization of telomere repeats by telomerase has been implicated in cancer, and the shortening of these repeats may be associated with ageing. It is thought that the human protein (TRF1) (van Steensel and de Lange, 1997) and its analogue Taz1p in the yeast *S. pombe* (Cooper *et al.*, 1998) bind to the telomere repeats and prevent the elongation of the telomere by inhibiting telomerase.

Telomerase activity in normal human tissues can only be detected in regenerative tissues that contain stem cells (e.g. skin, blood and intestine) (Hiyama *et al.*, 1995). and in actively dividing cells, such as germ line cells (testis and ovary) (Wright *et al.*, 1996).

Figure 1.3 *A model of telomere length regulation*



Loss of telomerase activity occurs naturally during mammalian development. Most human and some mouse somatic cells do not express telomerase at detectable levels and, as a consequence, cell division results in telomere shortening (Zakian, 1997).

The hypothesis that telomerase was involved in telomere length regulation was confirmed by work carried out in 1998 by Bodnar and her colleagues. They took two telomerase-negative normal human cell types, (retinal pigment epithelial cells and foreskin fibroblasts) and transfected them with vectors encoding the human telomerase catalytic subunit. In contrast to the telomerase-negative control clones, which exhibited telomere shortening and senescence, the telomerase-expressing clones had elongated telomeres even though they were dividing vigorously.

1.2.5 Telomere Shortening

1.2.5.1 *In vivo*

Foetal tissues have been shown to have telomeres of similar length within an individual (variation was obvious when like-tissues from different foetuses were compared), but this foetus specific tissue synchrony is lost after birth (Youngren *et al.*, 1998). This work suggests that all tissues from a single foetus undergo similar cell division whilst *in utero* and/or the possible presence of an intrauterine telomerase activity, which is lost post partum.

Hastie *et al.*, in 1990, demonstrated that the telomeres of somatic cells decreased in length as the donor age increased. It has been calculated that there is approximately a 33 bp loss in telomere length per year in normal blood and colon mucosa (Hastie *et*

al., 1990) and approximately 41 bp in peripheral blood lymphocytes (Vaziri *et al.*, 1993).

The work of these groups shows that after birth there is a intra-variation in telomere length. This occurs when the synchrony seen in foetal tissues ceases to govern telomere length, resulting in different tissue types from the same individual having varying telomere lengths. It is possible that the mechanism that protects foetal tissue from the telomere shortening which would be expected in such rapidly dividing cells is the presence of telomerase (Wright *et al.*, 1996) and also specific proteins (TRF1 in humans) which bind to telomeres and play a role in the feedback control of telomere length (van Steensel and de Lange, 1997). Therefore, it is possible that these protective control mechanisms are lost or silenced after birth.

Although considerable variation in telomere length has been demonstrated in individuals of the same age who are genetically unrelated (Slagboom *et al.*, 1994), studies of 115 twin pairs (2-63 years of age) showed that there was a 78% heritability for mean telomere length in that cohort. This data suggests that telomere length within individuals may be a highly genetically determined trait.

Also, the twin study shows that in non-genetically related individuals, there is an inter-variation in telomere length, but the loss per year seen *in vivo* is similar and can be compared in genetically different subjects, showing that the starting point may vary, but the subsequent shortening occurs at similar rates.

This difference in starting points may possibly be explained in a number of way. The first being the intra-uterine activity of telomerase which ultimately preserves telomere length. This may vary between individuals in either the efficiency or expression of the protein, resulting in genetically un-related newborns having differing telomere lengths (Youngren *et al.*, 1998). Another explanation for the inter-variation seen in telomere lengths of different subjects is related to the "Barker hypothesis" (1995). Here it is suggested that low birth weight due to under nutrition on the middle to late periods of gestation results in a 'catch-up' period *post-partum*. It is possible that this accelerated cell doubling *post-partum* results in the varying telomere lengths seen in adults of similar ages, because the telomerase and specific protein protection normally around to reduce the telomere attrition in the womb will have stopped. This theory will be discussed in more detail later.

1.2.5.2 *In vitro*

Telomeric loss due to donor age is not isolated to *in vivo* tissue. It has also been shown that cultured human cells lose telomeric DNA at a rate which is proportional to the population doublings that the cells have undergone. Previous work has demonstrated that cultured human stem cells, purified from adult bone marrow, had shorter telomeres initially compared with cultured cells from human foetal liver or umbilical cord. The adult tissue also lost more telomeric DNA per population doubling compared with the foetal tissue (35bp vs. 19bp and 23bp respectively).

However, this similar shortening seen in *in vitro* and *in vivo* tissues, does differ because *in vitro* experiments have demonstrated a much more rapid loss of telomeric

DNA, compared to the loss seen *in vivo*, with an estimated 85-1555 bp reduction with every population doubling (Vaziri *et al.*, 1994).

1.2.6 Telomere measurement

Telomere length is determined by the digestion of genomic DNA with restriction enzymes which do not cut within the telomeric repeat array, such as *Rsa I* and *Hinf I*. The digested DNA is then processed using an adaptation of the Southern blotting technique (Southern, 1975), and finally probing with a simple oligonucleotide, complementary to the telomere repeat sequence. Due to the nature of the telomere, a characteristic smear of hybridised products is obtained, reflecting the heterogeneity of length. These products are referred to as the Telomere Restriction Fragments (TRF's).

The mean TRF is then determined with the use densitometry. The use of molecular weight markers enables the allocation of molecular weight at any point in the smear and then the equation used for the calculations takes into account the fact that a greater amount of telomeres in any one area will bind more of the labelled probe and thus, appear darker on the auto-radiograph. The point on the gel where there is optimal probe binding and thus the greatest density, is the point at which there is the greatest distribution of telomere repeats and thus the mean of that population. Using the molecular weight markers, this area can then be assigned a length which is the mean TRF.

1.2.7 Senescence and Immortality

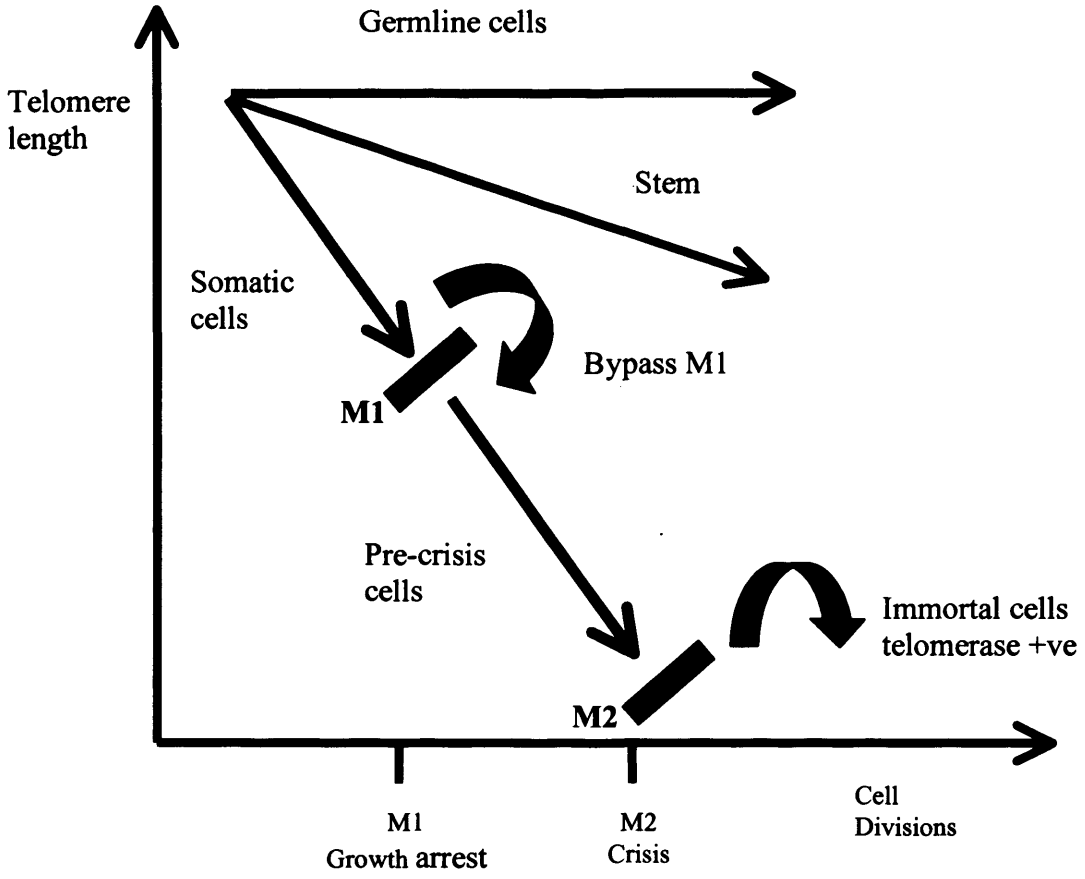
Senescence is the point in the cell cycle which results in an irreversible arrest of cell proliferation and altered cell function, a process controlled by multiple dominant

genes and depends on the number of cell divisions undergone, as opposed to the chronological age of the cell. Cellular senescence may reflect processes that occur during the ageing of an organism and may even be an underlying cause of ageing. Also, cellular senescence can be involved in the suppression of tumours by preventing cells from acquiring the multiple mutations necessary for malignant transformations to occur.

For cells to be 'rescued' from their senescent phase they must escape the M1 and subsequent M2 checkpoints of the normal cell cycle. If successful in doing this they become immortal. It is during this situation that the progression of cancer may occur, which is a common disease state, despite cellular survival beyond the M2 checkpoint being very rare. It is proposed that a unique enzyme is necessary for the genome to be stable after the M2 checkpoint has been survived (Shay and Wright, 1995). This enzyme is telomerase, which has already been described. See Figure 1.4 (adapted from Chui and Harley, 1997.)

Telomeres shorten with every cell division in normal somatic cells until they reach the M1 growth arrest stage and enter senescence. Stem cells express low levels of telomerase and have extended proliferative capacity, but telomeres still shorten, just at a slower rate than somatic cells. Cells can bypass M1 if they are transfected with tumour viruses resulting in a loss of tumour suppressor genes or the activation of oncogenes. These cells have an extended lifespan, but telomere shortening continues.

Figure 1.4 *Telomere hypothesis of cellular ageing and immortalisation.*



The M2 phase is entered by cells with an unstable genome, resulting in cell death for most of these cells. Cells that escape M2 have mutations and are termed immortal.

It has been suggested that the process of cellular senescence may be an anti-cancer mechanism, preventing cells from undergoing enough clonal expansions to form a tumour by having their growth haltered after a certain number of divisions (Shay and Wright, 1995). It is thought that telomerase must be activated to allow for tumour cells to escape the M1 and M2 checkpoint (Shay and Wright, 1996). Assays on a vast number of cancers demonstrated telomerase activity in 90% of the tumour samples but not in the normal or benign tissue adjacent or near to the tumour site. (Counter *et al.*, 1994; Kim *et al.*, 1994). Also, tumour cells have been demonstrated to have short but stable and consistent telomeres (Counter *et al.*, 1994; Broccoli *et al.*, 1995).

Halvorsen, Leibowitz and Levine demonstrated in 1998 that telomerase is indeed sufficient to allow transformed cells to escape from the crisis checkpoint. In their experiments, they introduced the simian virus 40 large T antigen (SVLT) into human primary cells which were subsequently able to proliferate beyond their normal replicative life span. However, this escape from senescence was temporary and the cells eventually entered crisis, during which they ceased growing or died. Following these initial observations, they tested the hypothesis that telomerase activation is the critical step in the immortalisation process by studying the effects of telomerase activity in two mortal SVLT-transformed human pancreatic cell lines. They introduced the telomerase catalytic subunit, hTRT, into late-passage cells via retroviral gene transfer. In all of the telomerase-positive cell lines, the cells formed rapidly dividing cell lines while control cells entered crisis.

It is as a result of the findings of these groups that research was subsequently undertaken to investigate the possible therapeutic use of telomerase inhibition in malignant cells, as a target for specific anti-cancer therapy (Harley *et al.*, 1990).

It is worth mentioning at this point that the work carried out by Halvorsen, Leibowitz and Levine in 1998 is of particular interest to this thesis with respect to the increased proliferative potential of cells which have been transfected with the SVLT virus. This is due to earlier suggestions by Benditt, who hypothesised that the atherosclerotic plaque is monoclonal in nature. This theory was put forward following the observation that eighty percent of discrete raised atherosclerotic plaques are of a single phenotype, suggesting excessive cell proliferation, perhaps to a greater extent to what would be expected if the telomere hypothesis is accepted. Benditt has suggested that these cells are capable of continued cell division due to a mutagenic stimulus, perhaps of viral origin (Benditt EP, 1977). If this is the case, then it is possible that a similar phenomenon to that noted by Halvorsen and colleagues, occurs at the site of the atherosclerotic plaque in that viral infection of arterial cells induces an extended proliferative capacity.

1.2.8 Telomeres and Disease

Telomere shortening has been shown to be accelerated in patients with premature ageing syndromes, such as Hutchinson-Gilford progeria, where the cells from these patients had a reduced life span potential compared to cells from age-matched normals (Allsopp *et al.*, 1992). Also, lymphocytes from Down's Syndrome patients lose telomeric repeats far faster than their age-matched normals. This was

accompanied by the fact that these patients have a much higher incidence of leukaemia (Vaziri *et al.*, 1993).

Accelerated telomere attrition has also been demonstrated in patients with chronic myeloid leukaemia (Brummerdorf *et al.*, 2000), patients suffering with ataxia telangiectasia (Metcalfe *et al.*, 1996); a disease which is characterised by neurological deterioration, immunodeficiency, spontaneous chromosomal instability and a predisposition to cancers and in insulin dependent type one diabetics (Jeanclos *et al.*, 1998).

The work carried out on patients with chronic myeloid leukaemia, a multilineage myeloproliferative disorder characterised by the Philadelphia chromosome (Ph), indicated that overall telomere length was significantly reduced in Ph^+ cells from patients with CML compared to blood leucocytes from normal individuals or normal (Ph^-) T lymphocytes from the same individuals (Brummendorf *et al.*, 2000).

The research using ataxia telangiectasia patients demonstrated accelerated telomere shortening in peripheral blood lymphocytes and the group proposed that the telomeric fusion seen in the patient samples were as a result of this telomere attrition (Metcalfe *et al.*, 1996).

Similarly, patients with insulin dependent diabetes mellitus, have shorter mean TRF's from white blood cells compared with age-matched normals and age-matched patients diagnosed with non-insulin dependent diabetes mellitus. It was suggested that this difference in telomere length could be due to the way that IDDM manifests – the

inflammatory infiltration of the pancreas by the circulating T cells – as opposed to the resulting hyperglycaemia and insulin dependency after the beta cells of the pancreas have been destroyed; this is supported by the fact that a similar pattern was not seen in NIDDM (Jeanclos *et al.*, 1998).

It is possible that the advanced and accelerated telomere attrition that has been associated with these pathological conditions may not be isolated to just the five disease states mentioned here. Suggestions have been made, as has already been eluded to, that based upon preliminary work, telomere attrition may also be linked to the atherosclerotic process. Indeed, Chang and Harley investigated the possible involvement of telomere length as a biomarker of cellular turnover in tissues implicated in atherogenesis. They demonstrated that cultured endothelial cells from iliac arteries were statistically shorter than the same cell type from iliac veins (102 bp/yr vs. 47 bp/yr, respectively, $P < 0.05$). This is consistent with increased cellular turnover *in vivo* of endothelial cells from iliac artery and the higher haemodynamic stress in this area. Preliminary data also showed that, in general, mean TRF lengths of medial tissues from plaque forming iliac arteries were shorter than those from non-plaque forming internal thoracic arteries (147bp/yr vs. 87 bp/yr, respectively, $P < 0.05$) (Chang and Harley, 1995). This possible telomeric involvement in atherosclerosis is further supported by the histological findings of giant endothelial, senescent like cells with bizarre or multiple nuclei in atherosclerotic lesions. The presence of such cells suggests that advanced cell turnover has occurred in this region and thus accelerated telomere shortening may be present in this disease process, as it has been shown to be in others.

1.3 Atherosclerosis

Atherosclerosis and its complications are important causes of morbidity and mortality in most Western societies and involves the thickening and fibrosis of the larger artery walls. Ischaemic heart disease (IHD) is the greatest single contributor to death in the industrialised world, including the United Kingdom. These deaths are often at a relatively early age, accounting for about 18,000 deaths (a third of all deaths) in men and 7,000 deaths (one fifth of all deaths) in women aged less than 65 years in the UK (Our Healthier Nation, 1998).

Atherosclerosis is a complex and indolent histopathological process, which is considered to be the most common underlying process in cardiovascular morbidity and mortality (Sherer and Shoenfeld, 2002). It involves the thickening and hardening of the walls of arteries, resulting in narrowing of the artery and reduced elasticity. The consequence of narrowing of vessels is poor tissue perfusion with blood and thus, oxygen, whilst the loss of elasticity results in a predisposition to vessel rupture and thrombosis (Ross, 1993)

1.3.1 The Endothelial Cell

The endothelium consists of a single layer of tightly apposed cells, which lines the luminal surface of the entire cardiovascular and lymphatic systems. It constitutes a continuous and dynamic interface between the blood and the rest of the body tissues, with many complex biochemical interactions occurring within the cell and at the cell surface (Fishman, 1982; Cryer, 1983). Endothelial integrity is critical for normal

vascular function, particularly in its role of blood compatibility and maintenance of blood flow (Gimbrone, 1974; Jaffe, 1984).

Each endothelial cell has a single nucleus, surrounded by scant cytoplasm. An endothelial cell is about 25 to 50 microns long and 10 to 15 microns wide. Cell thickness on cross sectioning is approximately 0.2 microns through the cytoplasm and 3 microns through the nucleus (Simionescu and Simionescu, 1978). This 'bulging' nucleus gives the endothelium a 'cobblestone' appearance in scanning electron micrographs of *en face* preparations.

Endothelial cells contain all the organelles that are common to eukaryotic cells, with the addition of Weibel-Palade bodies. These are electron dense, rod-shaped organelles, which consist of unit membrane bound bundles of tubules. The bodies have a maximum length at around 3.2 microns and their diameter ranges from 0.1 to 0.5 microns. (Weibel and Palade, 1964). These organelles are often clustered near the periphery of the cell cytoplasm. Their function is not entirely clear, but are basically storage granules for von Willebrand Factor (vWF) and other molecules (analogues to platelet alpha granules) and contain P-selectin in their membrane (Jaffe *et al.*, 1973; Wagner *et al.*, 1982).

Endothelial cells differ from most other cell types because they have a growth saturation point, which is independent of the culture conditions (Schwartz *et al.*, 1979). This suggests that the endothelial cell intrinsically controls its own growth. Endothelial cells will not grow past a monolayer. Studies many years ago discovered the presence of a cell surface protein, CSP-60, which was only present on confluent

endothelial cells, and could not be found on sub-confluent endothelial cell surfaces (Vlodavsky *et al.*, 1979). However, it is unlikely that cell contact is the only mechanism by which endothelial cell growth is controlled. Membrane extracts from confluent endothelial cells could arrest the growth of non-confluent cultures of the same cell type (Whittenberger and Glasser, 1977). Work by Folkman & Moscona (1978), showed that cell substrate adhesion regulated DNA synthesis and consequent cell division. When an endothelial cell culture predominantly consisted of adherent flattened, elongated cells, in a sub-confluent state, DNA synthesis was active. However, in cultures where the cells were still sub-confluent, but had been provided with a growth medium, which allowed the cells to partially detach from the culture vessel, DNA synthesis ceased.

1.3.2 Endothelial Cells and Atherogenesis

Vascular endothelial cells play an important role in maintaining normal vascular homeostasis and haemostasis by producing a balance of paracrine factors such as nitric oxide (NO) and angiotensin II. These factors are known not only to play an important role in the regulation of vessel tone, but also have dramatic effects on vascular structure. Nitric oxide, for example, is not only a potent vasodilator, but it also inhibits platelet adhesion and reactivity, smooth muscle proliferation, and leukocyte adhesion (Cooke *et al.*, 1997).

Nitric oxide is produced from the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). In endothelial cells, the constitutive isoform, eNOS (NOSIII) is responsible for endothelium-derived NO production. NO is produced in response to not only the specific level of shear but also to acute changes

in blood flow. Because NO has an important regulatory function in atherogenesis, increased eNOS expression by laminar shear stress has been hypothesised to be a factor in the resistance of specific vascular segments to atherosclerosis. Matsushita *et al* demonstrated that eNOS mRNA, protein and activity decreased in endothelial cells at senescence as compared with young human aortic endothelial cells *in vitro*. This effect was not seen in cells that had been infected with human telomerase reversed transcriptase (hTERT). They observed that in all cells, shear stress induced greater expression of eNOS, with the greatest levels of the protein being observed in the hTERT treated cells. Basal monocyte adhesion was also noted on aged endothelial cells compared with younger and hTERT treated cells. These studies demonstrated that replicative ageing results in decreased endothelial expression of eNOS. Stable expression of hTERT results in endothelial cells with a younger phenotype with greater amount of eNOS and NO activity (Matsushita *et al.*, 2001).

Insult to the endothelial cell precipitates an inflammatory response characterized by an abnormal expression of adhesion molecules on the surface of the endothelial cell (VCAM-1, ICAM-1, P-selectin and E-selectin) which support the adhesion of platelets, neutrophils, monocytes/macrophages and T lymphocytes to the site of injury (Chang and Harley, 1995). These adherent cells, and the altered endothelium, form and release numerous growth-regulatory molecules (reactive oxygen species, platelet derived growth factor cytokines and chemokines (PDGF)), which in turn act upon the adherent monocytes to cause them to migrate through the endothelial cell lining, localising sub-endothelially.

Once under the endothelium, the monocytes differentiate into macrophages which internalise ox LDL, turning into lipid rich foam cells. These result in the earliest recognizable lesion of atherosclerosis the so-called 'fatty streak' (Ross, 1993).

The growth molecules also act upon the smooth muscle cells in the media and adventitia, causing smooth muscle cell proliferation. An increase in the number of smooth muscle cells together with the formation of a fibrous coating, gives rise to the more advanced lesion of atherosclerosis, the fibrous plaque (Munro and Cotran, 1988).

The plaque contents can continue to accumulate, which may lead to protrusion into and often occlusion of the artery. Plaque rupture can occur, followed by thrombosis during which platelets aggregate and promote thrombin generation. Such thrombi could lead to partial/total artery occlusion and thus, ischaemia and if severe enough, myocardial infarction.

1.3.3 Atherosclerotic risk factors

Many subjects with coronary artery disease have a history of cigarette smoking, hyperlipidaemia, hypertension and diabetes mellitus and much attention has been focused on these 'traditional' coronary risk factors. However, a large proportion of subjects with clinical cardiovascular events do not fall into any of the above categories. This has led to the investigation into other possible risk factors for atherosclerosis such as homocysteinaemia and oxidised lipids.

1.3.3.1 *Lipids*

Lipids are an important factor in atherosclerosis and was noted to be a problem as early as childhood where by deposits of cholesterol in arterial macrophages and smooth muscle cells form fatty streaks (Stary, 1989). The role of lipids was further investigated in a study called Pathological Determinants of Atherosclerosis in Youth in which, 1079 men and 364 women between the ages of 15 to 34, who had died due to external causes, were autopsied in forensic laboratories. The group quantitated atherosclerosis of the aorta and right coronary artery by assessing the extent of intimal surface involved with fatty streaks and raised lesions. A post mortem serum was also analysed for lipoproteins and cholesterol. The amount of intimal surface involved in fatty streaks and raised lesions increased with age in both the sexes and race groups. VLDL (very low density lipoprotein) plus LDL (low density lipoprotein) cholesterol concentration was associated positively and HDL (high density lipoprotein) cholesterol was associated negatively with the extent of fatty streaks and raised lesions (McGill *et al.*, 1997). In addition to this work, other groups have further identified the role of lipids as a risk factor by demonstrating that therapeutic lowering of serum cholesterol, LDL and oxidised LDL (which occur when the oxidative capacity of the endothelium is exhausted) resulted in improvement in endothelium-dependent dilation in the forearm vasculature of patients with hypercholesterolaemia (Tamai *et al.*, 1997). Further to this *in vivo* work, studies have also demonstrated that when human endothelial cells were cultured in the presence of mildly oxidised lipoproteins, a significant induction of endothelial growth was observed. Further treatment of endothelial cells with mildly oxidised LDL modulated the expression of cytokines and growth factors. Endothelial cells which were chronically exposed to mildly oxidised LDL underwent a more rapid onset of cellular senescence (Maier *et*

al, 1996) and subsequent deranged behaviour. These three sets of investigations, in addition to many others, strongly suggests an involvement of lipoprotein levels, in particular those which are oxidised, in the formation and progression of atherosclerosis via interactions with the endothelial cells which form the lining of the arteries.

1.3.3.2 *Homocysteine*

Homocysteine is a highly reactive sulphur-containing amino acid. Homocysteinuria, one of the classical inborn errors of metabolism, is an autosomal recessive metabolic disease which has a frequency of between one in 70 and one in 200 of all live births (Boers *et al.*, 1989). The effects of homocysteine on endothelial morphology was first demonstrated by Harker and Ross in 1976 in primate models. They noted that homocysteinaemia caused patchy endothelial desquamation and atherosclerotic lesions (Harker and Ross, 1976). Since these first reports, homocysteinaemia has been seen to frequently accompany accelerated atherosclerosis (Cacciari and Salardi, 1989). In cell culture experiments, the addition of homocysteine into culture medium induces cell detachment from endothelial cell monolayers and functional abnormalities in the release of endothelium derived nitric oxide (Wall *et al.*, 1980). Also, Xu and colleagues demonstrated that exposure of cultured endothelial cells to homocysteine significantly accelerated the rate of endothelial senescence by increasing telomere loss per population doubling, both of which were inhibited by treatment of the cells with the peroxide scavenger, catalase (Xu *et al.*, 2000). In non-human primates, a continuous homocysteine infusion for 3 months resulted in patchy endothelial desquamation, amounting to 10% of the aortic surface (Lentz *et al.*, 1996). This work was taken further, with the use of human studies, which demonstrated that

patients who were defined as hyperhomocysteinaemic (mean plasma homocysteine, 34.8 +/- 8.5μmol/L), but were non-smoking, healthy individuals aged 53 +/- 9 years, had a much reduced endothelium-dependent flow-mediated dilation compared with age matched normals with a low plasma homocysteine level (9.9 +/- 3.2μmol/L). This work was statistically sound and demonstrated that hyperhomocysteinaemia is an independent risk factor for arterial endothelial dysfunction in healthy middle-aged adults (Woo *et al.*, 1997).

1.3.3.3 *Oxidative stress*

Oxidative stress has been implicated in the development of endothelial dysfunction. Fennel *et al* explored their ability to over express anti-oxidant genes (superoxide dismutase; SOD) *in vitro* and *in vivo* to assess their potential for reversing the effects of oxidative stress in a rat model, the stroke prone spontaneously hypertensive rat (SHRSP). They demonstrated that over expression of extracellular SOD but not mitochondrial SOD *in vivo*, resulted in improved endothelial function by increasing the nitric oxide availability (Fennel *et al.*, 2002), thereby underlying the fact that oxidative stress does indeed affect the endothelial cell and an increase presence of antioxidants protects this cell type.

1.3.4 Endothelial Cell Replication *in vivo*

Physical trauma to the artery also affects the role of the endothelial cell. When the vascular endothelium is damaged, revealing the underlying tissue, the adjacent endothelial cells will try to bridge the gap by stretching out to each other. If the gap is too wide, the closest cells to the site of damage will divide to produce daughter cells which cover the wound. It is common in vascular damage that injury to the

endothelium often re-occurs at the same site, which may result in some cells undergoing more population doublings compared with other cells situated further away from the damaged area. Using Hayflick's hypothesis, it is possible that if the endothelial cells have to divide many times in order to repair continued vascular damage then they will approach accelerated senescence and thus lose their replicative ability. If this physical trauma were to continue occurring at this now senescent site, the cell would no longer be able to replicate and there would be un-repaired damage to the vessel lining resulting in alterations to the surface of the artery at the injured site. This theory is supported by work which demonstrated that endothelial cells from both the lining of the cap of atherosclerotic plaques, and those seen in saphenous veins from elderly patients had altered morphology and were senescent-like. (Davies *et al.*, 1988; Burrig, 1991). Therefore it is highly possible that damage to the endothelium leads to endothelial cell proliferation and ultimately shortened telomere and thus, possibly related senescence.

It is also possible that damaged endothelial layers are repaired partially as a consequence of circulating endothelial stem cells. These cells may localise to the site of injury and proliferate to produce a new endothelial cell lining.

1.3.5 Endothelial Cell Culture

In 1963 Maruyama described a method of isolating and culturing human endothelial cells from umbilical cord vein. This was an easily obtainable and abundant source of human material that could yield large numbers of cells. However, Maruyama was limited by a difficulty in growing and maintaining a heterogonous culture for more than a few weeks. The cells they were culturing were never positively identified as

being endothelial. Jaffe *et al* (1973) and Gimbrone (1974) extended the work of Maruyama and devised the method for isolating and culturing human umbilical endothelial cells which is widely used at present. They also established the morphological and immunological criteria on which endothelial identification is based.

This work set the standard for the development of methods of isolating and culturing of endothelium from a variety of animal sources. Schwartz (1978) provided the method of growing bovine aortic endothelium, which is very easy to maintain for long periods in culture, and can be grown very quickly in large quantities without any special growth factor requirements. A simple method for cloning bovine aortic endothelial cells developed by Gadjusek & Schwartz (1983), allows growth of cell lines for much longer periods of time by eliminating contaminating and senescent cells.

This approach has been used to study the effects of oxidative stress on endothelial cells in culture.

1.4 Oxidative stress

Oxidant by-products of normal metabolism cause extensive damage to DNA, proteins and lipids and it is argued that this damage is a major contributor to ageing and to degenerative diseases of ageing such as cancer and cardiovascular disease (Ames *et al.*, 1993).

There are four endogenous sources of oxidants:

(i) Normal aerobic respiration, which involves the reduction of mitochondrial consumed O_2, producing H_2O. By-products of this process are $O_2^{\cdot-}$ (superoxide anion), H_2O_2 (hydrogen peroxide) and $^{\cdot}OH$ (hydroxyl radical)

(ii) The respiratory burst of phagocytic cells destroying bacteria and viruses. The by-products of this process are mainly NO (nitric oxide), $O_2^{\cdot-}$ and H_2O_2

(iii) The degradation of fatty acid by peroxisomes which produces H_2O_2

(iv) The activity of cytochrome P-450 enzymes, which are present in the body as a defence mechanism against natural toxins from plants and other dietary matter.

In addition to these generic endogenous sources of oxidants, Cosentino *et al* have demonstrated that endothelial derived nitric oxide synthase (eNOS) can lead to the production of reactive oxygen species when tetrahydro-biopterin (H_4B) is deficient. In mice which were severely lacking in H_4B, there was a decrease in the production of nitric oxide from eNOS in favour of the production of superoxide radicals (Cosentino *et al.*, 2001).

The oxidants, which are produced by these mechanisms are collectively termed reactive oxygen species (ROS). ROS are reactive chemical entities, which are classified into two categories: free radicals (e.g. O_2·- and ·OH) characterised by having one or more unpaired electron, and non-radical derivatives (e.g. H_2O_2). When in low concentrations, ROS can be useful signalling chemicals, but if they are not cleared from the system high concentrations can occur and they can be cytotoxic.

1.4.1 Effects of ROS on the Cell

The redox state in which these ROS are involved is finely tuned to preserve cellular homeostasis through expression and regulation of oxidant and antioxidant enzymes. Mammalian cells have a complex network of antioxidants such as superoxide dismutase which catalyses the reduction of superoxide anions to hydrogen peroxide, and catalase along with glutathione peroxidase which remove the hydrogen peroxide, converting it to water and oxygen. (Okuda *et al.*, 2001).

In certain circumstances, the concentration of ROS exceeds the antioxidant capacity of the cell resulting in oxidative damage to cellular constituents such as the mitochondria (Knight-Lozano *et al.*, 2002).

Another major feature of ROS-induced cellular injury is lipid peroxidation due to the effect of ROS on polyunsaturated fatty acids. This results in reduced membrane fluidity which alters membrane-bound protein functions. ROS can also cause damage to nucleic acids by interacting with base and sugar groups, resulting in single- and double stranded breaks and cross-links with other molecules.

1.4.2 Oxidative stress and Atherosclerosis

Oxidative stress induced by ROS has been implicated in the pathogenesis of a variety of vascular diseases including hypertension, coronary artery disease and atherosclerosis. It is thought that this oxidative stress may be involved in atherosclerosis by inducing smooth muscle cell proliferation and inactivating endothelium derived nitric oxide causing endothelial cell dysfunction (Griendling, 1998)

Much work has been carried out, both in this laboratory and others into the mechanisms associated with this atherosclerosis associated endothelial cell dysfunction following oxidative stress. In 1989, Tokunaga cultured cells from human aortas and inferior venae cavae of autopsied subjects ranging in age from infancy to 85 years old. Endothelial cells in the primary cultures were classified into two types; typical endothelium and variant endothelium. Typical endothelium consisted of cells which were small, round to polygonal shaped, and were arranged uniformly. Their diameter ranged from 50 to 70 μm. Variant endothelium consisted of cells which were larger, ranging from 100 to 200 μm in diameter and giant endothelial cells measuring more than 250 μm in diameter were scattered among them. Variant endothelial cells were usually multinucleated and possessed endothelial markers of von Willebrand factor and Weibel-Palade bodies. The ratio of variant endothelial cells to typical endothelial cells correlated well with the severity of atherosclerosis. Also, there were more variant endothelial cells in the cultures from aorta tissues than the vena cavae tissue.

Work in this laboratory has also associated a similar presence of variant endothelial cells following oxidative stress. Yang demonstrated a dose and time dependent response of bovine aortic endothelial cells following exposure to hydrogen peroxide and the morphological changes were similar to those seen by Tokunga. In Yang's series of experiments, cultured bovine aortic endothelial cells were briefly exposed to low concentrations of hydrogen peroxide which did not result in immediate lysis. Delayed cell death occurred over the following 24 hours accompanied by changes in nuclear morphology including nucleosomal fragments in extracted nuclear DNA, and the appearance of numerous DNA strand breaks (demonstrated by 3'OH *in situ* end-labelling) compatible with apoptotic cell death. Cells which survived apoptosis showed inhibition of cell division on subsequent culture for up to 15 days, and there were morphological changes, with the formation of uninucleate or multinucleate giant cells. These effects were suggested by Yang to be relevant to the mechanisms by which brief exposure to oxidative stress causes progressive vessel wall damage (Yang and de Bono, 1997).

In an attempt to understand the mechanisms by which oxidative stress may cause this endothelial dysfunction and thus initiate the progression of atherosclerosis, telomere lengths have been studied. Chang and Harley had previously demonstrated that endothelial cells cultured from iliac arteries had a significantly greater rate of telomere attrition compared with cells cultured from iliac veins (102 bp/yr vs. 47 bp/yr respectively, $P < 0.05$), which is consistent with the higher haemodynamic stress and increased cell turnover known to occur in arteries (Chang & Harley., 1995).

More recently Minamino *et al* have published work which suggests that the telomere has an important role in the recognised endothelial dysfunction associated with atherosclerosis. They demonstrated a loss of telomere function following the transfection of a defunct TRF-2 molecule (a telomeric protein essential for the formation of duplex loops at the telomere end, which have been shown to protect the telomere structures). This resulted in endothelial growth with phenotypic characteristics of cellular senescence including the expression of ICAM-1 with reduced activity of NOS as well as eNOS, all three of which have been induced with oxidative stress (Minamino *et al.*, 2002).

This work therefore suggests that oxidative stress may be involved in atherosclerosis by altering telomere length, which subsequently results in endothelial cell dysfunction.

1.5 Aims of the study

Previous work has suggested that telomere biology, with particular reference to length, is associated with certain disease processes. More specifically, the vascular ageing observed during the progression of atherosclerosis has indeed been linked to telomere shortening (Chang and Harley, 1995; von Zglinicki *et al.*, 2000).

Possible mechanisms by which such telomere shortening may occur have been investigated and many groups have reported that telomere interactions with

compounds capable of causing oxidative stress may be important to the changes seen in telomere length.

Using the observations made by these groups as a basis, the worked carried out for this thesis was designed to investigate what changes to telomeres, if any, occur in patients with coronary artery disease. Are these changes ubiquitous to all the cells involved in the disease process or isolated/exaggerated in certain cell types. By what mechanism are the telomeres of these cell types affected. In order to answer these questions, the following work was carried out:

1: Development of a robust and reproducible technique which could be used for the measurement of telomere length of cells from human and bovine tissue.

2: Investigations designed to show whether accelerated telomere shortening was seen in peripheral white blood cells of subjects with advanced coronary atherosclerosis compared with normal individuals.

3: Investigations designed to show whether telomere lengths from a heterogeneous population of cells contained within an atherosclerotic plaque were different to those of circulating white blood cells from the same patient. This was carried out to investigate what level of cell division, if any, was occurring at the site of an atherosclerosis-affected area of the vasculature, compared with the circulating white blood cells. The results would demonstrate whether or not the changes seen in atherosclerosis were similar in both tissue types and therefore as a result of a genetic

factor acting alone, or increased at the site of the plaque, suggesting the additional role of an exogenous factor.

4. An *in vitro* model was designed to test a possible hypothesis – that the mechanism by which telomeres shortened and thus caused atherosclerosis primarily targeted the endothelial cell and is initiated by oxidative damage. The model involving culturing bovine aortic endothelial cells under oxidative stress and the telomeres of the cell were measured over a period of time.

Chapter 2: Materials & Methods

2.1 Human tissue

Written informed consent was obtained from all participating volunteers prior to any specimens being taken and approval for all studies was granted by the UHL Trust Ethics Committee. Surgical procedures and sample collections were carried out by members of the Department of Surgery at the Leicester Royal Infirmary.

2.1.1 Peripheral Blood

Blood samples were taken from three different sources: consenting patients under the age of 60 undergoing endarterectomy procedures at the Leicester Royal Infirmary; consenting patients of any age undergoing investigations for coronary heart disease at the Glenfield General Hospital; apparently healthy volunteers (comprising of students, staff and/or relatives) between the ages of 20 and 80 years from within the University Clinical Sciences Wing, Glenfield General Hospital.

Blood samples were collected from patients into vacutainers containing EDTA (Sarstedt Ltd, Leicester, UK).

The blood samples were kept at room temperature until they could be processed which was usually within 4 hours of phlebotomy.

2.1.2 Atherosclerotic plaques

Atherosclerotic plaques were obtained from consenting patients undergoing a carotid endarterectomy at the Department of Surgery, Leicester Royal Infirmary. The only inclusion criteria for the study was the patient had to be 60 years or younger. No exclusion criteria were used.

Pieces of plaque were placed immediately in ice cold sterile Hank's balanced salt solution (HBSS) (GibcoBRL, UK), by the surgical team. The samples were transferred to the laboratory within 4 hours of the operation, where they were snap frozen in liquid nitrogen and stored at –80°C, until they could be processed.

Prior to anaesthesia for the endarterectomy procedure a 10 ml blood sample was also taken from each of the patients and processed as described 2.3.3.

2.2 Bovine Aortic Endothelial Cells

The techniques used in the following section were based on the methods of Jaffe (1973), Schwartz (1978) and Gadjusek and Schwartz (1983).

2.2.1 Tissue Culture Materials

Cells were routinely grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS). Cells were washed with Dulbecco's phosphate buffered saline (PBS), and detached from the culture vessel using trypsin-EDTA solution. All tissue culture reagents were obtained from GibcoBRL. Tissue

culture plates, flasks and dishes were supplied from Nunc (Hereford, UK) and the University of Leicester stores.

2.2.2 Primary Culture of Bovine Aortic Endothelium

Bovine aortas were obtained from the local abattoir as soon as possible after slaughter. Segments of descending thoracic aorta, approximately 30 cm in length, were transferred to the laboratory on ice and processed immediately under sterile conditions. Although times varied, it was usually about an hour from obtaining the aortas at the slaughterhouse and the start of the culture procedures. Any aortas found to have cuts prior to process were discarded.

The proximal end of the aorta was ligated, and a clamp was used to close the distal end. Any fat or connective tissue was carefully removed with a pair of scissors and a scalpel, taking great care not to damage any of the roots of the intercostal arteries near the aorta. All of the intercostal arteries were ligated with suture cotton. The clamped end of the vessel was cut off and a kwill filling tube inserted in the distal end and secured. The vessel was washed through thoroughly with saline and filled with 10 ml of 0.1% (w/v) collagenase (Sigma, Dorset, UK) HBSS using a syringe. The syringe was left in place and the vessel was then wrapped in cling film (to prevent evaporative cooling and desiccation) and a layer of aluminium foil (to aid the transfer of heat around the vessel), then incubated at 37°C for 15 to 20 minutes.

Once removed from the incubator, the vessel was gently massaged in order to aid the release of loosened cells. The collagenase/cell solution was removed using the syringe, which was still attached to the artery, and placed in a sterile universal tube. In

order to ensure that all cells were removed, the lumen was rinsed with 10 ml of culture medium. This rinse solution was added to the universal tube. The cell suspension was centrifuged at 1500g for 10 minutes and then the cell pellet was re-suspended in culture medium and re-centrifuged. The resultant cell pellet was re-suspended in culture medium and plated in an $80cm^2$ tissue culture flask.

Collagenase treatment removes the cells from the aorta wall in large sheets, which make an accurate cell count impossible, therefore the cells obtained from one such length of aorta were initially cultured in 20 ml of culture medium in one $80cm^2$ tissue culture flask, or re-suspended in 40 ml of medium and plated in two flasks, depending on the yield, estimated by eye under the microscope.

Most of the endothelial cells would adhere to the culture flask within 2 hours. Thereafter the cells were gently washed and fresh medium was added.

If the cells were free from contamination and in good condition, they normally reached confluence after 48 hours reaching a density of about $8 \times 10^6/80cm^2$ or $10^5/cm^2$. At this point the cells were cloned as described in section 2.2.3.

2.2.3 Cloning of Bovine Aortic Endothelial Cells

Primary cultures of bovine aorta endothelial cells (BAEC) which had reached approximately 90% confluence were checked under a microscope. Old growth medium was removed from batches of cells with high quality and purity. These cells were rinsed with 5 ml trypsin-EDTA solution (0.02% trypsin, 1mM EDTA and 10mM sucrose in 20mM Hepes buffered PBS without Ca^{2+} and Mg^{2+}, pH 7.4) and then

incubated with 5 ml fresh trypsin-EDTA solution for 5 minutes at 37°C. The cells were aspirated through a 25-gauge needle (whilst still suspended in the trypsin-EDTA solution) 2 to 3 times in order to break up any cell clumps. The cells were diluted with 15 ml of serum-containing growth medium to inactivate the trypsin and recovered by centrifugation at 1500g for 5 minutes. They were washed once with culture medium before finally being re-suspended in 20 ml of culture medium.

The cell suspension was counted under a microscope with the use of a haemocytometer and diluted with culture medium so as to achieve a final cell density of 10 cells/ml. The cells were then plated out in a 96 well plate at 100µl/well; equivalent to 1 cell/well. After 24 hours, each well was visually scored for cell number and examined daily for the following 3 days. The wells, that contained a single cell at 24 hours after plating were identified as a possible clone. After 4 days in culture, any wells which contained a single colony consisting of 8 to 12 cells, were considered to be derived from one single progenitor cell. The culture medium was changed every 4 to 5 days using a 50:50, fresh: conditioned medium mixture, until the cells formed a monolayer. It usually took about 12 to 18 days for the cells to reach confluence at a density of about 4 x 10^4 cells/well; during this time the cells were estimated to have undergone 13 to 15 cell doublings.

The cells were then transferred on a well-to-well basis to a 24 x 16mm well plate in normal growth medium, using standard sub-culturing techniques. It usually took about 4 to 6 days for the cells to reach confluence in these wells, reaching a density of about 2 x 10^5 cells/well (about $10^5/cm^2$), during which time the cells were estimated to have undergone another 3 to 5 cell doublings (16 to 20 in total).

Those cells that had retained the cobblestone morphology of normal endothelium on reaching confluence were assumed to free of any contamination from fibroblasts and smooth muscle cells (a limitation of endothelial culture in the absence of cloning procedures) and were stored in liquid nitrogen until they were used for all of the *in vitro* experiments described in this chapter.

2.2.4 Bovine Aortic Endothelial Cell Sub-culturing

The old growth medium was aspirated from the 80-cm^2 flasks, and the monolayer was rinsed with 5 ml PBS and then 5 ml of trypsin solution was added. The cells were incubated for 5 minutes at 37°C. Gentle tapping of the flask was used when the cells did not release from the flask efficiently. As soon as the cells had detached from the culture surface of the flask, the cell suspension was pipetted vigorously in order to remove any cell clumps and then added to 15 ml of serum-containing growth medium to inactivate the trypsin. The cell suspension was transferred to a sterile universal tube and then centrifuged at 1500g for 10 min, and washed once with the culture medium. The resultant pellet of cells was re-suspended in culture medium and counted using a haemocytometer. The cells were plated out at a density of between 3 and 5 x 10^4 cells/cm^2 (2.5–4 x 10^6 cells/80cm^2 flask), depending on the experiment.

2.2.5 Liquid Nitrogen Storage of Cells

When good primary culture and cloned cell lines were obtained, a quantity was stored in liquid nitrogen for future use.

Confluent cloned cells, which had been sub-cultured twice were trypsinised, washed thoroughly and re-suspended in PBS. The cells were recovered by centrifugation at

1500g for 5 minutes and then re-suspended in 90% foetal calf serum (FCS) (Gibco): 10% dimethyl sulphoxide (DMSO) (Sigma) at a density of approximately 2 x 10^5 cells/ml. The cells were dispensed in 1 ml aliquots into sterile cryotubes, which were cooled slowly in a well-insulated container to –70°C and kept at this temperature for 12 to 24 hours before being transferred to vapour phase liquid nitrogen (Oi & Herzenberg, 1980)

2.2.6 Recovery of Cells from Liquid Nitrogen Storage

Vials were removed from the liquid nitrogen immediately before use and thawed quickly, but not completely, in a 37°C water bath. 5 ml of cold growth medium was added drop wise. Slow addition of cold medium at this stage prevents rupture of the cells through heat or osmotic shock. The cell suspension was added to 13 ml of cold growth medium. The cells were recovered by centrifugation for 5 minutes at 1500g and re-suspended slowly in 20 ml of medium at 37°C. All of the cells from one cryotube were plated out in an 80cm^2 tissue culture flask.

The cells were then left for 24 hours, at which time the medium was changed in order to ensure that all of the DMSO had been removed. Confluence was reached after 5 to 10 days depending on the number remaining viable after storage and recovery.

2.2.7 Endothelial Cell Identification

2.2.7.1 *Morphological Criteria*

Endothelial cell cultures were mainly identified on their morphological criteria. Bovine aortic endothelial cells tend to be polygonal in shape and form a tight cobblestone appearance when confluent.

Pure endothelial cells grow in a single uniform monolayer with cells showing contact inhibition. At confluence, cell growth slows down and becomes static, and cultures of pure bovine endothelium can be maintained for long periods of time without any sub-culturing.

By contrast, fibroblasts and smooth muscle cells, the two common contaminants of endothelial cultures from large vessels, are elongated and less well defined in shape. Fibroblasts grow in multiple layers and very quickly overgrow any endothelial cells present in a culture. The striking difference in morphology between these cells helps to eliminate contaminated cultures at an early stage.

2.2.7.2 *Immunofluorescence staining of endothelial cells for vWF*

The most widely used immunological marker for endothelial cells is the presence of von Willebrand Factor (vWF) in the intracellular Weibel Palade granules. Cells incubated with a fluorescently labelled anti-vWF antibody can be identified by fluorescence microscopy. Staining for vWF was used to confirm the identity of the cells used, prior to any experiments.

Bovine aortic endothelial cells were plated out at a density of approximately $1x10^5$ cells per well, in culture medium, in an 8-well chamber slide (Nunc) and incubated overnight. The next day, the cells were washed three times with 300 µl HBSS/1% bovine serum albumin (BSA) (Sigma) solution. The cells were then incubated at 4°C with 200 µl 2% (w/v) paraformaldehyde (Sigma) in PBS for a minimum 16 hours.

If von Willebrand Factor within the Weibel Palade bodies were to be stained, a PBS solution containing 2% (w/v) BSA and 0.1% (w/v) saponin (Sigma) was used. Saponin is a detergent, which permeabilises the cell membrane and allows antibody and other exogenous agents to enter the Weibel Palade bodies. This therefore allows for the intracellular vWF to be measured. The wells were washed three times with 300 µl 2% (w/v) BSA, 0.1% (w/v) saponin in PBS solution and then incubated for 30 minutes at room temperature with 50 µl of a 1:500 dilution of ascitic fluid containing mouse anti-von Willebrand factor monoclonal antibody RFF-VIII:R/1 (Goodall *et al*, 1985), with gentle agitation. The optimum dilution of RFF-VIII:R/1 was determined by titration.

The wells were washed three times with 300 µl 2% BSA, 0.1% saponin in PBS solution the cells were then incubated with 50 µl FITC conjugated-anti-mouse IgG monoclonal antibody (Dako, Ely, UK), diluted 1:10 in 2% BSA, 100nM Hoechst (final concentration) (Sigma) in PBS solution, for 30 minutes at room temperature with gentle agitation.

The wells were then washed three times with 2% BSA, 0.1% saponin in PBS solution, at which point, the staining was complete.

The plastic wells were then carefully removed and the gasket sealant was peeled from the glass slide. Two drops of Dako anti-fade Fluorescent mounting medium (Dako) was added to a cover slip and the glass slide was lowered down gently onto the cover slip. The covered slide was then left in the dark for 10 minutes, before viewing under a Leitz Diavert inverted microscope with a Cosina camera and attachments.

For extended storage, the edges of the cover slip were sealed with clear nail varnish.

All photographs were taken at 100 x magnification unless otherwise stated, with phase contrast optics and a blue filter. Photomicrographs were taken on Kodak-Colour gold 400 or 200 ASA film.

2.2.8 Treatment of BAEC's with Hydrogen Peroxide

Bovine aortic endothelial cells were cultured in $80cm^2$ flasks. The cells were seeded at a density that would allow them to be approximately 85% confluent (approximately 5-6 x 10^6) on the day the exposure to oxidative stress would be carried out.

Hydrogen peroxide was chosen because biological membranes are permeable to this compound. Hydrogen peroxide (H_2O_2) is able to cross the cell membrane because of its neutral charge, however as no unpaired electrons are present, hydrogen peroxide cannot act as a free radical alone. The superoxide anion would be the natural choice, but because it is charged it cannot cross biological membranes. However, any biological system which generates superoxide anions will produce hydrogen peroxide by either non-enzymatic or SOD - catalysed dismuatation:

$$2\ O_2. + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Therefore these experiments are attempting to model free radical interactions outside the cell producing hydrogen peroxide as a by-product of dismutation, which then crosses the biological membranes of cells and interacts with the cell internally. Due to the antioxidant content of the serum in the culture medium, the oxidative exposure

was carried out in Hank's buffer, in the absence of proteins and amino acids. The medium also did not contain phenol red because this would react with hydrogen peroxide.

The BAEC's were subjected to oxidative stress by a single dose exposure to hydrogen peroxide (Sigma), for 1 hour at 37°C. The hydrogen peroxide was diluted to the working concentration with Hank's buffer and then 30 ml of this solution was added to the cells. Cell number, and thus the actual amount of the oxidant available to each individual cell determined the final concentration of the hydrogen peroxide (Yang., 1999).

For example, to achieve a target concentration of 0.5 pico moles per cell:
For a flask initially containing 6 x 10^6 cells and the required hydrogen peroxide concentration is 100μM, the amount per 10^6 cells is 0.5μmol (0.5 pico moles per cell). The total amount of hydrogen peroxide needed is [(6 x 10^6) x (0.5μmol/10^6 cells) = 3.0μmol]. It can then be calculated that a final concentration of 100μM can be made by using 30 ml Hank's solution [(3.0 μmol/30 ml) = (100 μM)].

After the 1-hour exposure time, the hydrogen peroxide/Hank's solution was aspirated from the cells and the normal culture was re-introduced to the cells. When the growth curve reached plateau, the experiment was stopped. The time at which this point was achieved varied between each experiment.

DNA was extracted from these cells as described in section 2.3.2.

2.2.9 Apoptosis Assay

This procedure was carried out to ensure viability of the cells during the oxidative stress experiments i.e. to make sure that the hydrogen peroxide was not just killing the cells and thus, forcing them into apoptosis.

One of the changes that occur in cells undergoing apoptosis is 'flipping' of the negatively charged phospholipids (predominantly phosphatidyl serine; PS) to the outer layer of the membrane bi-layer. These negatively charged phospholipids can be detected with the use of fluorescent-labelled Annexin V; a protein with a high affinity for PS. Annexin V binding is highly calcium dependent and so incubations are performed in calcium-containing buffer.

Annexin V-FITC (Alexis Biochemicals, Nottingham, UK) was diluted 1 in 40 with Annexin binding buffer (Alexis Biochemicals). This dilution was used as a result of a titration experiment, which demonstrated that with the number of cells expected to be present at this point in the experiment, this dilution is the lowest needed to give a positive result. Hoechst 33528 was used as a nuclear stain in order to help with orientation of the cells during the analysis procedure. The Hoechst dye was used at a final concentration of 100nM.

Cells were grown in eight well slide chambers and treated with hydrogen peroxide as described previously.

Cells were incubated with 40 μl Annexin V-FITC solution for 10 minutes at room temperature. This solution was removed and the cells were rinsed with PBS. The cells

were then incubated with acetone/methanol for 20 minutes at room temperature, rinsed with PBS and then incubated with 40 µl Hoechst stain for 10 minutes at room temperature. Finally, the cells were rinsed and mounted with a cover slip.

The cells were analysed using a fluorescent microscope with the use of suitable filters for both the FITC and Hoechst fluorochromes.

Control cells (with respect to the staining procedure) were left in normal culture medium through out the staining procedure for the other cells. The medium was removed and the cells rinsed with PBS immediately prior to the cover slip application.

A phase contrast/fluorescent microscope (Zeiss) was connected to a CCD camera (Hamanamatsu) from the image analysis system KS100. This system allowed images of cells to be produced using both phase microscopy and fluorescence.

Images were created at (x100) and the excitation and emission wavelengths used for the blue and green fluorescence were 360nm, 470nm and 499nm, 525nm respectively. Exposure time for phase contrast images was approximately 0.5 seconds with a 6dB gain and the exposure time was increased to 20 seconds for the blue and green fluorescence, with a gain of 12dB. Images were then taken into Paint Shop Pro 5, and the blue and green fluorescence for each image was superimposed onto its phase contrast image.

2.3 Isolating Genomic DNA

2.3.1 Phenol-Chloroform Extraction of DNA from Plaque samples

The frozen tissue sample was taken from –80°C and placed in a sterile petri dish. When the sample had thawed sufficiently so that it could be cut, enough was cut off for the preparation and the remaining piece, if any, was re-frozen as described above. On average, a 0.5cm piece of plaque was used.

The sample was cut into small pieces using a razor blade and a pair of forceps to keep the sample in place. When the pieces were thought to be small enough (approximately 1-2mm long on all sides), they were placed into their screw top eppendorf tube containing 700 µl TNE buffer (50mM Tris-HCl (pH 8.0), 150mM NaCl, 100mM EDTA) (Sigma) and put on ice.

To each tube, 35 µl of 10mg/ml proteinase K (Sigma) and 35 µl 0f 10% (w/v) SDS (sodium dodecyl sulphate) (Sigma) (i.e. to a final concentration of 0.5%) were added.

The tubes were placed 'head to head' in 30 ml universal tubes and rotated on a mixer (Spiromix) overnight at 37°C. 5 µl of (10mg/ml) RNase A (Promega, Southampton, UK) was added to the digested slurry which was incubated for 1 hour at 37°C.

After the RNase treatment 700 µl of phenol (Sigma) was added to each tube. The tubes were taped to a rack and shaken vigorously for 10 minutes. The tubes were then spun at high speed in a microfuge 1200g for 10 minutes.

The upper aqueous layer was transferred to a fresh tube, taking care to avoid including any of the interface. The aqueous layer was then re-extracted with 700 µl phenol: chloroform (Sigma) as before. The upper, aqueous layer was transferred to a fresh tube, making sure to avoid the interface and re-extracted with 700 µl chloroform (Fisher, Loughborough, UK): Iso Amyl Alcohol (IAA) (Fisher). The shaking and spinning steps were repeated. 500 µl of the aqueous layer was transferred to a fresh tube and 2 volumes of 100% ethanol (Fisher) were added. The tube was inverted slowly, to mix the contents. The DNA precipitated out of solution and appeared as a visible, long, white string. The DNA was 'hooked' out of the ethanol mixture using a P200 Gilson pipette with a flame sealed tip.

The DNA was washed first with 100% (v/v) and then 70% (v/v) ethanol and 'hooked' from each solution as already described. The DNA was then placed into a clean eppendorf and allowed to air dry for a few minutes to allow any excess alcohol to evaporate. Finally, the DNA had 300 µl of TE buffer (0.1mM Tris-HCl:0. 01mM EDTA) added and left at 4°C overnight to slowly re-suspend the DNA.

2.3.2 Phenol-Chloroform Extraction of DNA from BAEC

BAEC samples were trypsinised as described previously (2.2.4). After centrifugation, the resultant pellet was re-suspended in the cell lysis buffer used in 2.3.1 [700 µl TNE buffer (50mM Tris-HCl (pH 8.0), 150mM NaCl, 100mM EDTA)] and gentle rotated for 15 minutes on a Spiromix at room temperature. The rest of the protocol is the same as that used in 2.3.1

2.3.3 PureGene® Extraction of DNA from Whole Blood Samples

The DNA from the blood samples was extracted with the use of the PureGene® (Gentra Systems) kit reagents and protocol, which were supplied by the manufacturer.

The blood was transferred into sterile 50 ml universal centrifuge tubes containing 27 ml red blood cell (RBC) lysis solution. The tube was inverted several times before being placed on a Spiromix at room temperature for 20 minutes. The tube was inverted once during the incubation time.

The sample was then centrifuged for 10-20 minutes at 2000g. The supernatant was carefully decanted from the tube, leaving behind a visible white cell pellet and 300-600 µl of residual liquid. The white cell pellet was vortexed vigorously to re-suspend the white cells and 9 ml cell lysis solution was added. The tube was inverted to aid the cell lysis. At this stage the samples are stable at room temperature for 18 months.

A 45 µl aliquot of RNase A (10mg/ml) solution was added to the white cell lysate and mixed by inverting the tube 25 times. The tubes were incubated at 37°C for 15 minutes then placed on ice to cool to room temperature which usually required 5 minutes. At this point 3 ml of protein precipitation solution was added to the cell lysate, which was vortexed vigorously for 20 seconds and then centrifuged at 2000g for 10 minutes. The precipitated proteins formed a tight dark brown pellet at the bottom of the tube. The supernatant containing the DNA was decanted into a clean 50 ml tube containing 9 ml 100% isopropanol (Fisher). The tube was inverted 50 times to mix the sample, by which time white DNA threads were visible. The DNA was pelleted to the bottom of the tube by centrifugation at 2000g for 3 minutes, the

supernatant was decanted off and the inside of the tube was cleaned using a 'tissue-covered finger sweep' method. 9 ml 70% (v/v) ethanol was then added to the DNA pellet and the tube was inverted several times to wash the DNA pellet. The DNA was recovered by centrifugation at 2000g for 1 minute. The ethanol was carefully decanted and the tube drained on clean absorbent paper. The sample was allowed to air dry for a maximum of 10 minutes. DNA hydration solution (750 µl) was added to the pellet and the tube was placed in an incubator for 1 hour at 65°C. The tube was tapped a few times during this time to aid re-suspension. The sample was the left overnight at room temperature to ensure complete re-suspension.

Samples were aliquoted. Half was stored at –20°C and the other half was stored at between 2 and 8°C for long and short term storage, respectively.

2.3.4 Analysis of DNA Samples

2.3.4.1 *Spectroscopy Method for Quantitation*

The concentration of every DNA sample extracted was determined by spectroscopy, from the absorption at 260nm. To achieve this, 5 µl of the sample was added to a 1ml quartz cuvette, containing 1 ml distilled water (dH_2O). The solution was mixed thoroughly by inverting the cuvette slowly, several times.

The samples absorbance was then read at 260nm and 280nm, using a deuterium lamp. The concentration of the DNA sample was calculated using the following equation:

$$\text{Conc}^{n} \text{ of Double Stranded DNA } (\mu g/\mu l) = \frac{OD_{260} \times \text{dil}^{n} \text{ factor} \times 50}{1000}$$

The purity of the DNA was obtained by calculating the OD 260/280 nm ratio. This should be approximately 1.8.

2.3.4.2 *Electrophoresis Method for Measuring DNA Integrity*

The integrity of all DNA samples was analysed once the extracted DNA had re-suspended fully and before the sample was used in any telomere analysis experiment.

5 µl loading buffer (0.25% (w/v) bromophenol blue (Sigma); 0.25% (w/v) xylene cyanol FF (Sigma); 15% (w/v) Ficoll (Sigma)) was added to 2 µg of genomic DNA and the solution was loaded on to a 1% (w/v) agarose gel (ICN, Basingstoke, UK), made with 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. A 1kb ladder (GibcoBRL) was also loaded on to the gel to determine the size of the genomic DNA. Both the gel and the running buffer contained 0.05µl/ml of 10mg/ml ethidium bromide (Sigma).

The gel was run at 80 volts in 1 x TAE buffer for roughly 1 hour and analysed using a UV light box. Any smearing down the gel suggested that the DNA was not of good quality. It was also possible to see, at this point, whether the RNase treatment had been successful. If any RNA was still present it would be possible to see it on the gel at around 500bp.

2.4 Analysis of Telomere Lengths

8 µg genomic DNA was digested to completion with 15U of *Rsa I* (GibcoBRL) and *Hinf I* (GibcoBRL) overnight at 37°C. The digestion always took place in a buffer

containing a final concentration of 1 x One Phor All buffer (OPA) (Amersham, Buckinghamshire, UK), made to the correct volumes with distilled water.

A fluorometer reading was taken in order to determine the concentration of digested DNA after restriction enzyme digestion. This was carried out using a Hoefer Dynaquant fluorometer. 2 µl of digested DNA was added to 2 ml of a solution containing (1 x TNE (50mM Tris-HCl (pH 8.0), 150mM NaCl, 100mM EDTA) 1µg/ml Hoechst). The excitation emission was then calculated and the machine would produce a concentration of the DNA in ng/µl. This procedure relies on the fact that Hoechst 33528 intercalates with double stranded DNA to produce a fluorescent profile, but does not bind to single stranded, and thus any digested fragments will not be visible.

2.4.1 Electrophoresis

The gel electrophoresis apparatus (horizontal submarine gel tank) was set up according to the manufacturers instructions and a running buffer of 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) used to fill the electrophoresis tank. Electrophoresis tanks were manufactured in-house and power packs supplied by Bio-Rad. A 0.5 % (w/v) agarose gel was made in 1 x TAE. The agarose suspension was heated in a microwave until the agarose dissolved. The agarose solution was allowed to cool to 60°C before adding 0.05µl/ml of 10mg/ml ethidium bromide and pouring into a gel mould. 2 µg of digested DNA in 4 µl of loading buffer was loaded onto the gel. 1 µg of 1 kb ladder was run in a separate lane along side the samples to determine the size of the DNA.

The gel was run overnight in the 1 x TAE running buffer with 0.05µl/ml of 10mg/ml ethidium bromide in a temperature-controlled room set to 4°C. The samples were initially run from the wells at 150V for about 1 hour and then the voltage was reduced to 50V for the continuation of the overnight procedure.

The gels were examined with the use of an ultraviolet transilluminator to ensure that the genomic 'smear' had run to the bottom of the gel and this smear was also used to ensure equal loading of the samples. It was also important that the 12 and 11 kb bands in the DNA ladder were easily distinguishable from each other.

The gel was depurinated in 250ml of 0.25M HCl (Fisher) (6.25ml/250ml) depurinating solution for 20 minutes with gentle shaking. The solution was changed for fresh after 10 minutes. The usual method of depurinating until the bromophenol blue dye in the loading buffer turns from blue to yellow was not possible as this had usually run off the gel completely.

The gel was then briefly rinsed in distilled water and then immersed in a denaturing solution containing 1.5mol/L NaCl, 0.5 mol/L NaOH (Sigma) for 30 minutes with gentle shaking.

The gel was rinsed again with distilled water and then washed in a neutralising solution containing 1.0 mol/L Tris-HCl, 1.5 mol/L NaCl pH 7.2 for 30 minutes, with gentle shaking. The gel was rinsed for the last time in distilled water and left in this water until the drying apparatus had been prepared.

A sheet of 3MM paper was placed onto a BioRad gel drier. Hybond-N (Amersham) was placed on top of this filter paper and then the gel was very carefully placed on top of the Hybond-N, avoiding any distortion.

Saran wrap was placed over the gel and the drier was programmed to initially dry stringently without heat, with a vacuum pump, for one hour and then to dry with heat (65°C) for 45 minutes. The filter paper was changed after the first drying process was complete.

When the drying process was complete, the membrane was wrapped in Saran. The desiccated agarose was removed by carefully peeling it away from the Hybond-N. The DNA was then covalently cross-linked to the membrane by exposure to 7 x 10^4 J/cm^2 of UV light by placing the membrane for 1min 10sec on a UV transilluminator.

The membrane was stored at 4°C until the hybridisation process was carried out.

2.4.2 Membrane hybridisation

Pre-hybridisation and hybridisation were performed at 42°C in a Hybaid hybridisation oven. 'fixed' membranes were sandwiched between two nylon meshes in 3xSSC (0.45M NaCl; 0.045M NaCitrate (Sigma)), rolled up and placed into a hybridisation tube with 20 ml hybridisation buffer containing 0.5% (w/v) Marvel dried milk (non-specific blocking agent), 6% (w/v) polyethylene glycol (PEG) 8000 (Sigma), 0.75X SSPE (235mM NaCl; 15mM $NaH_2PO_4.H_2O$; 1.5mM EDTA) pH 7.4, 1% (w/v) SDS. The membrane was pre-hybridised at 42°C for a minimum of 1 hour.

Two methods were used for radio labelling probes, one was random priming of DNA probes longer than 100 bp, and the other was 3'–TdT labelling of oligonucleotides.

2.4.2.1 *Random primed labelling*

This method is a variation of the random priming method previously described by Fernberg and Vogelstein (1983), and is the protocol employed by the Rad Prime kit. 20ng of 1 kb or 5 kb DNA ladder (in a 21µl volume) was denatured by heating at 100°C for 10 minutes and placed immediately on ice. The probe was labelled by adding 20µl of 2.5 x random priming buffer (Promega), 3µl un-labelled dATP/dGTP/dTTP mix (Promega), 50 µCi ^{32P}[dCTP] (ICN) and 40 Units Klenow (GibcoBRL) polymerase; and was incubated for at least 15 minutes at 37°C.

Unincorporated radio labelled nucleotides that could hybridise non-specifically to the membrane were removed by isolating the probe on a Promega DNA-binding spin column. The reaction was passed through a column allowing the radio labelled probe to bind to the silica-based matrix and the unincorporated nucleotides to pass through; any nucleotides that bind to the column are eluted by two ethanol-based washes. The column is dried and the radio labelled probe is eluted by addition of TE buffer to the column.

2.4.2.2 *TdT labelling*

Short oligonucleotides were labelled by the enzyme terminal deoxynucleotidyl transferase (TdT), which catalyses the incorporation of radio labelled deoxynucleotides onto the 3' –OH terminus of DNA. 20 pico moles of the

complimentary telomere oligonucleotide probe ($AATCCC_3$) was incubated at 37°C for 1 hour in 1 x One-Phor-All buffer, 10μCi ^{32}p[dCTP], 50 Units TdT, 10mM $MgCl_2$.

Oligonucleotides were synthesised in-house (Dr. K. Lilley, Protein and Nucleic Acids Laboratory (PNACL), University of Leicester, UK). Oligonucleotides were ethanol precipitated and re-dissolved in distilled water before use.

The radio-labelled probe was added to 25ml of hybridisation solution (0.5% (w/v) Marvel dried milk (non-specific blocking agent), 6% (w/v) polyethylene glycol (PEG) 8000, 0.75X SSPE (235mM NaCl; 15mM $NaH_2PO_4.H_2O$; 1.5mM EDTA) pH 7.4, 1% (w/v) SDS) this solution then replaced that which the membrane had been pre-hybridising, and was incubated overnight at 42°C. After hybridisation, the membranes were washed with controlled levels of stringency. Membranes were routinely washed twice in two low stringency washes at 42°C for 10 minutes. The first wash was performed in 5xSSC, 0.1% (w/v) SDS, followed by one wash in 3xSSC, 0.1% (w/v) SDS.

After washing, the membranes were dried lightly on 3MM paper, wrapped in saran wrap and exposed to photographic film in the presence of intensifying screens at –70°C for as long as required (the membrane was usually left exposing to the film for 48 hours and depending on the result obtained, another exposure procedure would be carried out using the initial time as a guideline). The film was developed in a Kodak auto-radiography developer.

The membrane was also exposed to a Phosphor Imager plate (Molecular Dynamics, Amersham, UK)

For safe storage of the membrane the radio-labelled probe was stripped from the target nucleic acid. This was carried out by incubating the membrane in a 0.4M NaOH solution at room temperature for 20 minutes. The membrane was then neutralised in water, dried lightly on 3MM, wrapped in saran wrap and stored at 4°C.

2.4.3 Image Quant Analysis of TRF

The position of the size markers was used to calculate the distances migrated in the gel by fragments of known molecular weight. The lanes occupied by the DNA samples were divided into 20 regions of equal size between 3 kb and 18 kb by means of Image Quant software (Molecular Dynamics) and the molecular weight of each of these regions calculated from the inverse logarithmic relationship between molecular weight and the distance migrated and the known position of the size markers. The telomeric DNA smear was quantified and the signal recorded for each of these regions (optical density). The mean telomere length was then calculated as the sum of (optical density x molecular weight) divided by the sum of optical densities.

$$TRF = \Sigma OD_i/(OD_i/MW_i)$$

Where OD_i is the optical density at a given position in the lane and MW_i is the molecular weight at that position. This formula accounts for the fact that longer telomere bind more labelled probe and consequently appear darker on the x-ray film and Phosphor Imager image.

Chapter 3: Assay Optimisation

3.1 Introduction

The use of adapted Southern blotting techniques to measure mean telomere length was common practice previous to this project. The assays were generally very similar with only small differences between the groups. However, many of these groups were using the assay for varying purposes and not all intended to quantitate their findings. Therefore it was felt necessary to optimise the techniques which were being used in order to provide an assay which would routinely provide a result which could be quantitated.

The main priority at the beginning of this project was to establish a reproducible telomere assay capable of producing both accurate and precise results. This was achieved by using combinations of previously used conditions and comparing the results.

The assay was optimised using DNA which had been extracted from peripheral white blood cells. White blood cell DNA was the material of choice for these preliminary experiments because blood could be easily obtained from donors, the extraction process yields good amounts of DNA from relatively small volumes of blood and most importantly, would be the cell type of interest for a large part of the investigations to be carried out for this thesis.

Two samples were used for these preliminary experiments from donors aged 24 and 80 years. These two samples were used because previous work has demonstrated that telomeres from white blood cells are shorter in older subjects (Vaziri *et al.*, 1993; Slagboom *et al.*, 1994; Wynn *et al.*, 1998) and thus, by using the widest age gap possible, a large difference in telomere length should be obtained. Theoretically, the bigger the difference in mean TRF, the easier it would be to identify and thus calibrate the assay.

3.2 Telomere Assay Optimisation

Following isolation (see Chapter 2, section 2.3), the peripheral blood cell DNA from the two donor blood samples were processed as described in Chapter 2, section 2.4. Initially, differing conditions to those described in section 2.4 were tried and tested, before a final decision on the actual technique to be used was made. The parameters of the method which were assessed were:

1. Choice of restriction enzymes:

   Rsa I / Hae III or *Rsa I /Hinf I*

2. Concentration of agarose:

   0.5% or 1.0%

3. Choice of voltage and temperature for running the gel:

   50V at RT or 50V at 4°C

4. Choice of transfer method:

Classic blotting or Stringent vacuum drying

5. Choice of probe and labelling method:

Rad-prime labelled PCR product $(TTAGGG)_n$ or TdT labelling of a oligo probe $(TTAGGG)_3$

Those which are underlined were the conditions of choice and are those described in Chapter 2, section 2.4. The decision to use these conditions was based on the following results:

1. Both of the samples were digested, under identical conditions, with the two restriction enzyme cocktails and the telomere fragments analysed. There was no detectable difference between the resulting telomere smears. The autoradiogram shown in Figure 3.1 demonstrates this fact and also highlights a difference between the telomere smear obtained from the DNA of the 24 year old donor compared with the telomere smear obtained from that of the 80 year old donor. However, this visual difference cannot be confirmed as the telomere lengths in this assay were not quantitated. The decision to use *RsaI/HinfI* was due to the manufacturer product data, which stated that these two enzymes worked optimally together in the buffers which would be optimal for DNA experiments compared with *RsaI/HaeIII.*

2. The decision to use a 0.5% gel as opposed to the 1% option was based on the separation of the telomeric DNA obtained. This appeared to be consistently superior when a 0.5% agarose gel was used. Figure 3.2 demonstrates the increased distance

between the frontline of the smears on the 0.5% agarose gel compared to that obtained with the 1.0% gel.

3. The decision on temperature and thus voltage, was made on no other criteria than that of handling the gel itself. When run at room temperature, the heat generated from the current made the low percentage gel very fragile and thus subsequent handling was extremely difficult. The cooler running conditions kept the gel more rigid and therefore facilitated the subsequent processing of the gel.

4. The transfer method was chosen based upon two facts. The stringent vacuum drying of the gel onto the Hybond-N was quicker compared with the classic blotting technique and thus reduced the time needed to run the assay, allowing an experiment to be completed in a week. Also, as can be seen from the autoradiograms in Figure 3.3, the classic blotting technique did not appear to transfer the telomere fragments as well or as completely as the vacuum method. As this figure demonstrates, the Classic Blot results are of far poorer quality compared with the Stringent Vacuum results. There is non-specific binding with the classic blot, demonstrated by the speckling obtained after developing. Furthermore even though identical labelling procedures were used, stringent vacuum protocol provided results of greater intensity suggesting that a greater proportion of the telomeric DNA was transferred using this method. It must be noted that these results were obtained from one gel which was cut in half and then processed differently. Therefore the changes observed cannot be attributed to differences in running conditions earlier on in the assay.

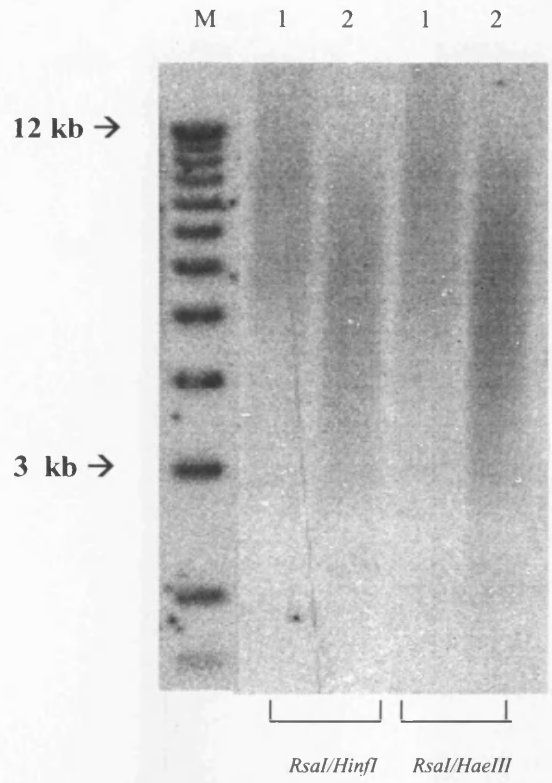
5. The choice of labelling method was based on which method proved easiest to perform, as there was no apparent difference between the results from either of the techniques. The oligo probe and thus TdT labelling was chosen as this was an easier technique to use. The probe could be generated at the University's PNACL, which meant that a lengthy and often temperamental PCR method would not be required to generate the $TTAGGG_{(n)}$ probe in this laboratory. Also, the signal generated by the oligo probe with TdT labelling was much stronger and thus required a shorter exposure time.

With all of the running conditions defined, an assay was carried out using the two DNA samples used throughout the optimisation process (DNA extracted from white bloods cells of two donors aged 24 and 80 years). The results of this assay are demonstrated in Figure 3.4.

The results obtained from these two peripheral blood cell DNA samples during the optimisation process were consistent with those of the other groups in that we had also demonstrated an age related shortening of mean telomere length in peripheral white blood cells. This difference was clearly visible with the naked eye, as can be seen in Figures 3.1, 3.2, 3.3 and 3.4

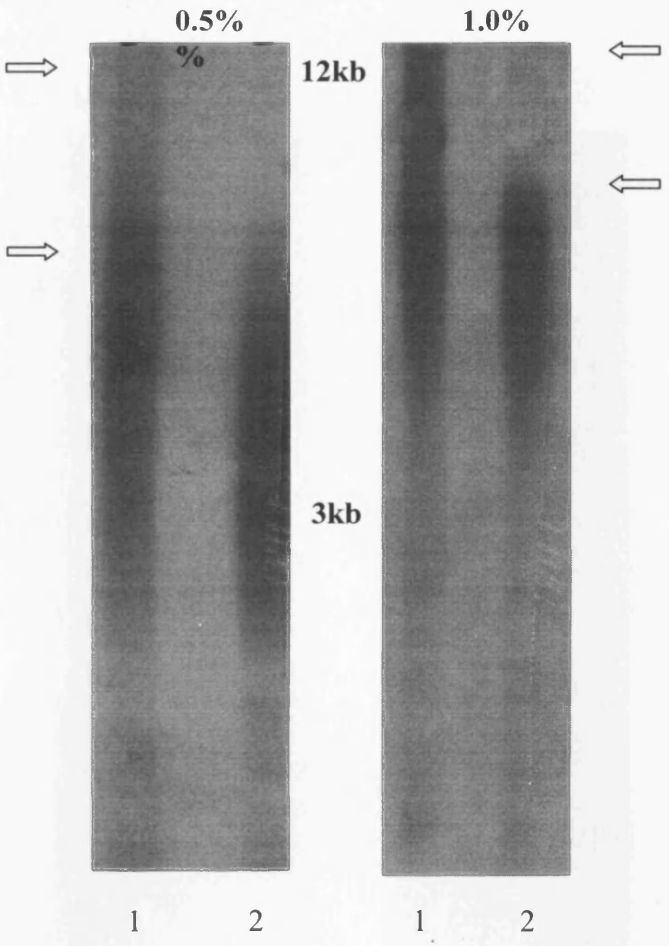
Also, having carried out the assay 30 times or more during the optimisation process, it became clear that the current applied to the gel should be continued until a clear gap had occurred between the 12 kb and 11 kb fragments in the molecular weight marker since resolution of the telomere smear was greatest when this separation occurred.

Figure 3.1 *Digestion of peripheral blood DNA using RsaI/HinfI or RsaI/HaeIII*



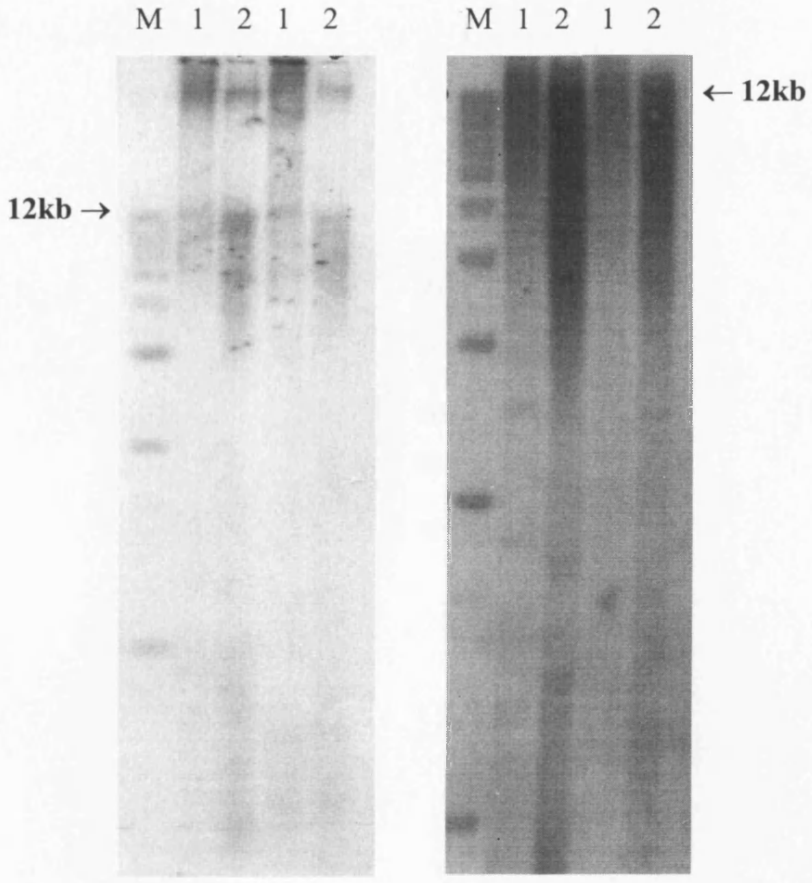
A representative autoradiogram showing Southern hybridisation of telomeric DNA isolated from human white blood cells (1) 24 year old subject (2) 80 year old subject (M) molecular weight marker. (0.5% agarose; 50V at RT)

Figure 3.2 *Separation of telomeric DNA using two agarose concentrations*



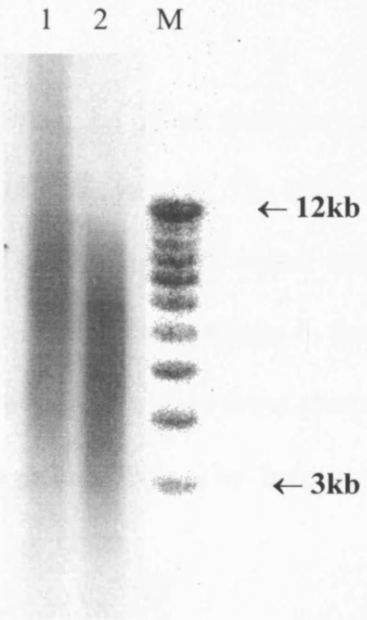
A representative autoradiogram showing Southern hybridisation of telomeric DNA isolated from human white blood cells (1) 24 year old subject (2) 80 year old subject. The gels consisted of (left) 0.5% agarose, (right) 1.0% agarose. Arrows at the side of the gels denote where the telomeric smears begin. (50V at RT)

Figure 3.3 *Transfer of telomeric DNA from agarose gel to Hybond-N using classic blotting or stringent vacuum techniques*



A representative autoradiogram showing Southern hybridisation of telomeric DNA isolated from human white blood cells (1) 24 year old subject (2) 80 year old subject. DNA transfer methods used were (left) Classic blotting (right) Stringent vacuum. (M) molecular weight marker. (0.5% agarose; 50V at RT)

Figure 3.4 *Telomeric DNA from human white blood cells after Southern hybridisation using conditions of choice.*



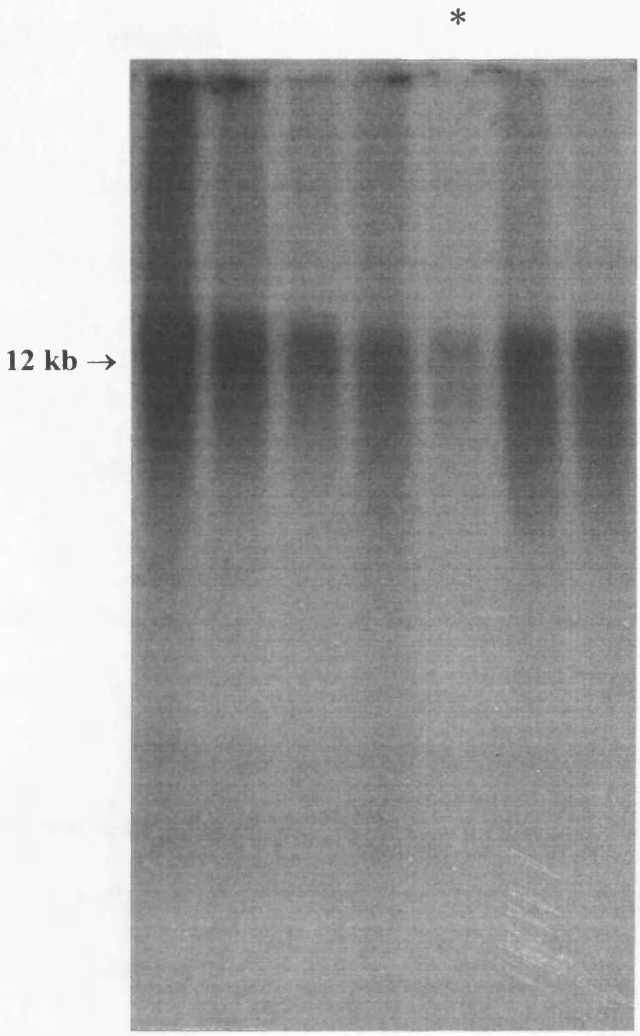
A representative autoradiogram showing Southern hybridisation of telomeric DNA isolated from human white blood cells (1) 24 year old subject (2) 80 year old subject. (M) molecular weight marker (0.5% agarose; 50V at RT).

During the optimisation process it was evident that digested DNA required quantitation prior to being loaded onto the gel. This became apparent when viewing autoradiograms from gels which had been loaded with DNA that had been quantitated before the digest procedure (using a spectrophotometer), but not following it. This omission produced results which consisted of lanes with obvious differences in DNA concentration. It was thought that this irregularity may be due to loss of DNA during the various stages of processing, incomplete digestion and pipetting for example.

The autoradiogram shown in Figure 3.5 is an example of one of the very first experiments to be carried out during this project. The DNA used in this experiment was extracted from bovine aortic endothelial cells which were being cultured under normal conditions within the department (see Chapter 2, Section 2.2.4). There is a obvious difference in the density obtained in some lanes compared with others despite each lane containing 2μg pre-digested DNA. The lane marked by * is the most noticeable.

To eliminate this problem, digested DNA was quantitated using a fluorometer and the volume loaded into each lane was calculated based upon this value.

Figure 3.5 *Separation of telomeric DNA which had not been quantitated after the digestion process.*



A representative autoradiogram showing Southern hybridisation of BAEC telomeric DNA. Each lane consists of 2μg (pre-digest concentration) of identically sourced DNA. (0.5% agarose; 50V at RT)

The final stage of the assay optimisation was the analysis and quantitation of the telomere smears. The method was adapted from another group (Wynn *et al*, 1998) and used a PhosphorImager (Molecular Dynamics, Amersham, UK) and ImageQuant software (Molecular Dynamics) to calculate the mean telomere lengths.

3.3 Inter-assay variation

Another factor which needed to be addressed was the degree of inter-assay variation. The initial idea was to produce an internal control of telomere origin which was of a known length. This control could be loaded onto all gels and used as an internal reference standard. In order to produce this internal control of known length, two primers were synthesised by the PNACL:

Tel 1: TTAGGGTTAGGGTTAGGG

Tel 2: CTAACCCTAACCCTAACC

These two probes were then used in a PCR reaction to make a double stranded telomere probe of unknown length. The following PCR conditions were used:

Telomere probe 1ng/μl	1μl
10μM Tel 1	4μl
10μM Tel 2	4μl
11.1x PCR buffer	3.6μl
dH_2O	26.6μl
Taq polymerase 5U/μl	0.8μl

Cycling conditions: 96 C 40 secs

65 C 40 secs

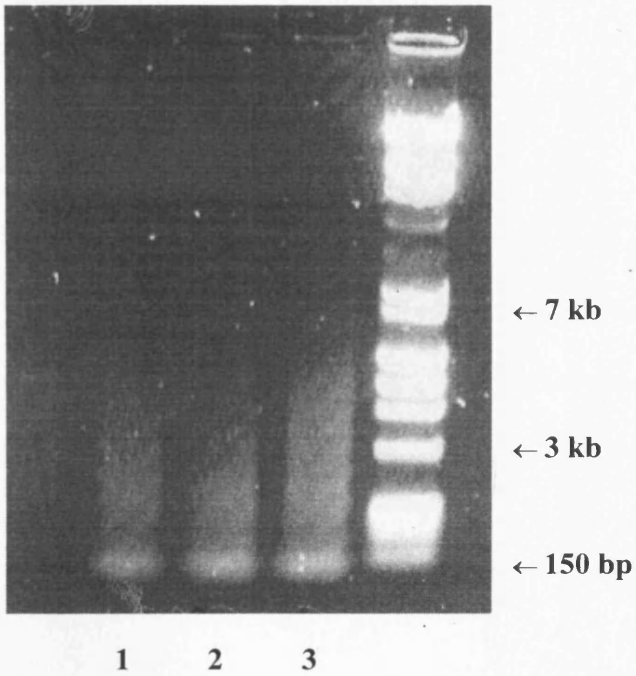
70 C 5 mins

(25-30 cycles) in an MJ Research PTC200 DNA engine, with heated lid

The PCR products were separated and analysed on a 1% HGT agarose gel in 1xTAE buffer. Based on previous PCR preparations carried out in the Department of Genetics, it was expected that a smear of products from approximately 150bp to 7kb should be seen on a denatured gel. Figure 3.6 shows the results of this PCR procedure. The molecular weight marker in the far right hand lane can clearly be seen and shows that the prediction of products ranging in length from 150bp to 7kb was correct. It is clear that a greater signal was obtained from lane 3 (25 cycles) compared with lanes 1 and 2, when the gel was viewed with the use of a UV light box. Lanes 1 (primers only) and 2 (20 cycles) do not appear any different, suggesting that 20 cycles was insufficient.

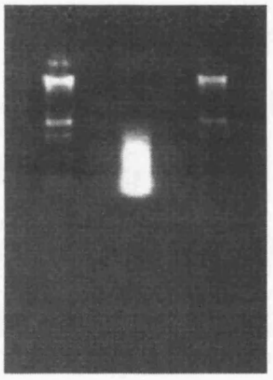
Densitometry demonstrated that although no great difference in intensity was present, the products obtained from using 25 cycles did produce a slightly stronger signal which directly correlates to an increased concentration, and agrees with what was seen on the UV light box with the naked eye. Based upon these results, it was decided that the contents of this lane in the region of 1kb would be excised and used in the gel extraction process using the Qiagen Qiaquick Gel Extraction Kit to isolate the PCR product for further use. The results of this gel extraction procedure can be seen in Figure 3.7. The gel shows that the purified PCR products formed a nice band which was positioned in the area on the gel which corresponded with the 1 kb size marker.

Figure 3.6 *Separation of resultant PCR products using a 1% HGT agarose gel*



The PCR products were loaded into lanes 1 (primers only), 2 (20 cycles) and 3 (25 cycles). (50V at RT)

Figure 3.7 *Separation of purified PCR products using a 1% HGT agarose gel*



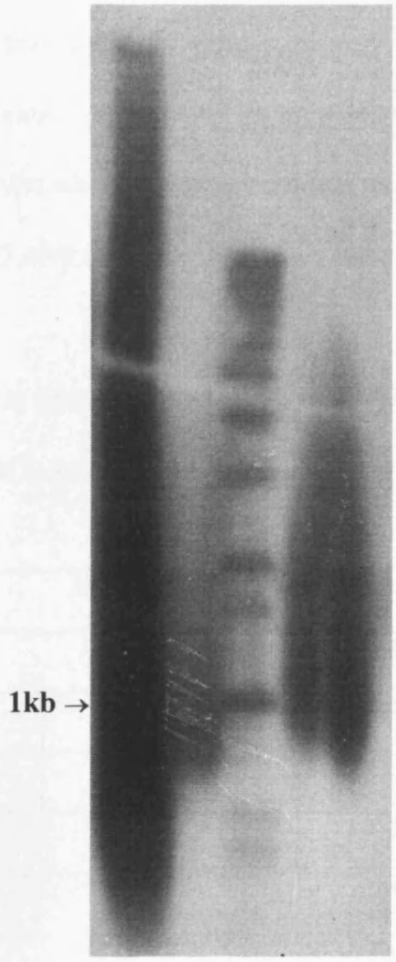
This gel was loaded as (M) molecular weight marker, (1) purified PCR products. (50V at RT)

The purified PCR products were subsequently analysed using the telomere assay to investigate whether it could be used as a viable internal reference standard. This would be possible if the subsequent quantitation process gave a mean TRF result of 1 kb.

However, having analysed the PCR products and quantitated the mean TRF, the result was not 1 kb. In addition to this, there was not the presence of a discrete band at the region comparable with the 1kb fragment in the molecular weight marker. Instead there was a very large smear which extended the whole length of the gel (See Figure 3.8). It was thought that this was due to the tandem repeat nature of the purified product. Concatamers would form when complimentary DNA folds on itself and joins together thus producing a new product of an unknown, random length, capable of forming the smear which is synonymous of telomeres with a heterogeneous length.

Sometimes it is possible to overcome this concatamer structure with a boiling step. This involves heating the DNA in a boiling water bath for 1 minute prior to gel loading. This should denature the bonds between the DNA base pairs resulting in the strands separating. However, this procedure did not change the results obtained from the telomere assay. Neither did the loading of a smaller concentration of the PCR product. Although 2μg of the product produced a smaller smear, the problem was not over come completely and can be seen in Figure 3.8.

Figure 3.8 *Southern Analysis of PCR products.*



The gel was loaded as follows: (1) 4μg PCR products which had not been boiled. (2) 2μg PCR products which had not been boiled. (3) 2μg PCR products which had been boiled prior to loading. (M) Molecular weight marker. (0.5% agarose; 50V at RT)

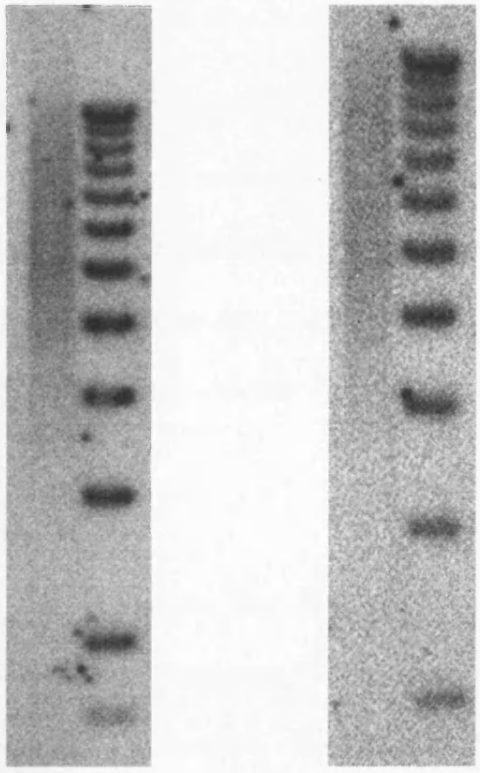
To calculate the degree of inter-assay variation, a sample (S21) was run eight separate times and its average mean TRF from these eight runs was calculated as 7.47 ± 0.054 kb (See Table 3.1). Figure 3.9 shows an example of two of these runs. This sample was then run on all subsequent gels and depending on the sample's mean TRF on a gel, all study data on that gel were proportionately adjusted using this value. For instance, if S21 gave a mean TRF of 8.02 kb on a gel which contained study samples, all of the study data results would be adjusted using the following equation:

(7.47/8.02) x Study sample mean TRF (kb)

Table 3.1 *A table to show all eight results which were obtained after the S21 DNA had been analysed using Southern hybridisation.*

S21	Mean TRF
1	7.49
2	7.42
3	7.56
4	7.39
5	7.44
6	7.46
7	7.51
8	7.45
AVE	**7.47**
STDEV	**0.054**
CV (%)	**0.720**

Figure 3.9 *Southern analysis of S21 telomeric DNA*



The gel was loaded as follows: (1) S21 DNA (M) molecular weight marker.

(0.5% agarose; 50V at RT)

With the assay optimised and an internal control established to take into account any possible inter-assay variability, it was now decided that the assay was ready to be used in the investigation and determination of mean telomere lengths in a variety of tissues.

3.4 Validation of the telomere assay

A preliminary study was carried out to test the fidelity of the assay. It was completed so the method could be scrutinised, by seeing if it was specific enough to identify the age related white blood cell telomere shortening that have been previously reported by other groups. This preliminary study was carried out using a cohort of normal volunteers (i.e. with no known heart or vascular disease).

3.4.1 Experimental Protocol

Preliminary Study Colleagues and their relatives (n=7) from within the Department of Clinical Sciences, University of Leicester, who were in a state of general good health, volunteered to donate a 10 ml sample of blood for the extraction of leukocyte DNA. The age range for this cohort was 21 to 80 years and consisted of both men and women. All volunteers were of white, Caucasian ethnicity.

DNA extraction, assessment of integrity, and analysis of telomere lengths were carried out as detailed in Chapter 2: Materials & Methods.

3.4.2 Preliminary Study Results

Figure 3.10 shows a representative blot of the findings and the data for all subjects is shown as a bar chart in Figure 3.11

These seven white blood cell samples produced results which had been expected, based on the work of other groups. With increasing age, the mean telomere length reduced, with the exception of one of the volunteers.

The results demonstrate an average shortening of 35 bp per year of the mean telomere length. This is consistent with the findings of Hastie *et al.*, who reported in 1990 that normal blood and colon mucosa underwent a 33 bp loss in telomere length per year.

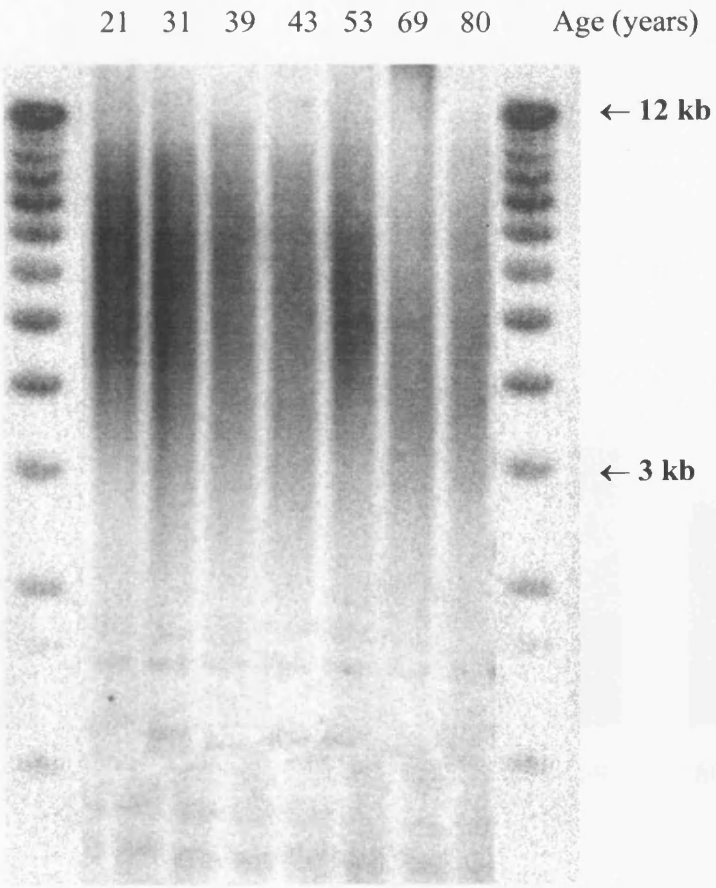
In this study we report findings that are consistent with other groups (Vaziri *et al.*, 1993; Hastie *et al.*, 1990) in that there is an observed age related telomere shortening in white blood cells, *in vivo*, equating to an attrition rate of approximately 35 bp per year.

The results obtained from this preliminary study therefore validates the assay developed as described in this chapter.

Special Note

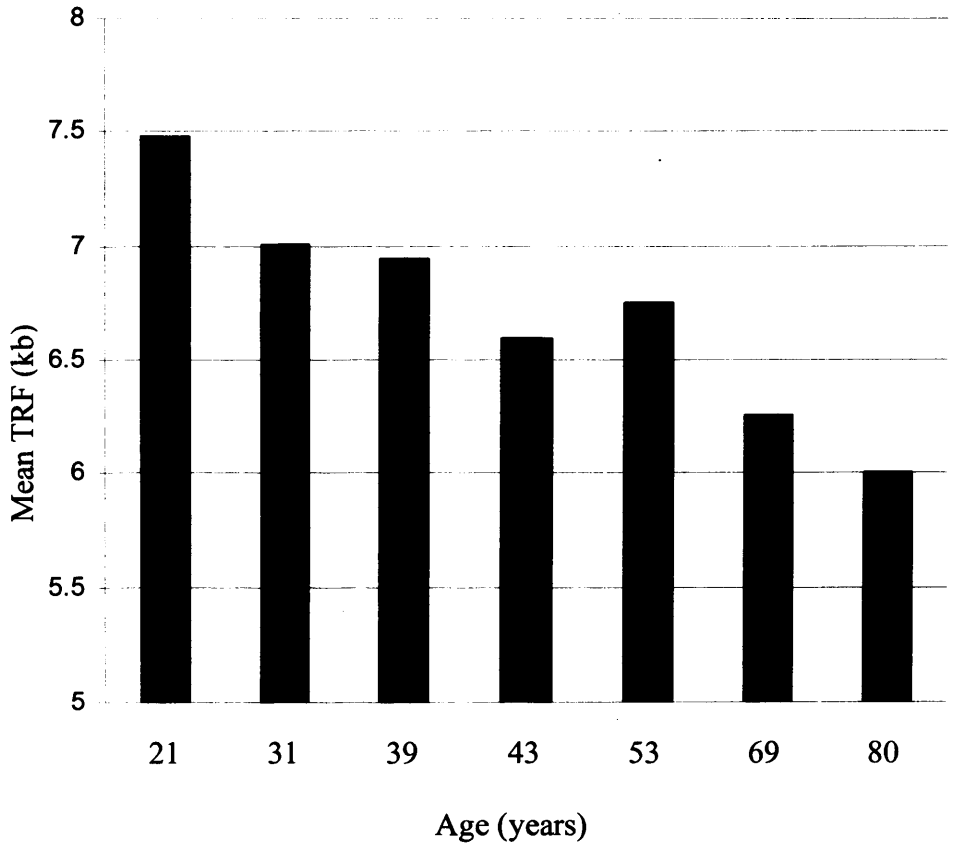
Page 89 missing from the original

Figure 3.10 *A representative autoradiogram for the Preliminary Study*



A representative autoradiogram obtained from the analysis of the samples from 7 healthy subjects. (0.5% agarose; 50V at RT).

Figure 3.11 *A bar chart to show the mean TRF results obtained in the Preliminary Study*



SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN

Chapter 4: Comparison of Telomere Length in Peripheral White Blood Cells

4.1 Introduction

The proliferative life-span of the stem cells that sustain haematopoiesis throughout life is not known. Most mature cells of the haematopoietic system that are released into the peripheral circulation are relatively short-lived and as a result, need to be replaced continuously throughout life. The high production rate of the haematopoietic cells, estimated at 10^{11} per day in a healthy human adult, are produced by a relatively small population of haematopoietic stem cells. Despite telomerase being active in these stem cells (Hiyama *et al*, 1995), shortening of telomeres during ageing *in vivo* has been observed in peripheral blood leukocytes (Hastie *et al.*, 1990; Vaziri *et al.*, 1993).

An acceleration of this telomere shortening in white blood cells has been demonstrated in patients with insulin dependent diabetes mellitus (IDDM) (Jeanclos et al., 1998). This work was suggesting that either the pathophysiology of the disease affects this subset of cells directly, causing aberrant behaviour and subsequent increased cell turnover and thus accelerated telomere loss, or that the reduced telomere length was inherited and ultimately a primary role for the affected white blood cells was to promote the disease. This may be mediated by white blood cells becoming activated following telomere shortening, resulting in a chronic

inflammatory response. This may lead to destruction of the pancreas and indeed, inflammation has been associated with the disease process.

Atherosclerosis is a co-morbidity of diabetes and is a known complication of the disease. (DCCT, 1993). This suggests that the mechanism by which diabetes manifests has a systemic affect and may be linked to atherosclerosis. It is therefore possible that inflammation could give rise to not only insulin-dependent diabetes, but also atherosclerosis and maybe telomere interactions during the inflammatory process occur in both disease states. Assuming this statement is correct, it is possible that associated diseases of insulin-dependent diabetes may demonstrate a similar pattern of telomere shortening in white blood cells.

Although atherosclerosis is manifested focally, there is increasing evidence that it is accompanied by more generalised perturbations, and in particular systemic inflammation (Ridker *et al.*, 1997; Ridker *et al.*, 2000). The work of Ridker *et al* has since been expanded and inflammatory mediators such as c-reactive protein (CRP), serum amyloid A (SAA), alpha-1-antichymotrypsin (ACT), and the soluble cellular adhesion molecule sVCAM have been recorded as being raised in patients with cardiovascular disease (Glurich *et al.*, 2002) In addition to this, the Bruneck study noted that chronic infections amplified the risk of atherosclerosis development in carotid arteries. Thus, the presence of atherosclerosis could be marked by increased cell turnover of white blood cells as a result of this inflammatory response and subsequently, increased biological age (i.e. telomere shortening) in haematopoietic cell types may be a feature of the disease.

Therefore the purpose of this investigation was to determine whether patients with advanced coronary atherosclerosis show systemic evidence of telomeric shortening in white blood cells compared with age-matched subjects with normal coronary arteries. This was called the 'White Blood Cell study'.

4.2 Experimental Protocol

White blood cell study Cases (n = 10) for this study were patients between 40 - 72 years of age with clinical ischaemic heart disease, who were attending the Department of Cardiology, Glenfield Hospital, Leicester and had been confirmed as having severe triple vessel coronary artery disease (CAD) by coronary angiography. The broad age range was deliberately chosen to allow for an assessment of age on telomere length, in addition to any possible affects of coronary artery disease, therefore allowing a comparison of the two parameters to be made.

Each case was matched with two control subjects (n = 20) who had also been investigated by coronary angiography, but had been shown to have normal coronary arteries. The control subjects had undergone angiography for investigation of either valvular heart disease (n = 9) or chest pain of uncertain origin (n = 11).

For all subjects age, sex, body mass index and history of cardiovascular risk factors (smoking, diabetes, hypertension, dyslipidemia) was noted at the time of clinic attendance by the doctor carrying out the appointment. However, racial group was not noted.

The study had approval from the local ethical review board, and all subjects provided informed written consent.

A 10 ml sample of blood for leukocyte DNA extraction was collected into an EDTA containing vacutainer at the time of angiography. DNA extraction, assessment of integrity, and analysis of telomere lengths were carried out as detailed in Chapter 2: Materials & Methods.

4.3 Results

The clinical characteristics derived from the patient information given in clinic is summarised in Table 4.1. A representative auto-radiogram showing the distribution of the TRF's in white cells of cases and controls for the first fifteen subjects is shown in Figure 4.1, and the second fifteen subjects in Figure 4.2. The mean TRF length for each subject, plotted by age, is shown in Figure 4.3.

It is difficult to see a difference in the telomeres on the autoradiogram with the naked eye, however, subsequent analysis of the smears showed that there was a highly significant ($p < 0.001$) effect of age on mean TRF with an average decrease in length equivalent to 35±4 bp per year of life. Adjusting for age and sex, cases had mean TRF lengths 300±90 bp shorter than those of controls ($p = 0.002$). There was no independent effect of gender ($p = 0.543$). The age-related decrease in mean TRF length was greater in cases (39±5 bp) than in controls (33±5 bp) but the difference was not significant ($p = 0.43$). There was no gender difference in the age slope ($p = 0.34$). Within the constraints of the limited numbers, there was no significant effect of

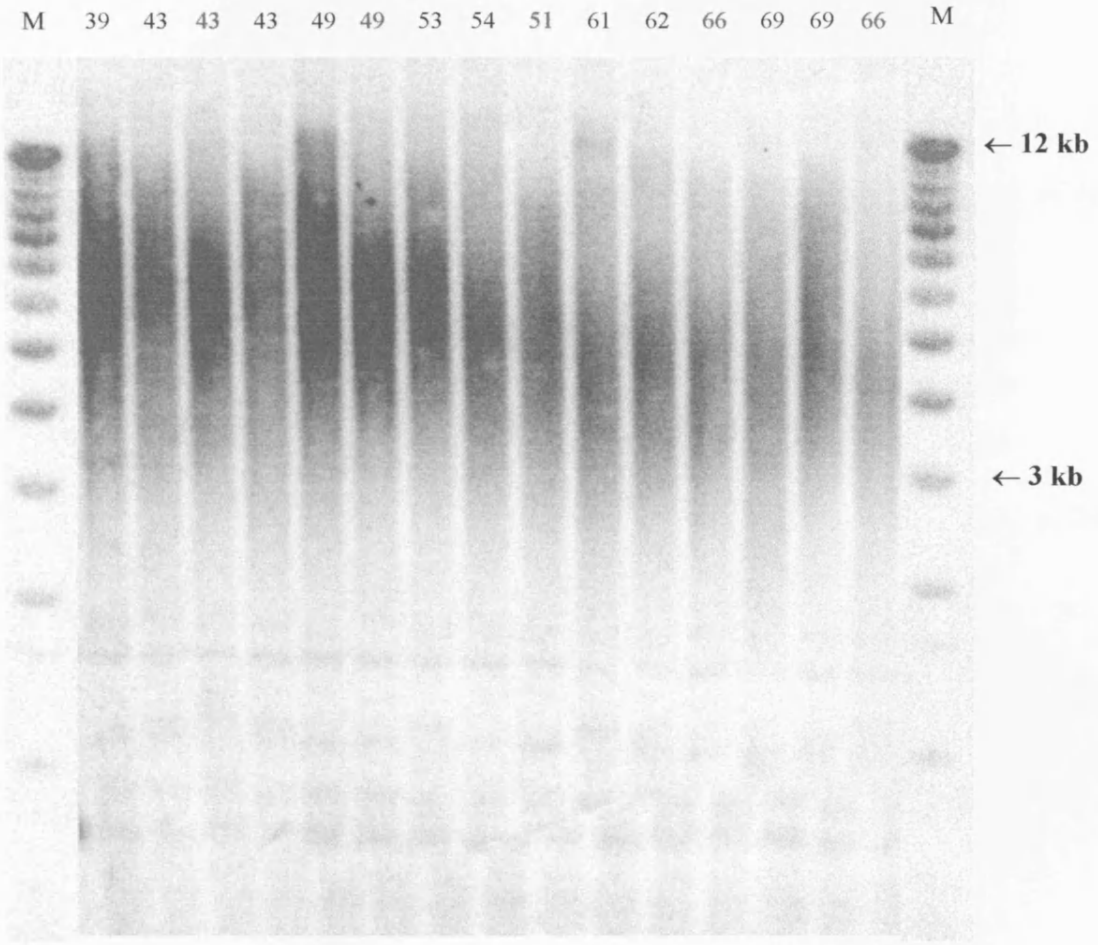
diabetes (p=0.61), hypertension (p=0.75), body mass index (p=0.92), serum cholesterol (0.07), or family history (p=0.57) on either the difference in mean TRF lengths between cases and controls or the decrease in mean TRF length with age. Only statin therapy (p=0.07) and current smoking (p=0.038) appear to have a relationship with telomere length. These comparisons were made using regression analysis in the first instance. This was followed by accumulated analysis of variance so that a multifunctional assessment could be made. This process tests the sex of a subject, then adjusts for age and finally status so that an overall estimate can be made as to the affect the risk factor has on the mean TRF. It is possible that this lack of affect of associated risk factors on telomere length is due to the small numbers analysed. Confirmation would be obtained in studies of larger cohorts.

Table 4.1 *Characteristics of cases and controls in the white blood cell study.*

	Cases [10]	Controls [20]	
Age range (yrs)	43 - 72	39 - 72	
Mean	59.2 (+/- 9.9)	60.0 (+/- 10.7)	p = 0.363
Male	9	10	
Female	1	10	
Smokers	6	8	
Diabetes	4	1	
Hypertension	4	8	
Cholesterol	*	*	
BMI (Mean)	28.8 (+/- 5.0)	27.7 (+/- 4.7)	p = 0.30
Family History	4	3	

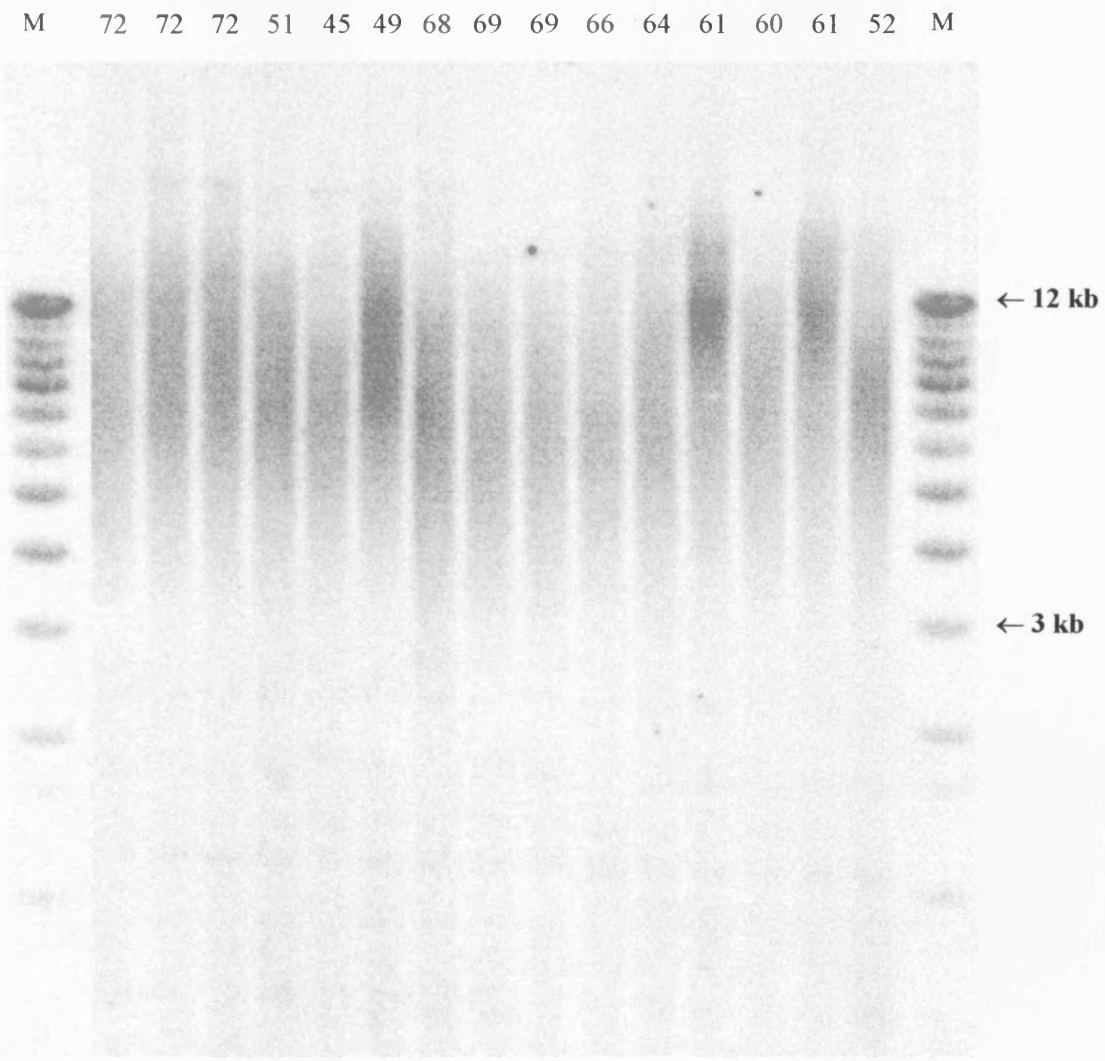
A summary of the information obtained from both cohorts of subjects used within this study. There was a statistically significant ($p = 0.032$) difference in the distribution of male and female subjects.

Figure 4.1 *Representative autoradiogram for the first cohort in the white blood cell study.*



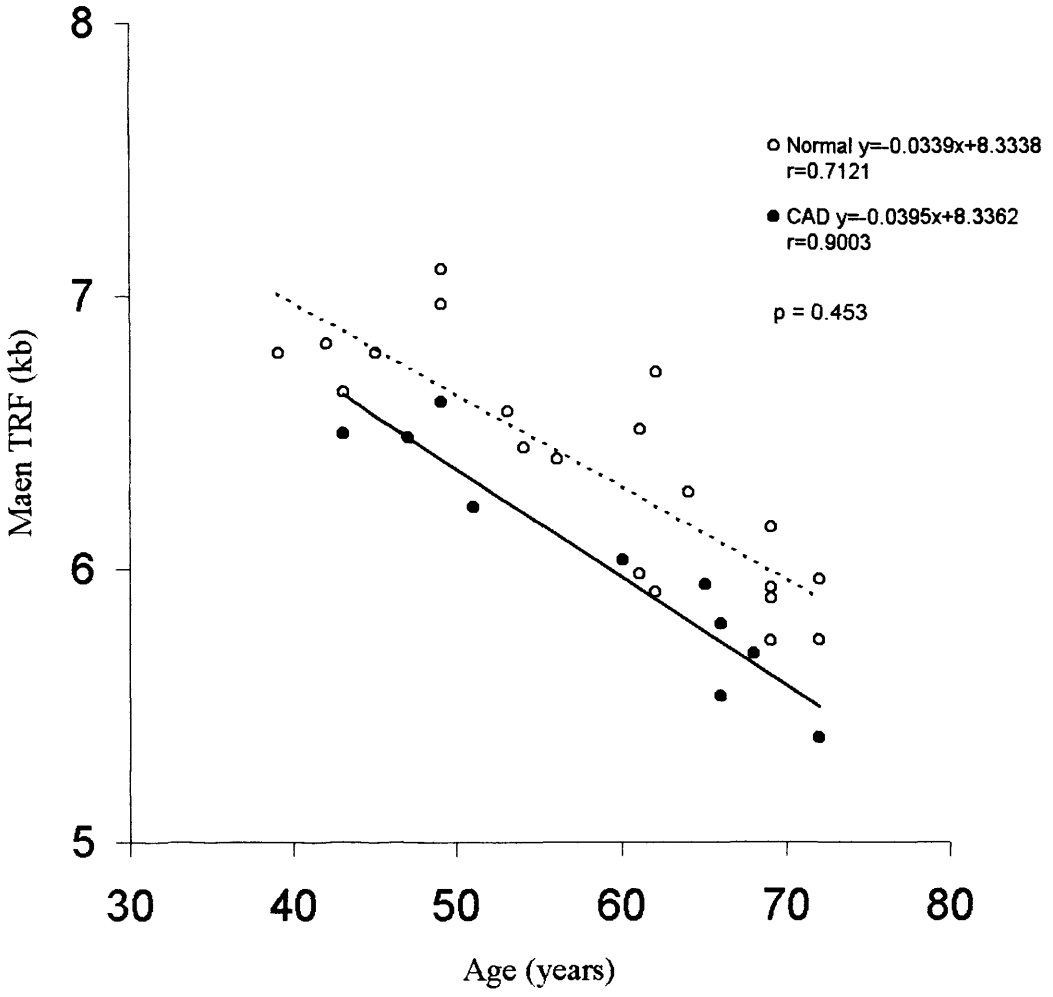
Representative autoradiogram showing Southern hybridisation of telomeric DNA from subjects with angiographically normal coronary arteries (black numbers) or with severe triple vessel disease (red numbers). M, DNA marker ladder. The number above each lane indicates the age of the patient in years.

Figure 4.2 *Representative autoradiogram for the second cohort in the white blood cell study.*



Representative autoradiogram showing Southern hybridisation of telomeric DNA from subjects with angiographically normal coronary arteries (black numbers) or with severe triple vessel disease (red numbers). M, DNA marker ladder. The number above each lane indicates the age of the patient.

Figure 4.3 *Plot of mean terminal restriction fragments (TRF) lengths of white blood cells from subjects with severe coronary artery disease and normal coronary arteries.*



The continuous line is the regression line for change in TRF length with age in CAD subjects and the dotted line the regression line for normals. This graph demonstrates an age related telomere shortening process for both sets of volunteers, with a consistently smaller telomere length being observed in those with severe coronary artery disease, compared with age matched normals.

On average, mean white cell TRF lengths in subjects with CAD were similar to subjects with normal coronary arteries that were 8.6 years older. This is calculated from the mean difference in TRF's between cases and controls (300 base pairs) and the mean annual decrease in telomere length (35 base pairs) (i.e. 300/35 = 8.6).

4.4 Discussion

This study presents a novel *in vivo* observation which demonstrates that compared with age-matched subjects with normal coronary arteries, those with severe coronary artery disease have significantly shorter telomeres in their circulating white cells.

Although haematopoietic stem cells do express telomerase (Hiyama *et al.*, 1995; Leber and Bachetti, 1996), the activity does not remain constant once the cells have left the bone marrow. As a result of this, telomere integrity is not lifelong in these cell types, with an average of 30-40 base pair loss per year (Hastie *et al.*, 1990; Vaziri *et al.*, 1993; Vaziri *et al.*, 1994; Slagboom *et al.*, 1994), a rate which correlates with the findings of this study. What is interesting from these results is that this 'normal' telomere attrition due to chronological ageing appears to be accelerated in the subjects with coronary artery disease from 30-40 base pairs per year to 300 base pairs per year. It is possible that this 'accelerated' shortening can be explained by looking at the manifestations of the disease.

Atherosclerosis has been documented to be preceded by inflammation (Ridker *et al.*, 1997; Ridker *et al.*, 2000) and oxidative stress (Hoeschen, 1997). Ridker *et al* demonstrated a link between atherosclerosis and inflammation by measuring plasma

C-reactive protein (CRP), a marker for systemic inflammation, in 543 apparently healthy men in whom myocardial infarction, stroke, or venous thrombosis subsequently developed, and in 543 study participants who did not report vascular disease during a follow-up period exceeding eight years. Base-line plasma CRP concentrations were higher among men who went on to have myocardial infarction (1.51 vs. 1.13 mg/L, $P<0.001$) or ischaemic stroke (1.38 vs. 1.13 mg/L, $P = 0.02$), but not venous thrombosis (1.26 vs. 1.13 mg/L, $P = 0.34$), than among men without vascular events. The men with the highest CRP values had three times the risk of myocardial infarction (relative risk, 2.9; $P<0.001$) and two times the risk of ischaemic stroke (relative risk, 1.9; $P = 0.02$) of the men with the lower CRP levels.

Atherosclerosis has also been strongly linked with diabetes, a systemic disease which is associated with autoimmunity and thus inflammation (DCCT, 1993). The hyperglycaemic state used to diagnose this disease occurs as a consequence of the insulin deficiency due to pancreatic impairment or, as in type II diabetes mellitus, due to insulin resistance, and is an important source of oxidative stress (Nourooz-Zadeh *et al.*, 1997; Davi *et al.*, 1999). Hyperlipidaemia is also a risk factor for atherosclerosis and an important source of oxidative stress.

It is therefore possible to attribute the telomere shortening in white blood cells observed in this study to a combination of the associated inflammation and oxidative stress, which are common to the atherosclerotic disease state. The mechanism by which the telomeres are affected in atherosclerosis is not clearly understood, but could possibly be due to one or more of the following:

Increased white cell turnover as a consequence of the inflammatory process. Increased stem cells activity is required to fulfil the demand thus resulting in increased telomere loss during this period.

Increased telomere loss per mitotic division during the normal cell cycle due to an exogenous factor directly affecting the structure of the telomere. This could cause telomere fragility and an increase in the number of base pairs lost with each round of mitosis. A possible factor which may support this model would be oxidative stress which has been documented as affecting telomeric regions preferentially compared with the other chromosomal material (von Zglinicki, 2000).

These two mechanisms may not by mutually exclusive but instead may act in combination.

However if cumulative injury from inflammation and/or oxidative stress is the cause of the shorter leukocyte telomeres, via one of the suggested mechanisms, it is sensible to expect the regression slopes for telomere loss with age to increasingly diverge between the groups. This is because you would expect telomere shortening as a result of normal chronological ageing in addition to additional telomere attrition due the underlying mechanism of the disease.

As chronological ageing cannot be halted, and assuming the disease process continues throughout life, the difference between white blood cell telomere length of a 60 year old with disease compared with an age-matched control, should be greater than the difference between white blood cell telomere length of a 50 year old with disease

compared with an age-matched control. This is because the effects of the disease has been in process for 10 years more and therefore would be expected to be deleterious to the telomere for longer. However, in this study, we demonstrated that although there does seem to be some divergence of results this was not at all significant and was possibly due to the narrow age range examined. Thus, statistically, the difference in white cell telomere length between CAD patients and normal subjects was similar at age 40-50 as at age 60-70, suggesting a constant loss with age as opposed to a cumulative loss, possibly suggesting that the longevity of the disease has no additional affect.

It is accepted that the number of subjects used for this study is very small, and further work using larger cohorts would be needed to confirm our findings. Though this initial study does suggest that the difference in white cell telomere length between those prone to develop CAD and those likely to have healthy coronary arteries is, in the main, set at an early stage and maintained throughout life i.e. the shortened telomere length may precede the onset of the disease and may even be the cause.

In support of the results and suggestions here, similar findings were observed in a much larger study involving diabetes mellitus patients. Jeanclos *et al* 1998 demonstrated that although the telomeres of white blood cells from both insulin dependent and non-insulin dependent diabetics were shorter than those of normal subjects, this shortening was not a simple function of hyperglycaemia, because it was not related to the duration of IDDM and because the TRF length of white blood cells from patients with NIDDM was not statistically different from the normals. In addition, hypertension and micro- and macroalbuminuria, which in IDDM are

indicators of nephropathy and predictors of cardiovascular complications, did not correlate with the TRF length of white blood cells. They stated that the shortened TRF length in IDDM did not appear to reflect the duration and severity of IDDM, but that it may be intrinsic to white blood cells of patients with IDDM. This group also accepted that this suggestion would require re-evaluation following a prospective, longitudinal monitoring of telomere attrition before a definite reason could be suggested, which is similar for the results reported in this chapter of the thesis.

However, if the telomere shortening seen here in patients with coronary artery disease (and also the insulin dependent diabetes mellitus patients) does precede the disease, it raises the possibility of two other interesting explanations. First, that the difference is genetically mediated. This suggestion is supported by the twin study which suggested that to a large extent (78%), individual differences in mean TRF length in blood cells, in subjects between 2-63 years is genetically determined (Slagboom *et al*, 1994). These results put forward the thought that those who have gone on to develop coronary artery disease inherited a shorter telomere length than those who remained healthy. This inheritance would cause a genetic disadvantage which when added to by risk factors associated with coronary artery disease, means that the disease manifests itself because the telomeres cannot survive the increased loss. Furthermore, it is possible that a predisposition to excessive inflammatory responses, a phenomenon associated with atherosclerosis, may be genetically mediated. Prospective family studies would possibly be able to distinguish between these two hypotheses.

Secondly, it is also possible that a shortened telomere length is acquired early on in life, as suggested in the "Barker hypothesis", which links dysfunctional growth *in*

utero and late onset cardiovascular disorders including hypertension and atherosclerosis (Eriksson *et al*, 1999; Barker, 2000). Barker suggests that a low birth weight initiates rapid growth *post partum* in order to compensate. This growth period would occur without the presence of telomerase, or at least at a lower activity, as its expression in non-germ line cells has all but been diminished by birth and thus telomere shortening will occur until this growth phase ceases, leaving these babies with what may be described as a 'sub-standard' telomere length. Providing that telomere length has an important role in the pathophysiology of disease, these babies who undergo this 'catch up' period of growth may then be at greater risk of going on to develop complex disorders such as diabetes mellitus and atherosclerosis.

The implication of either explanation is that, if true, the shorter telomeres in white cells moves from being a marker of the atherosclerotic process to a primary abnormality, providing a substrate for accelerated cellular ageing response to atherosclerotic risk factors.

Chapter 5: Comparison of Telomere Length in Atherosclerotic Plaque Cells

5.1 Introduction

The results from the White Blood Cell Study in Chapter 4 demonstrate that there is telomere involvement in the atherosclerotic process and so to follow on from this, the experiments conducted for this chapter were designed to investigate whether the cells that formed the atherosclerotic plaque undergo similar telomere changes to those seen in the peripheral blood cells of patients with coronary artery disease, and if so, to what extent are these changes present. This was called the 'Plaque Study' and involved a direct comparison of mean TRF in cellular constituents of carotid plaques and white blood cells from the same subject.

5.2 Experimental Protocol

Plaque study Patients (n=9) for this study consisted of 7 male and 2 female subjects and had a mean age of 68.1 years (SD = 6.1) with an age range spanning 62 – 75 years. All subjects were undergoing carotid endarterectomy for symptomatic or severe carotid artery stenosis, in the Vascular Surgical Unit at Leicester Royal Infirmary. Inclusion required the availability from a subject of both a suitable endarterectomy specimen from which high molecular weight DNA could be extracted, and a 10 ml sample of blood for extraction of leukocyte DNA.

Endarterectomy samples were placed immediately in ice cold sterile HBSS and transferred to the laboratory within 4 hours where they were snap frozen in liquid nitrogen and stored at –80°C until processed. Blood samples were transferred along with the plaque and processed immediately. Blood samples were never frozen.

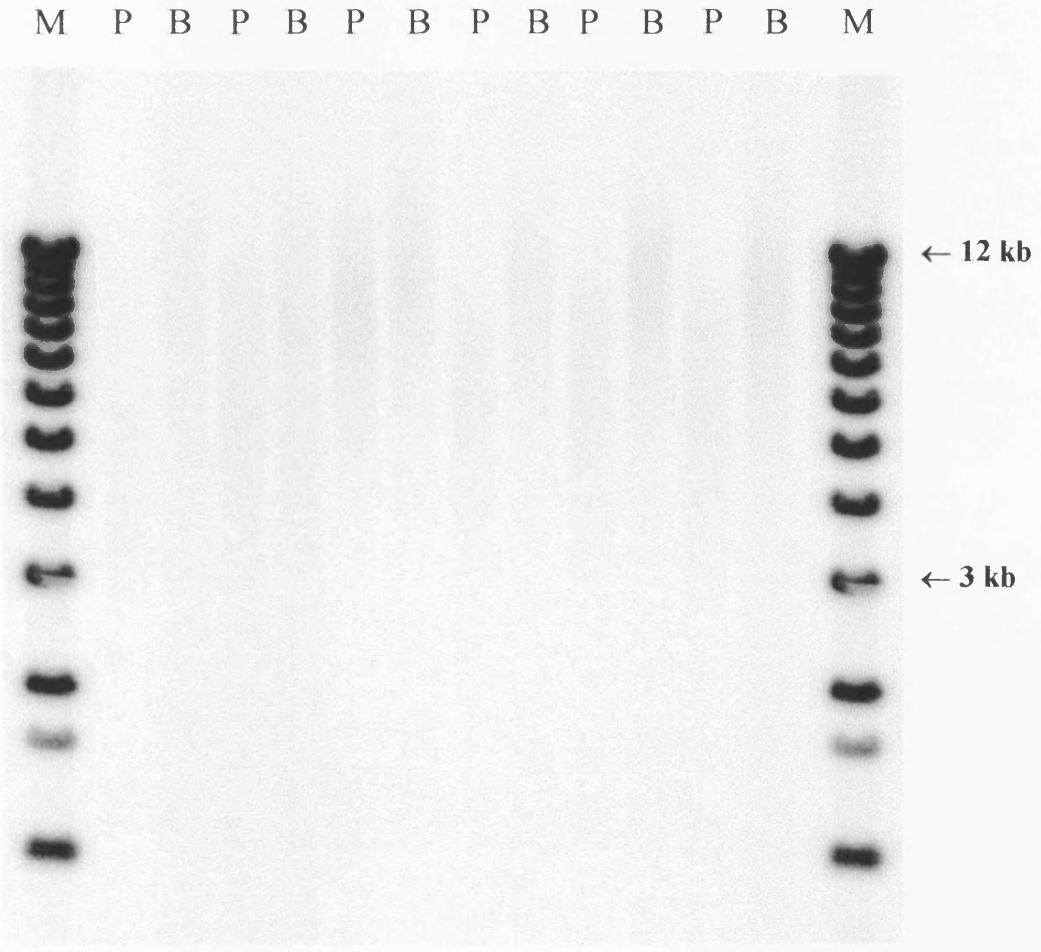
DNA extraction, assessment of integrity, and analysis of telomere lengths were carried out as detailed in Chapter 2: Materials & Methods.

In the plaque study, the mean TRF lengths in leucocytes and plaque were compared using a paired t-test.

5.3 Results

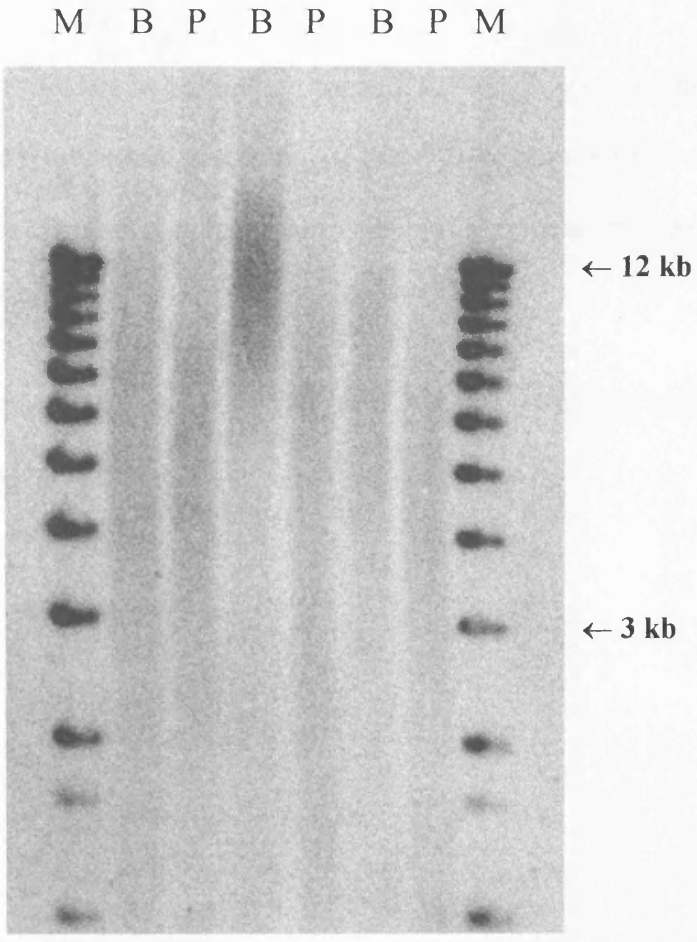
Figure 5.1 & Figure 5.2 both show a representative autoradiogram of the findings. Both of the autoradiograms clearly demonstrate a significant difference in the telomeres from the white blood cells compared with the plaque cells in the same subject. In all subjects the telomeric smear from the white blood cells begins much higher up the gel compared with the plaque cell smear. This directly correlates with a greater mean TRF in the blood cells and therefore longer telomeres. In addition to the white blood cell telomere being higher up the gel, the autoradiograms also demonstrate that the smears from the plaque cells extend much further. This again suggests that the cells from the plaque regions have a population of telomeres which are much shorter.

Figure 5.1 *Plaque Study Autoradiogram 1*



A representative auto radiogram showing Southern hybridisation of telomeric DNA from carotid plaque (P) and white blood cells (B) of the first six subjects collated for the study. M, DNA marker ladder.

Figure 5.2 *Plaque Study Autoradiogram 2*



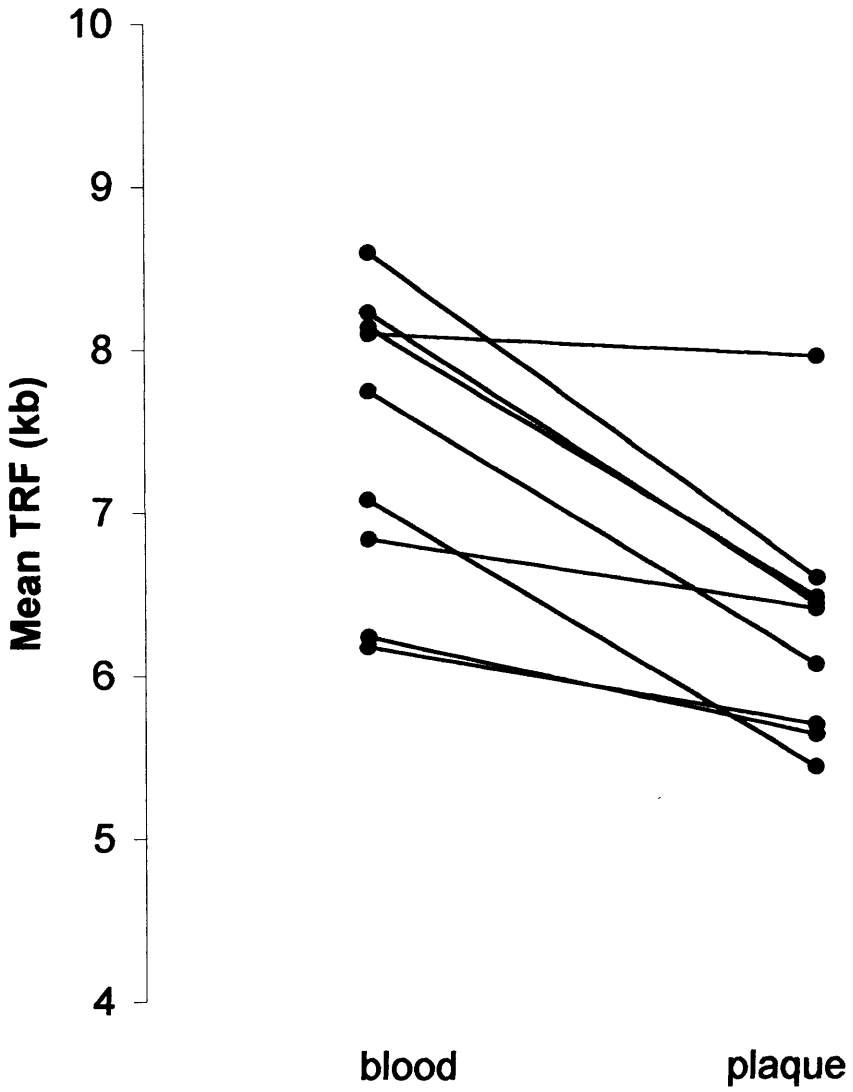
A representative auto radiogram showing Southern hybridisation of telomeric DNA from carotid plaque (P) and white blood cells (B) of the last three subjects collated for the study. M, DNA marker ladder.

These observations were confirmed when the smears were quantitated and the data for all subjects is plotted in Figure 5.3. In all but one subject there is a noticeable difference in the telomere length of the DNA isolated from the white blood cells compared to the DNA from the cells isolated from within the plaque region.

Although there was some variability, mean TRF length was significantly lower in DNA from a heterogeneous population of carotid plaques cells 6.30 kb (SD = 0.75), compared with the corresponding heterogeneous population of white cells 7.46 kb (SD = 0.90) ($p = 0.001$).

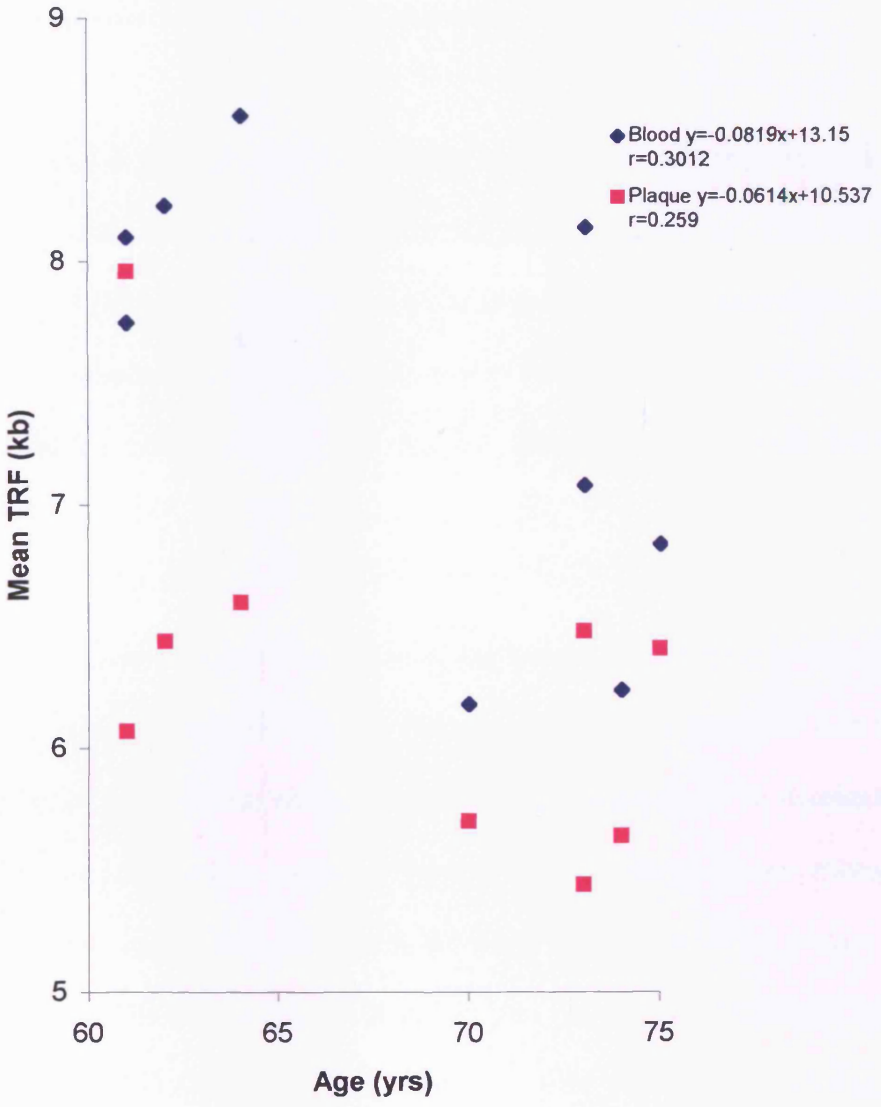
Figure 5.4 demonstrates the mean TRF as a function of age for both the blood and plaque samples.

Figure 5.3 *Plaque study plot of telomere analysis results obtained from white blood cells vs. plaque cells*



Mean terminal restriction fragment length of DNA from white blood cells (7.46 kb SD = 0.90) and carotid plaques (6.30 kb SD = 0.75) of 9 subjects undergoing carotid endarterectomy. ($p = 0.001$)

Figure 5.4 *Plaque study graph plotting the Mean TRF of the plaque and blood specimens for each subject.*



Mean TRF results following Southern hybridisation of plaque cells and white blood cells from the same subject

5.4 Discussion

Based on the findings from the work carried out in this chapter, a novel *in vivo* observation can be reported in that the cellular constituents of atherosclerotic plaques (the highest proportion being made up of smooth muscle cells) have significantly shorter telomeres than the white bloods cells of the same subjects.

It is therefore possible that the findings reported here and those of Chang & Harley who, in 1995, who observed a greater rate of telomere loss as a function of donor age in intimal DNA from iliac arteries, a site exposed to greater haemodynamic stress and prone to atherosclerosis compared to that of internal thoracic arteries which do no not demonstrate atherosclerosis, can explain some of the processes involved in this disease.

The "response to injury" prediction of augmented cell turnover in the atherosclerotic plaque region partly explains this reduction in telomere length in that the increased number of cell divisions associated with the disease results in the accelerated telomere shortening seen here. In particular, smooth muscle cells proliferate extensively during atherosclerosis and this cell type is the major constituent of the plaque and thus the telomere attrition seen here will be, in part, as a result of this increased mitotic activity. In addition to the role smooth muscle cells play in atherogenesis, other groups have noted that telomere attrition may also play a part in the morphological changes consistent with senescence in endothelium overlying atherosclerotic plaques (Davies *et al*, 1988; Burrig, K.F., 1991). However, it is also known that circulating

stem cells migrate to areas of damaged endothelium and differentiate into endothelial cells, becoming a part of the artery lining.

But this augmented cell turnover of the plaque cells cannot be the only cause of the telomere attrition. This is true because the plaque cell telomeres were significantly shorter than those in the white blood cells which are also undergoing rapid cell division as a result of the inflammatory process. This suggests that there could be another factor which is affecting the plaque cells in addition to the cell turnover and causing the increased telomere attrition in these cells only.

To help determine if this is the case it is interesting to note that in *in vitro* studies, oxidative stress, which is increasingly recognised as an important trigger for atherosclerosis (Harrison, 1998), has been shown to accelerate telomere shortening (von Zglinicki, 2000), and homocysteine, a recognised risk factor for atherosclerosis, to increase the amount of telomere length lost per population doubling in endothelial cells through a redox-dependent pathway (Xu *et al.*, 2000). This oxidative damage hypothesis certainly seems a likely cause for the additional telomere loss.

Another interesting feature is that if telomere length can directly affect cellular function as suggested earlier, their length may have an involvement in other changes seen such as cells producing increased amounts of PAI-I, ICAM-I, and fibronectin (Gonos *et al*, 1998) and could conceivably contribute to the cellular synthesis of molecules such as osteopontin that promote plaque calcification (Shanahan *et al*, 1994), a hall mark of advanced atherosclerosis.

The development of atherosclerotic plaques has already been described but is generally characterised by increased local cell turnover as a response to injury (Ross, 1993). As somatic cells have the capacity for a finite number of divisions, this increased local cell turnover leads to the biological ageing of the cells involved as described by Moss and Benditt (1975) who demonstrated that the replicative life-spans of cell cultures which comprised of cells from arterial plaques was less than the replicative life-spans of cells isolated and cultured from non-plaque areas. Other changes have been noted, which can also be linked to this senescent like loss of replicative capacity. Increases in the expression of plasminogen activator inhibitor type 1 (PAI-I) (Xu *et al.*, 2000), intracellular adhesion molecule (ICAM-1) (Comi *et al.*, 1995), and fibronectin (Gonos *et al.*, 1998), have all been observed in endothelial cells and are signs of cellular dysfunction, which have been linked with atherosclerosis (Ross, 1993).

It is therefore possible that the telomere not only governs the replicative capacity of the plaque cells, but also loss of length may initiate the activation of cellular proteins in the plaque cell, which are markers of cellular dysfunction and go on to promote the disease.

Further work needs to be carried out to determine all of these suggestions. The hypothesis of augmented cell turnover seems likely. However, the contribution of another exogenous factor is not certain. In order to confidently state oxidative stress as an additional factor affecting the cells in atherosclerosis, cell culture experiments involving cell exposure to oxidative stress and subsequent telomere analysis would be required. Also, the link between telomere shortening and cellular expression of

molecules associated with the disease progression cannot be confirmed by the results shown here. This is because the results of this study were obtained from a heterogeneous cell source including smooth muscle cells, endothelial cells and fibroblasts. To confirm this hypothesis, further work into the individual cells concerned would be necessary, in particular endothelial cells which have been demonstrated to express certain cell molecules on their surface during the atherosclerotic disease.

Assessment of mean TRF in individual cells types (Rufer *et al.*, 1998). could be achieved with the advent of fluorescent *in situ* hybridisation techniques, which are now being used for the determination of telomere lengths in specific cell types. Also, the use of more careful isolation techniques of DNA from the plaque could be used. This would involve the separation of the cellular subsets from the plaque prior to the DNA extraction procedure. This way, the cells which make up the plaque structure could be analysed independently in order to demonstrate whether the telomere shortening seen in this study is consistent throughout the plaque, or concentrated to individual cells and thus giving clearer information on the mechanisms of the disease.

However, it must be mentioned that even though the work in this chapter does suggest a possible mechanism of telomere involvement in atherosclerosis, it is difficult to draw conclusions from these results alone because the numbers in this study are very small. Many more subjects need to be investigated to provide more concrete evidence. Also, there is the issue that white blood cells cannot really be used as a control tissue. as work from the previous chapter shows that this cell type is also affected by the disease. However, the collection of a true disease free sample of tissue for a reliable

comparison of telomere length is unethical. The removal of healthy tissue at the time of the endarterectomy procedure would never by justified, nor granted by an ethics board, and therefore the white blood cells were the best control tissue available at the time. Even though it was agreed that this was not an ideal situation.

In summary, we have shown that telomere loss is accelerated in cells of atherosclerotic plaques compared to white blood cells of the same patient. It is unlikely that this is due to a genetic mechanism alone, but more likely as a consequence of an exogenous factor, such as oxidative stress, or a combination of the two.

Chapter 6: Oxidative Stress, Telomeres and Endothelial Cells

6.1 Introduction

The results from the Plaque Study in Chapter 5 demonstrate that there is increased telomere shortening in the cells which form the atherosclerotic plaque, compared to the telomere shortening of white blood cells of the same coronary artery diseased subjects. Although most of the cells which form the plaque will be smooth muscle in origin, nevertheless, this was a heterogeneous cell population and therefore the telomere results observed in Chapter 5 could not be attributed to any one cell type specifically.

However, shortened telomere lengths have been observed in endothelial cell dysfunction, which is known to be associated with the progression of atherosclerosis, and hydrogen peroxide has been used in *in vitro* studies to induce similar changes to the endothelial cells which have been observed in plaque tissue. Therefore it is suggested that the cause of the endothelial cell dysfunction seen in atherosclerosis is due to direct interactions between the ROS and the endothelial cell telomere, resulting in a defunct telomere structure which can no longer protect the cell during mitosis, causing progression of the disease.

In an attempt to demonstrate this, bovine aortic endothelial cells were exposed to hydrogen peroxide and subsequent morphological changes were noted, in particular, those which are synonymous to cells which have undergone augmented cell turnover (senescence) and/or oxidative stress. DNA was then extracted from these cells and analysed using the telomere assay in order to determine whether the morphological changes were linked to any loss in telomere length.

6.2 Experimental Protocol

6.2.1 Bovine Aortic Endothelial Cells

Endothelial cells have a limited replicative capacity *in vitro*. The exact life span varies from cell to cell, but generally, bovine aortic endothelial cells (BAEC) are capable of undergoing approximately 60 to 100 population doublings before they enter senescence. This is compared to human umbilical vein endothelial cells (HUVEC) which have a much shorter *in vitro* lifespan and are capable of 10 to 20 population doublings before senescent behaviour is seen.

BAEC were the cells of choice for these experiments because the aortas were much more readily available, and in greater numbers, compared with human umbilical cords. In addition to this, the number of cells which could be harvested from one bovine aorta was far greater than the number of cells that could be harvested from one human umbilical cord. As a result of these considerations it was decided that BAEC's would be a more useful source than HUVEC's for the experiments planned. However, the negative issue for using BAEC's is the results obtained cannot be expected to be exactly the same as human cells under identical conditions.

6.2.2 Endothelial Cell Identification

Endothelial cell cultures were mainly identified on their morphological criteria. Bovine aortic endothelial cells tend to be polygonal in shape and form a tight cobblestone appearance when confluent. These characteristics are demonstrated in Figure 6.1a.

In addition to the morphological identification described, cells were investigated for the presence of von Willebrand Factor (vWF) in the intracellular Weibel Palade granules, which is a marker commonly used for positive identification of endothelial cells.

Cells were 100% positive for vWF as seen in Figure 6.1b which demonstrates the FITC-labelled (green) von Willebrand factor, present in the cytoplasm and on the cell surface of the cells, and the Hoechst (blue) counter stain for the nuclei.

6.2.3 Effects of long term culture on BAEC

An initial procedure was carried out prior to the planned hydrogen peroxide treatment in order to investigate the effects of long term passage and thus replicative senescence on these cells types. A batch of BAEC's were cultured under 'normal' conditions for as long as they remained capable of replication. This was monitored with population doubling calculations using the following equation:

$$\text{Population Doubling} = [\log_{10} N_t - \log_{10} N_0] / \log_{10} 2$$

Cells were photographed and any morphological changes were noted.

Figure 6.1 *Identification of Bovine Aortic Endothelial Cells using (a) Morphological criteria (b) von Willebrand factor staining*

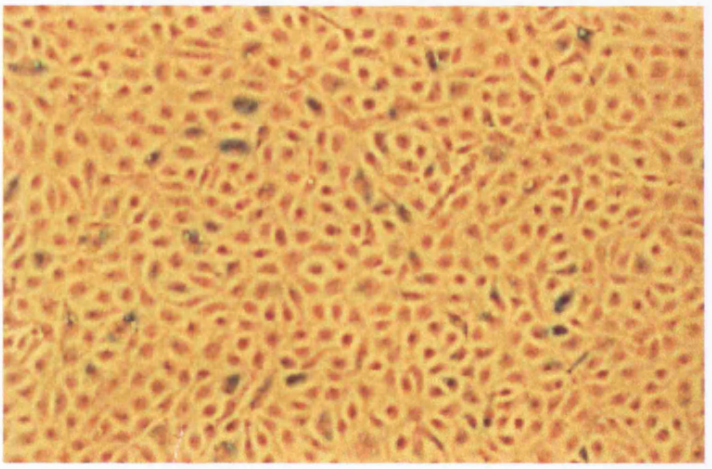


Figure 6.1a Cultured bovine aortic endothelial cells showing typical cobblestone morphology at confluence. Magnification x100

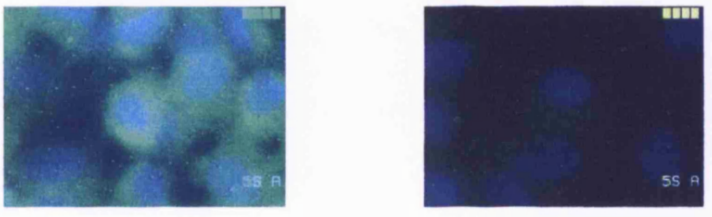


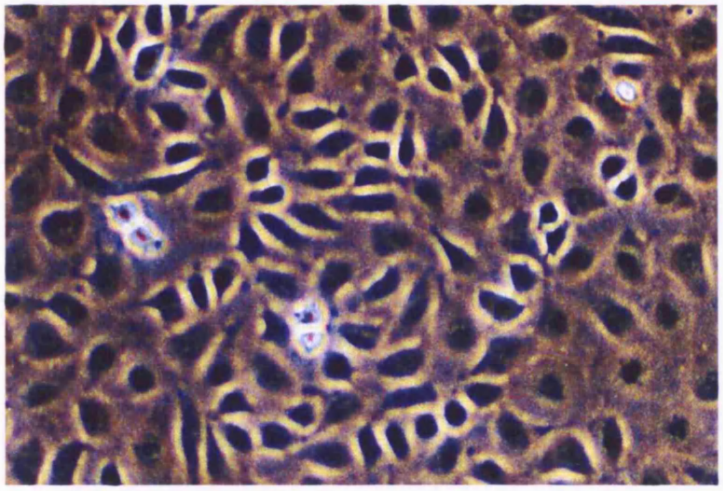
Figure 6.1b Cultured bovine aortic endothelial cells showing typical von Willebrand staining on the left. Negative control (no primary antibody) on the right. Magnification x400

Figure 6.2 demonstrates the morphological changes seen in the BAEC's throughout the progression of the experiment. Figure 6.2a is a photograph of the cells after they had undergone 10 population doublings. It shows a population of typical BAEC's with a normal morphology. They are small, uniform in size and round to polygonal in shape and all cells possess a single nucleus. Figure 6.2b is a photograph of the cells on the last day of the culture. At this point the cells had undergone 60 population doublings. The culture no longer consists of uniform cells. Many of the cells are no longer small, but giant in size. It is these cells which tended to be multinucleated. They are no longer round to polygonal but instead they have a bizarre and irregular shape. At this point the cells were unable to continue growing. They had lost their ability to adhere to the culture vessel and the experiment was halted. From start to finish, this experiment lasted for 72 days.

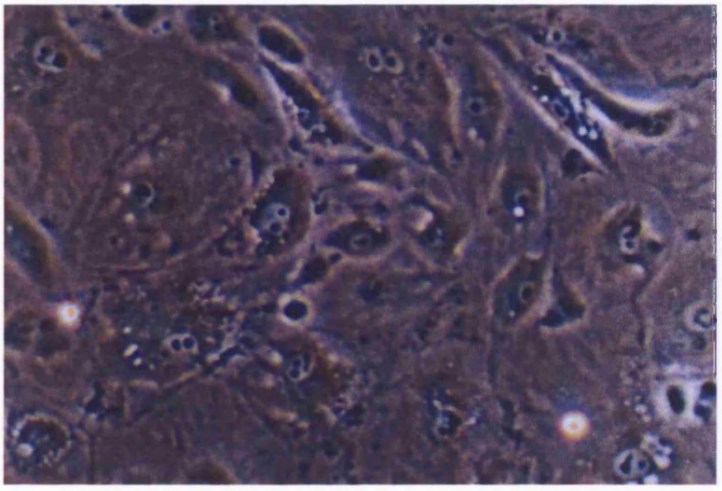
6.2.4 Hydrogen Peroxide Treatment

BAEC's that were approximately 85% confluent (~6 x 10^6/80cm – at the time of seeding) were subjected to oxidative stress by a single dose exposure to hydrogen peroxide (H_2O_2) for 1 hour at 37°C in Hank's solution. After this 1 hour exposure, the cells were returned to normal medium and continued in culture. Twenty four hours following the H_2O_2 treatment, the medium was aspirated from one flask at each concentration and a cell count was carried out (using a haemocytometer) in order to calculate the number of cells that had died following the treatment with H_2O_2. This step provided a true starting cell density with which to initiate accurate population doubling calculations. This was a vital step as the population doubling that the cells had undergone was crucial to the hypothesis. When the number of dead cells was

Figure 6.2 *Replicative senescence of BAEC following long term passage.*



a



b

a: Endothelial cells after 10 population doublings; b: Same batch of endothelial cells after 60 population doublings.

Phase contrast microscope. Magnification x100

counted, this number was subtracted from the initial seeding density. It was this number which was then used as the start point so that the population doubling for the next stage of the experiment could be calculated.

This experiment was carried out twice with a slightly differing protocol for each (the changes were made based on the results obtained from the first experiment and will be discussed later).

In Experiment 1, the cells were maintained under culture conditions following the treatment process and on day 2 the cells were sub-cultured. The experiment continued for 7 days, with cells also being sub-cultured and harvested on days 5 and 7. Tissue culture flasks were re-seeded at a density of 2×10^6 cells/flask. The cells were photographed at the time of sub-culture.

In Experiment 2, the cells were maintained under culture conditions following the treatment process and on day 4 the cells were sub-cultured. The experiment continued for 25 days, with cells being sub-cultured and harvested on days 8, 13, 18 and 25. Tissue culture flasks were re-seeded at a density of 1×10^6 cells/flask. The cells were photographed at the time of sub-culture. At the end of this experiment the cells had been cultured for a total of 36 days.

The decision to sub-culture the cells on the days stated was based upon when the cells appeared to be confluent or almost confluent. This was so that the cell numbers in the flask were sufficient to seed new flasks and also provide enough DNA for the

telomere analysis. The cells were not allowed to remain at a confluent state as a constantly dividing population of cell was required.

These two experiments were carried out only once, although five flasks per concentration were harvested for DNA analysis at each subculture. Therefore the telomere results are from a collection of all five flasks at each given time point.

6.2.5 Annexin Staining

As this study was intended to investigate the effects of oxidative stress on endothelial cells and in particular whether such treatment induced accelerated senescence, it was important to know that these cells had altered morphology and possibly telomere length attrition because they genuinely had undergone induced senescence as opposed to the H_2O_2 causing apoptosis.

Cells used within these experiments were investigated for apoptosis twenty four hours following H_2O_2 and also on the last day of the experiment. Samples of cells from each of the experimental groups were examined.

To aid the orientation of the cells, the nuclei were counterstained with Hoechst, a fluorescent dye which binds to double stranded DNA, and thus highlights the nucleus.

6.3 Results

6.3.1 Experiment 1

Observations shoed that following incubation with HBSS in the absence of hydrogen peroxide, the control cells appeared normal, with a typical cobblestone appearance

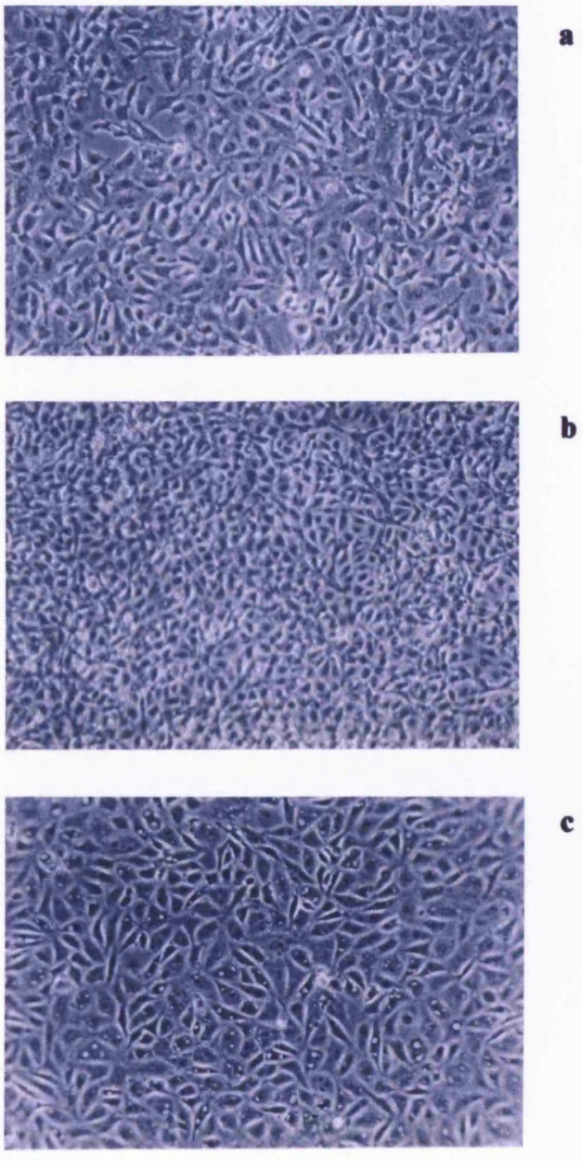
that remained throughout the culture process (Figure 6.3). It is evident from Figure 6.3b that these cells were over confluent (i.e. the density of these cells was much greater than that seen when a confluent flask of cells is observed) at this stage. This was both due to the seeding density decided upon being too high and because the cells had been left for 3 days before sub-culturing, which was obviously too long. This resulted in the cells compressing against each other as they continued to divide, squashing their cytoplasm, making them appear very small.

In contrast to the control cells, the treated cells responded to the hydrogen peroxide treatment in a dose dependent manner.

In the cell population treated with 50mM hydrogen peroxide there was a loss of cells ($1.4x10^6$ out of the 5.7×10^6 treated) following the oxidative exposure and the sparse number of cells remaining at day 2 can be seen in Figure 6.4a. At this stage, no morphological abnormalities can be seen. At day 5 there was presence of abnormal, giant cells, approximately 10 times bigger than normal cells in the culture. These variant cells were randomly dispersed within the culture and some appeared to have more than one nucleus. These cells can be observed in Figure 6.4b. Surprisingly, however, by day 7 of the experiment, the cells within this culture appeared to all be of normal morphology. None of the giant cells were visible, but instead, only small, regular shaped endothelial cells were present This is shown in Figure 6.4c.

Similarly, in the cell population treated with 100mM hydrogen peroxide there was an immediate loss of cells following the oxidative exposure. This loss was even greater than that seen in the 50mM population ($3.0x10^6$ out of the $5.6x10^6$ treated) and the

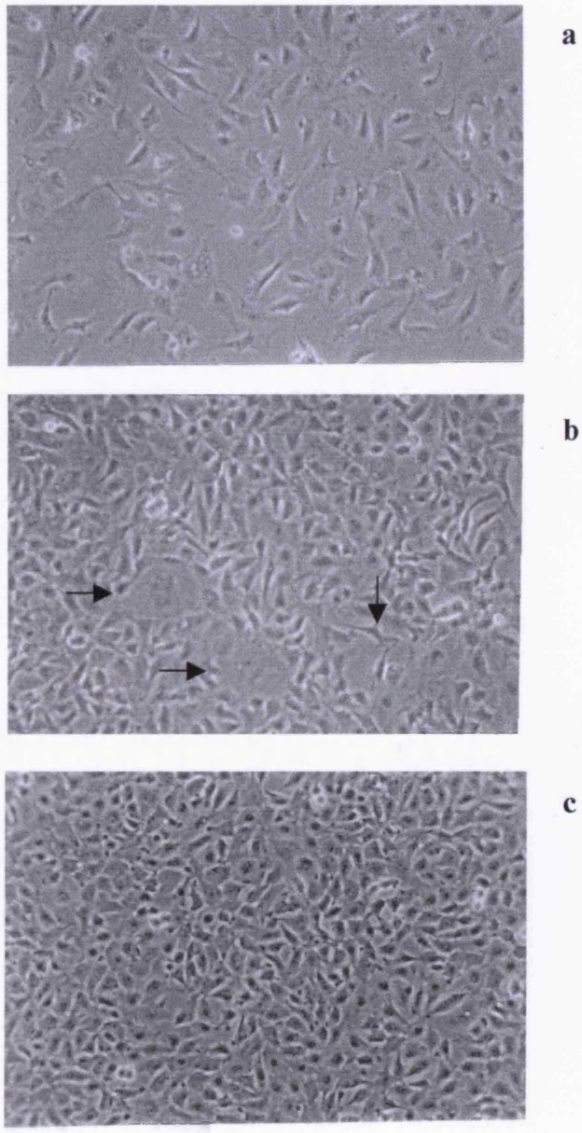
Figure 6.3 *Experiment 1: Control BAEC*



a: BAEC at day 2; b: BAEC at day 5; c: BAEC at day 7

Phase contrast microscope. Magnification x100

Figure 6.4 *Experiment 1: BAEC treated with 50mM hydrogen peroxide*

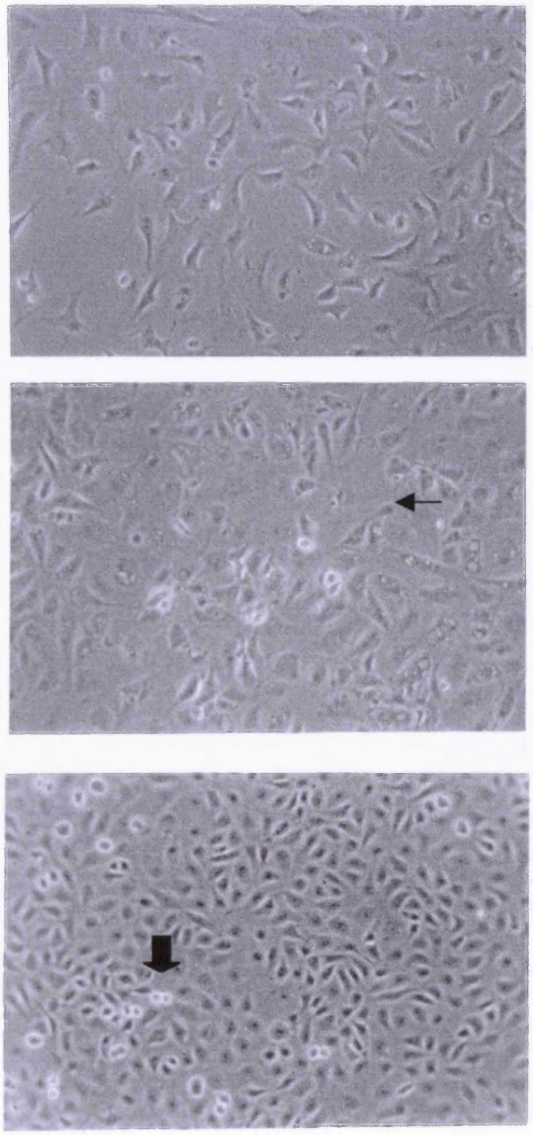


a: treated BAEC at day 2; b: treated BAEC at day 5; c: treated BAEC at day 7

Phase contrast microscope. Magnification x100

Giant cells →

Figure 6.5 *Experiment 1: BAEC treated with 100mM hydrogen peroxide*



a: treated BAEC at day 2; b: treated BAEC at day 5; c: treated BAEC at day 7

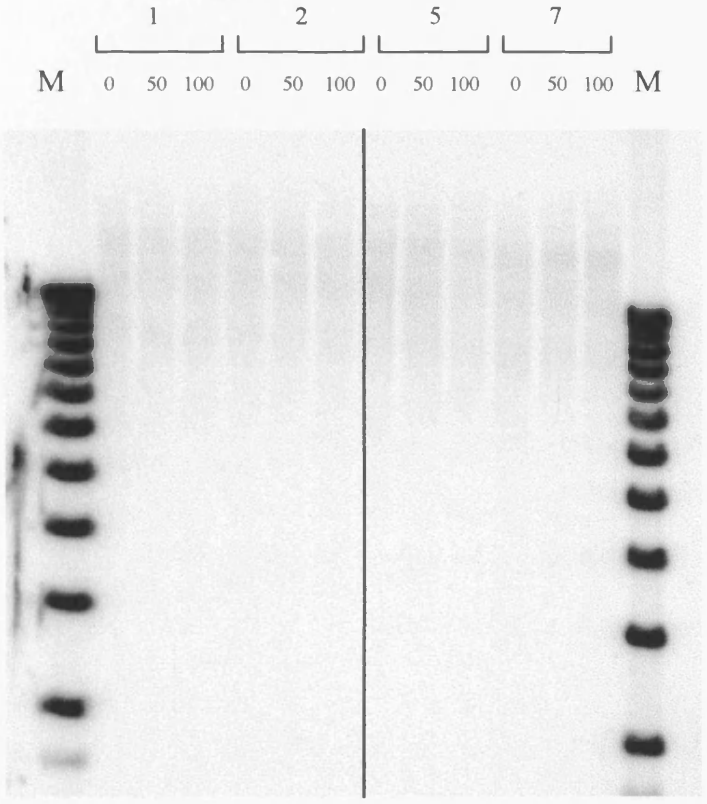
Phase contrast microscope. Magnification x100

Giant cells → Rounded up cells ➡

abnormal than normal which can be seen in Figure 6.5b. These abnormal cells were similar to those seen as a result of 50mM hydrogen peroxide. By day 7 the majority of the cells present in culture were of normal appearance, although there were still a small number of the variant cells remaining (Figure 6.5c). An additional observation of rounded up endothelial cells can be seen in both the day 5 and 7 cultures although more were present by day 7. These cells lifted up off the culture flask wall where they floated in the culture medium.

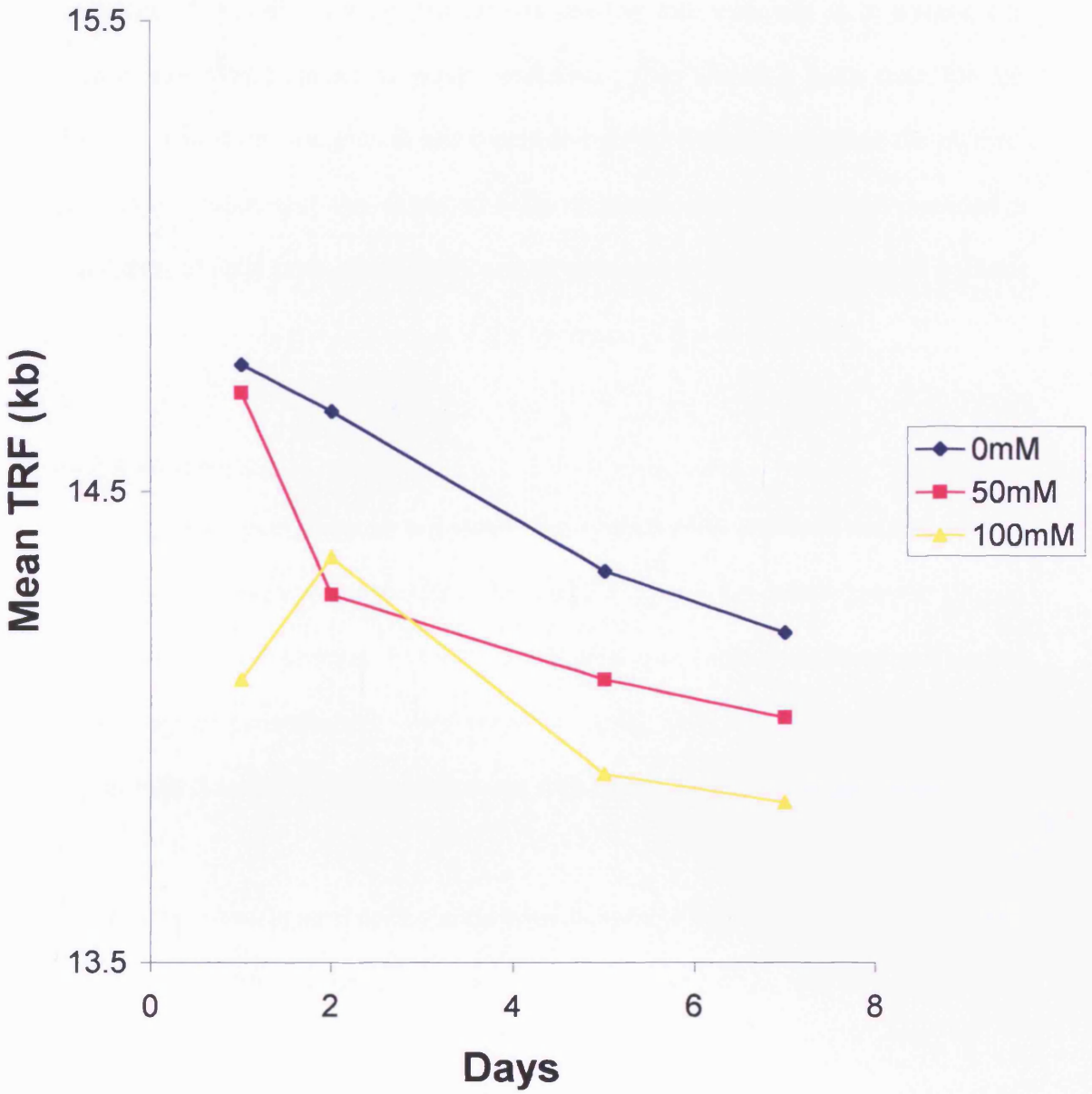
The return to normal morphology which was seen by day 7 in each of the 50 and 100mM cell populations was unexpected. An explanation for this situation is that the un-affected cells continued to grow at their normal rate, whilst the cells which were affected by the hydrogen peroxide took time before resuming normal cell division. During this 'rest' period, it is possible that the normal cells took over and dominated the population and therefore the affected cells were overgrown. In addition to this, the severely damaged cells would have rounded up and floated off from the culture vessel. In both instances, the normal cells predominated in the subsequent populations. This also helps understand the telomere results obtained, whereby there was an immediate shortening in telomere length following hydrogen peroxide treatment, but this loss then reached a plateau following the first sub-culture at day two (50mM) and five (100mM). Therefore due to the dominance of the normal cells and the loss of severely damaged cells, following subculture only unaffected cells remained for the DNA harvest. The telomere results obtained for this experiment are demonstrated by an autoradiogram in Figure 6.6 and graphically in Figure 6.7. The graph demonstrates a sudden loss of telomeres immediately after the hydrogen

Figure 6.6 *Representative autoradiogram of telomere assay using DNA harvested from BAEC (Experiment 1).*



An autoradiogram demonstrating the resultant telomere smears of BAEC after treatment with hydrogen peroxide. Numbers along the top denote the day of harvest. The smaller numbers denote the concentration of H_2O_2. The black line identifies where sub-culturing was started. M Molecular weight marker

Figure 6.7 *Mean TRF for hydrogen peroxide treated BAEC (Experiment 1)*



peroxide treatment. Following this, the subsequent telomere loss rate was similar for all three cell populations, regardless of hydrogen peroxide concentration

Due to these results experiment 2 was designed with slightly different culture conditions. A smaller seeding density was used so that with less cells present, the culture would take longer to reach confluence, thus allowing more time for the affected cells to resume growth and therefore become established within the culture. This would mean that the damaged cells remained and subsequently provided a population of cells from which DNA can be extracted to see if the telomeres in these affected cells are shorter as a result of the hydrogen peroxide treatment.

6.3.2 Experiment 2

Following hydrogen peroxide treatment, the control cells appeared normal, with a typical cobblestone appearance that remained throughout the culture process (Figure 6.8). Again, as in experiment 1, these control cells soon became confluent and similar morphological consequences were noted i.e. small cells due to continued growth (Figure 6.8c,d and e). Following treatment with HBSS, <1% of these cells died.

In contrast to these control cells, the cultures exposed to hydrogen peroxide responded in a dose dependent manner.

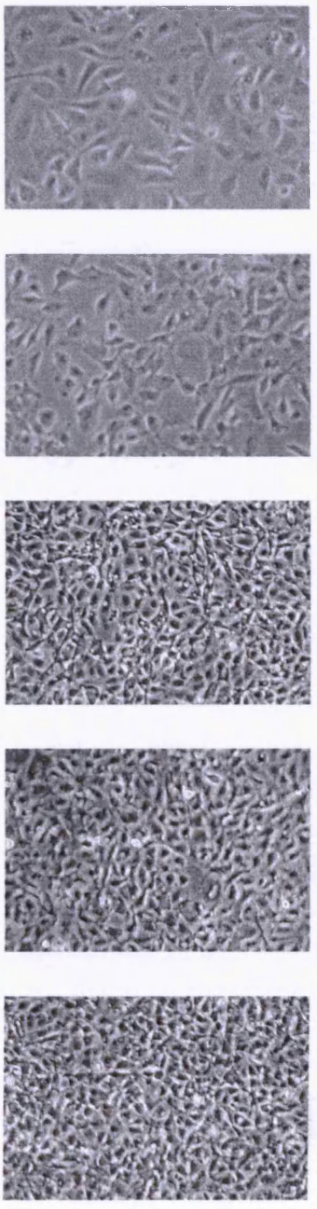
In the cell population treated with 25mM hydrogen peroxide there was a loss of cells (1.2×10^6 out of the 5.75×10^6 treated) following the oxidative exposure. The reduced number of cells remaining at day 2 can be seen in Figure 6.9a. At this stage, no morphological abnormalities can be seen. The cells appear to be active, with a number

of mitotic bodies present. At day 4 there was presence of vacuoles within some of the cells, however, most of the culture was of normal appearance, with a cobblestone morphology still present. At day 8, abnormalities were starting to appear. Some of the cells were looking somewhat larger and by day 18 there were a few giant cells dispersed among what seem liked normal endothelial cells. These cells can be observed in Figure 6.9b, c, and d.

In the cell population treated with 50mM hydrogen peroxide there was also a loss of cells (1.7×10^6 out of the 5.75×10^6 treated) following the oxidative exposure. The even greater reduction in cells remaining at day 2 can be seen in Figure 6.10a. The cells in this figure appear normal and are attempting to make contact with adjacent cells by spreading out which accounts for the loss of a normal polygonal shape. At day 4 the cells do not look a great deal different to those seen at day 2, however, by day 8 very irregular cells are present. These are much larger than the normal cells in the culture. Day 18 provided even greater numbers of these giant cells, which looked as almost 15-20 times bigger than the normal cells. These cells can be seen in Figure 6.10b, c and d.

Similarly, in the cell population treated with 100mM hydrogen peroxide there was an immediate loss of cells following the oxidative exposure. This loss was even greater than that seen in the 25mM and 50mM populations (3.53 out of the 5.75×10^6 treated) and the even more sparse number of cells remaining at day 2 can be seen in Figure 6.11a. Very little cell division took place following the first subculture resulting in little difference in the cell number by day 4 and rounded up endothelial

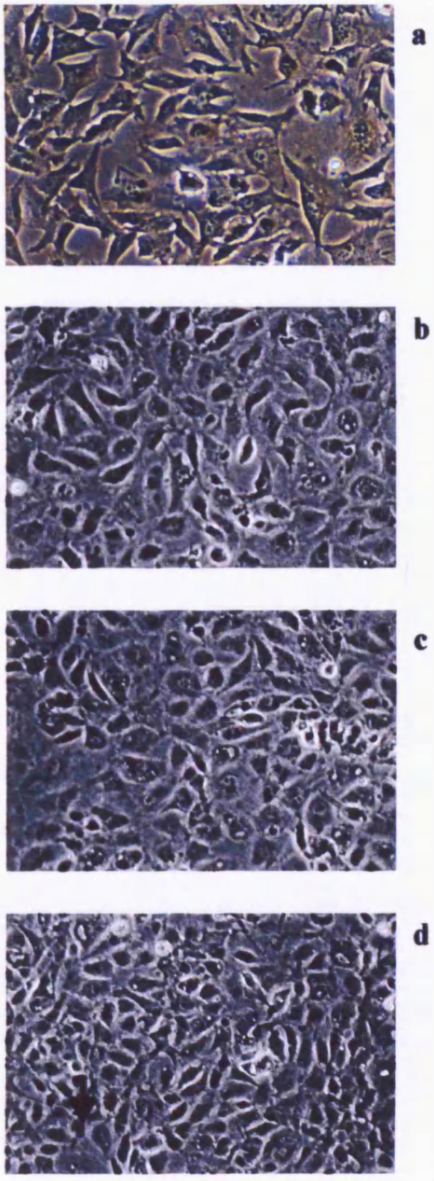
Figure 6.8 *Experiment 2: Control BAEC*



a: Day 0; b: Day 2; c: Day 4; d: Day 8; e: Day 18

Phase contrast microscope. Magnification x 100

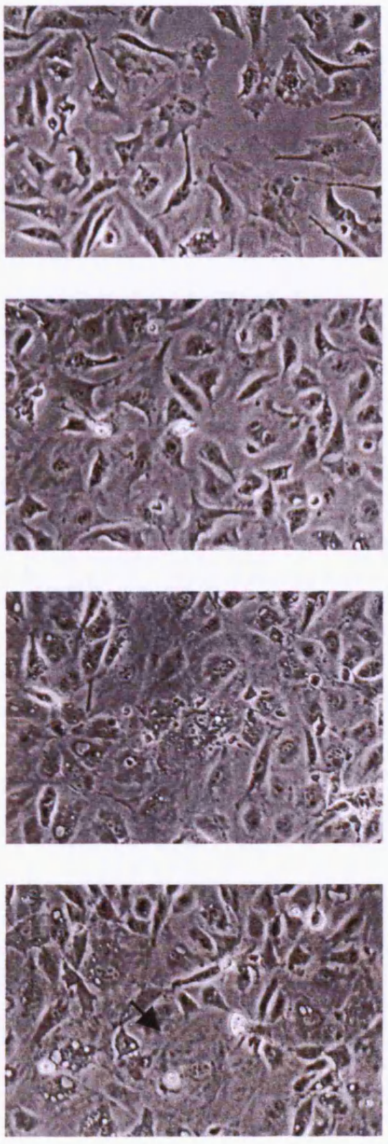
Figure 6.9 *Experiment 2: BAEC treated with 25mM hydrogen peroxide*



a: treated BAEC at day 2; b: treated BAEC at day 4; c: treated BAEC at day 8; d day 18. Phase contrast microscope. Magnification x100

Giant cells →

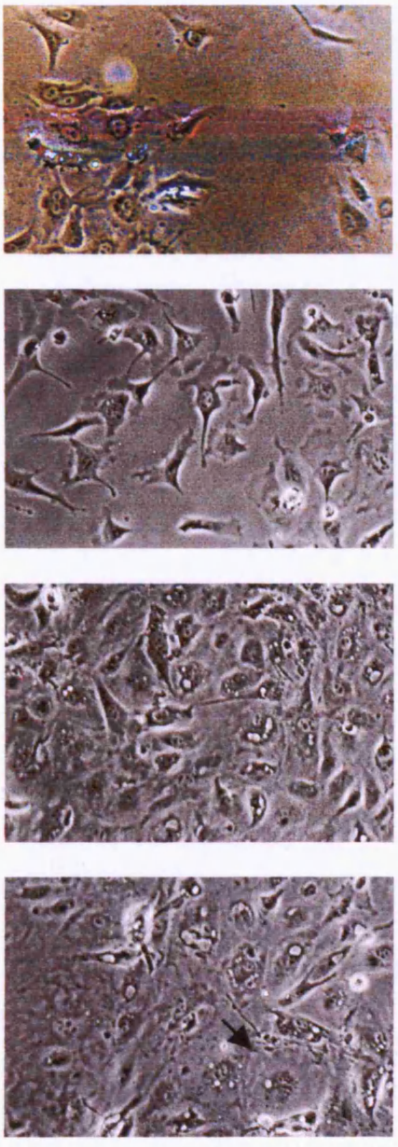
Figure 6.10 *Experiment 2: BAEC treated with 50mM hydrogen peroxide*



a: treated BAEC at day 2; b: treated BAEC at day 4; c: treated BAEC at day 8; d day 18. Phase contrast microscope. Magnification x100

Giant cells →

Figure 6.11 *Experiment 2: BAEC treated with 100mM hydrogen peroxide*



a: treated BAEC at day 2; b: treated BAEC at day 4; c: treated BAEC at day 8; d day 18. Phase contrast microscope. Magnification x100

Giant cells →

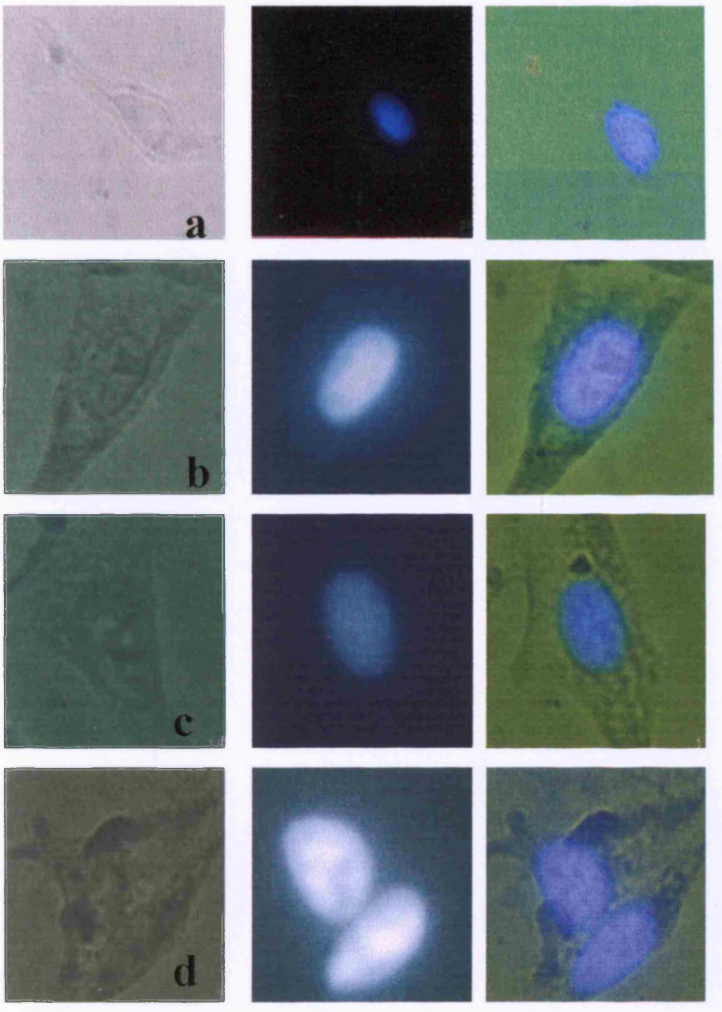
cells were already present. Very abnormal cells were present by day 8, with vacuoles and irregular nuclei in most cells. By day 18 no normal looking cells were present in the population and cells which covered the culture flask surface were the largest seen in any of the cultures. The cells treated with 100mM hydrogen peroxide can be seen in Figure 6.11b, c and d. These cells were so big that only 2.3 x 10^6 were required to cover the entire flask surface, compared to 11.3 x 10^6 in the control cell populations.

On day 25 a sample of the cells which had been treated with each concentration of hydrogen peroxide was taken for the Annexin V staining. This was carried out because we wanted to make sure that the cells we were harvesting DNA from were senescent, not apoptotic. This method for detecting apoptosis was used as it was being run routinely elsewhere in the department.

These results can be seen in Figure 6.12 The nuclei which were counterstained with Hoechst are blue. In the middle lane of images, only the nucleus is visible. If membrane flipping had occurred, a green outline of the cell would be seen here. There was no evidence of membrane flipping in any of the cell samples and therefore it can be assumed that the change in morphology is not due to induced apoptosis, but as a result of the hydrogen peroxide either affecting the cell directly or via the telomere.

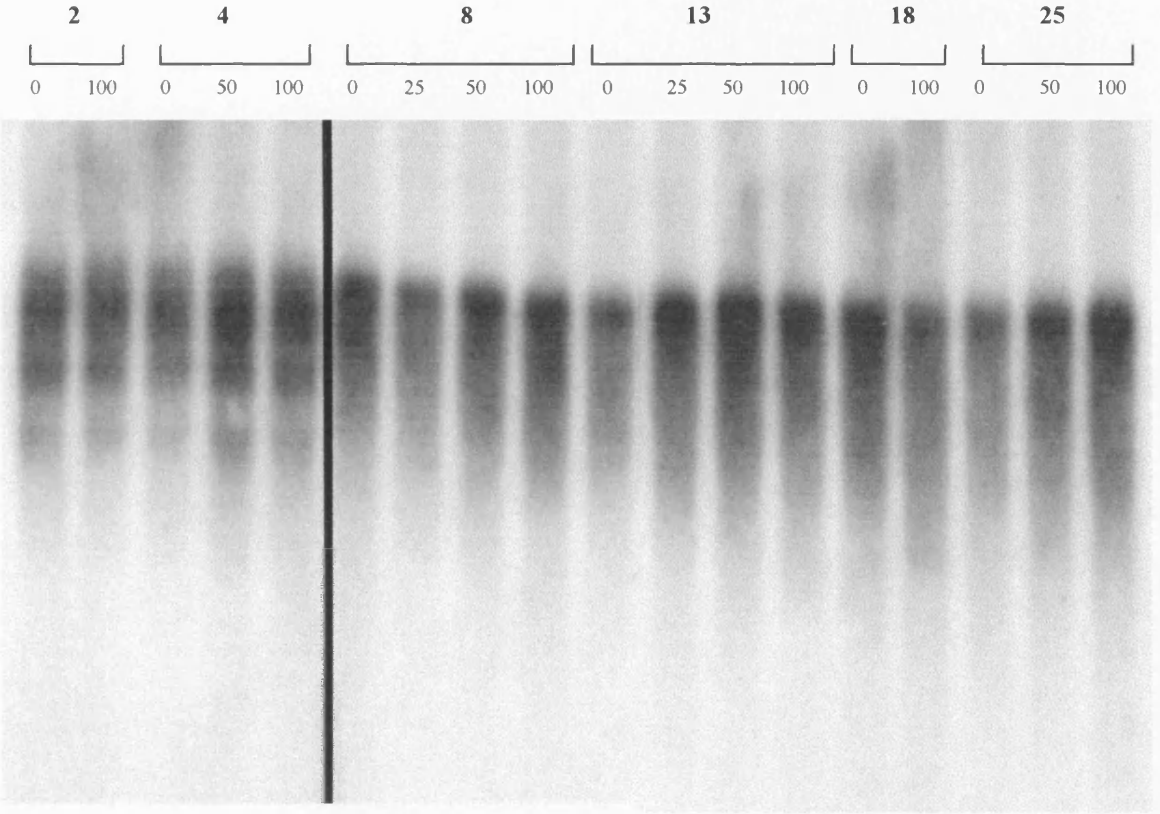
A positive control for this experiment was carried out using colchicine which causes cycle arrest and induced membrane flipping. However, the cells did not withstand the treatment so results are not available. However, this phenomenon was observed in another experiment being carried out by a colleague within the department on the same cell type.

Figure 6.12 *Annexin staining of BAEC for identification of apoptosis*



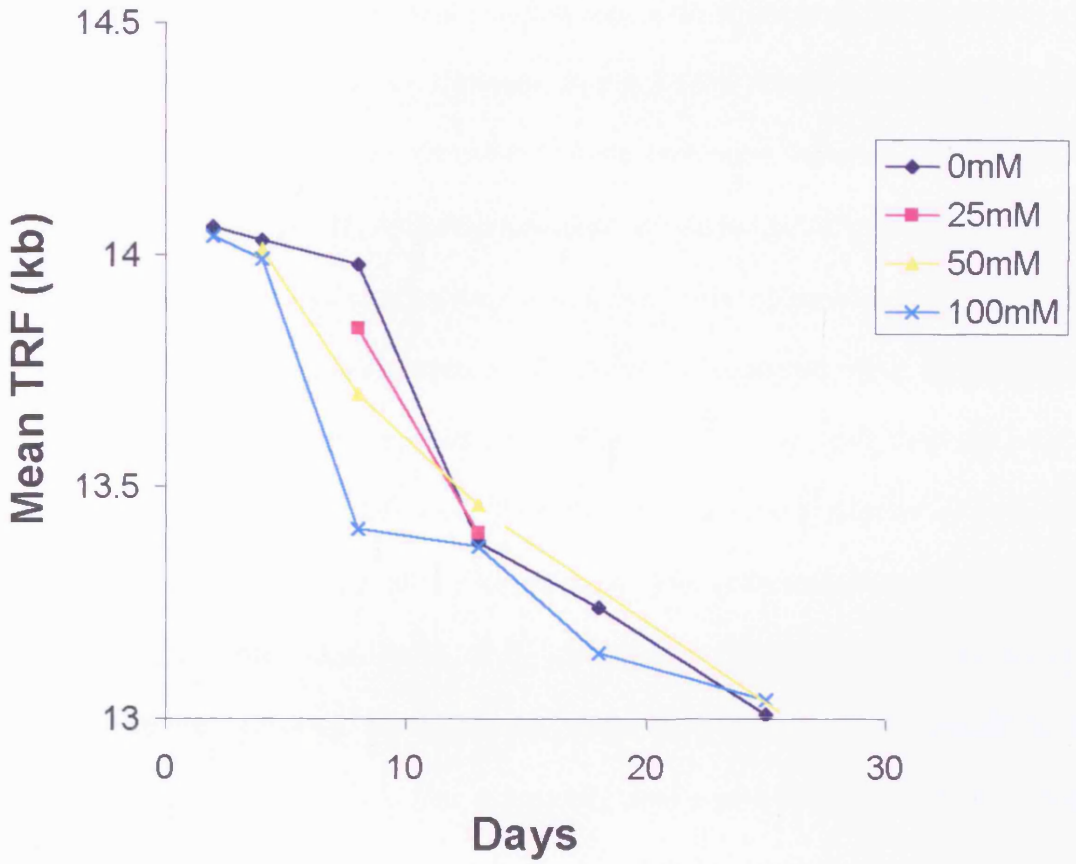
BAEC treated with a: Control cells (Day 0); b: 25mM H_2O_2 (Day 25); c: 50mM H_2O_2 (Day 25); d: 100mM H_2O_2 (Day 25). Left hand image: Phase contrast. Middle image: Hoechst positive nucleus. Right hand image: The two other images superimposed for orientation. An apoptotic cell would have been FITC positive on the middle giving rise to a green cell membrane. Magnification x 400

Figure 6.13 *Representative autoradiogram of telomere assay using DNA harvested from BAEC (Experiment 2).*



An autoradiogram demonstrating the resultant telomere smears of BAEC after treatment with hydrogen peroxide. Numbers along the top denote the day of harvest. The smaller numbers denote the concentration of H_2O_2. The black line identifies where sub-culturing was started.

Figure 6.14 *Mean TRF for hydrogen peroxide treated BAEC (Experiment 2)*



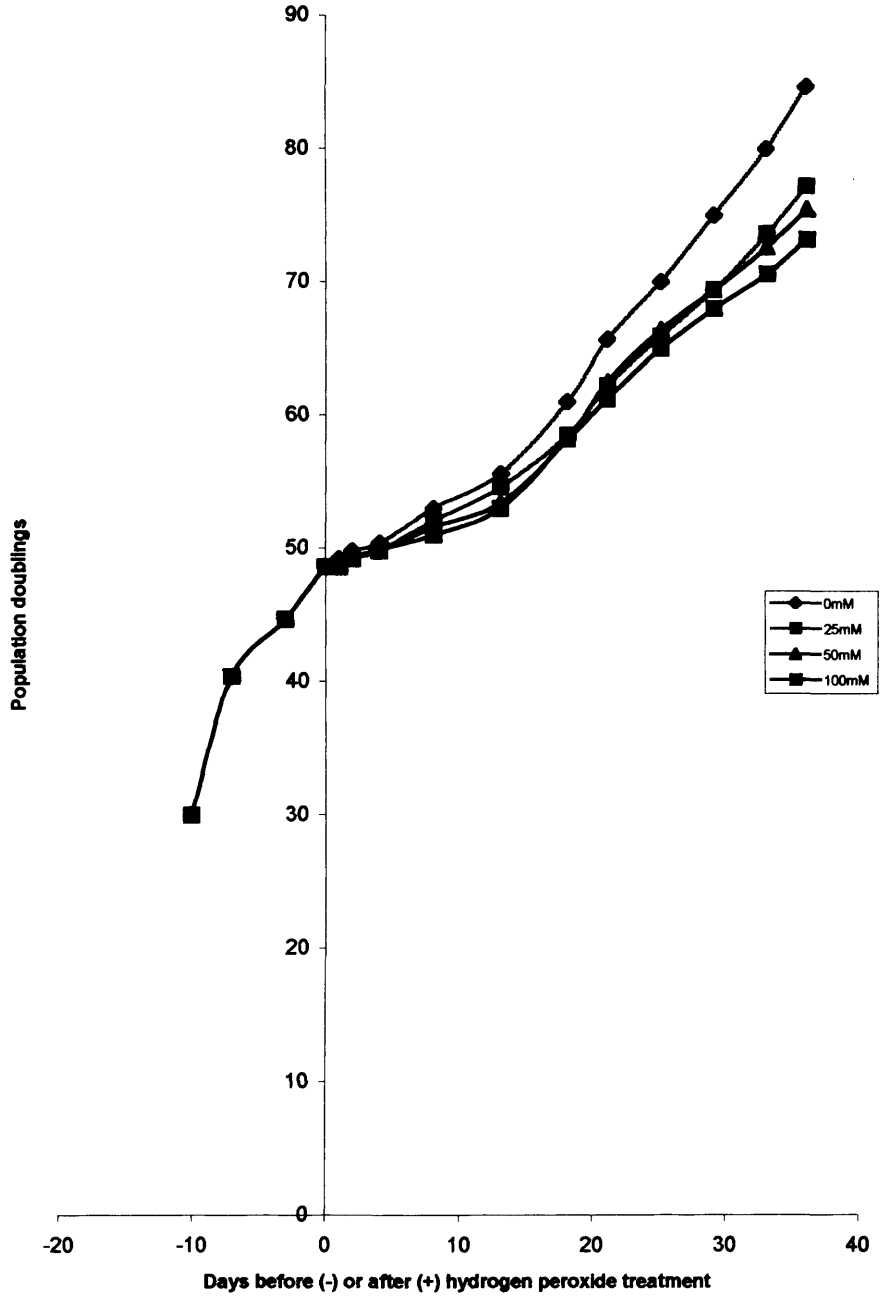
The reproducibility of the *in vitro* experiments cannot be stated as they were carried out only once. This explains the omission of error bars.

Telomere analysis of the DNA harvested was then carried out and the calculated mean TRF plotted as a function of time (days). An autoradiogram for this experiment can be seen in Figure 6.13 and a graph illustrating the mean TRF length throughout the experiment per hydrogen peroxide concentration can be seen in Figure 6.14.

Analysis of the telomere length demonstrates that in the control cells there is a telomere attrition which occurs as a function of normal cell division. At day 7 this shortening appears to occur at a greater rate in the hydrogen peroxide treated cells in a dose related response. However, by day 13 this acceleration in telomere loss in the treated cells does not seem to have continued. In fact by the end of the experiment, at day 25, All cell populations appear to have a very similar telomere length, regardless of whether they were treated with hydrogen peroxide or not and irrespective of the concentration. This may be explained when the cumulative population doublings are examined in Figure 6.15. It is visible from the graph in this figure that the cells went through a different total number of population doublings by the end of the experiment. The cells underwent the following population doublings: 0mM 42.6, 25mM 39.2, 50mM 36 and 100mM 35.8. Therefore following the treatment, those cells which were exposed to the hydrogen peroxide were less mitotically active in a dose dependent response compared with the control cells, suggesting that the treatment directly affected the cell growth and population doubling capacity. Therefore, this increased number of population doublings may account for the telomere loss seen in the control cell.

However, it is also possible that the apparent 'slow down' of telomere attrition in the treated cells after day 13 is due to the over-population of the culture by a small

Figure 6.15 *Cumulative population doublings of hydrogen peroxide treated BAEC (Experiment 2)*



number of cells which were not affected by the hydrogen peroxide. Therefore these unaffected cells went on to dominate the culture and thus influenced the telomere length attained.

6.4 Discussion

The results obtained in this study are consistent with the findings of other groups who also demonstrated senescent like behaviour in bovine aortic endothelial cells as a result of hydrogen peroxide exposure. These changes included an altered morphology and the presence of giant, multinucleated cells. These changes were proportional to the concentration of oxidative stress used (von Zglinicki *et al.*, 2000).

In addition to these morphological changes, telomere attrition has been linked with oxidative stress. This was demonstrated when antioxidants were shown to slow down age-dependent telomere loss (Furomoto *et al.*, 1998) and fibroblasts from patients with Fanconi anaemia, a disease known to be associated with chromosomal instability, disturbances in oxygen metabolism and a high burden of intracellular reactive oxygen species, were shown to have accelerated telomeric loss compared with healthy controls (Adelfalk *et al.*, 2001). Both of these studies have demonstrated a link between telomere loss and oxidative stress.

The results in this study are very preliminary. It has to be pointed out that experiment two was only carried out once and therefore would need repeating to add confidence to the findings.

In spite of this, the telomere loss per population doubling which was seen in experiment 2 demonstrated that as the hydrogen peroxide concentration increased, so did the base pair attrition per cell division. Comparing the control cells and the 100mM cells, there is almost a 50% increase in telomere loss per population doubling. This finding suggests that the hydrogen peroxide may have a direct affect on the extent of telomere damage during cell division. In support of this, Furomoto *et al* in 1998, also suggested that oxidative damage acts by increasing the telomere attrition rate per population doubling.

The results presented in this chapter must, however, be interpreted cautiously. It was evident from the telomere assay that the telomere length from these cells of bovine origin were much longer than those cells from human donors. On average, the bovine cells had an initial telomere length of approximately 14kb compared to the range of 5.5 to 7kb from human cells, depending on age. This increased telomere length may well have biased the results, especially as the telomere hypothesis is based upon the Hayflick limit suggestion that cells continue to replicate until the telomere is too short to protect the cell from the deleterious consequences of mitosis. Therefore, this means that the bovine cells may well have an increased replicative capacity compared with human endothelial cells and are not an ideal model for this study.

In addition to this, the extended telomere length of these bovine cells also created an analytical problem. Although an extra molecular weight marker was used to take into account that the telomere smear extended beyond the 12kb fragment of the molecular weight ladder used in the human studies, during this work it was not possible to radio-label this extra ladder. This meant that the difference in position of the fragments

from 15kb to 12kb on the UV light box, had to be extrapolated onto the densitometer image, ready for quantitation. Obviously this is by no means an ideal situation and given more time, this problem could have been overcome.

However, the mechanism by which the oxidative stress affects the endothelial cells to cause senescence and telomere loss remains unclear. It had been suggested that the telomere may be a preferential site for oxidative DNA damage. Petersen and colleagues suggested that accelerated telomere shortening is due to preferential accumulation of oxidative damage in telomeres. They demonstrated that chronic hyperoxic treatment accelerated fibroblast loss and the rate of telomere shortening in parallel. They suggested that the mechanism causing the effects they observed was due to a free radical-mediated generation of single-strand breaks. They hypothesised the accumulation of single strand breaks or single-stranded regions in telomeres under oxidative stress prior to DNA replication would significantly contribute to the shortening of telomeres in the daughter cells (Petersen, 1998). Their work demonstrated an increase in this type of region with hydrogen peroxide treated fibroblasts, in particular it was noted that this damage occurred in a G specific manner along the chromosome which may explain why the G rich telomere is preferentially damaged under such situations.

To summarise the results in this chapter, a dose dependent telomere shortening was observed in bovine aortic endothelial cells. This was accompanied by similar morphological changes which have been demonstrated in cells isolated from atherosclerotic plaques. The mechanism by which these changes occur was not investigated during the work for this chapter, but the increased base pair loss per

population doubling, as the concentration of hydrogen peroxide increased, suggests that the telomere is directly affected by this oxidative damage in some way.

Chapter 7: Discussion

Telomeres have been demonstrated to be essential in providing a protective 'capping' and thus, stability to the chromosome terminus. In addition to this role, they have also been shown to influence the nuclear organization of the cellular DNA during division. However, possibly their most important biological role within the cell is involved with mitosis, during which incomplete DNA synthesis occurs. This is due to the nature of the constraints associated with the actions of DNA polymerase during the synthesis of DNA. During this process, the telomere appears to provide a 'buffering zone' through which the loss of DNA incurred due to the DNA replication problem is prevented from being deleterious to the cell. Instead of losing vital DNA important for the production of genetic information, there is a loss of telomeric DNA which is not translated into proteins. While the telomere is intact and of a sufficient length to cope with the subsequent effects of mitosis, the cell can continue to divide. However when the telomere falls below a predetermined length the integrity of vital DNA cannot be guaranteed and the cell is susceptible to entering a period of senescence (Hayflick & Moorhead, 1961) or apoptosis (Ahmad & Golic, 1998). It appears that the fate of the cell is dependent upon the chromosome affected and whether this incomplete DNA replication leads to the production of lethal fragments which are detected by cell checkpoint proteins and induce cell death. Therefore, the telomere has been seen as an internal biological clock, controlling the mitotic activity of the cell and thus the chronological age (Olovnikov, 1973).

This senescent behaviour and shortened telomere length has been observed in many different cell types, although the order in which these two situations occur remains unclear. In particular, senescence has been observed in endothelial cells as a result of oxidative stress *in vitro*. Morphological changes were noted during lengthy experiments in which endothelial cells were exposed to a one-off, short term treatment of an oxidative agent. The changes observed were time and dose dependent and included the presence of vacuoles, apoptotic bodies and bizarre, multi nucleated giant cells (de Bono & Yang, 1995).

The clinical significance of these changes is that similar oxidative damage to the endothelium is thought to occur during atherosclerosis. As a result of this, both the endothelium and leucocytes are activated, increasing their adherence to each other, culminating in migration of the leucocytes out of the circulation to underlying tissues, where they localise and ultimately result in the formation of plaques and eventually stenosis of the artery.

The experiments carried out for this thesis were designed to explore the length of the telomeres from these cells involved in atherosclerosis by inducing the oxidative stress related changes in cultured bovine aortic endothelial cells and measuring telomere length. In addition to this, two separate studies were also carried out to see if the hypothesis could be tested *in vivo* by measuring telomere length in leucocytes from subjects with known coronary artery disease and comparing the results obtained with from age matched normals and measuring telomere lengths in the cells which form plaque regions obtained from subjects undergoing an endarterectomy procedure.

These results were compared with the telomere lengths of leucocytes from the same subject.

The results of these studies have shown that there is telomere shortening in peripheral white blood cells from patients with known coronary artery disease compared with age-matched controls. In addition to this, the cells which form the atherosclerotic plaque had even shorter telomeres than the white blood cells taken from the same subject. Finally, i*n vitro* studies suggest that there may be a direct affect of oxidative damage on the telomeres of endothelial cells, which correlated with the morphological changes observed. Both occurred in a time and dose dependent response.

7.1 Atherosclerosis and senescence

Cells involved in the atherosclerotic process, including leucocytes, smooth muscle cells, the cellular constituents of plaques and endothelial cells which have been subjected to oxidative stress, have all been demonstrated to have shortened telomeres. Therefore by the nature of the telomere hypothesis, these cells are more likely to enter a period of senescence, suggesting that atherosclerosis may be a disease associated with accelerated cellular aging. The accelerated telomere shortening observed within the studies of this thesis may be accounted for in a number of ways.

Firstly, the telomere results obtained from analysing white blood cells from subjects with coronary artery disease may be due to increased cell turnover following an inflammatory response to circulating oxidative stress. High reactive oxygen species (ROS) levels have been note in atherosclerosis (Irani, 2000) and are deleterious in

many ways. Patel *et al* (1991) demonstrated that low dose exposure to hydrogen peroxide induced the adherence of polymorphonuclear neutrophils to the endothelial cells, which was completely blocked by antibodies to P-selectin. This interaction of leucocytes with the endothelium results in loss of this cell type via migration from the peripheral circulation to the underlying tissue (Patel *et al.*, 1991; Patel *et al.*, 1992; Prescott *et al.*, 2002). This results in an increased requirement for leucocyte production to balance the levels in the circulation, leading to haematopoietic stimulation and ultimately a raised leucocyte count (Bickel *et al.*, 2001). This increased cell turnover in itself could explain the shortened telomere lengths seen in white blood cells from subjects with coronary artery disease.

Secondly, the results observed from telomere analysis of plaque cells may be explained by observing what happens to the leucocytes which migrate through the endothelium. In addition to this initial oxidative stimuli which is suggested here to promote haematopoietic proliferation and thus telomere shortening in white blood cells, there is further oxidative stress. Once the leucocytes are tethered to the endothelium, the endothelial cells secrete vesicles containing oxidised phospholipids which interact with the neutrophils (Smiley *et al.*, 1991). Also, the leucocytes themselves release ROS into their surroundings in response to the tethering process (Harlan *et al.*, 1991). With such exposure to oxidative stress it is possible that this has a direct affect on the telomeres of the leucocytes and endothelial cells involved, causing loss in addition to that which occurs as a result of the extra mitotic activity.

In addition to these two hypotheses, once under the endothelium, the migrated white blood cells continue to proliferate which would also contribute to the loss of telomere length in the cellular constituents of the plaque.

This theory has been supported by work carried out by Petersen *et al* who demonstrated that telomeres are vulnerable to oxidative damage. They observed a preferential accumulation of single strand breaks in the telomeres of fibroblasts following oxidative exposure, which remained un-repaired for at least 19 days. They hypothesise that these single strand breaks may be the cause of the telomere shortening which accompanied the fibroblasts. The shortening that was observed was up to one order of magnitude greater following free radical stress (Petersen *et al.*, 1998).

As these affected cells form the plaque itself, this may explain why these cells demonstrated much shorter telomeres than the circulating white blood cells from the same subject. This is because the cells which form the plaque are exposed to a greater amount of telomere damage via localised oxidative stress in addition to the increase mitotic activity, whereas the circulating white blood cells are affected by the initial short burst of oxidative stress and subsequent mitotic activity only.

Another explanation as to what is happening at the plaque site is augmented cell turnover as a result of damage. Davies *et al* noted large areas of endothelial denudation with exposure of underlying collagen in plaques from patients with atherosclerosis (Davies *et al.*, 1998). This exposed collagen is very thrombogenic and as a result of this the endothelial cells spread out or divide to bridge the gap, resulting

in increased telomere loss in these cells. Burrig also observed the presence of giant endothelial cells on the plaque surface, probably indicating accelerated endothelial cell senescence attributable to sustained non-denudating injury in the region of disturbed blood flow (Burrig, 1991). These results are consistent with endothelial senescence and excessive cell replication, which would account in part for the telomere attrition seen in the cells isolated from the plaques of subjects undergoing carotid endarterectomy surgery. However, it must be mentioned that a very small proportion of the plaques isolated would be endothelial in origin and thus, this hypothesis would not contribute greatly to the telomere attrition observed.

It is not only the endothelial cells which appear to contribute to this overall telomere shortening within the plaque site. Moss and Benditt demonstrated that extracted smooth muscle cells from atherosclerotic plaques had reached their finite replicative life span when it was attempted to culture these cells *in vitro* (Moss & Benditt, 1975) suggesting that the telomere capacity of these cells had been exhausted. This work was supported by Burrig who reported that patients who had undergone carotid endarterectomy surgery had experienced rapid proliferation of the smooth muscle cells in the damaged area (Burrig 1994). Again, such extensive cell replication would contribute to the telomere loss of the cells which made up the plaque region, a theory which supports the results noted in this thesis. Furthermore, it is possible that the macrophages and leucocytes which have migrated through the endothelium forming the plaque, may go on to replicate. Again, this so far unproven hypothesis, would also contribute to the telomere attrition demonstrated here.

In addition to these suggestions, it must also be noted that the plaque cells live longer than the leucocytes in circulation and as a results of this, they are more likely to suffer further insult and subsequently shorter telomeres compared with leucocytes which may only experience acute attacks.

7.2 Atherosclerosis and telomere inheritance

Many suggestions have been made regarding the causes of the telomere attrition observed during the disease process, including augmented cell turnover and oxidative stress. However, it is likely that other interactions and influences may also be of vital importance, such as the genetic inheritance of telomere capacity.

Inheritance of telomere length has been demonstrated in twin studies (Slagboom *et al.*, 1994) and as such it is possible that the subjects with coronary artery disease, investigated within these studies may have all inherited a shorter telomere length than those who were free of the disease. This would mean that the cells involved in the disease process had a reduced replicative capacity at the beginning of disease onset and therefore were not able to cope with the increased cell division that accompanies the progression of the atherosclerotic process and ultimately became symptomatic. It is also possible that these individuals may lose a greater amount of telomere DNA with each round of mitosis, which again would lead to the susceptibility of disease.

To test this hypothesis, a prospective study would need to be carried out, involving recruitment of subjects (with and without coronary artery disease) and their families. Telomere assessment of young children whose parents or grandparents had gone on to

develop coronary artery disease would be required The results would have to be compared with age matched children with no known family history of coronary artery disease. If the telomeres of those children from families with a history of known coronary artery disease were shorter than the children from healthy families, free from the disease, then this would suggest that coronary artery disease may occur, in part, due to the inheritance of shorter telomeres. It would also be of value to continue monitoring these two groups of children into adulthood and maybe even until the onset of disease, if this is the case. With this information, the telomere lengths from affected family members could be compared to investigate if there is a common telomere length or percentage telomere loss at age of onset.

Another hypothesis was suggested by Barker in 1995. He suggested that foetal under nutrition in middle to late gestation, which leads to disproportionately low foetal growth, programmes the onset in later life of coronary artery disease. This was supported by studies in humans which showed that men and women whose birth weights were at the lower end of the normal range, who were thin or short at birth, or who were small in relation to placental size have increased rates of coronary heart disease. It is possible that birth size and atherosclerosis are linked because of a suggested 'catch-up' period, whereby the new-born undergoes rapid growth *post partum* via cell division to balance out the inadequate growth which occurred *intra uterine*. It is hypothesised that this happens without the protection of *intra uterine* telomerase and therefore telomere loss occurs. Such events may result in shorter telomere lengths even before the disease process has begun and as with the telomere inheritance theory, the reduction in replicative capacity means that as the risk factors are encountered, the cells involved are less able to cope. To investigate this possible

genetic involvement, a retrospective analysis would be required, which would include collating data from the individuals who collectively formed the cohort of coronary artery disease subjects. Time of gestation and birth weight of these individuals could be compared with those subjects formed the normal cohort to identify any trends, such as whether births occurred earlier on in pregnancy in those who ultimately developed coronary artery disease and if these patients had a below average birth weight, compared with those in the normal cohort.

7.3 Atherosclerosis and oxidative stress

Whilst much work has been undertaken in the measurement of telomere loss, and indeed the work for this thesis had concentrated solely on this aim. Little work has been carried out to investigate how the oxidative stress associated with atherosclerosis actually exerts its affects upon the telomere.

It has been suggested that these alterations in telomere length are attributed to age-associated functional changes in vascular cells, but several other factors, such as oxidative stress and DNA damage, have been shown to cause cellular senescence *in vitro* as well. Indeed, the cull culture experiments carried out in this thesis may support this, but the mechanism by which these changes occur is still not known. However, one study has shown that endothelial cells become senescent and their functions altered by the inhibition of TRF-2 alone. The duplex array of TTAGGG repeats at mammalian telomeres is bound to by two related proteins, the TTAGGG repeat binding factors TRF-1 and TRF-2 (Chong et al., 1995; Bilaud et al., 1997; Broccoli et al., 1997). TRF-2 is essential for the formation of large duplex loops

which appear to protect the telomere end. Inhibition of TRF-2 results in activation of the ATM/p53 dependent checkpoint pathway, leading to cell cycle arrest and apoptosis (Karlseder et al., 1999). Although the telomeric repeat array remains largely intact, the single-stranded protrusion of TTAGGG repeats found at all mammalian telomeres (Makarov et al., 1997) is lost upon inhibition of TRF-2, suggesting that the G-strand overhangs are important for telomeric protection (van Steensel et al., 1998). TRF-2 is shown to protect these G-strand overhangs by altering the structure of the telomere. Electron microscopy has identified that telomeric DNA is isolated as large duplex loops called t-loops, in which the terminus is embedded with the double stranded part of the telomeric tract. TRF-2 can generate t-loops *in vitro* and its *in vivo* function is consistent with t-loops as the main mechanism by which mammalian cells mask natural chromosome ends (Griffith *et al.*, 1999).

The introduction of TRF2 D/N induced growth arrest with phenotypic characteristics of cellular senescence, such as enlarged cell shapes and increased senescence-associated B gal activity. The expression of ICAM-1 was significantly increased with reduced levels of NOS end eNOS proteins (Minamino 2002), all signs of endothelial cell dysfunction, which have been shown to be inducible directly by oxidative stress (Fennel, 2002). It is therefore possible that oxidative stress may exert its affects on the cell and in particular the telomere directly by inhibiting the binding of TRF-2, preventing t-loop formation and thus, telomere protection.

In addition to this suggested interaction with TRF-2, it is also possible that oxidative stress may damage the G-rich overhang directly, further affecting the t-loop formation, which has previously been suggested by Petersen.

However, the mechanism by which oxidative stress exerts its actions during atherosclerosis may in fact be far more simple than either of the TRF-2 or G-strand alternatives. Some cells may respond to the exposure to oxidative stress by dying, a situation which was noted in the cell culture experiments carried out for this thesis. In *in vivo* situations, particularly involving the endothelial cells, this would lead to denudation which would be repaired by increased cell division and therefore telomere loss by normal mitotic process.

It appears that there may be more than one mechanism by which oxidative stress may exert its actions on the telomere and is by no means a simple process.

7.4 Improvements & Future work

7.4.1 Telomere assay

The assay optimised during this study was adequate for the measurement of telomeric DNA obtained from human sources, where great differences in length were present, as observed when white blood cell telomeres were analysed and compared with plaque cell telomeres. However, it is probably too crude for the identification of very small differences in telomere length (i.e. just a few base pairs) which may be the case when applying the method to *in vitro* studies such as the bovine aortic endothelial cell experiments, whereby some cell populations may only differ by one or two population doublings and therefore the telomere changes would be very small, especially as bovine cells have much longer telomeres compared with human cell lines.

To overcome this problem of sensitivity, measurement of telomere lengths using fluorescent in situ hybridisation (FISH) techniques as opposed to the adapted Southern blot methods adopted for use in this thesis are being implemented. Indeed, several groups (Hultdin et al., 1998; Rufer et al., 1998; Brummendorf et al., 2000; Gomez et al., 1998) have used FISH assays to label the telomeres of specific individual cells and measured the fluorescent signal by flow cytometry. The levels of sensitivity has allowed for the estimation of very small changes in telomere length from subpopulations of human lymphocytes (Rufer et al., 1998). Also, differences in telomeres from normal and malignant cells in chronic myeloid leukaemia have been measured (Brummendorf et al., 2000) Both of these studies used a single blood sample without any prior separation procedures being required. The Southern hybridisation technique used in the studies carried out for this thesis would not have been able to offer such results.

Implementing this FISH technique to measure telomeres from the cells isolated from the atherosclerotic plaque samples would have eliminated the problem of having a heterogeneous population of cells which included endothelial cells, smooth muscle cells and fibroblasts. This is because the flow cytometry would enable the separate identity of the cells to be characterised and thereby allow for telomere measurement of each cellular population. Thus a comparison for all of the arterial constituents would be possible making it possible to identify if all the cell telomeres of the plaque region were affected by the atherosclerotic process equally, or one cell type more than the others. The results obtained from the plaque specimens by the methods used cannot offer this information and as such the results of the telomere lengths have to be

attributed to the plaque as a whole and a possible mechanism for the disease cannot be suggested.

Similarly, in the tissue culture experiments where heterogeneity of cells was present following hydrogen peroxide treatment, the FISH technique could have been used to divide the cells into normal and altered populations. This would therefore have demonstrated whether telomere shortening occurred preferentially in the morphologically altered cells compared with the ones which appeared to have normal endothelial cell characteristics.

7.4.2 Specimen selection

The main criticism of the human studies has to be the small numbers of volunteers used in the both the white blood cell and plaque cell studies. To validate the results further and provided greater significance, the number of subjects included in the cohorts needs to be increased. This is a situation that can be rectified simply with more time for subject inclusion and future experiments could be carried out with greater numbers. Since the completion of this thesis, further work on much larger cohorts has been completed. The results obtained support those presented here and therefore add confidence.

The lack of any normal tissue for the use as a reliable comparison with the plaque results is also a problematic situation. This means that there was no true negative control. This will be much more difficult to overcome as requesting normal tissue to be removed in addition to the plaque region would not have been approved by the

ethics committee and it is therefore very difficult to see how this problem could be eliminated.

Cultured endothelial cells were used in this thesis as a model system for vasculature lining. The development of endothelial cell culture has contributed greatly to the current status of knowledge on these cells. Cells in culture provide a means of examining cell-cell and cell-molecule interrelationships, during artificially generated circumstances, such as that of oxidative stress

The technique of tissue culture also provides the required amount of experimental material and reduces the need initially for animal experiments. Other advantages are that the technique greatly reduces the cost of research, and in some cases it is the only experimental material practically available.

However, limitations of this approach have to be recognised, with the realisation that the cell culture technique removes endothelial cells from their normal, quiescent *in vivo* state (0.1% replications per day) to an activated phenotype (1% to 10% replications per day). This problem has yet to be overcome and so far, no model for the resting endothelium *in vitro* has been developed. Thus, all experiments carried out and results obtained should be considered with this in mind in addition to the absence of shear forces, and factors which are released into the blood that alter the behaviour of the endothelium in vivo.

Bovine aorta endothelial cells were used in these experiments but to investigate what changes are occurring in human atherosclerosis, human artery endothelial cells should

have been used. Whilst is was possible to obtain small sections of human arteries from surgery (i.e. coronary artery by-pass grafting) bovine aorta endothelial cells are very robust in culture and can undergo about 70 to 100 population doubling times before showing sign of senescence. This is compared with approximately 10 to 15 population doublings for human endothelial cells. So whilst this tissue type was not perfect, it suited the experimental protocol.

7.4.3 Alternative oxidative exposure

Hydrogen peroxide was used as the source of oxidative stress as this had been used previously in the department for similar experiments. Also, it is readily available as well as being a physiological source of reactive oxygen species. However, hydrogen peroxide in itself is not a free radical, but the by product of other ROS such as the superoxide anion. It is produced when superoxide is converted to hydrogen peroxide and oxygen by an enzyme from the dismutase family. The hydrogen peroxide is then free to enter the cell via the cell membrane because it expresses no charge. Therefore these experiments were designed to mimic what occurs following the elimination of the free radical resulting in an accumulation of the by-product, hydrogen peroxide.

Alternative sources of ROS could have been used which would have been more physiological and relevant to atherosclerosis, such as oxidised lipoproteins. However, this would not have been without difficulty because the cell membrane would not assist free access of such a molecule to the genetic material because of the size and solubility.

The results obtained were consistent with the morphological changes reported by others in that the bovine aortic endothelial cells expressed signs of senescent behaviour which was time and dose dependent. As far as the telomere results were concerned, it was not known before hand what would be the outcome of the experiments. Certainly, previous studies have demonstrated that antioxidants activate telomerase and delay endothelial senescence (Vasa et al, 2000). Also, cells with a high antioxidant capacity such as human foreskin fibroblasts express slow telomere shortening and a long replicative lifespan (Lorenz et al, 2001). Age dependent telomere shortening has been demonstrated to be slowed down by the enrichment of intracellular vitamin C via the suppression of oxidative stress (Furomoto et al, 1998).

However, the link between senescent behaviour, altered morphology and telomere shortening following hydrogen peroxide exposure had not been demonstrated.

In this study, a link was seen but the results were not entirely convincing. The technique used to measure the telomeres may not have been sensitive enough to identify the changes brought about by the hydrogen peroxide and population doubling, although a reduction as a function of cell division was observed

The way in which the cells were exposed to oxidative damage may not have been ideal. Recurrent exposure to the hydrogen peroxide instead of the single exposure used in this study would present a far more physiological system, similar to that present in the atherosclerotic plaque. This is because it is a chronic disease and subsequent treatment would provide a better model for what occurs in the vessel *in vivo* at the time of insult.

7.5 Summary

Atherosclerosis does appear to have an affect on the telomeres of the cells involved with the disease process. In addition to this, oxidative stress appears to be involved in the manifestations and progression of the disease.

However, the exact mechanism by which the disease occurs is still not known. It is likely to be multifactorial, involving telomere shortening as a result of increased cell division following inflammation, cell damage and oxidative stress. It is also possible that oxidative stress has a direct affect of the telomere causing DNA damage via structural interactions. Additionally, some people may be genetically predisposed to atherosclerosis due to short telomeres as a result of inheritance of post partum cell division due to a low birth weight.

It is suggestive that one or more of these situations causes the premature senescence observed in endothelial cells, white blood cells and the plaque cells from the affected individuals.

Adelfalk C, Lorenz M, Serra V, von Zglinicki T, Hirsch-Kauffmann M, Schweiger M. Accelerated telomere shortening in Fanconi anemia fibroblasts – a longitudinal study. *FEBS Letters* 2001; **506**: 22-26

Ahmad K, Golic KG. The transmission of fragmented chromosomes in Drosophila melanogaster. *Genetics* 1998; **148**: 775-792

Allshire RC, Dempster M, Hastie ND. Human telomeres contain at least three types of G-rich repeat distributed non-randomly. *Nucleic Acids Research* 1989; 17: 4611-4627

Allshire RC, Gosden JR, Cross SH, Cranston G, Rout D, Sugawara N, Szostak JW, Fantes PA, Hastie ND. Telomeric repeat from T. thermophila cross hybridizes with human telomeres. *Nature* 1988; **332**: 656-659

Allsopp RC, Vaziri H, Patterson C, *et al.* Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci USA* 1992; **89**: 10114-10118

Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of ageing. *Proc Natl Acad of Sci U SA* 1993; **90**: 7915-7922

Barker DJ. Fetal origins of coronary heart disease. *BMJ* 1995; **311**: 171-174

Barker DJ. In utero programming of cardiovascular disease. *Theriogenology* 2000; **5**: 555-574

Benditt EP. Implications of the monoclonal character of human atherosclerotic plaques. *Beitr Pathol* 1976; **158(4)**: 405-416

Bickel C, Rupprecht HJ, Blackenberg S, Espiniola-Klein C, Schlitt A, Rippin G, et al., Relation of markers of inflammation (c-reactive protein, fibrinogen, von Willebrand factor and leucocyte count) and statin therapy to long-term mortality in patients with angiographically proven coronary artery disease. *Am J Cardiol* 2002; **89**: 901-908

Bierman EL. Atherosclerosis and ageing. *Federation Proceedings* 1978; **37**: 2832-2836

Biessmann H, Kasravi B, Jakes K, Bui T, Ikenaga K, Mason JM. The genomic organization of HeT-A retroposons in Drosophila melanogaster. *Chromosoma.* 1993; **102**: 297-305

Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gibson E. Telomeric localization of TRF2, a novel human telobox protein. *Nat Genet* 1997; **17**: 236-239

Blackburn EH, Gall JG. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *Journal of Molecular Biology* 1978; **120**: 33-53

Blackburn EH. The molecular structure of centromeres and telomeres. *Annual Review of Biochemistry* 1984; **53**: 163-94

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S,, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; **279**: 349-352

Boers GH. Carriership for homocystinuria in juvenile vascular disease.] *Haemostasis* 1989; **19 (Suppl 1)**: 29-34

Bourgain FM, Katinka MD. Telomeres inhibit end to end fusion and enhance maintenance of linear DNA molecules injected into the Paramecium primaurelia macronucleus. *Nucleic Acids Research* 1991; **19:** 1541-1547

Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America* 1995; **92**: 9082-9086

Brummendorf TH, Holyoake TL, Rufer N, Barnett MJ, Schulzer M, Eaves CJ, Eaves AC, Lansdorp PM. Prognostic implications of differences in telomere length between normal and malignant cells from patients with chronic myeloid leukemia measured by flow cytometry. *Blood* 2000; **95:** 1883-1890

Burrig KF. The endothelium of advanced atherosclerotic plaques in humans. *Arteriosclerosis & Thrombosis* 1991; **11**: 1678-1689

Burrig KF. The morphology of the carotid artery after uncomplicated endarterectomy. *Journal of Cardiovascular Surgery* 1994; **35**: 413-418

Cacciari E, Salardi S. Clinical and laboratory features of homocystinuria. *Haemostasis* 1989; **19 (Suppl 1)**: 10-13, 1989

Campisi J. Replicative senescence: an old lives' tale? *Cell* 1996; **84**: 497-500

Chang E, Harley CB. Telomere length and replicative aging in human vascular tissues. *Proc Natl Acad Sci USA* 1995; **92**: 11190-11194

Chiu CP, Harley CB. Replicative senescence and cell immortality: the role of telomeres and telomerase. *Proceedings of the Society for Experimental Biology & Medicine* 1997; **214**: 99-106

Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Tempst P, de Lang T.. A human telomeric protein. *Science* 1995; **270**: 1663-1667

Coleman MJ, McHale MT, Arnau J, Watson A, Oliver RP. Cloning and characterisation of telomeric DNA from Cladosporium fulvum. *Gene* 1993; **132**: 67-73

Comi P, Chiaramonte R, Maier JAM. Senescence-dependent regulation of type 1 plasminogen activator inhibitor in human vascular endothelial cells. *Exp Cell Res* 1995; **219**: 304-308

Consentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M, Luscher, TF. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydro-biopterin-deficient mice. *Arteriosclerosis, Thrombosis and Vascular Biology* 2001; **21(4)**: 496-502

Cooke JP, Dzau VJ. Derangements of the nitric oxide synthase pathway, L-arginine, and cardiovascular diseases. *Circulation* 1997; **96**: 379-382

Cooper JP, Watanabe Y, Nurse P. Fission yeast Taz1 protein is required for meiotic telomere clustering and recombination. *Nature* 1998; **392**: 828-31

Counter CM, Hirte HW, Bacchetti S, Harley C. Telomerase activity in human ovarian carcinoma. *Proc Natl Acad Sci USA* 1994; **91**: 29900-2904

Cryer A. Biochemical interactions at the endothelium. *Elsevier* 1983

Davi G, Ciabattoni G, Consoli A et al. In vivo formation of 8-iso-Prostaglandin F_2 alpha and platelet activation in diabetes mellitus: Effects of improved metabolic control and vitamin E supplementation. *Circulation* 1999; **99**: 224-229

Davies MJ, Woolf N, Rowles PM, Pepper J. Morphology of the endothelium over atherosclerotic plaques in human coronary arteries. *Br Heart J* 1988; **60**: 459-464

de Bono DP, Yang WD. Exposure to low concentrations of hydrogen peroxide causes

de Bono DP, Yang WD. Exposure to low concentrations of hydrogen peroxide causes delayed endothelial cell death and inhibits proliferation of surviving cells. *Atherosclerosis* 1995; **114**: 235-245

de la Sena C, Chowdhary BP, Gustavsson I. Localization of the telomeric (TTAGGG)n sequences in chromosomes of some domestic animals by fluorescence in situ hybridization. *Hereditas* 1995; **123**: 269-274

de Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM, Varmus HE. Structure and variability of human chromosome ends. *Mol Cell Biol* 1990; **10**: 518-527
delayed endothelial cell death and inhibits proliferation of surviving cells. *Arteriosclerosis* 1995; **114**: 235-45

Eriksson JG, Forsen T, Tuomilehto J, Winter PD, Osmond C, Barker DJP. Catch-up growth in childhood and death from coronary artery disease: a longitudinal study. *BMJ* 1999; **318**: 7-11

Fennel JP, Brosnan MJ, Frater AJ, Hamilton CA, Alexander MY, Nicklin SA, Heistad DD, Baker AH, Dominiczak AF. Adenovirus-mediated over expression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension. *Gene Ther* 2002; **9**: 110-117

Fishman AP. Endothelium: a disturbed organ of diverse capabilities. *Ann N Y Acad Sci* 1982; **401**: 1-8

Folkman J, Moscona A. Role of cell shape in growth control. *Nature* 1978; **273:** 345-349

Forney JD, Blackburn EH. Developmentally controlled telomere addition in wild-type and mutant paramecia. *Molecular & Cellular Biology 1988;* **8**: 251-258

Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sciences* 1998; **63**: 935-948

Gadjusek CM, Schwartz SM. Technique for cloning bovine aortic endothelial cells. *In Vitro* 1983; **19**: 394-402

Gimbrone MAJ, Cotran RS, Folkman J. Human vascular endothelial cells in culture. Growth and DNA synthesis. *J Cell Biol* 1974; **60**: 673-684

Glurich I, Grossi S, Albini B, Ho A, Shah R, Zeid M, Baumann H, Genco RJ, De Nardin E. Systemic inflammation in cardiovascular and periodontal disease: comparative study. *Clinical & Diagnostic Laboratory Immunology* 2002; **9**: 425-432

Gonos ES, Derventzi A, Kveiborg M, et al. Cloning and identification of genes that associate with mammalian replicative senescence. *Exp Cell Res* 1998; **240**: 66-74

Goodall AH, Jarvis J, Chand S, Rawlings E, O'Brien DP, McGraw A, Hutton R, Tuddenham EGD. An immunoradiometric assay for human factor VIII/von Willebrand factor (VIII:vWF) using a monoclonal antibody that defines a functional epitope. *British Journal of Haematology* 1985; **59**: 565-577

Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 1995; **43**: 405-413

Griendling KK, Ushio-Fukai M. Redox control of vascular smooth muscle proliferation. *Journal of Laboratory & Clinical Medicine* 1998; **132**: 9-15

Harlan JM, Vedder NB, Winn RK, Rice CL. Mechanisms and consequences of leucocyte-endothelial interaction. *Western Journal of Medicine* 1991; **155**: 365-369

Harker LA, Ross R, Slichter SJ, Scott CR. Homocysteine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. *J Clin Invest* 1976 **Sep;58(3)**: 731-741

Harley CB, Futcher AB, Greider CW. Telomeres shorten during aging of human fibroblasts. *Nature* 1990; **345**: 458-460

Harrison D. Oxidative stress and coronary artery disease. *Can J Cardiol* 1998; **14(Suppl D)**: 30D-32D

Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. Telomere reduction in human colo-rectal carcinoma and with aging. *Nature* 1990; **346**: 866-868

Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp. Cell Res* 1961; **253**: 585-621

Henderson ER, Blackburn EH. An overhanging 3' terminus is a conserved feature of telomeres. *Molecular & Cellular Biology* 1989; **9**: 345-348

Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, Shay JW. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nature Medicine* 1995; **1**: 249-55

Hoeschen RJ. Oxidative stress and cardiovascular disease. *Can J Cardiol* 1997; **13**: 1021-1025

Hultdin M, Gronlund E, Norrback K, Eriksson-Lindstrom E, Just T, Roos G. Telomere analysis by fluorescence in situ hybridization and flow cytometry. *Nucleic Acids Research* 1998; **26**: 3651-3656

Irani K. Oxidant signaling in vascular cell growth, death and survival: a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ Res* 2000; **87**: 179-183

Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; **52**: 2745-2756

Jeanclos E, Krolewski A, Skurnick J, Kimura M, Aviv H, Warram JH, Aviv A. Shortened telomere length in white blood cells of patients with IDDM. *Diabetes* 1998; **47**: 482-486

Karlseder J, Broccoli D, Dai Y et al. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science.* 1999; **283**: 1321-1325

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; **266**: 2011-2015

Kipling D, Cooke HJ. Hypervariable ultra-long telomeres in mice. *Nature* 1990; **347**: 400-402

Kirk KE, Harmon BP, Reichardt IK, Sedat JW, Blackburn EH. Block in anaphase chromosome separation caused by a telomerase template mutation. *Science* 1997; **275**: 1478-1481

Klobutcher LA, Swanton MT, Donini P, Prescott DM. All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus. *Proc Natl Acad Sci USA* 1981; **78**: 3015-3019

Knight-Lozano CA, Young CG, Burow DL, Hu ZY, Uyeminami D, Pinkerton KE, Ischiropoulos H, Ballinger SW. Cigarette smoke exposure and hypercholesterolaemia increase mitochondrial damage in cardiovascular tissues. *Circulation* 2002; **105**: 849-854

Leber B, Bachetti S. Telomeres and telomerase in normal and malignant haematologic cells. *Leukaemia & Lymphoma* 1996; **24**: 1-9

Lentz SR, Sobey CG Piegors DJ, Bhopatkar MY, Earaci FM, Malinow MR, Heistad DD. Vascular dysfunction in monkeys with diet-induced hyperhomocysteinaemia. *J Clin Invest.* 1996; **98**: 24-29

Lingner J, Cech TR. Purification of telomerase from Euplotes aediculatus: requirement of a primer 3' overhang. *Proceedings of the National Academy of Sciences of the United States of America* 1996; **93**: 10712-10717

Lorez M, Sarrtzki G, Sitte N, Metzkow S, von Zglinicki T. BJ fibroblasts display high antioxidant capacity and slow telomere shortening independent of hTERT transfection. *Free Radical Biology & Medicine* 2001; **31**: 824-831

Luke S, Verma RS. Telomeric repeat [TTAGGG]n sequences of human chromosomes are conserved in chimpanzee (Pan troglodytes). *Mol Gen Genet* 1993; **237**: 460-462

Maier JA, Barenghi L, Bradamante S, Pagini F. Induction of human endothelial cell growth by mildly oxidised low denisity lipoprotein. *Atherosclerosis* 1996; **123**: 115-121

Makarov V, Hirose Y, Langmore JP. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell* 1997; **88**: 657-666

Manuelidis L, Borden J. Reproducible compartmentalization of individual chromosome domains in human CNS cells revealed by in situ hybridization and three-dimensional reconstruction. *Chromosoma* 1988; **96**: 397-410

Matsushita H, Chang E, Glassford AJ, Cooke JP, Chiu CP, Tsao PS. eNOS activity is reduced in senescent human endothelial cells: Preservation by hTERT immortalization. *Circulation Research* 2001; **89**: 793-798

McClintock B. The stability of broken ends of chromosomes in zea mays. *Genetics* 1941; **26**: 234-282

McGill HC, McMahan CA, Malcolm GT, Oalmann MC, Strong JP. Effects of serum lipoproteins and smoking on atherosclerosis in young men and women. *Arterioscler Thromb Biol* 1997; **17**: 95-106

Metcalfe JA, Parkhill J, Campbell L, Stacey M, Biggs P, Byrd PJ, Taylor AM. Accelerated telomere shortening in ataxia telangiectasia. *Nature Genetics* 1996; **13**: 350-353

Meyne J, Ratliff RL, Moyzis RK. Conservation of the human telomere sequence (TTAGGG)n among vertebrates. *Proceedings of the National Academy of Sciences of the United States of America* 1989; **86**: 7049-7053

Minamino T, Komuro I. Role of telomere in endothelial dysfunction in atherosclerosis. *Current Opinion in Lipidology* 2002; **13**: 537-543

Moss NS, Benditt EP. Human atherosclerotic plaque cells and leiomyoma cells. Comparison of *in vitro* growth characteristics. *Am J Patho*l 1975; **78**: 175-190

Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J. Ratliff RL, Wu JR. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* 1988; **85**: 6622-6626

Muller H. The remaking of chromosomes. *The Collecting Net – Woods Hole* 1938; **13**: 181-195

Munro JM, Cotran RS. The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Laboratory Investigation* 1988; **58**: 249-261

Nourooz-Zadeh J, Rahimi A, Tajaddini-Sarmadi J, Tritscler H, Rosen P, Halliwell B, Betteridge DJ. Relationship between plasma measures of oxidative stress and metabolic control in NIDDM. *Diabetologica* 1997; **40**: 647-653

Okuda M, Inoue N, Azumi H, Seno T, Sumi Y, Hirata Ki, Kawashima S, Hayashi Y. Itoh H, Yodoi J, Yokoyama M. Expression of glutaredoxin in human coronary arteries: its potential role in antioxidant protection against atherosclerosis. *Arteriosclerosis, Thrombosis & Vascular Biology* 2001; **21**: 1483-1487

Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *Journal of Theoretical Biology* 1973; **41**: 181-90

Olovnikov AM. Principle of marginotomy in template synthesis of polynucleotides *Doklady Akademii Nauk SSSR* 1971; **201**: 1496-1499

Olovnikov AM. The immune response and the process of marginotomy in lymphoid cells. *Vestnik Akademii Meditsinskikh Nauk SSSR* 1972; **27**: 85-87

Patel KD, Zimmerman GA, Prescott SM, McEver RP, McIntyre TM. Oxygen radicals induce human endothelial cells to express GMP-40 and bind neutrophils. *J Cell Biol.* 1991; **112**: 749-759

Patel KD, Zimmerman GA, Prescott SM, McIntyre TM. Novel leukocyte agonists are released by endothelial cells exposed to peroxide. *J Biol Chem.* 1992; **267**: 15168-15175

Petersen S, Saretzki G, von ZT. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res* 1998; **239**: 152-160

Prescott DM. The DNA of ciliated protozoa. *Microbiological Reviews* 1994; **58**: 233-67

Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM. Platelet-activating factor and related lipid mediators. *Annu Rev Biochem.* 2002; **69**: 419-445

Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997; **336**: 973-979

Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentrations of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* 2000; **101**: 1767-1772

Ross R, Glomset JA. The pathogenesis of atherosclerosis (second of two parts). *New England Journal of Medicine* 1976; **295**: 420-425

Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; **362**: 801-809

Rufer N, Dragowska W, Thornbury G, Roosnek E, Lansdorp PM. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat Biotechnol* 1998; **16**: 743-747

Sandell L. Zakian V. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell* 1993; **75**: 729-741

Schwartz SM, Selden SC, Bowman P. Growth control in aortic endothelium at wound edges. Cold Spring Harbor 3rd Conference on Cell Proliferation In Hormones and Cell Culture. Ross R, Sato G (Eds) *Cold Spring Harbor NY* 1979; **6**: 593

Schwartz SM. Selection and characterization of bovine aortic endothelial cells. *In Vitro* 1978; **14**: 966-980

Shampay J, Szostak JW, Blackburn EH. DNA sequences of telomeres maintained in yeast. *Nature* 1984; **310**: 154-157

Shanahan CM, Cary NRB, Metcalfe JC, Weissberg PL. High expression of genes for calcification-regulation proteins in human atherosclerotic plaques. *J Clin Invest* 1994; **93**: 2393-2402

Shay JW, Wright WE. The reactivation of telomerase activity in cancer progression. *Trends in Genetics* 1996; **12**: 129-131

Sherer Y, Shoenfeld Y. Atherosclerosis. *Ann Rheum Dis* 2002; **61**: 97-99

Simionescu M, Simionescu N. Isolation and characterization of endothelial cells from the heart microvasculature. *Microvascular Research* 1978; **16**: 426-452

Slagboom PE, Droog S, Boomsma DI. Genetic determination of telomere size in humans: a twin study of three age groups. *AM J Hum Genet* 1994; **55**: 876-882

Slagboom PE, Vijg J. The dynamics of genome organization and expression during the aging process. *Annals of the New York Academy of Sciences* 1992; **673**: 58-69

Smiley PL, Stremler KE, Prescott SM, Zimmerman GA, McIntyre TM. Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. *J Biol Chem.* 1991; **266**: 11104-11110

Stary HC. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 1989: **9(Suppl I)**: I19-32I

Tokunaga O, Jianglin F, Watanabe T. Atherosclerosis and age-related multinucleated variant endothelial cells in primary culture from human aorta. *Am J Pathol* 1989; **135**: 967-976

van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature* 1997; **385**: 740-743

Vasa M, Breitschopf K, Zeiher AM, Dimmeler S. Nitric oxide activates telomerase and delays endothelial cell senescence. *Circulation Research* 2000; **87**:540-542

Vaziri H, Dragowska W, Allsopp R, Thomas T, Harley CB, Landsorp PM. Evidence for a mitotic clock in human haematopoietic stem cells: loss of telomeric DNA with age. *Proc Natl Acad Sci USA* 1994; **91**: 9857-9860

Vaziri H, Schachter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet* 1993; **52**: 661-667

Vlodavsky I, Johnson LK, Gospodarowick D. Appearance in confluent cell surface protein (CSP-60) not detected in actively growing endothelial cells or in cell types growing in multiple layers. *Proc Natl Acad Sci USA* 1979; **76**: 2306

von Zglinicki T. Role of oxidative stress in telomere regulation and replicative senescence. *Ann N Y Acad Sci* 2000; **908**: 99-110

Vourc'h C, Taruscio D, Boyle AL, Ward DC. Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. *Experimental Cell Research* 1993; **205**: 142-151

Wagner DD, Olmstead JB, Marder VJ. Immunolocalisation of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J Cell Biol* 1982; **95**: 355

Wall RT, Harlan JM, Harker LA, Striker GE. Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thrombosis Research* 1980; **18**: 113-121

Weber S, Schmid M, Meyer J, Cooke HJ, Lipps HJ. A linear vector carrying human telomeres is replicated in unfertilized eggs of Xenopus laevis. *Cell Biology International* 1993; **17**: 623-624

Weibel P, Palade GE. New cytoplasmic components in arterial endothelia. *J Cell Biol* 1964; **23**: 101-112

Whittenberger B, Glaser L. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. *Proc Natl Acad Sci USA* 1977; **74**: 225

Woo KS, Chook P, Lolin YI, Cheung ASP Chan LT, Sun YY, Sanderson JE, Metreweli C, Celermajer DS. Hyperhomocysteinaemia is a risk factor for arterial endothelial dysfunction in humans. *Circulation* 1997; **96**: 2542-2544

Wright WE, Piatyszek MA, Rainey WE Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. *Developmental Genetics* 1996; **18**: 173-179

Wynn RF, Cross MA, Hatton C, Will AM, Lashford LS, Dexter TM, Testa NG. Accelerated telomere shortening in young recipients of allogenic bone-marrow transplants. *Lancet* 1998; **351**: 178-181

Xu D, Neville R, Finkel T. Homocysteine accelerates endothelial cells senescence. *FEBS Letters* 2000; **470**: 20-24

Yang SS, Yeh E, Salmon ED, Bloom K. Identification of a mid-anaphase checkpoint in budding yeast. *Journal of Cell Biology* 1997; **136**: 345-354

Yang W, de Bono D. A new role for vascular endothelial growth factor and fibroblast growth factors: increasing endothelial resistance to oxidative stress. *FEBS Letters* 1997; **403**: 139-142

Youngren K, Jeanclos E, Aviv H, Kimura M, Stock J, Hanna M, Skurnick J, Bardeguez A, Aviv A. Synchrony in telomere length of the human fetus. *Human Genetics* 1998; **102**: 640-643

Zakian VA, Pluta AF. Telomere formation in yeast. *Nature* 1989; **338**: 468

Zakian VA. Life and cancer without telomerase *Cell* 1997; **91**: 1-3

Zakian VA. Telomeres: Beginning to understand the end. *Science* 1995; **270**: 1601-1607

Alexis Biochemicals
PO Box 6757 Bingham
Nottingham
NG13 8LS
UK

Amersham Biosciences UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA

Dako Ltd
Denmark House
Angel Drove
Ely
Cambridgeshire
CB7 4ET
UK

Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leicestershire
LE11 5RG
United Kingdom

Gentra Systems Inc
13355 10th Avenue N
Suite 120
Minneapolis
MN55 441
USA

ICN Pharmaceuticals Ltd
Cedarwood
Chineham Business Park
Crockford Lane
Basingstoke
Hants
RG24 8WD

Nunc
Unit 1a
Thorn Business Park
Hereford
HR2 6JT
UK

Promega UK Ltd
Delta House
Chilworth Research Centre
Southampton
SO16 7NS
UK

Sarstedt Ltd

Boston Road

Beaumont Leys

Leicester

LE4 1AW

Sigma-Aldrich Company Ltd

Fancy Road

Poole

Dorset

BH12 4QH

England