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Estrogen Receptor β Expression in Human Vascular Tissues

Submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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June 1999

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Abstract

Estrogens exhibit potent anti-atherogenic effects through mechanisms that may involve direct effects on the artery wall. The existence of the classical estrogen receptor (ERα) in vascular tissues has been established. Recently a new estrogen receptor (ERβ) has been discovered that represents a distinct gene product with homology to the classical ERα.

The purpose of this thesis was to explore the expression pattern of ERβ, in normal and diseased arteries. My experimental data demonstrate that ERβ mRNA is also expressed in human vascular tissue. Using semi-quantitative RT-PCR, there was no relative difference in ERβ mRNA expression in paired human vascular tissue from normal and diseased arteries. Southern blot and Northern blot analyses, as well as sequencing, were used to confirm these results. We also studied ERβ protein expression in the normal and diseased coronary arteries using immunohistochemisty. The results further confirm that ERβ protein is also expressed in both normal and diseased coronary arteries.

TABLE OF CONTENTS

Abstract.......... i

Table of Contents.......... ii

List of Tables and Figures.......... v

Dedication.......... vii

Acknowledgements.......... viii

Abbreviations.......... ix

1. Introduction

1.1 Overview of coronary heart disease.......... 1

1.2 Potential cardiovascular protective effects of estrogens.......... 3

1.3 Mechanisms of cardiovascular protective effects.......... 4

1.4 The estrogen receptor (ERα).......... 5

1.5 Estrogen receptor signaling.......... 7

1.6 The discovery of ERβ.......... 9

1.7 Estrogen receptors α and β: homology, differential ligand activation and tissue distribution.......... 11

1.8 Objectives of the current study 15

2. Materials and Methods

2.1 Reverse transcription and polymerase chain reaction.......... 16

2.1.1 Cell culture.. 16

2.1.2 Tissues collection.. 16

2.1.3 RNA isolation.. 17

2.1.4 Reverse transcription... 18

2.1.5 Primer design... 19

2.1.6 Polymerase Chain Reaction................................... 21

2.2 Southern Blot Analysis

2.2.1 Preparation of the agarose gel and PCR Sample............ 21

2.2.2 Blotting.. 22

2.2.3 Prehybridization... 22

2.2.4 Preparation of the probe... 23

2.2.5 Probe Labeling 24

2.2.6 Hybridization.. 25

2.2.7 Washes.. 25

2.3 Sequencing of PCR fragments.................................. 26

2.4 Northern Blot Analysis... 26

2.4.1 Agarose/Formaldehyde gel Electrophoresis.................. 26

2.4.2 Northern Blot analysis... 26

2.5 Immunohistochemistry.. 27

2.6 Statistical analysis.. 29

3 RESULTS

3.1 Northern Blot to detect estrogen receptor β..................... 31

3.2 RT-PCR.. 30

3.2.1 Primer design.. 31

3.2.2 Optimizing the PCR annealing temperature.................. 32

3.2.3 The proper template concentration............................ 40

3.2.4 Optimizing the number of PCR cycles........................ 41

3.3 Sequencing and Southern Blot Analysis.......................... 43

3.4 Immunohistochemistry.. 76

4. Discussion

4.1 General statement.. 78

4.2 ERβ mRNA expression in human cardiovascular tissues..................................... 79

4.3 Human ERβ protein expression............................ 82

4.4 Conclusions and future perspectives....................... 83

5. References

5. References.. 86

List of Tables and Figures

Table 1 Primers used for PCR together with expected size of amplicons 30

Table 2 ERβ over GAPDH ratio for RT-PCR reaction 53

Table 3 Mean and Standard error of ERβ over GAPDH ratio 55

Table 4 Results of immunostaining for ERβ in human arteries 73

Figure 1-1 Schematic diagram of human estrogen receptor cDNA and protein structure 6

Figure 1-2 Examples for cellular mechanisms for hormonal stimulation 8

Figure 1-3 Alternative estrogen signaling pathway 10

Figure 1-4 Comparison of the structures between ERα and ERβ 13

Figure 2-1 Primer's for ERs and GAPDH 20

Figure 3-1 Northern blot analysis for ERβ 35

Figure 3-2 Comparison of set 1 and set 2 primers specific for ERβ 37

Figure 3-3 Optimization of RT-PCR annealing temperature 39

Figure 3-4 Determination of the optimal quantity of starting RNA for RT-PCR 46

Figure 3-5 Determination of the proper number of PCR cycles for ERβ 48

Figure 3-6 Determination of the proper number of PCR cycles for GAPDH........ 50

Figure 3-7 Semi-quantification of RT-PCR results using GAPDH...... 52

Figure 3-8 The ratios of ERβ to GAPDH after RT-PCR........ 54

Figure 3-9 Comparison of normal and diseased arteries........ 56

Figure 3-10 RT-PCR products on 2% low melting agarose gel........ 58

Figure 3-11 ^{32}P labeled ERβ probe........ 60

Figure 3-12 Southern blot analysis for human ERβ........ 62

Figure 3-13 Sequence of RT-PCR fragment........ 64

Figure 3-14 Sequence alignment........ 66

Figure 3-15 Breast tumor was used as a positive control tissue ERβ expression........ 68

Figure 3-14 Immunolabeling of human internal mammary artery and normal Coronary artery with anti-ERβ polyclonal serum P3........ 70

Figure 3-15 Immunolabeling of human diseased coronary artery with Anti-ERβ polyclonal serum P3........ 72

Figure 3-16 Statistical results of immunostaining for ERβ in human arteries........ 75

Dedications

I would like to dedicate this thesis to my mother Xiuzhong, Wang for her love and support.

Acknowledgements

I would like to take this opportunity to thank my supervisor, Dr.O'Brien who provided an opportunity for me to work in his lab. Special thanks are due to the member of my advisory committee, Dr Ross Milne his advice and support, also to Harvey Miller, our laboratory technician for sharing his technical expertise.

I am grateful for the support and help from my family, especially my mother. I would not have finished my degree without her encouragement and help. I also thank my friend Heidi Bennet for her unfailing support when things got tough. Most of all, however, I thank my husband and my son for their patience and understanding during the long weeks and months that they hardly ever saw me.

List of Abbreviations and symbols

AF-1	transcription activation functions 1
AF-2	transcription activation functions 2
BSA	bovine serum albumin
CPM	counts per minute
DBD	DNA binding domain
DD	dimerization domain
ER	estrogen receptor
ERE	estrogen response element
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBD	hormone binding domain
hr	hour(s)
kb	kilobase
mg	milligram(s)
M	molar
min	minute(s)
ml	milliliter(s)

mRNA	messenger ribonucleic acid
ng	nanogram(s)
NLS	nuclear localization signal
nt	nucleotide(s)
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
TBE	Tris-borate-EDTA buffer
T	thymidine
U	units
µg	microgram

1 Introduction

1.1 Overview of coronary heart disease

The clinical manifestations of coronary heart disease (CAD), also known as ischemic heart disease, are diverse, with a spectrum that encompasses various forms of angina pectoris, myocardial infarction, sudden cardiac death, and chronic coronary heart disease. These syndromes result from complex interactions between the coronary circulation and the myocardium, usually with coronary atherosclerosis as a major factor. The major cause of CAD is coronary atherosclerosis, a process that develops as a response of the vessel wall to chronic, multifactorial injury and leads to the formation of atherosclerotic plaques. These plaques are regions of thickened intima that are composed of various mixtures of fibrous tissues, cells, and lipids.

The normal blood vessel wall is composed of three layers. The innermost layer, the intima, is made up of a single layer of endothelial cells. The middle layer, the media, is composed of layers of smooth muscle cells. The outermost layer, the adventitia, is composed of fibroblasts, collagen fibers and other components of loose connective tissues. Atherosclerotic disease leads to extensive remodeling of the vessel wall. Dilation of the vessel may occur, in such a way that the lumen is maintained despite the presence of

intimal plaque, and is referred to as arterial remodeling. Lumen narrowing occurs only when atherosclerotic disease is advanced. Approximately 70% narrowing of luminal diameter is needed before blood flow is affected.

Atherosclerosis is a chronic inflammatory condition of the vascular wall that is converted to an acute clinical event by the induction of plaque rupture, which in turn leads to thrombosis (Fuster et al, 1992). The lesions of atherosclerosis have been divided into three general categories: (a) the fatty streak, (b) the intermediate or fibrofatty lesion, and (c) the fibrous plaque or advanced complicated lesions (Ross R, 1995). The fatty streak is the earliest visible lesion and although not clinically significant, is most likely the precursor to the latter events that lead to clinically significant disease. The fatty streak consists primarily of an intimal collection of lipid-filled, monocyte-derived macrophages with a varying number of T-lymphocytes (Faggiotto et al, 1984) and smooth muscle cells. The accumulation of lipid-filled macrophages, or foam cells, represents the bulk of the lesion. These macrophages give the lesion a yellow discoloration when viewed en face, and hence it is called the fatty streak. Normally, fatty streaks are distributed randomly throughout the artery tree, but early lesions are commonly found at branches, bifurcation, and curves in the system, which are sites of low shear stress where changes in blood flow such as decrease of flow occurs. A hallmark of the conversion of fatty streaks to more advanced atherosclerotic lesions is the formation of a fibrous cap (Stary et al, 1994). This process involves an increase in the number of smooth muscle cells within the intima and probably requires both migration and proliferation as well as stimulation of connective tissue synthesis. The hallmark of an advanced atherosclerotic lesion is the formation of a

necrotic core. This necrotic core is an acellular region consisting of lipid, necrotic cell debris and in some case calcium-phosphate deposits that are centrally located at the base of plaque. Aortic calcification is often accompanied by the presence of cartilaginous metaplasia, and this may be generally controlled (Qiao et al, 1995). It has been reported that arterial calcification is a regulated process similar to bone formation, possibly mediated by pericyte like cells (Bostrom et al, 1993). Indeed, a subpopulation of calcification arterial wall cells has been identified (Balica et al, 1997). Interestingly, in vitro estrogen promotes the formation of calcific nodules by these cells.

1.2 Potential cardiovascular protective effects of estrogens

Coronary artery disease is a leading cause of death in North America. The gender difference in the incidence of morbidity and mortality from coronary heart disease is well known. Estrogens are steroid hormones that have profound effects on both the female and male reproductive systems, such as the mammary gland, uterus, ovary, testis, and prostate (Clark et al.1992). Estrogens are mainly produced in the ovaries and testis. Estrogens also play an important role in bone maintenance and in the cardiovascular system, where estrogens have certain cardioprotective effects (Farhat et al, 1996). The role of estrogens is being explored in large-scale epidemiological studies that show the incidence of coronary heart disease to be relatively low among premenopausal women, with a sharp rise after menopause (Barret-Connor and Bush, 1991) and (Hong et al, 1992). There are several lines of evidence suggest that the lack of estrogens in the circulation may enhance

arterial lesion formation. The beneficial effect of estrogen replacement therapy in postmenopausal women (Stampfer et al, 1989 and 1991) and (Grady et al, 1992) further supports the role for estrogens in protecting against the development of coronary artery disease. However, data from the recent HERS study suggest that the efficacy of hormone replacement therapy in women with established cardiovascular disease may not be as convincing as it plays in people who don't have cardiovascular disease (Hulley et al, 1998).

1.3 Mechanisms of cardiovascular protective effects

While it has been realized that estrogens have cardiovascular protective effects since the 1960's (Korach KS, 1994), the specific effects of estrogens on the arterial wall are not completely understood. A number of mechanisms have been postulated (Jensen et al, 1987) and (Subbiah MT, 1998). Estrogens have been shown to improve patient's lipoprotein profiles by raising the level of cardiovascular protective high density lipoproteins (HDL), and by decreasing plasma levels of risk factors for atherosclerosis such as low density lipoproteins (LDL), total cholesterol and lipoprotein Lp (a) (Bush et al, 1987). Estrogens have also been shown to prevent LDL from oxidative modification, which is thought to initiate the atherogenesis (Huber et al, 1990). However, the beneficial effects of estrogens on the blood lipid profile account for only 20-30% of the overall protection (Mikkola et al, 1998). Currently, there is increasing evidence that estrogens have beneficial effects through a direct action on the blood vessel wall itself (Mendelsohn

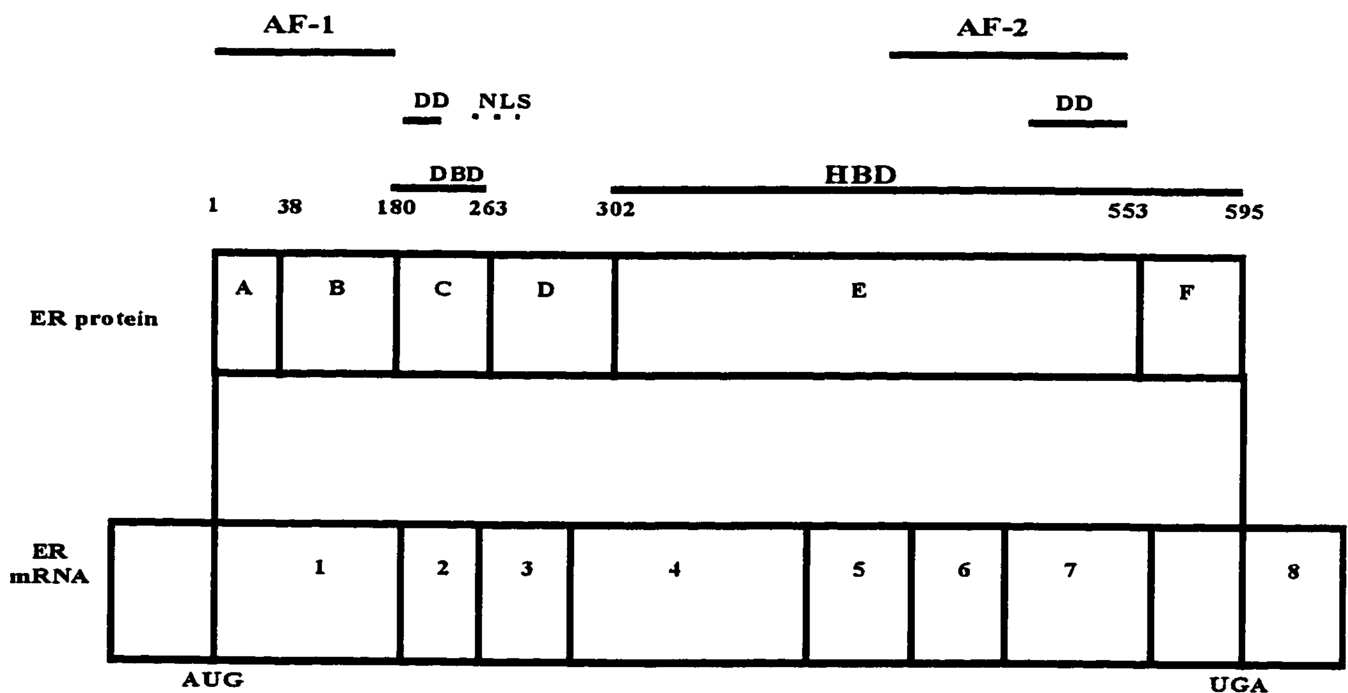
et al, 1994). The proliferation and migration of smooth muscle cells play a critical role in the pathogenesis of coronary artery disease. Some data show that estrogens can inhibit smooth muscle cell proliferation and migration in vitro and in vivo (Vargas R et al, 1993). Furthermore, the vascular wall contains specific high-affinity receptors for estrogens both in endothelial (Colburn et al, 1978) and smooth muscle cells (Karas et al, 1994).

Estrogen administration promotes vasodilation both in human and experimental animals, in part by stimulating prostacyclin (Corvazier et al, 1984) and nitric oxide synthesis (Moncada et al, 1991). Both prostaglandin synthase and the constitutive nitric oxide synthase are induced by estrogen treatment (Luscher et al, 1994) and ((Mikkolat et al, 1998). In addition, estrogen exerts a direct inhibitory effect on the smooth muscle by inhibiting calcium influx (Hughes et al, 1995). Recently, whole-cell patch-clamping techniques have been used to demonstrate an inhibitory effect of estradiol on voltage-dependent calcium currents in a cultured vascular smooth muscle cell line (Zhang et al, 1994). While all of these data support the concept that estrogens are cardioprotective, more information about estrogen receptors (ERs) in the vessel wall is needed (Baysal and Losordo, 1996).

1.4 Estrogen Receptor (ER)

The ER belongs to the nuclear receptor superfamily of structurally similar transcription factors that includes 30 receptors, among them steroid hormone and thyroid hormone receptors, as well as vitamin D and retinoic acid receptors, and also a number of

"orphan receptors" whose activating ligands have yet to be identified. Like other nuclear receptors, the ER can be subdivided into several functional domains by biochemical and mutational analysis. The N-terminal A/B domain is highly variable in sequence and length, and usually contains a transactivation function (AF-1), which activates target genes by interacting with components of the core transcriptional machinery. The C domain contains two type II zinc fingers, which are involved in specific DNA-binding and receptor dimerization. The E region of the ER is relatively large and complex. It includes the ligand-binding domain and another activation function (AF-2). In addition it harbours regions involved in receptor dimerization, and nuclear localization. (Figure 1-1).



(Chan et al, 1997)

Figure 1-1: Schematic diagram of the human ER mRNA and protein

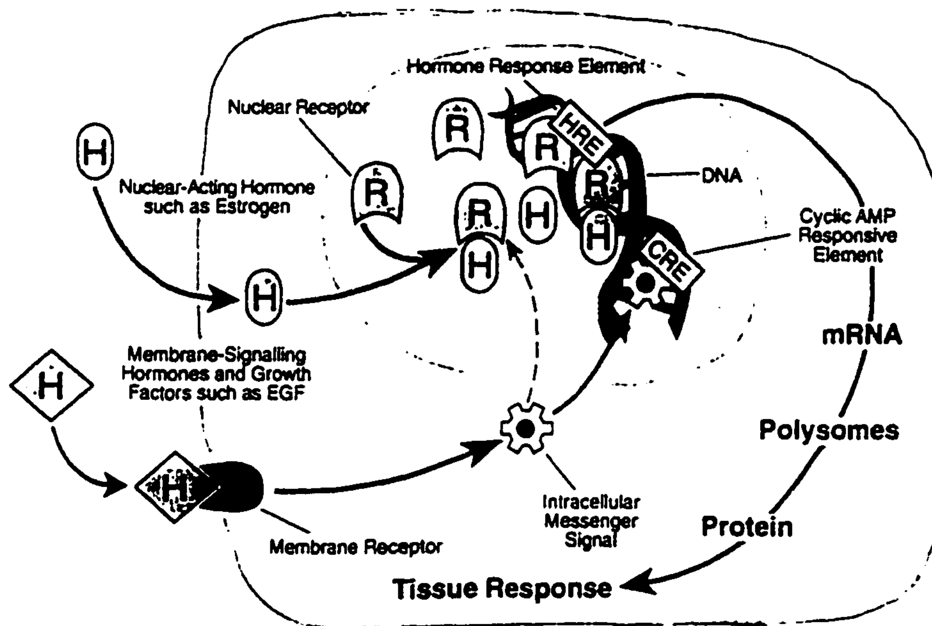
ER contains 8 different exons coding for the protein, and is divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in the trans-activating function 1 (AF-1). The DNA binding domain is located in the C region. Region E is implicated in the hormone binding and another activation function 2 (AF-2). DD, dimerization domain; NLS, nuclear localization signals.

1.5 Estrogen receptor signaling

Estrogens trigger a broad array of physiological responses which are tissue- and organ-specific by binding to a nuclear receptor within target cells. These responses include tissue differentiation, growth, protein synthesis, and secretion (Korach et al, 1981). ER is a ligand-inducible transcription factor that modulates target gene expression after binding hormone.

ER is associated with heat shock protein in the absence of estrogen. Once bound to ligand, ER undergoes a conformational change, which causes the dissociation of heat shock protein complexes and homodimerization of the receptor. The estrogen-bound, dimeric receptor complex exhibits a high affinity for a specific palindromic DNA sequence with the estrogen response element AGGTCAnnnTGACCT (ERE), that is involved in transactivation of transcription of specific target genes. Some specific growth

factors, such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), can also mimic estrogens by affecting nuclear estrogen receptor protein properties and stimulating biological responses (Ignar-Trowbridge et al. 1993). Mechanistically, growth factor activation of the ER appears to operate by coupling the ER to multiple signaling pathways that converge in a tissue specific response.



(Korach KS. 1994)

Figure 1-2 Examples of cellular mechanisms for hormonal stimulation

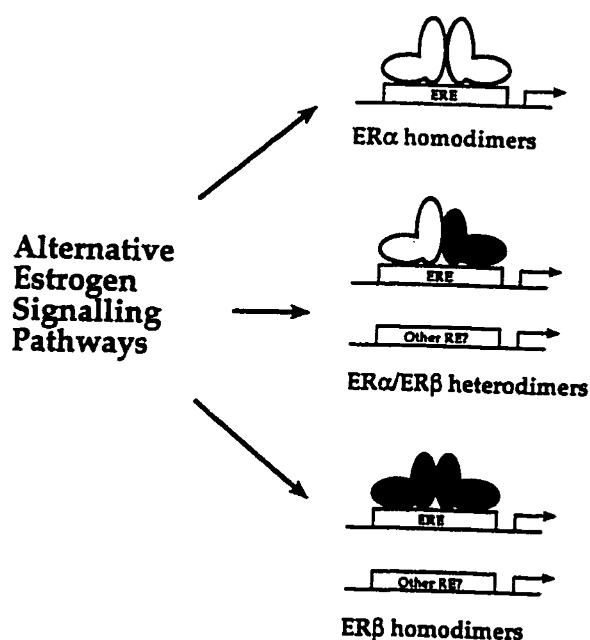
Steroid hormones diffuse into cells where they interact with nuclear receptor proteins that function as ligand-activated transcription factors. These receptor ligand complex dimerize and bind to specific DNA sequences (HRE) upstream of genes regulated by the hormone

Regulation results in an increase in transcription of specific genes that influence responses within target cells. Protein hormones and growth factors are examples of stimulants which interact with membrane receptors eliciting a cellular response mediated by an intracellular second messenger signaling pathway.

1.6 The discovery of a second type of ER

The ER-encoding cDNAs were cloned during 1986 from several species (Green et al, 1986) and (Greene et al, 1986). Since then it had generally been accepted that only one ER gene exists. However, in late 1995 a novel estrogen receptor was cloned from a rat prostate cDNA library (Kuiper et al, 1996). This novel ER protein was named ERβ to distinguish it from the classic ER, which has been renamed ERα. Recently, the mouse (Tremblay et al, 1997) and human (Mosselman et al, 1996) ERβ have also been cloned. The genes for the ERα and ERβ are located on different chromosomes. It has been known that human ERα was located in chromosome 6q (Magdelenat et al, 1994). Using PCR technique, the human ERβ gene was localized to chromosome 14, and using the FISH technique, ERβ was more precisely mapped to 14q22-24 (Enmark et al, 1997). The recent discovery of an additional estrogen receptor subtype ERβ has advanced significantly our understanding of the mechanisms underlying alternative estrogen signaling pathways (Giguere et al, 1998). In cells expressing only the ERα or ERβ subtype, homodimers of either subtype can interact with response elements in target genes to promote and influence transcription levels. The existence of two ER subtypes and their ability to form

pathways of estrogen signaling: 1) via ERβ in cells exclusively expressing this subtype and 2) via the formation of heterodimers in cells expressing both ER subtypes (Pettersson et al, 1997). This is shown schemetically in Figure 1-3



(Kuiper et al, 1997)

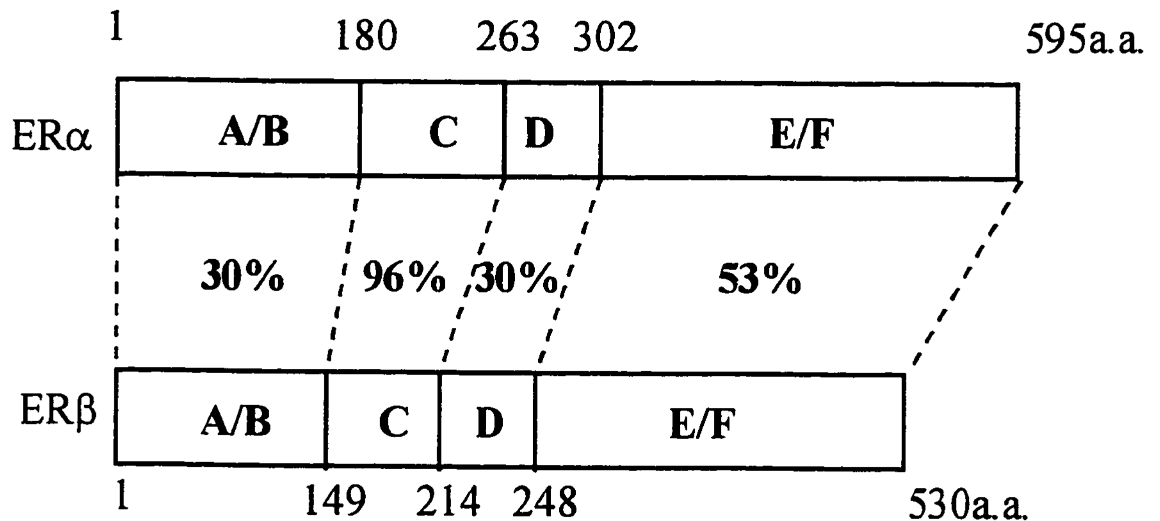
Figure 1-3 Alternative estrogen signaling pathway

The existence of two ER subtypes and their ability to form DNA-binding heterodimers, suggests the existence of three potential pathways of estrogen signaling

1.7 Estrogen receptors α and β: homology, differential ligand activation and tissue distribution of transcripts

There is very high homology between ERβ of different species, with human ERβ showing approximately 89% identity to rat ERβ, 88% to mouse ERβ and 80% to chicken (Krust et al, 1986). Furthermore, human ERβ is also highly homologous to ERα, particularly in the DNA-binding domain (96%) and in the ligand-binding domain (53%), but not in the A/B activation function-1 domains. Both receptors bind to estrogen response elements (ERE) consisting of two hexanucleotide repeats. In addition to acting as transcription activators by binding to estrogen response elements, estrogen activated ERα and ERβ, may also modify transcription by controlling an AP1 enhancer site at the N-terminal . It is important to note that ligand activated ERα and ERβ influence the AP1 enhancer elements quite differently (Pennisi et al, 1997): ligand-activated ERβ acts as a transcription inhibitor, which is in contrast to the transcriptional induction by ligand-activated ERα. Moreover, the antiestrogens tamoxifen, ICI 164384 (Zeneca, Delaware, USA) and raloxifene are potent transcriptional activators with ERβ at AP1 enhancer elements (Paech et al, 1997). These findings strongly suggest that the vascular antiproliferative properties of estrogens may be caused by their action on the AP1 enhancer element complexed with ERβ to inhibit transcription. However, ERα and ERβ have very different tissue distribution patterns (Kuiper et al, 1997). By semiquantitative RT-PCR, ERα was found to be most abundant in the uterus, and smaller quantities were

detected in the ovary, testis, skin and gut. ERβ mRNA was present at moderate to low levels in the thymus, pituitary gland, skin, lung, kidney, and brain cortex. High amounts of human ERβ are present in ovaries, testes, adrenals and spleen. In these tissues, the levels of ERβ mRNA were higher than ERα (Brandenberger et al, 1998). Human ERα and ERβ consists of 595 amino acids and 477 amino acids, respectively. However, at the beginning of 1998, a human ERβ cDNA that encodes the full-length amino acid sequence was isolated from testis poly (A)+ RNA by using both cDNA screening and reverse transcription polymerase chain reaction (RT-PCR). The amplified RT-PCR product with a size of 1740 bp, was subcloned and sequenced (Ogawa et al, 1998). The product was composed of an 1590 bp open reading frame and a segment of the 5'- and 3'-untranslated region (UTR), and encoded an additional 53 amino acids in the N-terminal region compared with the published sequence (Mosselman et al, 1996). The predicted ERβ protein consist of 530 amino acids, with a calculated relative molecular mass of 59.2 kDa counted from the ATG codon at nucleotide 99, which is preceded by an in-frame stop codon at nucleotide 33. Thus, this recently discovered cDNA is regarded as the full-length open reading frame of ERβ. Northern blot analysis showed a 7.5 kb ERβ transcript both in testis and ovary. Comparison of the structures among human ERα and ERβ is shown in Figure 1-4



(Ogawa et al, 1998)

Figure 1-4 Comparison of the structures between ERα and ERβ

The functional domains of ERα and ERβ are schematically represented, with the numbers of amino acid residues indicated. The percentage of amino acid homology is also shown.

Based on in vitro studies, the ERβ protein binds estradiol with an affinity similar to ERα and is able to mediate the effects of estradiol in transfected mammalian cell lines. In addition, the ERα antagonist ICI-164384 is a potent antagonist for ERβ (Mosselman et al, 1994) and (Barkhem et al, 1998). Furthermore, ERα and ERβ can form heterodimers thereby influencing each other's transcriptional activity (Pettersson et al, 1997).

Homezygous mutant mice with the ERα gene disrupted (Lubahn et al, 1993) were made at the time when it was thought that only a single ER gene exists. These mice appear healthy and with the exception of fertility problems in male and female, there are no obvious problems in pre-natal sexual development. It was quite surprising because of the known importance of estrogens in breast and uterine development and in preventing bone loss after menopause and after ovariectomy in mice (Turner et al, 1994). It was anticipated that mutations in the ER gene would be lethal. However, ERα knock-out mice lacked gross abnormalities other than reproductive defects. Estrogens have also been shown to inhibit the same degree of vascular injury in both normal and ERα knock out mice. The data suggest that estrogens inhibit vascular injury by a novel mechanism in dependent of the classic ERα. Therefore, the cardiovascular protective effects of estrogens may involve ERβ in ERα knock out mice (Iafrati et al, 1997). Either ERβ serves as a backup receptor in ERα knock out mice and mediates the cardiovascular protective effects of estrogens or ERβ plays a key role in mediating the effects of estrogen (Gustafsson et al, 1997). However, ERβ mRNA expression is not thought to be different in between wild type and ERα knock out mice (Couse et al, 1997). Nevertheless, it was found that estrogen is able to protect against vascular injury in ovariectomized female ERα knockout mice (Rosenfeld et al, 1998). These mice express the ERβ in their aortas (Lindner et al, 1998). Therefore, these data are consistent with a role for ERβ in mediating the direct vascular effects of estrogens.

1.8 Objectives of the current study of ERβ

We hypothesized that the newly discovered ERβ is expressed in human arteries and in the presence of atherosclerosis ERβ expression is reduced, compared to a non-diseased arteries from the same individual.

My research objectives were to use the RT-PCR technique to determine whether ERβ is expressed in both normal and diseased human vascular tissues. We compared the relative abundance of ERβ mRNA in human arteries with and without atherosclerosis by semi-quantitative RT-PCR. The house keeping gene, GAPDH, was used as an internal control. To confirm the results we have used, Southern blot analysis and sequencing of RT-PCR products. Finally, immunolabeling with anti-ERβ serum was used to examine ERβ protein expression in normal and diseased arteries.

2. Material and Methods

2.1. Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

2.1.1. Cell culture

MCF-7 cells, a human breast cancer cell line, were grown in DMEM with the following supplements: 30% glucose, 5% fetal bovine serum, 2 mM MEM non-essential amino acids, 2mM L-glutamine, 10,000 units/ml penicillin-streptomycin in an incubator filled with a mixture of 95% air, 5% CO_2 and maintained at 37°C. Cells were refed fresh media once every 2-3 days and split 1:10 when they reached 80-90% confluence. Newly plated cells take 3-4 days to become confluent. Total RNA was extracted from cells after 3 passages.

2.1.2. Tissues collection

ERβ expression was studied in normal and atherosclerotic human arteries obtained from 6 pairs of patients undergoing coronary artery bypass surgery at the University of Ottawa Heart Institute. The collection of these tissues for research purpose was approved by the Human ethics committee of the Ottawa Civic Hospital and informed consent was obtained from each patient prior to surgery. The normal internal mammary is used as a bypass conduct, and excess vascular tissue is often trimmed and discarded during the

normal course of an operation. We collected these specimens and used them as examples for normal vascular tissue. As well, each of these patients underwent removal of atherosclerotic plaque (or endarterectomy) from coronary arteries that required bypass grafting. These endarterectomy specimens were used as examples of atherosclerotic vascular tissue. Therefore, for each of these 6 patients we studied paired samples of normal (internal mammary artery) and diseased (endarterectomy tissue) vascular specimens. All specimens were frozen in liquid nitrogen immediately after surgically harvesting, and were stored at –80°C.

2.1.3. RNA isolation

Tissue samples were homogenized in 1 ml of TRIzol Reagent (GIBCOBRL, Cat. NO: 15596-018) per 100 mg of tissue using a power homogenizer (DREMEL MODEL 395). Cultured cells were lysed directly in the culture dish by adding 1 ml TRIzol to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIzol Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. An insufficient amount of TRIzol Reagent may result in contamination of isolated RNA with DNA. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml per 1 ml of TRIzol Reagent) was added to each. The tubes were mixed vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were centrifuged at 12,000 g for 15 minutes at 4°C.

Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, which was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, at a ratio of 0.5 ml of isopropyl alcohol per ml of TRIzol Reagent. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol. At least 1 ml of 75% ethanol was added per ml of TRIzol Reagent used for the initial homogenization. The sample was washed by vortexing and centrifuged at 7,500 g (eppendorf centrifuge 5415) for 5 minutes at 4°C. At the end of the procedure, the RNA pellet was briefly dried and then dissolved in RNase-free water (GIBCOBRL). The absorbance of the RNA solution at 260 nm was measured in a HEWLETT PACKARD spectrophotometer and the RNA concentration was calculated according to the formula:

$C = A_{260} \times \text{dilution factor} \times 40/1000\ \mu g/\mu l$ (Kingston RE, 1997)

RNA absorbance was also measured at 280nm. The A260/280 ratios were between 1.65 to 1.85. This is consistent with the RNA preparations being free of protein contamination.

2.1.4. Reverse Transcription (RT)

Total RNA 3 µg was used for reverse transcription reactions. Oligo $(dT)_{12\text{-}18}$ (1 µl, 500 µg/ml) was added to the RNA in a nuclease-free microcentrifuge tube. The mixture was heated to 70°C for 10 minutes and quickly chilled on ice. 4 µl of 5x First

Strand Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH=8.3), 1 μl 0.1 M DTT, and 1μl 10mM dNTP Mix (GIBCO/BRL) were then added. After the addition of 1μl (200 units) of SUPERSCRIPT II (GIBCO/BRL), the mixture (final volume 20 μl) was incubated for 50 minutes at 42°C. The reaction was inactivated by heating at 70°C for 15 minutes. The cDNA was precipitated by adding 50 μl of 100% ethanol and 2 μl glycogen, and stored at –20°C overnight. Glycogen is used as carrier for the precipitation of DNA. Then the solution was centrifuged at 12,000g for 30 minutes, the pellet was dissolved in 20 μl ddH_2O. Only 10% of the Reverse Transcription reaction solution was used to initiate the PCR reaction.

2.1.5 Primer's design

One critical parameter for successful amplification in PCR is the correct design of the oligonucleotide primes. Oligonucleotide primers were designed using a current DNA database which contains sequence information for human ERβ (O'Hara et al, 1991). Figure 2-1 shows the schematic diagram illustrates full length cDNA of ERα, ERβ and GAPDH, as well as the annealing sites of the primers.

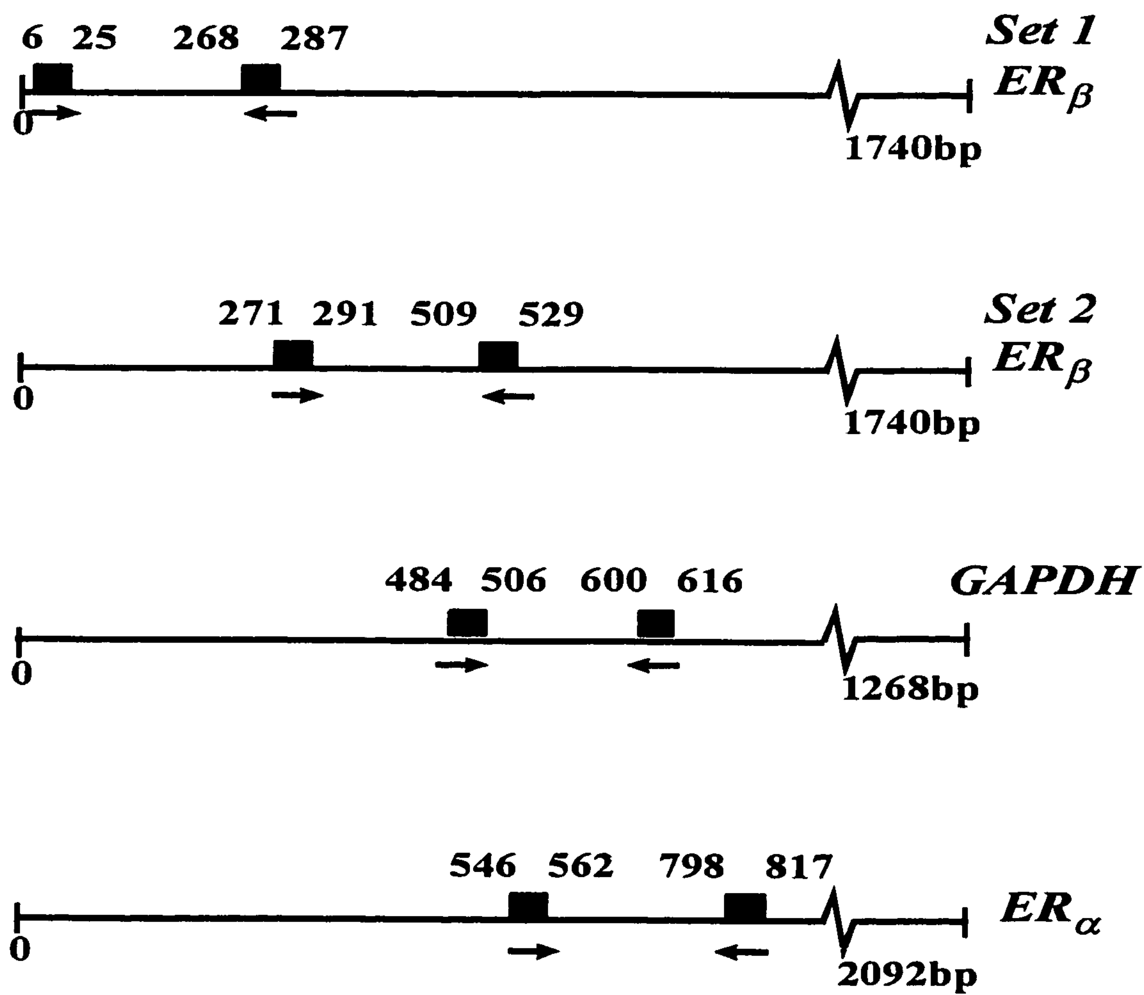


Figure 2-1 The annealing sites of primers for ERs and GAPDH

2.1.6. Polymerase Chain Reaction (PCR)

PCR (Coen DM, 1991) was performed to detect ERβ gene expression. The primers that were used for PCR together with the expected size of the amplicons are listed in Table 1. RT products were amplified in a DNA thermal cycler (AMPLITRON II). The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM dNTP (0.5 mM each), 50 pM of each (sense and antisense) oligonucleotides, and 1.75 units of thermostable Taq DNA polymerase (BOEHRINGER MANNHEIM) in a total volume of 100 μl. After mixing and spinning the PCR mixture down, 1 drop of mineral oil (about 50 μl) was overlayed. The step-cycle program was set up to hot start for 2 minutes at 95°C before adding Taq polymerase. The conditions for PCR varied for each set of primers. For example, with the β_1 and β_2 primers, PCR cycling was performed under the following parameters: denaturation for 1 minute at 94°C, annealing for 40 seconds at 57.5°C, and elongation for 40 seconds at 72°C. In the 32 and final cycle, the elongation step was extended to 7 minutes at 72°C.

In order to get the quantitative PCR results, GAPDH, a housekeeping gene was used as an internal control for the PCR reaction. The amplification of GAPDH mRNA was done in separate tubes at the same PCR conditions except using the specific GAPDH primers instead of ERβ primers. Co-amplification of ERβ and GAPDH in the same tube is not practical, because the GAPDH is expressed at such high levels in the RNA mixture that its amplification inhibits the amplification of ERβ.

To validate the RT-PCR products, the RT-PCR products and 100 bp DNA ladder (GBLCO/BLR) were loaded on 4% NuSieve agarose gel, electrophoresed at 100 V for 4hr and stained with EtBr. The NuSieve is a standard melting temperature agarose and gives fine resolution of DNA, RNA and PCR products < 1kb.

2.2. Southern Blot Analysis

2.2.1 Preparation of Agarose Gels and PCR Samples

A 1.5% agarose TBE gel was prepared by heating 1.5 g of agarose in100 ml TBE solution in a microwave oven for 2 min. The agarose solution was allowed to cool to 50°C before it was poured into the mold. PCR products (20 µl) were carefully removed and added to another tube, taking care not to transfer any of the mineral oil. Loading buffer (4 µl) was added to the samples, which were then heated for 5 minutes to 65°C. Samples were cooled on ice, centrifuged, and loaded on to the gel. After loading, the gel was run at 90 volts for 4 hours. The gel was stained with 0.5µg/ml ethidium bromide for 20 minutes and destained with distilled water for 30 minutes.

2.2.2. Blotting

DNA was denatured by placing the agarose gel in a container of 0.5 N NaOH, 1 M NaCl, pH 13 on a moving platform for 20 minutes at room temperature. The gel was then

neutralized by putting it in 0.5 M Tris-HCl, pH 7.4, 3 M NaCl for 30 min. Whatman 3MM paper and Zeta-Probe GT membrane were cut to the exact size of the gel. The Zeta-Probe GT membrane was placed in the ddH_2O for 5 min before using. Blotting the DNA to Zeta-Probe GT membrane was carried out in 10x SSC buffer overnight by capillary transfer (Chomczynski and Mackey 1994).

2.2.3 Prehybridization

After the transfer to the Zeta-probe GT membrane (BIO-RAD) was completed, the DNA was covalently attached to the membrane by UV cross-linking. The blot was placed in an ULTRAVIOLET CROSSLINKER (CL-1000, UVP) with the side that was in contact with the gel facing up. The blot was exposed to 1200 Joules of UV energy and marked with an indelible marker to show the approximate band position and orientation. Then the blot was incubated in prehybridization solution (0.5 M Na_2HPO_4, pH 7.2, 7% SDS) briefly at 65° C for 5 minutes.

2.2.4 Preparation of the probe

The radioactive probe that was used for the Southern blot was a PCR product generated with primer Set 1 and purified by low melting point agarose gel electrophoresis. After electrophoresis of the DNA, the gel was stained with 0.5μg/ml ethidium bromide for 20 minutes and destained in ddH_2O for 30 minutes. The gel was illuminated under long

wavelength UV light, then a sharp instrument was used to excise the band of interest. DNA was purified from agarose gel after PCR by using spin-X centrifuge tube filters. Spin-X centrifuge tube filters consist of a membrane-containing filter unit within a centrifuge tube. The gel slice was placed into the filter cup (pore size 0.22 μm) of the spin-X tube (Catalog # 8160, CORNING) with 200 μl TE buffer and incubated at 37°C for 30 minutes. The tube was spun at about 13,000 g (Eppendrof centrifuge) for 30 minutes at room temperature. The agarose gel was retained by the spin-X membrane and the filtrate containing the DNA was mixed with 500 μl 100% ethanol and the solution was placed at – 20°C overnight (Beltz GA, 1983). The samples were then centrifuged at 12,000 g for 30 minutes at 4°C to precipitate DNA. The supernant was discarded, the DNA pellet, visible on the bottom of the tube, was dried completely, and then dissolved in an appropriate volume of double distilled H_2O.

2.2.5. Probe Labeling

The gel-purified human ERβ DNA (100 μg) PCR product was denatured by heating at 90°C for 3 minutes, followed by cooling on ice. The labeling reaction was carried out with a reaction mixture containing 5 ul labeling buffer (10 x), 5 μl primer BSA (660 ng/μl), 2 μl enzyme (1U/μl), 5 μl [α-^{32}P] dCTP (10 μCi/ μl), and distilled water to a final volume of 50 μl. The reaction was performed for 2 hours at 37°C or overnight at room temperature. After the incubation was completed, the unincorporated label was removed using a G-50 sephadex spin column. The column was first rinsed with TE

equilibration buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA), allowing the equilibration buffer to completely enter the gel bed (approximately 3ml). The radio-labeled reaction product (50 μl) was added to the column. The test tubes for sample collection were placed under the column, 2 μl of the collected solution was added to the liquid scintillation vials with 10 ml of 30% scintisafe ™ advanced safety LSC-Cocktail (Fisher-Scientific) and the specific activity of the probe was determined by liquid scintillation counting (1219 RACKBETA, LKB WAIIAC).

2.2.6. Hybridization

The prehybridization solution was replaced with fresh solution and 10×10^6 cpm/ml of denatured probe was added per 10 ml of hybridization solution. Air bubbles were removed and the blot was allowed to hybridize overnight at 50°C with agitation in a hybridization incubator (Robbins Scientific, Model 1000).

2.2.7. Washes

The membranes were washed twice, at 55°C, for 30-60 minutes each, in 40 mM Na_2HPO_4, pH 7.2, 5% SDS. The membranes were washed twice at 55°C, 2 times, for 30-60 minutes each, in 40 mM Na_2HPO_4, pH 7.2, 1% SDS. After washing, the blotted membranes were covered with plastic wrap, placed together with the Kodak film in the cassette and stored in the –80°C freezer overnight before development.

2.3 Sequencing of PCR fragments

To confirm that the PCR product (from section 2.1.5) was ERβ cDNA, the band of interest was excised from low melting agarose gel and purified by Spin-X Centrifuge Tube Filters (described in 2.2.3). 100 ng DNA is enough for one sequencing reaction with one primer, so 200 ng total DNA is sufficient for both sense and antisense primers reactions. Sequencing of human ERβ cDNA was performed by the sequencing facility at University of Ottawa, using an ABI automated sequencer. The fragments were sequenced in both directions to confirm the location.

2.4 Northern Blot Analysis

2.4.1 Agarose/Formaldehyde gel Electrophoresis

Agarose (1.0 g) was heated in 36 ml water, cooled to 60°C and then 5ml of 10x MOPS running buffer and 10 ml of 12.3 M formaldehyde were added. The RNA samples were adjusted to11 μl with H_2O, then 5 μl of 10x MOPS running buffer, 9 μl of 12.3 M formaldehyde, and 25 μl of formamide were added before incubation for 15 min at 70°C. 10 μl of formaldehyde loading buffer was added to each sample. The gel was run at 50 volts for 3 hours in 1x MOPS running buffer (Kingston RE, 1997).

2.4.2. Northern blot

After electrophoresis, the RNA gel was rinsed in deionized ddH_2O then soaked in 0.4% NaOH for 10 to 20 minutes. The RNA was transferred by capillary transfer using 10x SSC overnight, following the same protocol described for Southern blotting analysis (section 2.2). The RNA was cross-linked to the membrane in a UV chamber and hybridized with human ERβ plasmid labeled with ^{32}P dCTP using a random oligonucletide system (Boehringer Mannheim). Hybridizing signals were quantified by densitometer scanning using a Molecular Dynamics phosphoimager.

2.5. Immunohistochemistry

A peptide (CLSKAKRNGGHAPRVLEL) corresponding to amino acids 96-113 of human ERβ was conjugated to keyhole limpet hemocyanin (Affinity, Exeter, UK) and used to immunize sheep according to standard methods (Saunders et al, 1997). Polyclonal IgGs were purified from serum on a Hitrap protein A sepharose column according to the manufacture's instructions (Pharmacia). Tissues obtained from human internal mammary arteries and some coronary endarterectomy specimens were fixed in 10% neutral buffered formalin. Before processing into paraffin wax, 5 μm thick sections were mounted on coated slides, dewaxed by immersing the sections for 2 x 10 min in xylene, 1 x 3 min in 100% ethanol, 1 x 3 min in 95% ethanol, 1 x 3 min in 75% ethanol, 1 x 3 min in 50% ethanol, and 1 x 3 min in dd H_2O. Immunocytochemistry was performed according to a

previously described protocol (O'Brien et al, 1993). Endogenous peroxidase was blocked using 3% hydrogen peroxidase in methanol for 30 min. After washing in water, sections were subjected to microwave antigen retrieval (Bremner et al, 1994) by performing 5 min incubations on full power, in 0.01M citric buffer pH 6 which had been prewarmed for 5 min (Saunders et al, 1998) Slides were washed twice in PBS 5 min per wash, and then dried before applying primary antibody at 1: 1000 dilution. After incubation in a moist area at room temperature for 24 hours, the slides were washed in PBS (twice, 5 min each) and sections were incubated with biotinylated anti-sheep immunoglobulins (Vector) for 30 min. The slides were again washed and incubated with a horseradish peroxidase avidin-biotin complex (Dako) for 30 min. After additional washes in PBS, bound antibodies were visualized using 0.05% (w/v) 3, 3'-diaminobenzidine tetra- hydrochloride (DAB, Sigma Chemical Co. Poole, Dorset) in 0.5 m Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide (Simonian et al, 1997). The sections were then washed in distilled water, lightly counter stained with haematoxylin, dehydrated in graded ethanol, cleared in xylene and coverslipped using Permount (Fisher Scientific).

In Figure 3-15A shows a cross section of severe lumenal narrowing due to atherosclerosis with Movat pentachrome stain. Movat pentachrome stain is a method for staining the vascular tissues which gives black colour in nuclei and elastin, yellow colour in collagen and reticulin, blue colour in mucin, red colour in muscle and deep red for fibrin. Hypocellular extra cellular matrix is substance of tissue with few cells.

2.6 Statistical Analysis

All data presented here are from experiments that were repeated at least 3 times. The results of the RT-PCR densitometry studies (Figure 3-9) were analyzed using one-way ANOVA. In the six pairs of internal mammary arteries and endarterectomy specimens, the means are not significantly different.

Table 1. Primers used for PCR together with the expected size of amplicons. Oligonucleotides were used to detect mRNAs for estrogen receptors and the housekeeping gene, GAPDH

mRNA	Sense and antisense primers	Expected size
ERβ Set 1	5' primer: 5'-CAGCCATTATACTTGCCCAC	
	3' primer: 5'-AGTGACATTGCTGGGAATGC	282 bp
ERβ Set 2	5' primer: 5'-TTCCCAGCAATGTCACTAACT	
	3' primer: 5'-CTCTTTGAACCTGGACCAGTA	259 bp
ERα	5' primer: 5'-AGGCTGGGCGTTCGGC	
	3' primer: 3'-AGCCATACTTCCCTTGTCAT	272 bp
GAPDH	5' primer: 5'-AACAGCCTCAAGATCATCAGCAA	
	3' primer: 3'-CAGTCTGGGTGGCAGTGAT	133 bp

3. Results

3.1 Northern blot analysis

Six pairs of tissues from 6 patients were numerically coded: 103, 106, 108, 110, 113, and 114 respectively. "I" represents a human internal mammary artery, and "E" represents a human coronary endarterectomy specimen. Among these six patients only one was a female patient, 106. Total RNA was extracted from all samples, and quantified on the basis of absorbance. From 3-20 μg of total RNA was recovered from individual tissue samples. Northern analysis was first carried out on internal mammary artery and endarterectomy samples from patients 108 and 114 (Figure 3-1). 8μg RNA was electrophoresed on a formaldehyde 1% agarose gel, after electrophoresis, the agarose gel was transferred to nylon membrane and hybridized with ^{32}P end-labeled to human ERβ, which was got from the RT-PCR fragment. Unfortunately, no ERβ was detected by Northern blot analysis, thereby suggesting that expression of ERβ in human vascular tissues may be very low or absent.

3.2 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Reverse Transcription, coupled with the Polymerase Chain Reaction (RT-PCR), is the most sensitive method available for the in vitro study of gene expression.

Theoretically, the abundance of a transcript can be determined by knowing the amount of RNA used in the initial cDNA synthesis reaction (Wang et al, 1989), the amount of cDNA used in the PCR reaction, and the number of PCR cycles necessary to generate enough product for detection either by direct visualization on ethidium bromide stained agarose gel or by autoradiograph or by Southern transfer and hybridization with a labeled probe. It is very important that the PCR be performed at optimal conditions. Therefore, careful consideration must be given to primer design, annealing temperature, template concentration, and the number of PCR cycles.

3.2.1 Primer design

The selection of oligonucleotide primers is often critical for the overall success of PCR amplification reaction. Well-designed primers can help avoid the generation of background and nonspecific products and allow one to distinguish between cDNA and genomic templates in RT-PCR. Primer design also greatly affects the yield of the product. When poorly designed primers are used, no or very little product is obtained. Computer-assisted primer design is more effective than manual or random selection. Some of the factors that affect the performance of primers used in PCR are melting temperatures and possible homology among primers. These factors are well defined and can be easily encoded in computer software. The speed of computers allows calculations of all possible permutations of the primer's placement, length, and relation to other primers that meet conditions specified by user (O'Hara et al, 1991). Primers are normally 15-30 bases long, in principle, it is not likely a 20bp set of primers will find another perfect match in the

genome. Primers were designed for specific and reproducible amplification of target mRNA without interference from inadvertent amplification of DNA. As I described in Chapter I (Introduction), ERβ is a nuclear receptor, and has a high homology with the classic ERα protein, particularly in the DNA binding domain (95%) and the hormone binding domain (55%). As a consequence, the primers were designed to anneal to sequence that encoded the N-terminal A/B region. This region has the lowest similarity (30%) between ERα and ERβ. Two sets of ERβ primers were used to detect mRNA expression in the vascular tissue. The Set1 primers, which correspond to the A/B region were designed by myself, whereas the sequence of the Set 2 primers, which correspond to the C region are already published (Dotzlaw et al, 1996). As shown in Figure 3-2, the Set 1 primers gave better results than the Set 2 primers. Therefore, the Set 1 primers were used in all subsequent experiments.

3.2.2 Annealing Temperature

Optimization of annealing temperature begins with calculation of the T_m values of the primer-template pairs by one of several methods, the simplest being $T_m = 4\ (G+C) + 2\ (A+T)$. More complex formulae can also be used (Sambrook et al, 1989), but in practice, because the T_m is variably affected by the individual buffer components and even by the primer and template concentration, any calculated T_m value should be regarded as an approximation. Most of the time, the annealing temperature (T_a) is about 5°C lower than

Figure 3-1 Northern blot analysis

Human internal mammary artery tissues and endarterectomy tissue RNA (8 μg) from patients 108 and 114 respectively, was electrophoresed on a formaldehyde 1% agarose gel containing ethidium bromide.

8 μg RNA was electrophoresed on
a formaldehyde 1% agarose gel

patent's # 108 114

I E I E

28S →

18S →

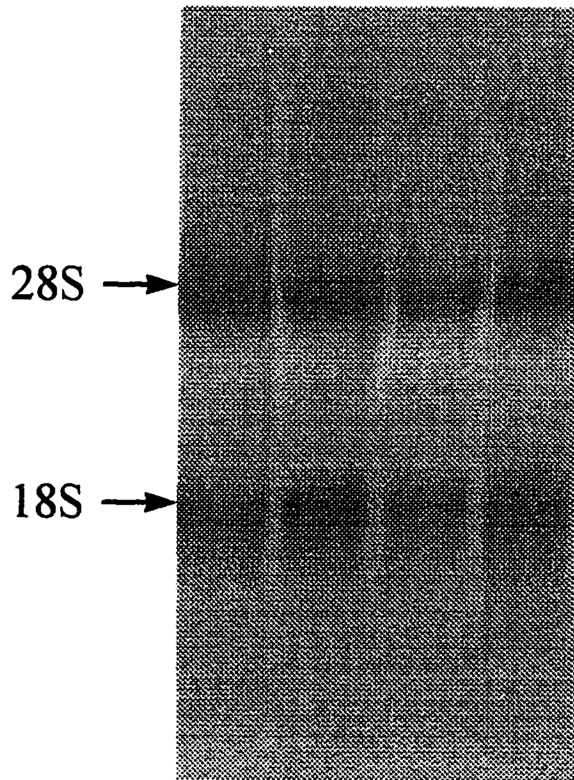


Figure 3-2 comparison of Setl and Set 2 primers for ERβ

Set 2 primers yield a 259 bp PCR product and Set 1 primers give a 282 bp PCR product. The GAPDH primers produce 133 bp PCR product.

The PCR results with the Set 2 primers (published primers) were not as good as the Set 1 primers which I designed.

\

Set 2

Set 1

DNA ladder

ERβ plasmid

108 I

MCF-7

108 E

108 I

MCF-7

108 E

500 bp

400 bp

300 bp

259 bp

200 bp

100 bp

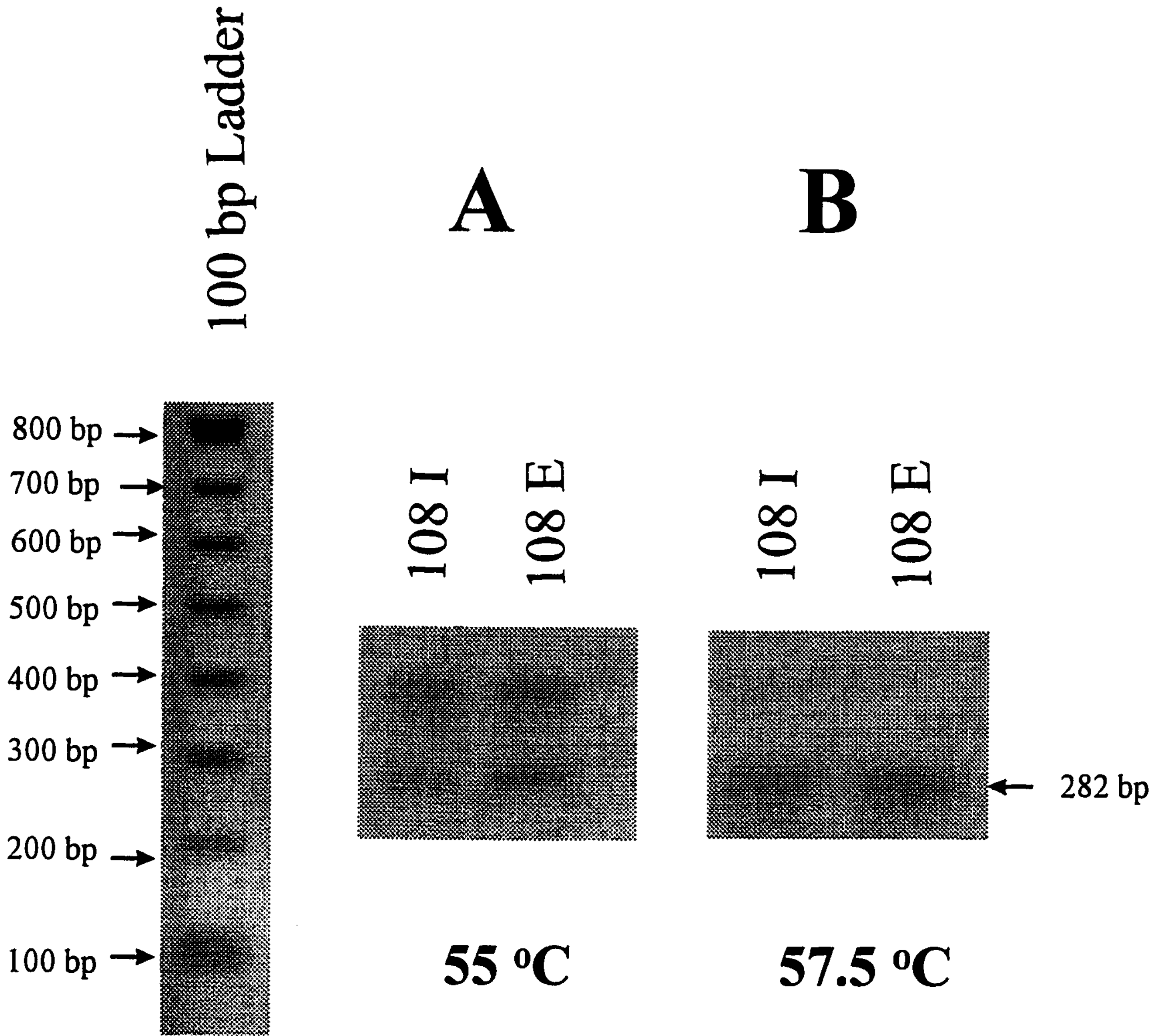
282 bp

133 bp

Figure 3-3 Optimization of RT-PCR annealing temperature for ERβ

A RT-PCR products were analyzed in a 4% NuSieve agarose gel, then visualized with ethidium bromide. 108 I represents human internal mammary artery tissue, 108 E represents endarterectomy tissue from the same patient The PCR cycles consisted of denaturing for 1 min at 94°C, annealing for 40 sec at 55°C, extension for 40 sec at 72°C. After 32 cycles, the final extension step was for 7 min at 72°C.

B PCR was performed under same condition except with an annealing temperature of 57.5°C instead of 55°C.


100 bp Ladder
A
B
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
108 I
108 E
108 I
108 E
282 bp
55 °C
57.5 °C

the T_m. Therefore, $T_a = 4\,(G{+}C) + 2\,(A{+}T) - 5$ (Berent et al, 1985), which was used as the starting annealing temperature for the PCR reaction. Based on the sequence of Set 1 primers, on my experiment, the annealing temperature for ERβ Set 1 primers was 55°C. The PCR reaction was carried out at an annealing temperature as 55°C. Unfortunately, two bands were seen instead of one specific band with each sample. When the annealing temperature was increased to 57.5°C, the larger nonspecific bands were eliminated (Figure 3-3). So 57.5°C was used as the annealing temperature in subsequent experiments.

3.2.3 The Proper Template Concentration

Typically, 0.1- 1 µg of mammalian genomic DNA is utilized per PCR reaction (Saiki et al. 1985). In general, the efficiency of PCR is greater for smaller-size template DNA (e.g., amplified fragment, plasmid, phage DNA) than for high-molecular-weight DNA (e.g., eukaryotic genomic). For bacterial genomic DNA or a plasmid DNA, which represent a much less complex genome, as little as picogram or nanogram quantities can be used per PCR reaction (Sambrook et al, 1989). The starting template concentration was changed from 0.2 µg to 1.0 µg. While theoretically, the more templates, the more PCR products, an excess amount of templates can increase the formation of nonspecific PCR products. The PCR reaction mixture must contain excess amounts of primers and dNTPs. The ratio between the primer and template is important with regard to the specificity of PCR. If the ratio is too high, PCR is more prone to generate nonspecific amplification products, and primer-dimers are also formed. If the ratio is too low, the efficiency of PCR

is decreased. In the PCR reaction, the free dNTPs concentration drops with time. This, in turn, has a deleterious effect on the overall efficiency of PCR. Thus, for amplifying a large target sequence, a higher concentration of dNTP is recommended. Therefore, 25mM dNTP was used instead of 10 mM concentration, and 2.5 mM $MgCl_2$ instead of 2.0 mM to optimize the efficiency of PCR. When different quantity of RNA were tested, the strongest band was observed with 0.8 μg RNA per reaction mixture (Figure 3-4). Consequently, 0.8 μg RNA was used in all subsequent experiments.

3.2.4 The proper number of PCR cycles for ERβ amplification

The number of PCR cycle is a very important parameter for PCR, especially when quantification is required. The number of cycles of PCR should be optimized with respect to the number of input copies. In a typical PCR, 10^{12} represents plateau respect to the maximum amount of amplification possible. From a single copy, the most efficient PCR would reach the plateau in 40 cycles ($10^{12}=2^{40}$). PCR may be 80-90% efficient, so the amplification factors are near $(1.9)^n$, where n is number of cycles. It is usually advisable to run the minimum number of cycles needed to see the desired specific product, since unwanted nonspecific products will interfere if the number of cycle is excessive. If the quantification is required, the number of PCR cycles must be less than that in which amplification reaches a plateau. Continuing PCR beyond this point often results in amplification of unspecific bands, the appearance of small deletion mutant bands, and, in certain instances, the disappearance of the specific product. To identify the highest

number of PCR cycles before the PCR reaction reaches a plateau, PCR was carried out using different numbers of cycles (Figure 3-5 A).

Amplification of GAPDH cDNA under the same condition was performed in parallel with ERβ using specific GAPDH primers (Figure 3-6A). The optimized conditions for PCR reactions that we have established earlier (Figure 3-3 and Figure 3-4) were used. Densitometry was used to obtain the relative optical density for each band, and Figure 3-5 A and Figure 3-6 A was analyzed by Sigma plot 4.0. The results are shown in 3-5 B and 3-6 B for ERβ and GAPDH respectively. Thirty-two cycles were determined to be the optimal number of cycles for PCR for both ERβ and GAPDH.

RNA isolated from 6 pairs of tissues was used to run the RT-PCR reaction followed by the EtBr staining. The results from one of three separate experiments are shown in the Figure 3-7. MCF-7 cells were used as the positive control and plasmid ERα as a negative control. The sizes of the expected PCR products are 282 bp for ERβ, 272 bp for ERα and 133 bp for GAPDH. In order to compare the relative abundance of the ERβ mRNA expression in normal and diseased tissues, the band representing the ERβ amplification product was quantified by densitometry and normalized to GAPDH. The data from 3 experiments of RT-PCR experiments for each tissue shows in Table 2. The mean ERβ / GAPDH ratios and the standard errors are presented in Figure 3-8. Table 3 is the result of statistical analysis of Table 2 using the one way AVOVA. The mean of

ERβ / GAPDH ratios and standard errors are also present in Figure 3-9, which the six pairs of internal mammary artery and endarterectomy tissues are compared. The means of ERβ mRNA expression are not significantly different between normal (internal mammary artery), and diseased arteries (endarterectomy). These RT-PCR results were further validated by hybridization and nucleotide sequencing.

3.3 Sequencing and Southern blot analysis

To confirm the identity of the PCR product amplified by the ERβ primers, we sequenced the PCR fragment shown in Figure 3-10, The band of interest (which is 282 bp long) was excised from the low melting agarose gel and purified using Spin-X Centrifuge Tube Filters (described in 2.2.3). Sequencing of human ERβ cDNA was performed by the sequencing facility at University of Ottawa, using an ABI automated sequencer. The sequence from RT-PCR of human vascular tissue (108 I) with ERβ pimer (β1) is shown in Figure 3-14. The automated sequencer was set up to read long sequence, it can not read well at the beginning of sequences. Therefore, even though the full length of sequence from my sample is supposed to be 282 nucleotides, the sequencing result is only 273 nucleotides, furthermore, in the 273 nucleotides, only 261 nucleotides are identity with the human ERβ cDNA. The alignment result (Figure 3-15) shows that there is 92.5% identity between the RT-PCR fragment and human ERβ cDNA (261/282=92.5%). In addition, the RT-PCR products were tested for their ability to anneal to an ERβ probe. The ERβ

ERβ oligonucleotide probe was prepared using the PCR-generated cDNA fragment from MCF-7 cells which was excised from the low melting agarose gel (Figure 3-10) as a template. To label with [α–^{32}P] dCTP, and the labeled ERβ DNA fragment was separated from unincorporated ^{32}P dCTP on the G-50 sephadex column (Fig 3-11), and a southern blot was carried out. The PCR cDNA fragments that were subjected to agarose gel electrophoresis (Figure 3-12A) were transferred to a charged nylon membrane and then hybridized with a specific ERβ probe. As shown in Figure 3-12B, PCR products of the 6 pairs of samples, amplified with ERβ primers gave very clear hybridization bands. We used ERα plasmid as a negative control, which gave no band in that position. This illustrates the specificity of the ERβ probe.

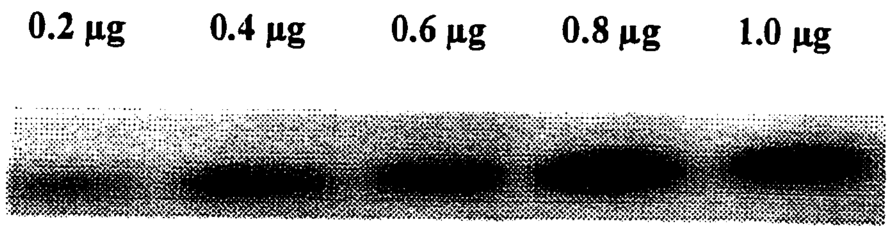
Figure 3-4 Determination of the optimal RNA concentration for RT- PCR

(A) 108 I (human internal mammary artery tissue) was used as a sample to amplify ERβ. The PCR protocol consisted of denaturation for 1 min at 94°C, annealing for 40 sec at 57.5°C, and extension for 40 sec at 72°C. The amount of RNA used for reverse transcription varied from 0.2 µg, to 1.0 µg / reaction.

(B) The relative density of the bands shown in Figure 3-5 was determined by densitometry and plotted as a function of the amount of starting RNA.

The Y axis unit is OD density times volume. Since the volumes are the same, the adjusted volume OD x mm^2 is equal to OD density.

A



B

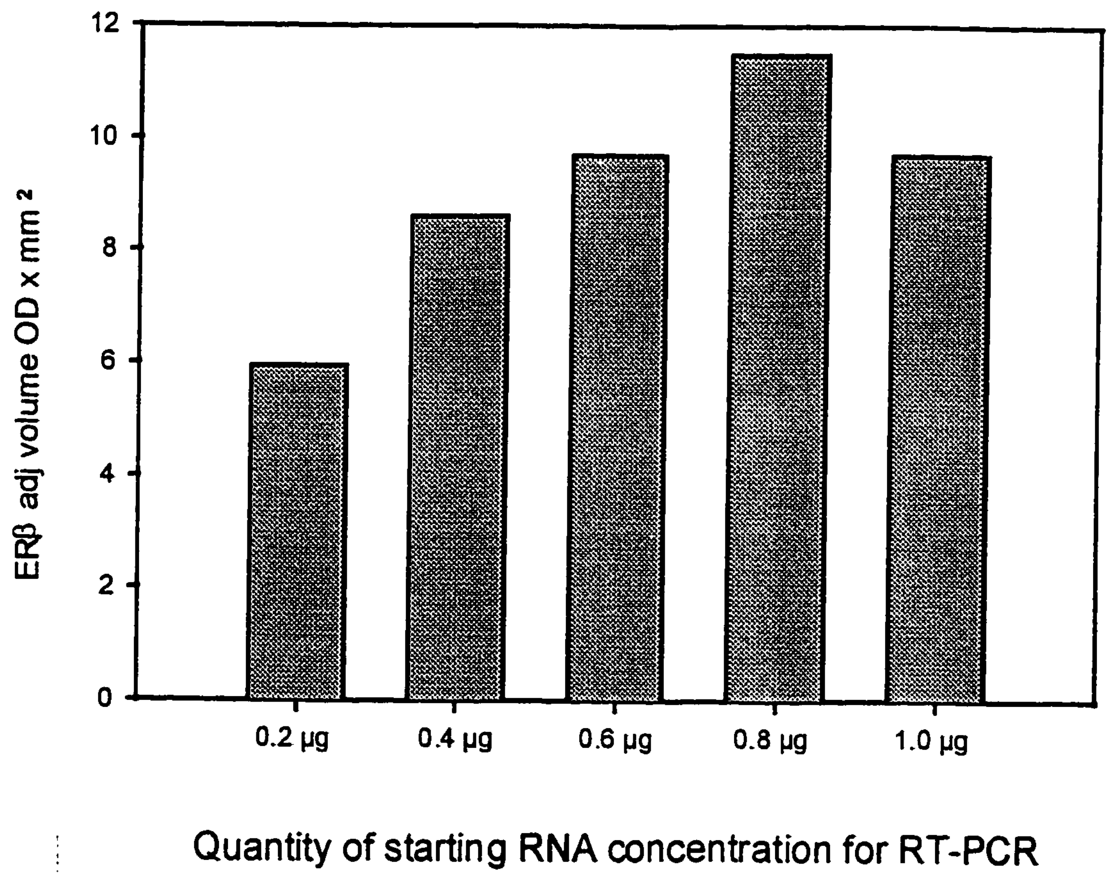
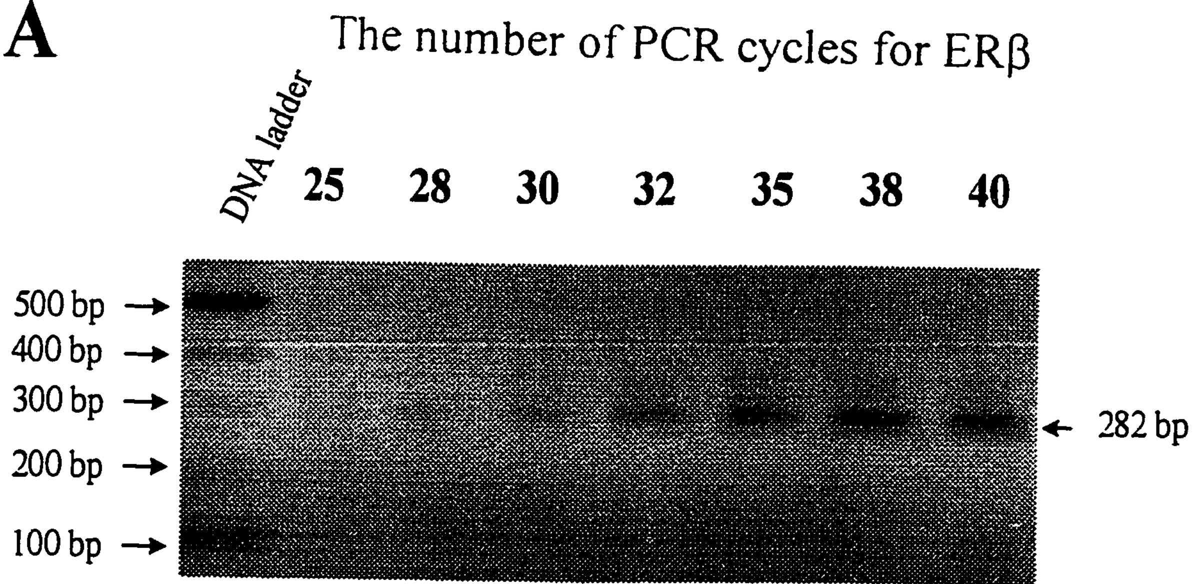


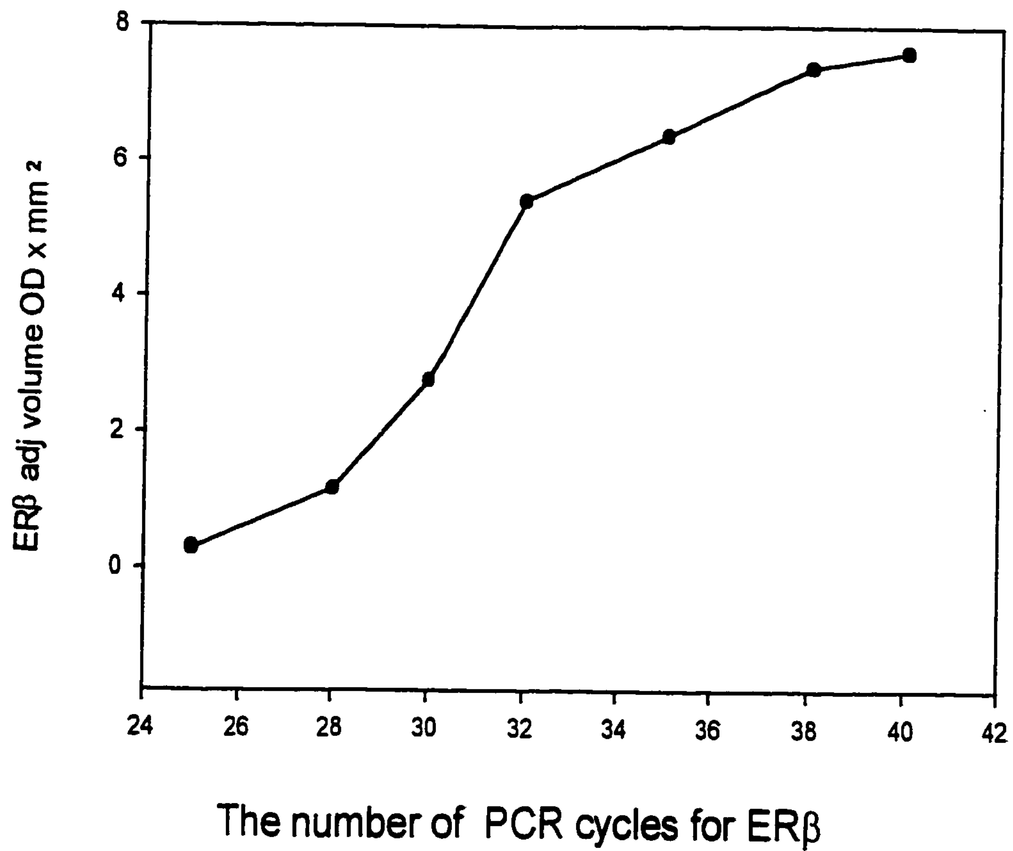
Figure 3-5 Determination of the optimal number of PCR cycles for ER β

(A) RNA from MCF-7 cells, a human breast cancer cell line, was reverse transcribed to establish the optimal number of PCR cycles with the Set 1 ER β primers. PCR was performed using the same conditions described in the figure 3-2. The PCR products were loaded at 4% NuSieve agarose gel.

(B) The relative density of the bands was determined and plotted as a function of the number of PCR cycles.



A
The number of PCR cycles for ERβ
DNA ladder
25
28
30
32
35
38
40
500 bp
400 bp
300 bp
200 bp
100 bp
282 bp

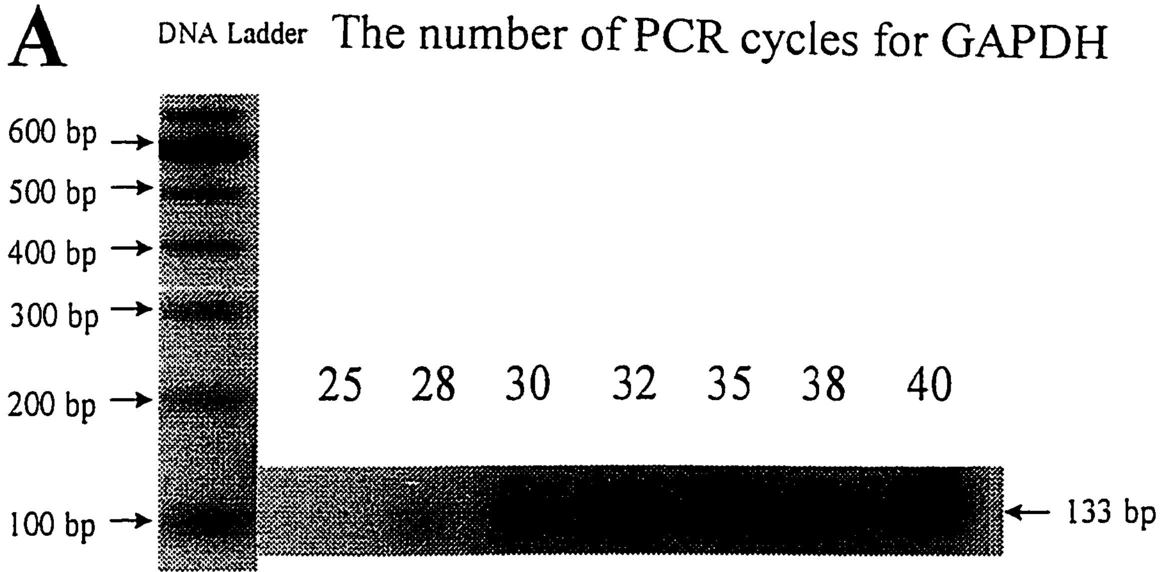


B
ERβ adj volume OD x mm²
8
6
4
2
0
24
26
28
30
32
34
36
38
40
42
The number of PCR cycles for ERβ

Figure 3-6 Determination of the optimal number of PCR cycles for GAPDH

(A) RNA isolated from the MCF-7 human breast cancer cell line was used to identify the optimal number of PCR cycles to quantify GAPDH mRNA. GAPDH, a housekeeping gene was used as an internal control for semi-quantitative PCR. PCR was performed under the same conditions as described for ERβ mRNA.

(B) The relative density of the bands shown in **A** are plotted as a function of the number of PCR cycles. It was determined that 32 cycles is optimal for quantification of the GAPDH transcript.


A
DNA Ladder
The number of PCR cycles for GAPDH
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
25
28
30
32
35
38
40
133 bp

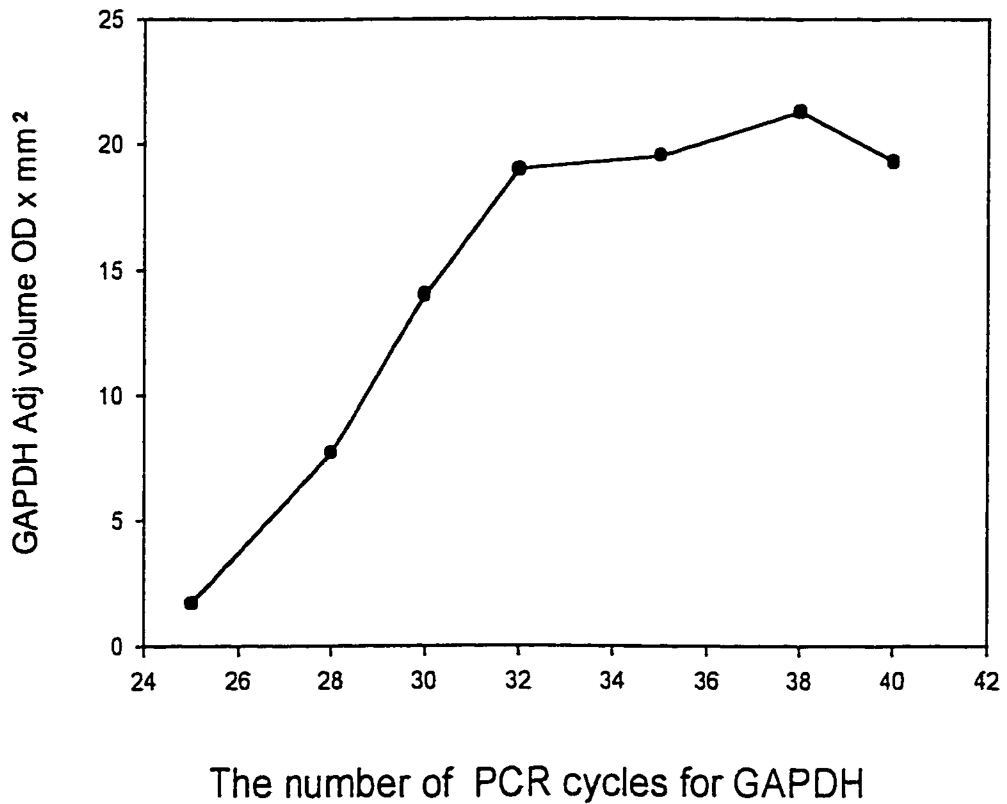

B
GAPDH Adj volume OD x mm²
0
5
10
15
20
25
24
26
28
30
32
34
36
38
40
42
The number of PCR cycles for GAPDH

Figure 3-7 Semi-quantitative RT-PCR products were loaded on the 4% NuSieve agarose gel staining with EtBr.

The relative abundance of ERβ mRNA in six pairs of human tissues (identified as 103, 106, 108, 110, 113, and 114) was determined. I: represents internal mammary artery, E: represents endarterectomy. PCR products with ERβ primers and GAPDH primers were obtained using the same protocol. After 32 cycles, the final elongation step was extended to 7 min at 72°C. RNA from MCF-7 cells was used as positive control, and an ERα plasmid was used as a negative control. The expected sizes of the PCR products are 282 bp for ERβ and 272 bp for ERα.

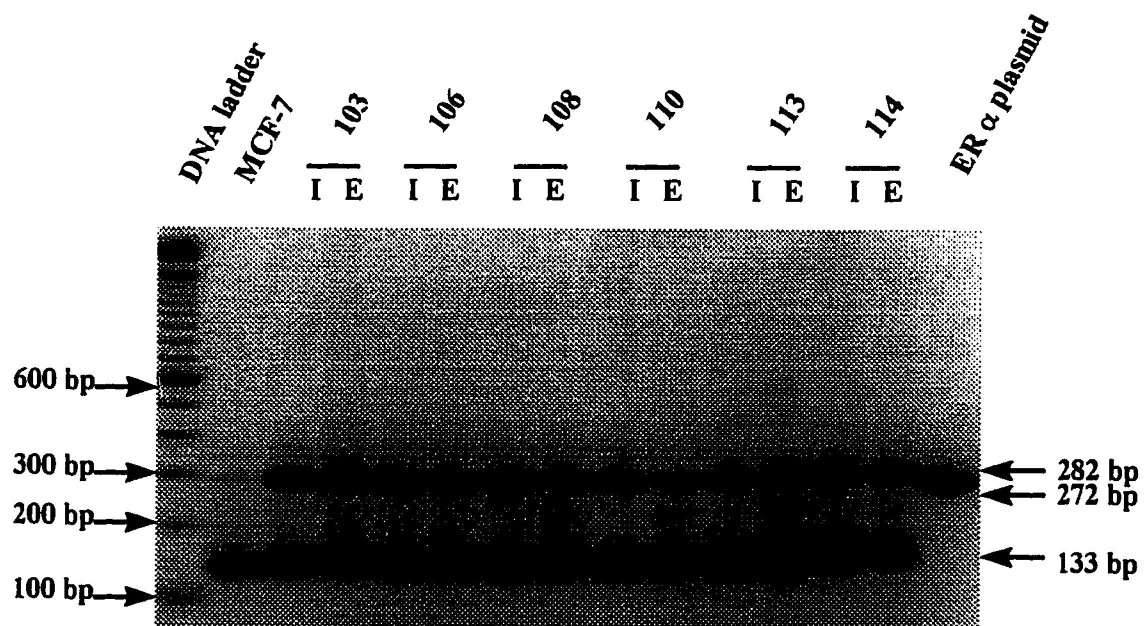

DNA ladder
MCF-7
103
106
108
110
113
114
I E
I E
I E
I E
I E
I E
ER α plasmid
600 bp
300 bp
200 bp
100 bp
282 bp
272 bp
133 bp

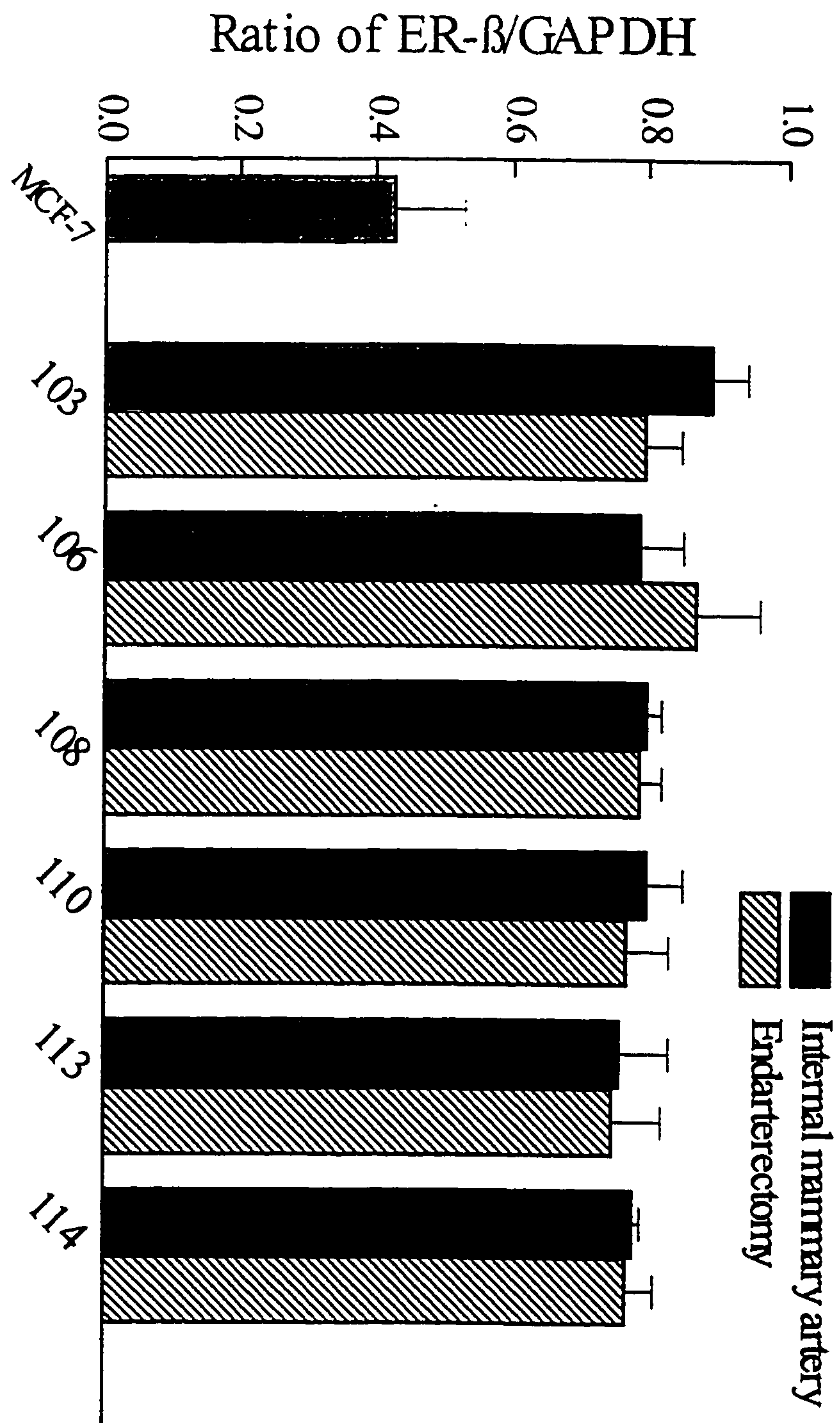
Figure 3-8 the ratios of ERβ to GAPDH in the agarose gel after the RT-PCR

Based on Figure 3-7. The RT-PCR agarose gel was analyzed using densitometry to obtain the relative optical density for each sample band. The results, normalized to the internal control (GAPDH) and the standard error, are presented.

Table 2. Mean and Standard Error of ER-ß/GAPDH Ratio.

Samples	MCF-7 (mean ± S.E)	I (mean ± S.E)	E (mean ± S.E)
MCF-7	0.429 ± 0.12		
103		0.892 ± 0.07	0.784 ± 0.08
106		0.779 ± 0.08	0.876 ± 0.11
108		0.792 ± 0.02	0.778 ± 0.03
110		0.794 ± 0.06	0.753 ± 0.07
113		0.764 ± 0.08	0.741 ± 0.08
114		0.783 ± 0.01	0.765 ± 0.04

Values are means ± SE of 3 experiments. MCF-7: Human breast cancer cell line as control. I: Internal mammary artery. E: Endarterectomy.



Ratio of ER-ß/GAPDH
0.0
0.2
0.4
0.6
0.8
1.0
MCF-7
103
106
108
110
113
114
Internal mammary artery
Endarterectomy

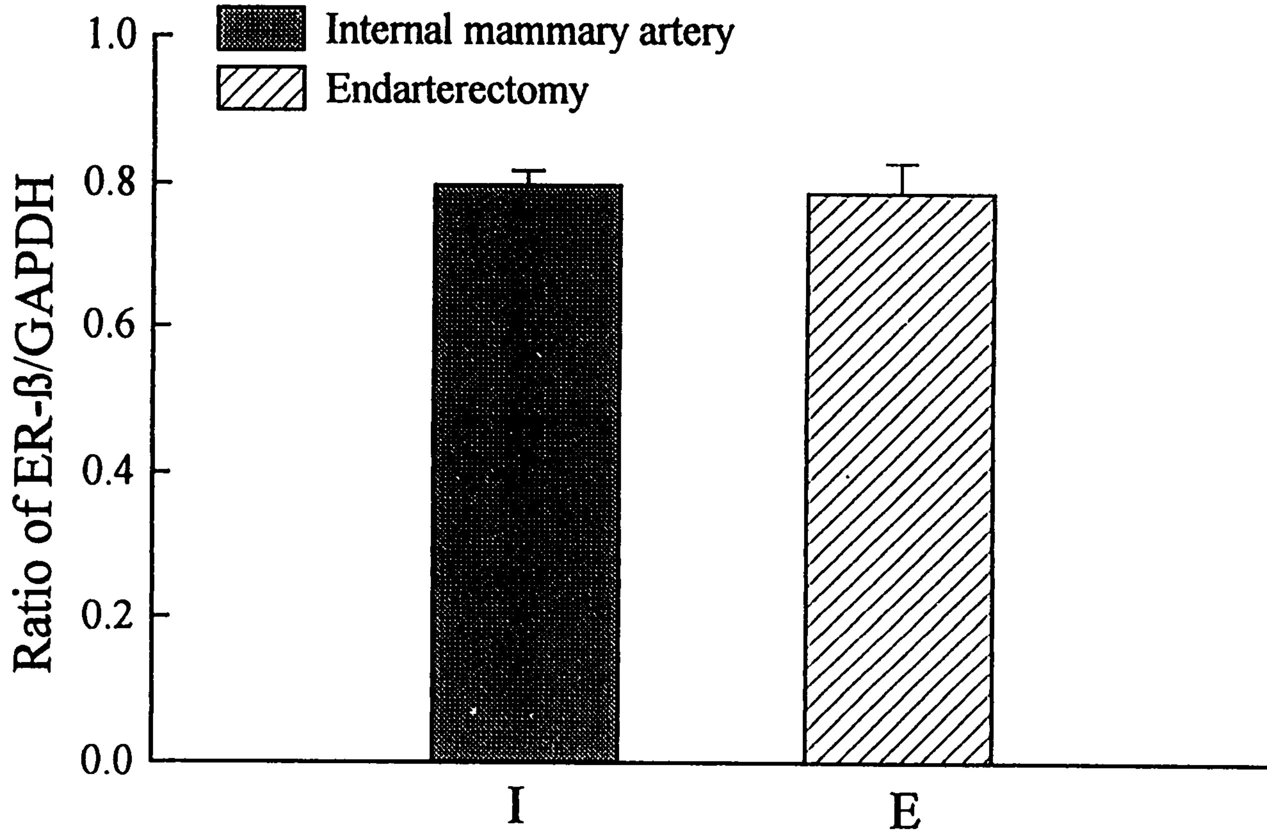
Figure 3-9 Comparison of the normal and diseased arteries.

The mean ERβ : GAPDH ratio of all the internal mammary artery and endarterectomy samples, respectively, from 3 experiments is presented.

Table 3. Mean and Standard Error of ER-ß/GAPDH Ratio.

	Mean	S.E	Number
I	0.801	0.02	6
E	0.783	0.05	6

Values are mean ± S.E of 6 samples of each group. I: Internal mammary artery. E: Endarterectomy.



Values are mean ± S.E. of 6 normal or disease patients.

Figure 3- 10 Three bands from MCF-7, 108I, and 108E tissues, were excised.

RT-PCR products were run on 2% low melting agarose gel

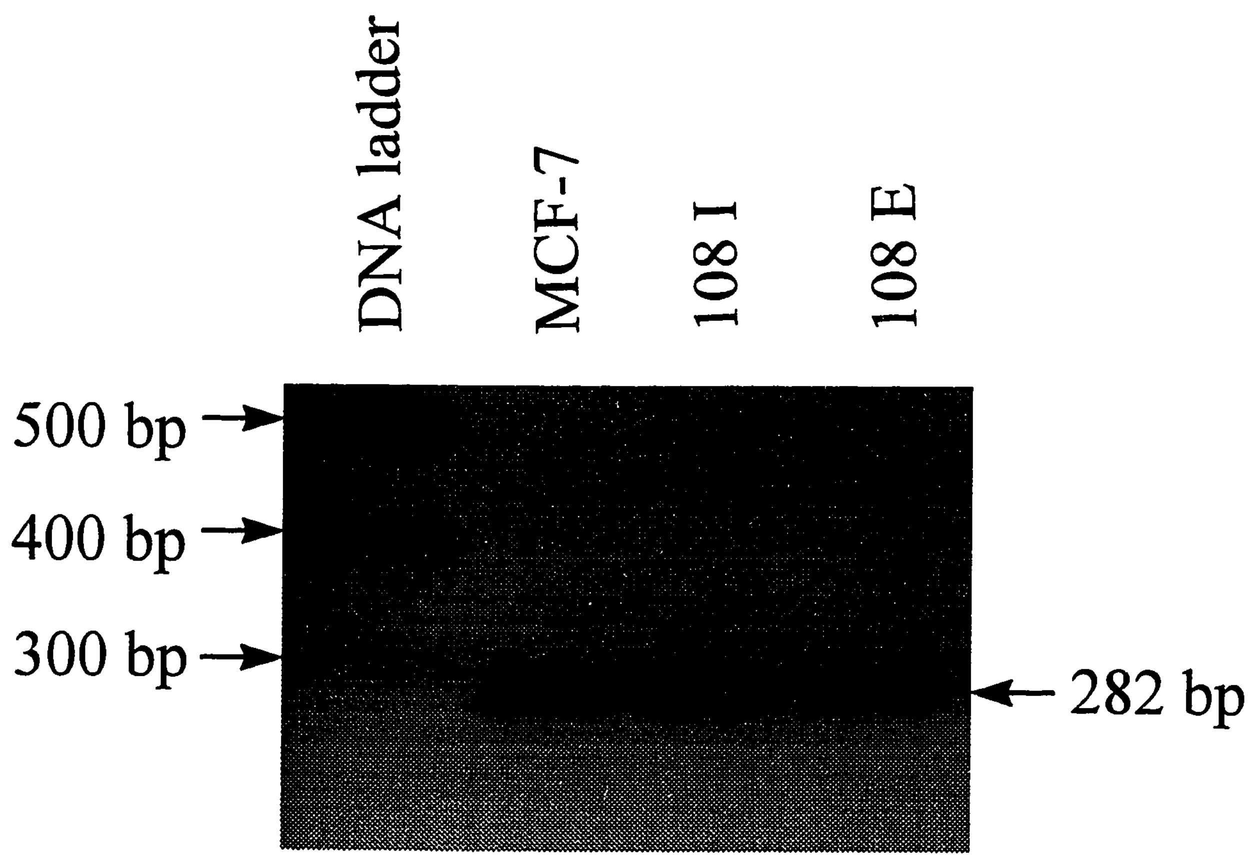
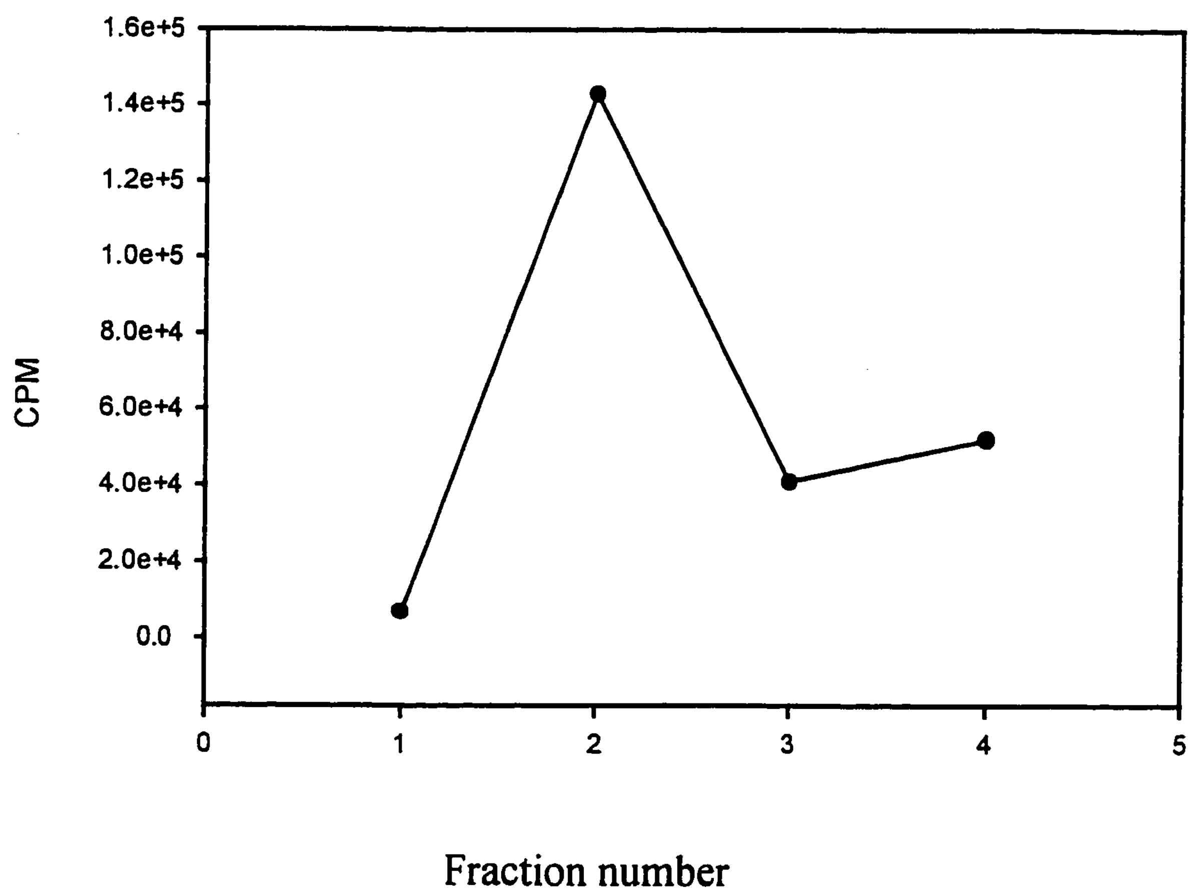

DNA ladder
MCF-7
108 I
108 E
500 bp
400 bp
300 bp
282 bp

Figure 3-11 ^{32}P end-labeled ERβ probe.

The ERβ fragment was obtained by RT-PCR. 100 ng of the purified DNA was labeled with 50 μCi ^{32}P dCTP, and the labeled fragment was separated from unincorporated [α-^{32}P] dCTP on a G-50 Sephadex column. The X is fraction number, the Y is the count per minute. The number 2 fraction was used as labeled probe.



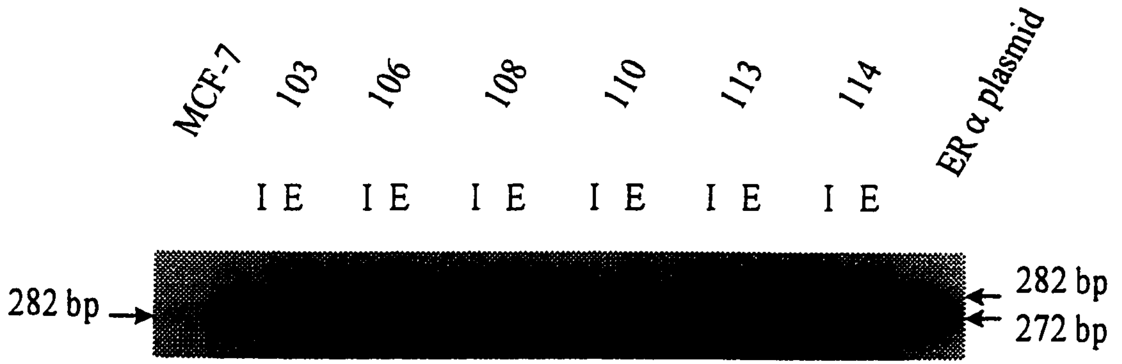
Fraction number

Figure 3-12 Southern blot analysis for human ERβ

(A) The bands that represent the ERβ RT-PCR products from Figure 3-7.

(B) The RT-PCR agarose gel was transferred to a charged nylon membrane and then hybridized with the specific ERβ probe. The MCF-7 cell line was used as positive control, and an ERα plasmid was used as negative control.

A



B

Figure 3-13. The sequence of RT-PCR fragment

Sequence of the 273 nt from human vascular tissue (108I) with specific ERβ primer (β1)

1 TTGNAACCCCNNTATNTTGGACACTTATAATGACCTTTGT 40

41 GCCTCTTCTTGCAAGGTGTTTTCTCAGCTGTTATCTCAAG 80

81 ACATGGATATAAAAAACTCACCATCTAGCCTTAATTCTCC 120

121 TTCCTCCTACAACTGCAGTCAATCCATCTTACCCCTGGAG 160

161 CACGGCTCCATATACATACCTTCCTCCTATGTAGACAGCC 200

201 ACCATGAATATCCAGCCATGACATTCTATAGCCCTGCTGT 240

241 GATGAATTACAGCATTCCCAGCAATGTCACTAC

Figure 3-14 Sequence alignment of human ERβ cDNA with the fragment sequence from RT-PCR product.

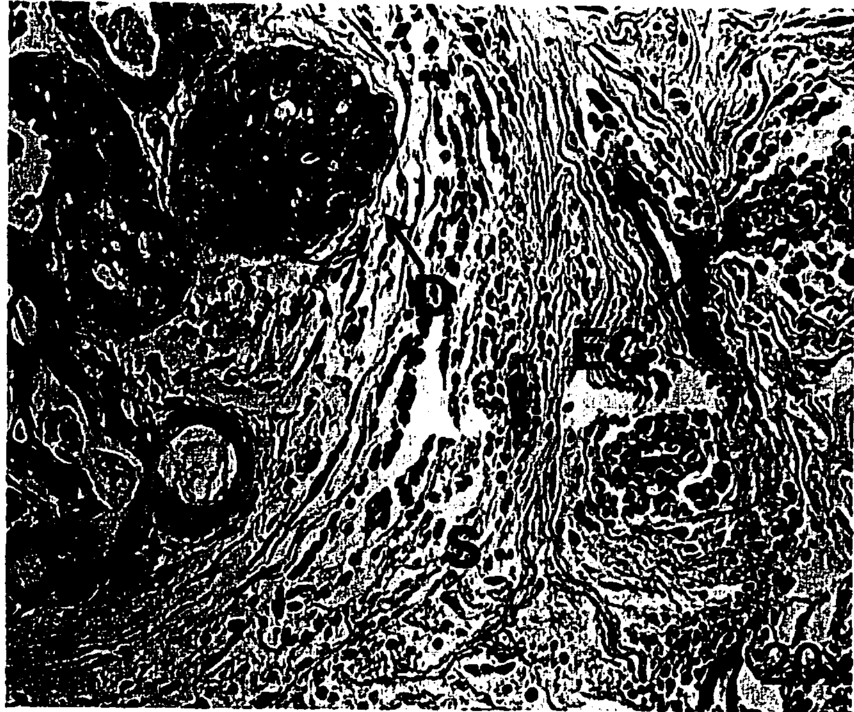
A Genebank database search identified the human ERβ cDNA 1740 nt (Genebank locus AB006590). Double dots show the identity of the 273 nt fragment RT-PCR product with ERβ. A number of gaps were introduced to obtain a optimal alignment. The numbering of ERβ is based on the published sequence (Ogawa et al, 1998).

```
                10        20        30        40        50        60
ERβ     GTTGACAGCCATTATACTTGCCCACGAATCTTTGAGAACATTATAATGACCTTTGTGCCT
         :  :   ::  :::  :::  :::               ::::::::::::::::::::
RT-PCR  TTGNAACCCCNNTATN-TTGGACAC---------------TTATAATGACCTTTGTGCCT
                10        20                        30        40

                70        80        90       100       110       120
ERβ     CTTCTTGCAAGGTGTTTTCTCAGCTGTTATCTCAAGACATGGATATAAAAACTCACCAT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RT-PCR  CTTCTTGCAAGGTGTTTTCTCAGCTGTTATCTCAAGACATGGATATAAAAACTCACCAT
                50        60        70        80        90       100

               130       140       150       160       170       180
ERβ     CTAGCCTTAATTCTCCTTCCTCCTACAACTGCAGTCAATCCATCTTACCCCTGGAGCACG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RT-PCR  CTAGCCTTAATTCTCCTTCCTCCTACAACTGCAGTCAATCCATCTTACCCCTGGAGCACG
               110       120       130       140       150       160

               190       200       210       220       230       240
ERβ     GCTCCATATACATACCTTCCTCCTATGTAGACAGCCACCATGAATATCCAGCCATGACAT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RT-PCR  GCTCCATATACATACCTTCCTCCTATGTAGACAGCCACCATGAATATCCAGCCATGACAT
               170       180       190       200       210       220

               250       260       270       280       290       300
ERβ     TCTATAGCCCTGCTGTGATGAATTACAGCATTCCCAGCAATGTCACTAACTTGGAAGGTG
        ::::::::::::::::::::::::::::::::::::::::::::::::
RT-PCR  TCTATAGCCCTGCTGTGATGAATTACAGCATTCCCAGCAATGTCACTAC-----------
               230       240       250       260       270
```

Figure 3-15 Breast tumour specimens were used as positive control tissues for ERβ protein expression, using immunohistochemistry study

A Immunolabeling with anti-ERβ polyclonal serum (P3) produced a brown colour reaction product with cytoplasmic localization pattern in ductal cells (D), as well as some stromal cells (S) and endothelial cells (EC) of tumour microvessels

B Immunolabeling with the pre-immune serum resulted in no colour reaction product (negative control)



P3 anti-ERβ antiserum

A

Pre-immune serum

B

Figure 3-16 Immunolabeling of human internal mammary artery and normal coronary artery using the anti-ERβ polyclonal serum P3

A Internal mammary artery: These is abundant immunolabeling of lumenal endothelial cells (EC), medial smooth muscle cells and some adventia (Adv) fibroblasts. Note the absence of intimal thickening in the artery

B Coronary artery, with modest intimal thickening (a normal developmental phenomenon). ERβ immunolabeling is primarily limited to medial smooth muscle cells. There is an absence of ERβ protein expression in lumenal endothelial cells and in the adventitial (Adv)

Figure 3-17 Diseased human coronary artery

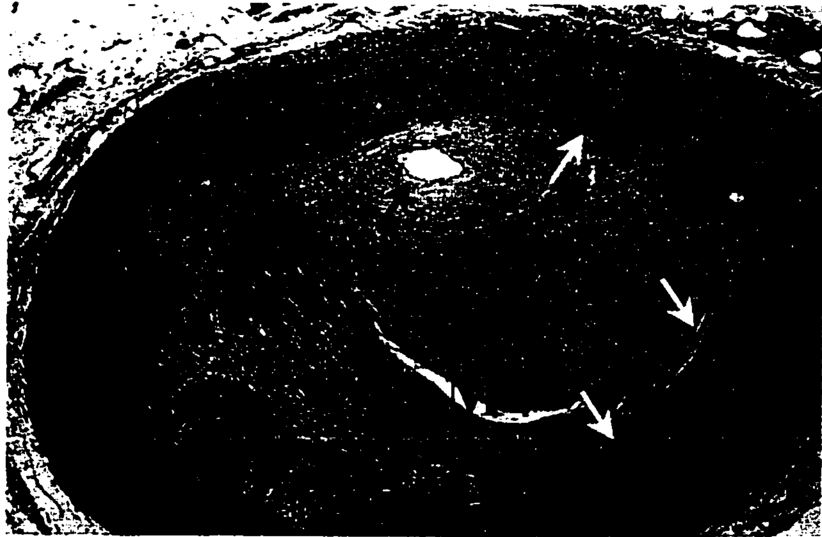
A 4x Movat pentachrome stain.

There is severe lumenal narrowing due to atherosclerosis. The lesion primarily consists of a dense, hypocellular extra cellular matrix and a necrotic core (nc). The white arrows indicate the location of the internal elastic lamina that separates the media from the atherosclerotic intima.

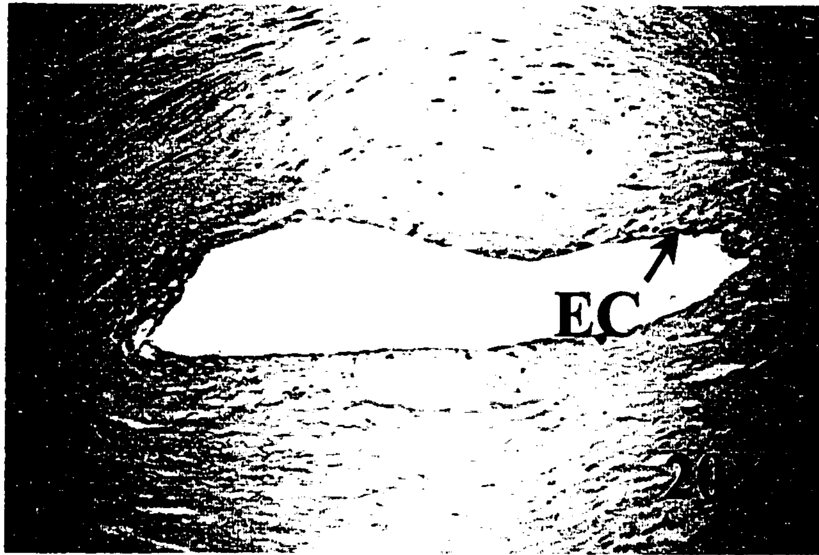
B (20x) and C (40x)

Immunolabeling with the anti-ERβ polyclonal serum (P3) demonstrates ERβ protein expression in lumenal endothelial cells (EC) and some smooth muscle cells (SMC) of fibrous plaque.

A



B



C

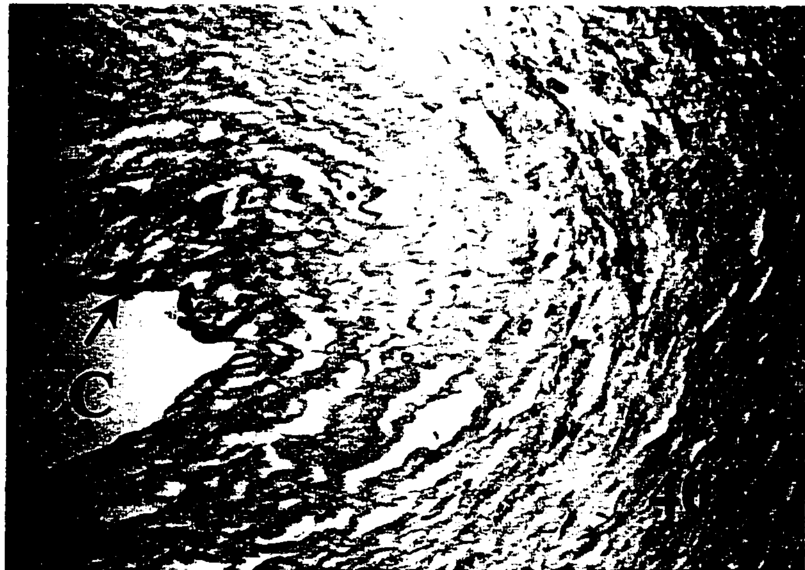


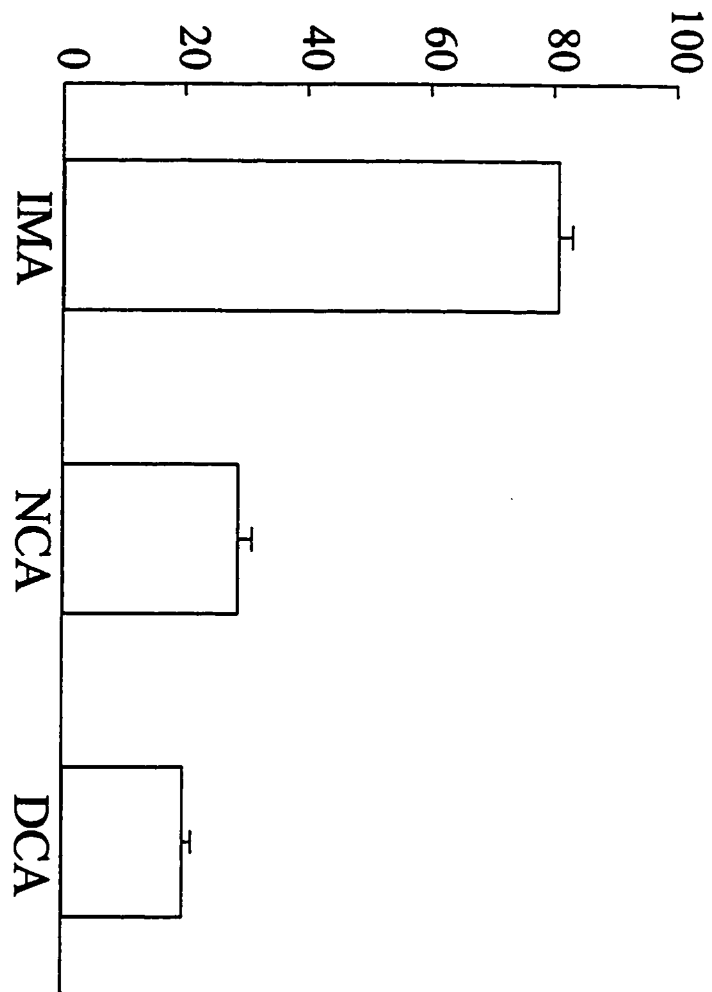
Table 4. Results of Immunostaining for Estrogen Receptor-ß in Human Arteries.

Samples	Number of immunoreactive cells per section		
	IMA	NCA	DCA
S8S-2591	76	27	25
29	84	31	19
VB 007I	92	40	20
VB 006	79	29	17
VB 063	75	30	20
VB 006D	80	21	16
VB 004 E	81	28	24
Mean ± SE	81 ± 2.2	29 ± 2.1*	20 ± 1.7*#

IMA: Internal mammary artery. NCA: normal coronary artery. DCA: Diseased coronary artery. *$p<0.05$ compare with IMA group. #$p<0.05$ compare with NCA group.

Figure 3-16 Statistical results of immunohistochemistry

The number of immunostaining positive cells per section with IMA (internal mammary artery), NCA (normal coronary artery) and DCA (diseased coronary artery).



Number of Immunoreactive cells
0
20
40
60
80
100
IMA
NCA
DCA

3.4 Immunohistochemistry

We have previously demonstrated by RT-PCR analysis that the ERβ transcript is present in human vascular tissues of both normal and diseased arteries. To more precisely define the distribution and localization of ERβ protein in the artery wall, we used the polyclonal antibody P3, which was kindly provided by Dr. Saunders. Breast tumour specimens, which are shown in Figure 3-15, were used as positive control tissues. Immunolabelling breast tumour specimens with anti-ERβ poyclonal serum (P3) showed (Figure 3-15A) the ERβ expression in ductal cells, stromal cells and endothelial cells of tumor microvessels. No immunolabelling was observed with the pre-immune serum (Figure 3-15B), thereby highlighting the specificity of the P3 anti ERβ polyclonal serum. The sections of human internal mammary artery and normal coronary artery (Figure 3-16) were also immunolabelled with P3. Figure 3-16A shows that the ERβ is expressed in all three layers, endothelial cells, smooth muscle cells and adventitial fibroblasts. The normal coronary artery tissue gave different results from internal mammary artery. In Figure 3-16B, immunoreactive ERβ is limited to medial smooth muscle cells. There is an absence of ERβ protein expression in lumenal endothelial cells and adventitial fibroblasts. Figure 3-17A, shows a section of severely atherosclerotic artery. In Figure 3-17B and 3-17C, the diseased coronary artery is illustrated at a 20x and 40x magnification, respectively. The diseased coronary artery was also immunolabeled with anti-ERβ serum P3. Artifact is due to folding of specimen during processing for histology. Some of the specimens also

stained with antibody to smooth muscle α–actin (results are not shown here) to confirm the presence of vascular smooth muscle cells in the region of the artery were also positive for ERβ expression. Table 4 shows the number of positive cells per section in 21 human tissue specimens with immunohistochemistry staining for ERβ protein. The positive stained cells with polyclonal anti-serum P3 produced brown colour reaction products with cytoplasmic localization patterns; the negative staining cells were lightly counterstained with hematoxylin, as they would otherwise be undetectable by light microscopy. Based on the data on Table 4, the statistical analysis results shown in Figure 3-18, There is significantly different ERβ protein expression between normal artery and diseased artery.

Discussion

4.1 General statement

Estrogens are known to have important effects in the reproductive system and also in many tissues outside the reproductive system, including bone (Turner et al, 1994), liver, and cardiovascular system (Katzenellenbogen BS 1996). It is generally recognized that estrogens appear to have cardiovascular protective effects. However; the underlying mechanism of the effect of estrogens on the artery wall are incompletely understood. Estrogens act via receptors, both the conventional ERα and recently discovered ERβ. We hypothesized that the newly discovered ERβ is expressed in human arteries and in the presence of atherosclerosis ERβ expression is reduced, compared to a non-diseased arteries from the same individual. In this study, we have demonstrated evidence for estrogen receptor beta (ERβ) expression in human cardiovascular tissues at both the mRNA and protein levels. Furthermore, we have investigated the relative abundance of ERβ mRNA in normal and diseased human arteries. The RT-PCR results have not confirmed the hypothesis, however the immunohistochemistry results are consistent with the hypothesis, that the atherosclerotic arteries have low human ERβ expression compared to the normal internal arteries. Estrogen receptors are controlled by the levels of the estrogens and progesterone in the blood, under the physiological condition, estrogens

increase the ER levels and progesterone decreases the ER levels. Estrogens and progesterone interact each other to control the growth, development and physiology of the female reproductive tract and other organ system.

4.2 ERβ mRNA expression in human cardiovascular tissues

ERβ has been detected in human breast tumor biopsy samples and human breast epithelial cell lines (Dotzlaw et al, 1997) and osteoblast SV-HFO cells (ARTs et al, 1997) using reverse transcription and the polymerase chain reaction analysis. Human ERβ has also been observed in human fetus (Brandenberger et al, 1997) and abdominal subcutaneous adipose tissue (Crandall et al, 1998), normal ovary, ovarian serous cystadenocarcinoma, ovarian cancer cell lines (Brandenberger et al, 1998) and pituitary gland (Wilson et al, 1998). This broad tissue distribution of ERβ suggest the important roles for ERβ. Traditionally, levels of individual mRNA have been analyzed by procedures such as Northern blots, RNA dot/slot blots, nuclease protection assay and in situ hybridization. Analyses of human artery specimens are often limited by the size of the samples. General speaking, 20 μg of total RNA is necessary to perform Northern blot. In my experiments (Figure 3-1), only two samples (108 and 114) were of sufficient size to permit a Northern blot analysis. Total RNA (8 μg) was subjected to electrophoresis and transferred to a nylon membrane and then hybridized to the ERβ probe. In neither case, were ERβ bands detected. As a consequence, a technique for the detection of ERβ mRNA expression in human vascular tissues that is more sensitive than Northern analysis is

required. Application of the RT-PCR technique provides another method of mRNA analysis, which is more sensitive than Northern blot. Therefore, RT-PCR was carried out in normal and diseased arteries from six patients. The RT-PCR data presented here (Figure 3-7) provide evidence for the expression of the ERβ in human arterial tissues. In this experiment, 110 E has a lower amount of PCR product compared to other samples. This was not the case in two other experiments (Figure 3-8).

The goal of these experiments was not only to detect but also to measure the relative abundance of ERβ in the normal artery (internal mammary artery) and diseased coronary artery (endarterectomy). Because of its ubiquitous and uniform gene expression, GAPDH, was selected as an internal control for the PCR reactions. The amplification of GAPDH cDNA was performed in parallel with ERβ using specific GAPDH primers, we have tested RNA isolated from 6 pairs of tissues in a single experiment. In Figure 3-7, MCF-7 cells were used as the positive control, a cell line that is known to express ERβ. The relative abundance of the ERβ transcript, as determined by densitometry and normalized to GAPDH transcript is presented in Figure 3-9. Statistical analysis showed there is no significant difference of ERβ mRNA expression between normal and diseased arteries. To our knowledge, these results are the first to compare human ERβ expression in normal and diseased arteries. My results clearly demonstrate that ERβ is indeed expressed in human vascular tissues and provides a new dimension for the study of estrogen action and hormone dependence in these tissues. In addition, although the role of ER in mediating estrogen-associated effects on cardiovascular risk remains uncertain, the

expression of ERβ mRNA in male and female coronary arteries suggests the possibility of targeting of these receptors for prevention or treatment of coronary artery disease in humans through the use of tissue specific selective estrogen receptor modulators (SERMS). Recently, ERβ has been shown to act in an opposite manner from ERα with respect to ligand-mediated transcription from an AP1 site (Paech K et al, 1997), highlighting the potential influence that alterations in the relative expression of ERα and ERβ might have on tissue responses to mammalian and plant estrogens, antiestrogens, and SERMS. Research addressing these issues will provide new insights into the tissue specific mechanisms of estrogen action.

The expression of ERβ has been examined in male rat aortas before and after vascular injury by in situ hybridization. Little or no change in ERα expression was observed after balloon injury. However, ERβ mRNA was found to be markedly expressed after balloon injury (Lindner V et al, 1998). In contrast, I did not observe such a difference in ERβ expression levels between normal and diseased arteries in my RT-PCR reaction (Figure 3-7). This discrepancy in results might be explained by species-specific differences between human and rats. In support of this explanation, this laboratory has recently noted that ERα protein expression is up-regulated after balloon injury of porcine coronary arteries (Hoffert et al, 1997). Alternatively, experimental balloon injury may not accurately reflect human atherosclerosis in terms of ERβ expression.

It is not clear whether it is of benefit to increase ERβ expression rather than ERα to achieve cardiovascular protection; However, it has been shown that estrogen can

inhibit the response to vascular injury in the carotid artery in both ERα knock out and normal mice with the level of inhibition being the same in both groups of animals. This suggests that estrogen can inhibit the vascular injury by a mechanism that is independent of ERα. This implicates the role of ERβ in the cardiovascular effects (Foegh and Ramwell, 1998) and (Katzenellenbogen and Korach, 1997) . Nevertheless, a man with a mutation in the estrogen receptor alpha gene and who was resistent to exogenous estrogen therapy (Smith EP et al, 1994) was recently reported to have premature coronary artery disease (Sudhir et al, 1997). These results underline the limitations in extrapolating observations from rodent models of experimental atherosclerosis to human disease.

4.3 Human ERβ protein expression

Norma and diseased coronary artery sections were examined for evidence of ERα expression in normal coronary arteries and the absence in diseased coronary arteries using monoclonal antibody immunohistochemical staining (Losordo et al, 1994). This investigation provides evidence of ERα expression in normal human coronary arteries and not in the atherosclerotic coronary arteries. The study of ERβ has largely been restricted to the RNA level. None of the published reports have investigated ERβ protein expression in different tissues, because of a lack of specific ERβ antibodies. Recently a polyclonal serum P3 was made by our collaborator, Dr. Saunders that reacts with A/B regions that are specific for ERβ. We studied ERβ expression in the internal mammary artery (IMA), and in normal and atherosclerotic coronary arteries using immunohistochemistry. The results

in Figure 3-16A, show that the internal mammary artery has relatively abundant expression of ERβ protein in all 3 arterial layers. There are very high levels of ERβ expression in endothelial cells that line the lumen of the artery wall. ERβ protein is also expressed in medial smooth muscle cells and in adventitial fibroblasts. In Figure 3-16B, less ERβ expression was observed in normal human coronary artery compared to the internal mammary artery. In Figure3-17, ERβ expression was lower in coronary arteries with atherosclerosis than in normal coronary arteries. The statistical analysis (Figure3-18) confirms that there are significant difference ERβ expression between internal mammary arteries and diseased coronary arteries. While normal and diseased coronary arteries showed similar levels of ERβ transcript by RT-PCR, there were lower levels of immunoreactive ERβ protein in diseased arteries compared to normal arteries. One possible explanation for the apparent independence of ERβ transcript levels and immunoreactive ERβ levels is that ERβ may be under post-transcriptional control. This could reflect translational control and or control at the level of catabolism. Alternatively, the observed differences in immunoreactive ERβ between normal and diseased tissues could reflect differences in cell density. The diseased artery specimens can be composed of extensive fibrous plaque and relatively few cells.

4.4 Conclusions and future perspectives:

By means of a sensitive, semi-quantitative RT-PCR technique, I have shown for the first time that the ERβ receptor mRNA is expressed in human vascular tissue.

Moreover, levels of the transcript were similar in normal and diseased arteries. I have used Southern blot analysis and DNA sequencing to verify that the amplified product that is detected is, in fact, generated from the ERβ mRNA. I have also demonstrated the presence of immunoreactive ERβ in normal internal mammary artery and in normal and diseased coronary artery specimens. While the levels of ERβ mRNA appear to be similar in the three tissues, levels of immunreactive ERβ are highest in internal mammary arteries, intermediate in normal coronary arteries and lowest in diseased coronary artery samples. While the apparent dissociation between ERβ mRNA and ERβ protein abundance in different tissues could reflect post-transcriptional control mechanisms, further experimentation will be required to determine if this is the case. It will be equally important to do a quantitative immunohistochemical analysis of normal and diseased vascular tissue to determine if the observed differences in ERβ protein expression could result from differences in the number of total cells or the relative numbers of individual cell types in the tissues. Furthermore it is essential to evaluate different anti-ERβ antibodies to assure that there is not differential expression of ERβ epitopes in different tissue samples. While the lower levels of immunoreactive ERβ in diseased coronary arteries would be consistent with our initial hypothesis, a rigorous quantitative immunohistological examination of many normal and atherosclerotic samples will be required to demonstrate that, this is a generalized phenomenon.

The major question that remains is the physiological role of the ERβ in vascular tissue in normal and pathological states. It is clear that the relative expression of ERα and

ERβ varies in different tissues and species. While some functional overlap between ERα and ERβ may exist and, under some circumstances, ERβ may serve as a backup for ERα, the differential expression of the two ER subtypes could be regarded as support for the hypothesis that ERα and ERβ have different physiological functions. The delineation of the function of ERβ should be facilitated by the availability of ERβ knockout mice (Krege et al., 1998). It has been shown that ERβ is essential for normal ovulation efficiency but is not essential for female or male sexual differentiation, fertility, or lactation. Future experiments will be required to define the role of the ERβ in normal bone and cardiovascular homeostasis and to determine the susceptibility of ERβ homozygote and heterozygote knockout mice to dietary and genetic (e.g. apoE deficiency, human apoB overexpression) atherosclerosis. It is not yet clear if the combined targeted disruption of the ERα and ERβ genes will yield viable mice. As estrogen has the same protective effects against vascular injury in ERα knockout mice and wildtype mice, it will be important to repeat these experiments in the double knockout mice, assuming that these latter are viable. It will also be important to extend these studies to other murine models of atherosclerosis that more closely resemble the human disease. The expression of other estrogen-sensitive genes whose products are potentially implicated in atherosclerosis (e.g. apoE, hepatic lipase, low density lipoprotein receptor etc,) should also be monitored in ERα-, ERβ-and ERα / ERβ-knockout mice. An important final question that remains is whether other estrogen receptors exist in addition to ERα and ERβ. Whether one or more additional estrogen receptors do, in fact, exist, the discovery of the ERβ has added a new dimension to our concepts of the mechanisms of estrogen action.

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Education

Sept. 1982-July 1987	**B. Sc.** Pharmaceutical Chemistry, Beijing Medical University, China.
Jan. 1997- Jun 1999	**M. Sc.** Department of Biochemistry, Microbiology and Immunology, University of Ottawa. Study on the mechanisms of estrogen's prevention in coronary artery diseases through the newly discovered estrogen receptor beta.

Technical Skills

Immunological Techniques

Immunization of Animals
Cell Fusion
Cloning of Hybridoma Cell Lines
Production of Monoclonal Antibodies
Purification of Monoclonal Antibodies

Radioimmunoassay
Mapping Epitopes of Monoclonal Antibodies
Label Protein with Isotopes
SDS-PAGE
ELISA
Western Blot
Gel Filtration
Two-Dimension Electrophoresis

Molecular Biology Techniques

Isolation of Plasmid DNA
Transfection Mammalian Cell Lines
Transformation of E.coli.
Production of Fusion Protein in E.coli.
Sequencing
RT-PCR
Northern and Southern blot analysis.

General Capabilities

Good inter-personal skills
Outgoing personality
Speaks and writes English fluently
Computer Knowledge, Such as WordPerfect 6.0, Excel, AmiPro, Microsoft Word, Power point, Sigma plot, etc.

Working Experience

Sept. 1987-June 1992	Research Assistant, National Vaccine and Serum Institute, Beijing, China. Working on the quality control and Pertussis Vaccine Purification of T-cell receptors and production of polyclonal antibodies.
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