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Pre-existing intimal hyperplasia and overexpression of TGF-β1 in saphenous vein grafts before myocardial revascularization in humans: Implications for aortocoronary saphenous vein graft disease

DISSERTATION

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Abbreviations

AEC	ε-Amino-Ethyl-Carbazole
Anti-Smad	Antagonistic Smad
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Grafting
cDNA	Complement DNA
Co-Smad	Common-Partner Smad
Cy	Cysteine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
HEPES	N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid
IEL	Internal Elastic Lamina
IGFII-R	Type II Insulin Like Growth Factor Receptor
IH	Intimal Hyperplasia
ITA	Internal Thoracic Artery
IMR	Intima Media Ratio
ITI	Intimal Thickness Index
kD	Kilo Dalton
LAP	Latency-Associated Protein
LTBP	Latent TGF-β-Binding Protein
M6P	Mannose-6-Phosphate
mRNA	Messenger RNA
PBS	Phosphated Buffered Saline
PL	Plasmin
PLg	Plasminogen

RII	TGF-β-Receptor type II
R-Smad	Receptor-regulated Smad
Smad	TGF-β Signal Transduction Proteins
SMC	Smooth Muscle Cell
SV	Saphenous Vein
Tgase	Transglutaminase
TGF-β1	Transforming Growth Factor-β1
TSP	Thrombospondin
TβR	TGF-β-Receptor
uPA	Urokinase Plasminogen Activator
uPA-R	Urokinase Plasminogen Activator Receptor
VSMC	Vessel Smooth Muscle Cell

Content

1. Introduction

Coronary artery disease accounts for half of all deaths in the developed world and approximately 400,000 coronary artery bypass grafting (CABG) procedures are performed annually in the United States alone. The advent and maturation of surgical techniques for the treatment of coronary artery disease using myocardial revascularization ranks among the most famous medical achievements of last century. The first aortocoronary saphenous vein (SV) graft implantation in a human being by Garrett and colleagues in May 1967 [Garrett et al. 1973] and the subsequent pioneering work of Favaloro [Favaloro 1969] ushered in the era of surgical revascularization for the global epidemic of ischemia heart disease.

Coronary artery bypass graft surgery is initially effective in relieving the symptoms of intractable angina and prolongs survival. The internal thoracic artery (ITA) graft is generally regarded as the standard conduit for CABG because of its excellent late patency and low prevalence of histopathologic changes [Loop 1996]. Underlying the unique benefits of ITA grafting is the striking resistance of this conduit to atheroma, with 10-year patency rates well above 90%. The structural and physical properties of the ITA confer this resistance to atheroma. Though in spite of this trend, the autologous saphenous vein has remained an important and convenient conduit used for more than 70% of grafts for multiple coronary diseases, because it has various advantages, such as availability and graft length, over the ITA graft [Fitzgibbon et al. 1996]. However saphenous vein grafts are prone to develop segmental stenoses, and vein graft failure is one of the primary reasons for coronary artery bypass reoperation. During the first year after bypass surgery, up to 15% of saphenous vein grafts occlude, between 1 and 6 years, the graft attrition rate is 1% to 2% per year, and between 6 and 10 years, it is 4% per year. By 10 years after surgery only 60% of vein grafts are patent (Figure1.1) and only 50% of patent vein grafts are free of significant stenosis [Fitzgibbon et al. 1996].

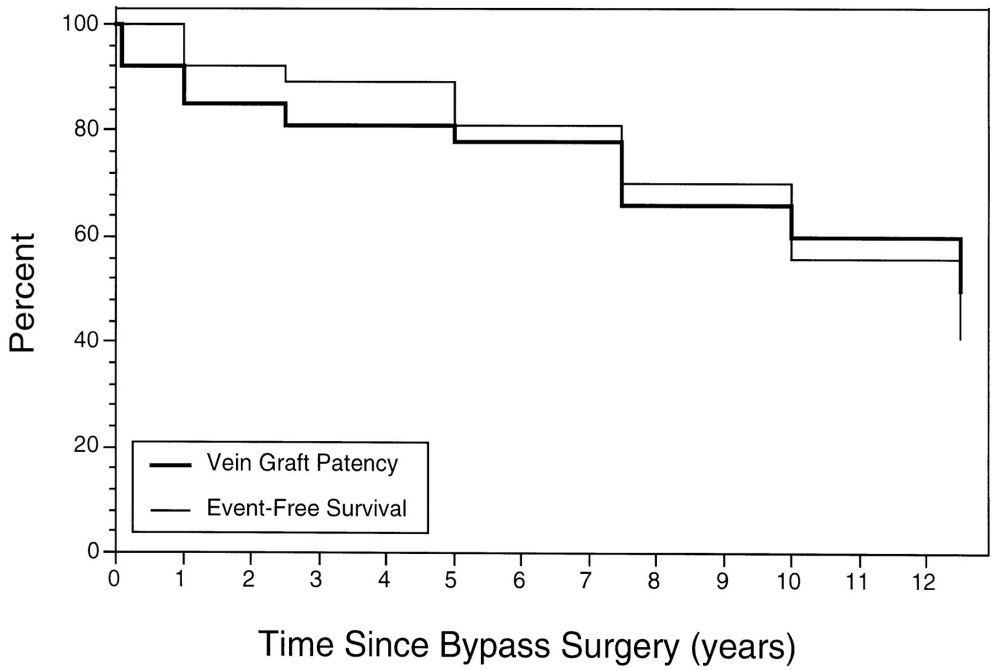

Figure 1.1 Long-term graft patency and event-free survival after saphenous vein bypass surgery. [Motwani et al. 1998]

The sequences of the histopathologic changes that occur in the saphenous vein grafts in humans can be divided into three stages: acute thrombotic changes (within 1 month), intimal hyperplasia (IH) (within 1 year), and atherosclerotic changes (after the first postoperative year). The stenosis and occlusion of the vein graft principally result from intimal hyperplasia and its secondary changes, such as superimposed atheroma and its rupture [Cox et al. 1991]. Vein grafts are susceptible to intimal hyperplasia and atherosclerosis for two main reasons. First, explanted vein grafts are prone to vessel wall ischemia owing to a loss of functional vasa vasorum; regrowth of these nutrient vessels appears as early as 5 days after graft placement, but a rich network sufficient to provide blood flow to the thickened neointima takes up to 6 months to develop [Ohta et al. 1997]. Second, an arterialized vein graft is subject to an acute, marked increase in wall stress. Intimal hyperplasia is the universal response of a vein graft to insertion into the arterial circulation and is considered to result from migration and proliferation of intimal smooth muscle cells (SMC) originating from the media [Bryan et al. 1996]. This phenomenon may represent an adaptation of a vein graft imposed by arterial hemodynamic environment, but, on the other hand, it underlies luminal stenosis and its secondary changes, which may lead to vein graft

failure. Histological studies have confirmed a range of pre-existing pathological changes, notably varying degree of saphenous vein wall thickening owing to pre-existing intimal hyperplasia, SMC hypertrophy and inflammatory cell infiltration and, these "pre-existing changes" before CABG have been linked to postoperative vein graft disease [Marin et al. 1993, Wilson et al. 1997]. Therefore, it is important to know the underlying mechanisms of this structural change that occurs in the saphenous vein grafts for establishment of the prophylactic and therapeutic strategies and to prevent vein graft stenosis and occlusion.

Growth factors are involved in the alteration of the vessel wall and endothelium. They induce and regulate numerous cell functions during the process of intimal hyperplasia and atherogenesis. They may act in cell recruitment and migration, cell proliferation, and the control of protein synthesis including extracellular matrix (ECM) proteins. Transforming growth factor-β1 (TGF-β1) is a homodimeric peptide integral in the early progression of intimal hyperplasia, especially smooth muscle cell proliferation, extracellular collagen matrix synthesis, and receptor formation important in binding of extracellular components.

1.1 General information of TGF-βs

The transforming growth factor-β (TGF-β) cytokines are a multi-functional family that exerts a wide variety of effects on both normal and transformed mammalian cells [Zimmerman et al. 2000]. TGF-βs were discovered by De Larco and Todaro [De Larco et al. 1978] in 1978. Originally called "sarcoma growth factors," they were first isolated from the supernatant fluids of Moloney MuSV-transformed mouse 3T3 fibroblasts and described as a family of growth-stimulating polypeptides. The further nomenclature "transforming growth factor" was adopted because of the ability of these molecules to confer functional properties associated with neoplastic transformation on untransformed indicator fibroblasts [De Larco et al. 1978, Roberts et al. 1980]. In the early 1980s, several members of the TGF-β superfamily were identified and subsequently shown to play important roles in many diseases. Now,

many components of the TGF-β family have been identified [Simmons et al. 1995]. It is widely recognized that TGF-βs play critical roles in growth and development, inflammation and host immunity. Indeed, these factors can exert either a positive or a negative effect on proliferation, differentiation, or cell death, depending on the developmental stage of the target cell, its in vivo environment, or the medium used for in vitro studies. More recently, efforts to understand how TGF-βs exert their effects led to identification of TGF-β-receptors and several downstream signaling pathways activated by this family of growth factors.

1.2 Biological effects of TGF-βs

Three highly similar isoforms of TGF-β, called TGF-β1, -β2, and -β3, were identified and cloned from mammals between 1985 and 1988. Two other isoforms, called TGF-β4 and -β5, have been cloned, respectively, in chicken [Jakowlew et al. 1988] and in xenopus. Each isoform is encoded by a distinct gene and is expressed in both a tissue-specific and a developmentally regulated fashion. TGF-β1 messenger RNA (mRNA) is expressed in endothelial, hematopoietic, and connective-tissue cells; TGF-β2 mRNA in epithelial and neuronal cells; and TGF-β3 mRNA primarily in mesenchymal cells. During development, TGF-β1 and TGF-β3 are expressed early in structures undergoing morphogenesis, and TGF-β2 is expressed later in mature and differentiating epithelium. All three isoforms are highly conserved in mammals, suggesting a critical biologic function for each isoform. These isoforms differ in their binding affinity for TGF-β receptors, and the deletion of individual isoforms in mice results in different phenotypes. Knock-out of TGF-β1 gene does not yield any gross embryonic abnormality, but newborn mice exhibit lethal multi-focal inflammation, starting three weeks after birth. Therefore, TGF-β1 appears to act as an essential regulator of the immune system. The phenotype of mice lacking TGF-β3 is different, as these animals are born with cleft palate and die within a few hours, showing the requirement for TGF-β3 for normal embryonic development [Proetzel et al. 1995].

Numerous reports have showed direct or indirect involvement of TGF-βs in

processes such as wound healing, angiogenesis, hematopoiesis, mammary gland development, bone metabolism, and skin formation as well as in multiple pathologies such as inflammatory and fibrotic disease and tumor development [Roberts et al. 1990]. Increases or decreases in the production of TGF-βs have been linked to many disease states, including atherosclerosis and fibrotic disease. TGF-βs influence atherogenesis via different mechanisms. TGF-βs can act as potent inducers of matrix biosynthesis [Mustoe et al. 1987, Waltenberger et al. 1993a, Waltenberger et al. 1996] and show immunosuppressive and anti-inflammatory effects [Shull et al. 1992, Waltenberger et al. 1993b]. In vivo, TGF-βs enhance intimal thickening, an effect that could experimentally be provoked by overexpression or administration of TGF-β1 [Nabel et al. 1993, Kanzaki et al. 1995] and that could be suppressed by the use of a neutralizing antiserum against TGF-β1 [Waltenberger 1997, Wolf et al. 1994]. The role of TGF-βs in fibrosis may be related to its effect on extracellular matrix. TGF-βs both enhances the synthesis of ECM components and downregulates the proteolytic activities responsible for ECM catabolism.

1.3 Structure of TGF-βs: latent and active forms

TGF-βs are synthesized as precursor proteins that are modified intracellularly prior to secretion. One of the most relevant intracellular modifications is the cleavage of the C-terminal pro-region from the N-terminal portion of the protein. The pre-pro-peptides require a 2-step process to give rise to active TGF-βs [Gentry et al. 1988, Lawrence et al. 1991, Gleizes et al. 1997]. A first proteolytic cleavage leads to the elimination of a hydrophobic signal peptide, in the N-terminal region of the precursor protein, yielding pro-TGF-β. A second cleavage leads to the separation of the pro-region of the protein from the TGF-β mature peptide (Figure 1.2).

In the case of TGF-β1, the TGF-β1 gene encodes for a 390 amino acid chain precursor (pre-pro-peptide). The signal peptide corresponds to amino acids 1 to 29, the pro-region of the precursor to amino acids 30 to 278, and the mature peptide to amino acids 279 to 390 [Gentry et al. 1988]. The bioactive forms of TGF-βs (25 kD)

are composed of 2 mature peptide chains linked by disulfide bonds. TGF-βs are usually produced as homodimers (TGF-β1.1, -β2.2, -β3.3), but natural heterodimeric molecules have also been identified (TGF-β1.2 and -β2.3) [Ogawa et al. 1992].

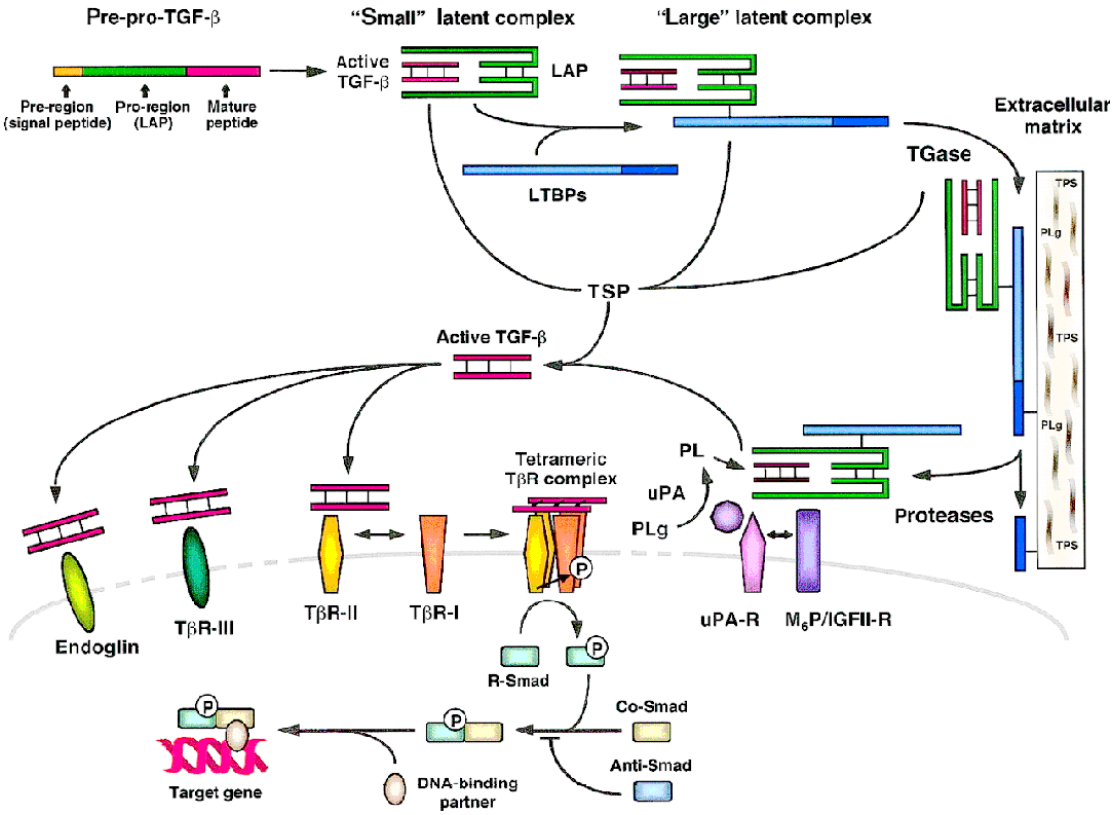

Figure 1.2 Structure, latency, activation, and receptors of TGF-β. LAP indicates latency-associated peptide; LTBP, latent TGF-β binding protein; M6P/IGFII-R, mannose-6-phosphate/type II insulin-like growth factor receptor; PLg, plasminogen; PL, plasmin; Smad, TGF-β signal transduction proteins; Anti-Smad, antagonistic Smad; Co-Smad, common-partner Smad; R-Smad, receptor-regulated Smad; Tgase, transglutaminase; TβR-I, -II, -III, TGF-β receptor type I, II, III; TSP, thrombospondin; uPA, urokinase plasminogen activator; and uPA-R, uPA receptor. [Fortunel et al. 2000]

Once processed, TGF-βs are released by cells as latent complexes, which are biologically inactive. Two forms of latent complexes have been described, the "small" and "large" latent complexes, as shown in Figure 1.2. In the small latent complex, one molecule of mature, active TGF-β is noncovalently associated with one disulfide-bonded pro-peptide dimer, called latency-associated protein or (LAP) (74 kD in the case of TGF-β1). In the large latent complex, LAP is linked by disulfide bonds to one member of a family of high-molecular-weight proteins (125-160 kD), called latent

TGF-β-binding proteins or LTBPs [Miyazono et al. 1988, Wakefield et al. 1988].

The secretion and activation of TGF-βs is regulated by their association with LAP and LTBPs. Over the past few years, four members of the LTBPs family have been identified: LTBP2 [Gibson et al. 1995, Moren et al. 1994, Bashir et al. 1996, Fang et al. 1997], LTBP3 [Yin et al. 1995, Li et al. 1995] and LTBP4, in addition to the prototype LTBP1 first sequenced in 1990 [Giltay et al. 1997]. Three of the LTBPs are expressed in a variety of isoforms as a consequence of alternative splicing. Recent data indicating that differential expression of LTBP1 isoforms occurs during the development of coronary heart disease is considered, together with evidence that modulation of LTBPs function, and hence of TGF-βs activity, is associated with a variety of cancers. LAPs are sufficient to render the mature homodimer inactive, and removal of both the LAPs and LTBPs or modulation of their interaction is essential for any of the TGF-β isoforms to function.

1.4 Biological effects of LTBPs

LTBPs are required for efficient secretion and correct folding of TGF-βs. The cDNAs of various related LTBPs have been cloned [Moren et al. 1994, Yin et al. 1995, Saharinen et al. 1998]. In the erythroleukemic cell line HEL, the synthesis of LTBPs has been found to be coordinated with that of TGF-β small latent complex to form the large latent complex, which is then secreted by cells [Miyazono et al. 1991]. The secreted large latent complexes associate covalently with the extracellular matrix via the N-terminal of TGF-βs. The LTBPs confer to this complex the ability to associate with the extracellular matrix, permitting the storage of TGF-βs in the ECM [Munger et al. 1997, Taipale et al. 1997]. LTBPs belong to the fibrillin-LTBP family of extracellular matrix proteins, which have a typical repeated domain structure consisting mostly of epidermal growth factor (EGF)-like repeats and characteristic eight cysteine (8-Cys) repeats. Currently four different LTBPs and two fibrillins have been identified. LTBPs contain multiple proteinase sensitive sites, providing means to

solubilize the large latent complex from the extracellular matrix structures. LTBPs are now known to exist both as soluble molecules and in association with the extracellular matrix. An important consequence of this is LTBP-mediated deposition and targeting of latent, activatable TGF-βs into extracellular matrices and connective tissues. LTBPs have a dual function, they are required both for the secretion of the small latent TGF-βs complex as well as directing bound latent TGF-βs to extracellular matrix microfibrils. However, it is not known at present whether LTBPs are capable of forming microfibrils independently, or whether they are a part of the fibrillin-containing fibrils. Most LTBPs possess RGD-sequences, which may have a role in their interactions with the cell surface. Analyses of the expressed LTBPs have revealed considerable variations throughout the molecules, generated both by alternative splicing and utilization of multiple promoter regions. Because LTBPs exist in several isoforms, the bioavailability of TGF-βs and its specific targeting to different organs may be regulated in part by the formation of different types of large latent complexes. The release of latent TGF-β from the extracellular matrix is triggered by proteolytic enzymes such as chymase, elastase, and plasmin, which are able to cleave LTBPs [Taipale et al. 1994, Taipale et al. 1995, Olofsson et al. 1995].

1.5 Activation of latent TGF-βs

TGF-βs are unique among growth factors in their potent and widespread actions. Almost every cell in the body has been shown to produce some form of TGF-βs, and almost every cell has receptors for TGF-βs. Therefore, it becomes apparent that this growth factor must be tightly regulated to prevent disease. The mechanisms of regulation of TGF-βs are extensive and complex.

Extracellular activation of the latent TGF-β latent complex is a critical process in the regulation of TGF-βs functions in vivo. The interaction between TGF-β and LAP is not covalent and can be disrupted in vitro by heat treatment or acidification [Brown et al. 1990]. Although physicochemical variables such as local acidification [Jullien et al. 1989] or exposure to active oxygen species [Barcellos-Hoff, et al. 1996] may

participate in the regulation of TGF-β activation, mechanisms involving proteolytic cleavage or conformational modification of LAP are more likely to operate in vivo.

Different mechanisms of activation are presented in Figure 1.2. Plasmin has been shown to promote the activation of latent TGF-β by proteolytic nicking within the N-terminal region of the LAP [Lyons et al. 1988, Lyons et al. 1990]. This disrupts noncovalent bonds and results in the release of active TGF-β [Lyons et al. 1990]. In monocytes, macrophages, and endothelial cells, cellular activation of latent TGF-β has been reported to involve the mannose-6-phosphate/type II insulin like growth factor receptor (M6P/IGFII-R) and the urokinase plasminogen activator receptor (uPA-R) [Dennis et al. 1991, Nunes et al. 1995, Godar et al. 1999]. One proposed mechanism is that M6P/IGFII-R, which binds latent TGF-β, complexes with uPA-R. Plasmin would be generated locally from plasminogen through the action of uPA and would allow the production of active TGF-β. Another enzyme, transglutaminase (Tgase), has been identified as an effector controlling both the deposition rate of LTBPs in the matrix [Verderio et al. 1999] and the cell-surface activation of latent TGF-β [Nunes et al. 1995, Kojima et al. 1993]. Tgase-mediated activation of latent TGF-β depends on interactions with specific residues of LTBP [Nunes et al. 1997]. Thrombospondin (TSP), a platelet α-granule and extracellular matrix protein, has also been shown to promote activation of latent forms of TGF-β. In contrast to what has been described for plasmin and transglutaminase, TSP-mediated activation of latent TGF-β occurs through a cell- and protease-independent mechanism, as demonstrated by in vitro studies. This effector induces a conformational change of LAP, which then results in the release of active TGF-β [Schultz-Cherry et al. 1994a, Schultz-Cherry et al. 1994b]. The role of TSP in the activation of latent TGF-β in vivo has been demonstrated by the generation of TSP-null mice [Crawford et al. 1998]. In this model, major histologic abnormalities have been observed and correlated with a lack of active TGF-β. These defects could be reversed by a treatment that activates TGF-β. Regulation of the glycosylation of LAP has also been proposed to participate in the control of TGF-β latency [Miyazono et al. 1989]. Recently, activation of latent TGF-β via interaction with the integrin α5β6 has been reported, providing a novel possible

mechanism regulating the activation of TGF-β [Munger et al. 1999].

1.6 TGF-β receptors (TβRs)

Original cross-linking studies revealed that TGF-β binds to several cell-surface proteins termed type I (TβR-I; 53 kD), type II (TβR-II; 70-100 kD), and type III receptors (TβR-III; 200-400 kD) according to their approximate sizes [Massague et al. 1990]. The type III receptors are the most abundant. They function by binding TGF-β and then transferring it to type I and type II signaling receptors [Cheifetz et al. 1992, Lopez-Casillas et al. 1993]. The nonsignaling role of type III receptors is shared by other abundant proteoglycan cytokine receptors, including syndecan for fibroblast growth factor, p75 for nerve growth factor, and the type II receptor for insulin-like growth factor. Another TGF-β receptor that is abundant on endothelial cells, endoglin, contains a transmembrane region and a cytoplasmic tail homologous to the type III receptor. The type III receptors, betaglycan and endoglin, might modulate ligand access to the signaling receptors, whereas receptors I and II are responsible for signal transduction. Both molecules contain serine-threonine protein kinases in their intracellular domains that initiate intracellular signaling by phosphorylating several transcription factors known as Smads.

Serine/threonine kinase receptors are glycoproteins composed of a short extracellular ligand-binding region, a single transmembrane segment, and an intracellular region with serine/threonine kinase activity. Based on structural and functional features, the receptor family can be divided into two subgroups termed type I and type II receptors. Although both types of receptors have functional extracellular ligand-binding and cytoplasmatic kinase regions, physical and functional interaction between the two receptors is required for signal transduction. Transfection studies with Mv1Lu cells that lack responsiveness to TGF-β provided evidence for the heteromeric receptor model, which postulates that receptor I requires receptor II for ligand binding, and receptor I and the kinase activity of receptor II are required for signaling [Wrana et al. 1992]. According to this concept, TGF-β binds first to the type

II receptor. This interaction triggers subsequent recruitment of the type I receptor into the complex, providing the basis for downstream signaling. This mode of sequential ligand binding is characteristic for TβR-II, ActR-II, ActR-IIb, and AMR-II, which are able to bind ligand on their own [Massague et al. 1998]. The alternative cooperative binding mode is typical of bone morphogenetic proteins (BMPs) receptors, which only bind ligand with high affinity when expressed together [Liu et al. 1995, Rosenzweig et al. 1995]. In view of the dimeric structure of the ligands, the receptor complexes are probably tetrameric structures of two receptor I and two receptor II molecules [Yamashita et al. 1994].

A general mechanism for TGF-β signaling is outlined in Figure 1.2. TGF-β binds either to type III receptors, which then present TGF-β to type II receptors, or directly to type II receptors. Once activated by TGF-β, type II receptors recruit, bind, and transphosphorylate type I receptors, thereby stimulating their protein kinase activity. The activated type I receptors phosphorylate Smad2 or Smad3, which then binds to Smad4. The resulting Smad complex then moves into the nucleus, where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of many genes. Because TβR-I and TβR-II exist in multiple forms, it has been proposed that homodimeric and heterodimeric forms of TGF-β may induce a specific response by interacting with different heterotetrameric receptor complexes of specific signaling capacities [Piek et al. 1999].

In addition to TβR-I and TβR-II, accessory TGF-β receptors, not necessarily required for signal transduction, can be expressed at the surface of cells responsive to TGF-β [Piek et al. 1999]. The TβR-III, a membrane-anchored proteoglycan [Segarini et al. 1988, Lopez-Casillas et al. 1991], and the 180-kD glycoprotein endoglin could function as regulators of ligand access to the signaling receptors. Although the precise roles of endoglin and TβR-III are not fully understood, some of their properties suggest distinct functions for these two TGF-β receptors. First, TβR-III is able to interact with TGF-β1, -β2, and -β3 [Kaname et al. 1996], whereas endoglin interacts with TGF-β1 and -β3 but not efficiently with TGF-β2 [Barbara et al. 1999]. Second, the role of TβR-III could be to present TGF-β to TβR-II and facilitate their binding

[Lopez-Casillas et al. 1993, Moustakas et al. 1993], whereas endoglin appears to diminish rather than enhance TGF-β responses in certain cell types [Lastres et al. 1996]. Third, endoglin and TβR-III possess a specific cell-distribution pattern, which may confer the ability of different cell types to respond differentially to TGF-β1, -β2, and -β3. Other cell-surface receptors have been identified for their ability to bind TGF-βand are classified as TβR-IV to TβR-VI [Massague et al. 1992]. However, the function of these other receptor families in TGF-β signaling remains to be clarified.

1.7 The aim of the present study:

1. The different incidence of pre-existing pathological changes in saphenous vein and internal thoracic artery grafts, and its relationship to the development of aortocoronary bypass vein graft disease.
2. The morphometric characteristics of saphenous vein and internal thoracic artery.
3. The expression of the TGF-β1, LTBP-1, RII and fibronectin in saphenous vein and internal thoracic artery grafts, the effects of them in the development of intimal hyperplasia.
4. To evaluate the correlation between the staining intensity of growth factors and the indices of intimal hyperplasia.
5. To explore the possible implications of these findings in cardiac surgery.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetone	Merck, Darmstadt, Germany
Bovine Serum Albumin (BSA)	Biomol, Hamburg, Germany
Bretschneider cardioplegic solution	Köhler Chemie, Alsbach-Hähnlein, Germany
Dimethylsulphoxide (DMSO)	Sigma, Deisenhofen, Germany
ε-Amino-Ethyl-Carbazole(AEC)	Sigma, Deisenhofen, Germany
Glacial acetic acid	Carl Roth, Karlsruhe, Germany
Haemalaun	Chroma, Münster, Germany
Hydrochloric acid	J.T.Baker, Deventer, Holland
N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES)	Carl Roth, Karlsruhe, Germany
30% Hydrogen peroxidase	J.T.Baker, Deventer, Holland
Methanol	Merck, Darmstadt, Germany
Mikroskopie	Merck, Darmstadt, Germany
Na-Acetat	Merck, Darmstadt, Germany
Phosphate buffered saline (PBS) tablets	Calbiochem, CA. U.S.A.
Sodium hydroxide	J.T.Baker, Deventer, Holland
Tissue freezing medium	Leica Instruments, Nussloch, Germany
Urea	Sigma, Deisenhofen, Germany

The other chemicals not mentioned above were obtained from the following companies:

Merck, Darmstadt, Germany

Sigma, Deisenhofen, Germany

2.1.2 Reagent Kit for immunoassay

Quantikine human TGF-β1 immunoassay kit: R&D Systems Inc, MN, U.S.A.

2.1.3 Reagent Kits for immunohistochemistry

Avidin-biotin blocking kit: Vector Laboratories, CA. U.S.A.

Vectastain avidin-biotin-peroxidase complex kit: Vector Laboratories, CA. U.S.A.

2.1.4 Antibodies and blocking normal serum for immunohistochemistry

Anti-human-LAP (TGF-β1)	R&D Systems, MN, U.S.A.
Anti-human-TGF-β-RII	R&D Systems, MN, U.S.A.
Anti-human-TGF-β banding protein 1	Santa Cruz, Heidelberg, Germany
Anti- fibronectin	Santa Cruz, Heidelberg, Germany
Anti-goat IgG-B	Santa Cruz, Heidelberg, Germany
Anti-mouse IgG-B	Santa Cruz, Heidelberg, Germany
Normal goat serum	Santa Cruz, Heidelberg, Germany
Normal donkey serum	Santa Cruz, Heidelberg, Germany

2.1.5 Instruments

Precleaned Polysine Microslides	R.Langenbrinck, Emmendingen, Germany
Sqare cover Glasses	Merck, Darmstadt, Germany
4°C refrigerator	Liebeherr, Germany
-80°C refrigerator	Heruaeus, Hanau, Germany
Centrifuger	Labofuge GL, Heruaeus, Hanau, Germany

Microtome	Leica, Jung CM 1800, Bensheim, Germany
Light microscope	Axiophot, Zeiss, Oberkochen, Germany
Photo camera	Leica, M35, Bensheim Germany
Photo film	Fujichrome, Tokyo, Japan
Microplate	R&D Systems, MN, U.S.A
Multi-channel pipette	Eppendorf, Hamburg, Germany
Squirt bottle	Eppendorf, Hamburg, Germany
Manifold dispenser	Eppendorf, Hamburg, Germany
12 mm x 75 mm polypropylene tubes	Fridenhousa, Greinen, Germany

2.1.6 Buffer and solution

1∗PBS

PBS	1 tablet
ddH_2O	1000ml

1% BSA

BSA	1g
1×PBS	100ml

30% Haemalaun

Haemalaun	30ml
ddH_2O	70ml

AEC stocking solution

AEC	100mg
DMSO	60ml

AEC working solution

AEC stocking solution	6ml
Acetat-buffer	54ml
Hydrogen peroxidase	15µl

1M Acetat-buffer

Na-Acetat	82.03g
ddH_2O	1000ml

20mM Acetat-buffer pH5.2

1M Acetat-buffer	10ml
ddH_2O	490ml

2.2 Methods

2.2.1 Patients selection and clinical parameters

From August to November 2000, 50 consecutive patients who received selective coronary artery bypass grafting operation at the University Hospital of Ulm were included in our study. Informed consent was obtained from all patients according to the permission issued by the Ethical Committee of the University Hospital of Ulm.

There were 38 (76%) male and 12 (24%) female patients. The mean age at operation was 65.1 ± 7.9 years (range 48 to 79 years). Catheterization and selective coronary artery angiogram were routinely performed pre-operatively in all patients to assess heart function and severity of coronary artery disease as well as native valve structure and function. The mean ejection fraction (EF; %) was 61.3±13.1% (range 27 to 81%). The mean LVEDP (mmHg) was 16.4±7.0 mmHg (range 5 to 38mmHg). Thirteen patients (26%) were in New York Heart Association (NYHA) functional class I and II; thirty-four patients (68%) were in class III and three patients (6%) were in class IV. The vessels diseased ranged from 1 to 3 arteries, including 9 left main stem stenosis. Concomitant cardiac diseases included aortic valve stenosis (n=3), aortic valve insufficiency (n=2), and mitral valve insufficiency (n=2). Cardiovascular risk factors were distributed as follows: diabetes (n=15), smoking (21), arterial hypertension (19), hyperlioproteinemia (24). 16 patients suffered from chronic obstructive pulmonary disease (COPD). The mean pre-operative serum creatinine level was 107.86±20.54 μmol/L. All patients accepted standard bypass operation with cardiopulmonary bypass (CPB) except one patient who received off-pump bypass operation. The mean CPB time was 112±42 minutes (range 38 to 246 minutes), the mean ischemia time was 63±26 minutes (ranged from 13 to 160 minutes), and the mean vessel bypassed was 2.78±0.47. Additional aortic valve replacement operations were performed in five patients. The other concomitant valve diseases did not have an indication for operative treatment.

2.2.2 Preparation of saphenous veins and internal thoracic arteries

A segment (approximately 2 cm) of SV and ITA (if available) grafts were obtained immediately after conventional surgical harvesting and collected in cold Bretschneider cardioplegic solution containing sodium heparin, 4 IU/ml. Tissue specimens were transported at 4°C to the laboratory and immediately snap frozen in cold tissue freezing medium cooled by liquid nitrogen and stored at -80°C until processed for immunohistochemistry.

2.2.3 Collection of blood samples

Blood samples were obtained pre-operatively from all patients. Blood was drawn from an indwelling venous line into a serum separator tube (SST) and samples were allowed to clot for 30 minutes at room temperature. For complete release of TGF-β1, samples were incubated overnight at 2-8°C before centrifugation. Samples were then centrifuged for 10 minutes at approximately 1000 rpm. Serum was removed and assayed immediately or stored at - 80°C until investigation.

2.2.4 Histological and Morphometric analysis

46 SV segments and 27 ITA distal segments were collected from 50 patients. There were 20 paired SV and ITA vessel segments from 20 patients. Multiple transverse slides of the vessels were cut at 5 μm. The slides were examined by two independent viewers blinded to the clinical data and origin of the vessels. SV and ITA vessel segments were recorded as normal if there was no cellular or stromal tissue between the endothelium and the internal elastic lamina (IEL). SV and ITA segments were recorded as with various degrees of intimal hyperplasia when the vessels showed fibrous tissue or intimal cells between the endothelium and the IEL.

For the purposes of quantitative measurement, 73 vessels were suitable for morphometric analysis. The morphometric measurement of vessels was analyzed with a Nikon microscope and a color image analysis system (Digital image analyzer, software from Bilany Consultants Gmbh). Each measurement was performed three times in a blinded fashion. The intimal area, medial area, width of the intima, and

width of the media were measured.

Previous investigators (Ruengsakulrach et al. 1999) have suggested that the intima-to-media ratio is the most sensitive method available for grading intimal hyperplasia and atherosclerosis. In this study, two methods were used to evaluate the degree of intimal thickening and atherosclerosis: (1) intimal thickness index (ITI) = intimal area/medial area, and (2) intima-to-media ratio (IMR) = width of intima at maximal intimal thickness/width of media at maximal intima thickness. The severity indices were calculated from the most severely diseased section of the specimens using these indices (Figure 2.1).

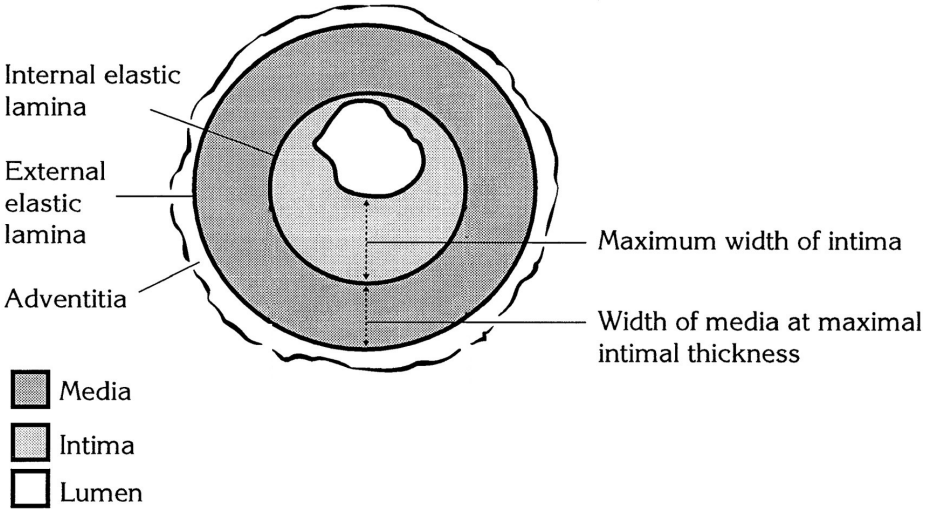

Figure 2.1 Schematic drawing depicting indices used to evaluate the severity of intimal hyperplasia. ITI=intimal area/medial area; and IMR=width of intima at maximal intimal thickness/width of media at maximal intima thickness. The area rather than the thickness of the intima was measured to allow accurate evaluation of eccentric or irregular disease. [Ruengsakulrach et al. 1999]

2.2.5 Immunohistochemistry

The vascular segments were cryosectioned into 5 μm slices, fixed in ice-cold 100% acetone for 10 min, air-dried and stored at -80°C. Endogenous peroxidase activity was quenched by 0.3% hydrogen peroxide in cold methanol. An avidin-biotin blocking kit (Vector Laboratories) was used to prevent non-specific reactions between endogenous biotin and the detection system. To prevent unspecific binding of the

primary antibodies, the sections were blocked with PBS containing 2% normal donkey serum (for anti-LAP and anti-RII) or 2% normal goat serum (for anti-LTBP1 and anti-Fibronectin). The sections were incubated overnight with the primary antibody at -4°C in a humidified chamber. The Vectastain avidin-biotin-peroxidase complex kit (Vector Laboratories) was used for detection of the immunoreaction. It contained either biotinylated donkey anti-goat or goat anti-mouse antibodies and an avidin-biotin peroxidase complex. We have stained control sections with the serum of the species the first antibodiy was derived from to confirm the specificity of our staining. The immunocomplexes were visualized by aminoethylcarbazole and 0.3% hydrogen peroxide. The sections were counterstained with 30% haemalaun.

Protocol for Immunohistochemistry:

1. Take out the specimen slides from the -80°C refrigerator, then put in the PBS solution wash for 5 minutes.
2. Rinse the slides in cold Methanol with 0.3% hydrogen peroxidase for 15 minutes. 600 µl 30% hydrogen peroxidase add in 60 ml cold Methanol.
3. Wash with three changes of PBS for 3 minutes each.
4. Blocking with 2% serum derived from the same species in which the secondary antibody is raised for 20 minutes. Use sufficient solution to cover the specimen (approximately 100 µl per slide is usually adequate).
5. Remove blocking serum from slides.
6. 1 drop cold Avidin-solution for each slide for 15 minutes.
7. Wash with PBS for 3 minutes.
8. 1 drop cold Biotin-solution for each slide for 15 minutes.
9. Aspirate solution from slides.
10. Cover each slide with 100 µl optimal concentration of the primary antibody, then the slides were incubated over-night at 4°C in a humidified chamber.
11. Remove antibody from slides.
12. Wash with three changes of PBS for 3 minutes each.
13. Blocking with 2% serum for 10 minutes.
14. Aspirate serum from slides, incubate with the optimal concentration of the second

antibody for 60 minutes in room temperature.

15. At the same time, begin to prepare the ELITE solution, it must be incubated for 30 minutes before use.
16. Wash with PBS for 3 minutes.
17. Rinse the slides in AEC working solution for 6 minutes.
18. Wash with three changes of water for 3 minutes each.
19. The slides were counterstained with 30% haemalaun for 10 seconds.
20. Wash with water for 3 minutes.
21. Slides were air-dried, and then covered with glyceringelatine and small square cover glasses.

2.2.6 Microscopic evaluation

The stained slides with immunohistochemistry were evaluated by two independent viewers blinded to the origin of the vessels and to the clinical data. Photomicrographs were taken with a light microscope (Axiophot, Zeiss, Oberkochen, Germany). A semiquantitative scheme as described earlier [Waltenberger et al. 1993] was applied to evaluate tissue sections referring to the intensity of the immunostaining.

0 = no staining

1 = weak staining

2 = moderate staining

3 = strong staining

2.2.7 Quantitative immunoassay of TGF-β1 serum levels

TGF-β soluble receptor type II, which binds TGF-β1, has been pre-coated onto a microplate and binds all TGF-β1 present in the sample. Standards and samples were pipetted into the wells. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 was added to the wells to form a "sandwich" with the TGF-β1-TβR-complex immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate

solution was added to the wells and color develops in proportion to the amount of TGF-β1 bound in the initial step. The color development was stopped and the intensity of the color was measured.

2.2.7.1 Reagents to activate serum samples

2.5 N acetic acid/10 M urea (250 ml): to 100 ml deionized water, add 150.2 g urea. Mix well until dissolved. Slowly add 35.9 ml of glacial acetic acid. Mix well. Bring final volume to 250 ml with deionized water.

2.7 N NaOH/1 M HEPES (250 ml): to 140 ml deionized water, add 67.5 ml 10 N NaOH. Mix well. Add 59.5 g HEPES. Mix well. Bring final volume to 250 ml with deionized water.

2.2.7.2 TGF-β1 sample activation procedure

To activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the Quantikine TGF-β1 immunoassay, the activation procedure outlined below was followed. Samples were assayed after neutralization (pH 7.2 - 7.6) and polypropylene test tubes were used.

1. To 0.1 ml serum, add 0.1 ml 2.5 N acetic acid/10 M urea.
2. Mix well.
3. Incubate 10 minutes at room temperature.
4. Neutralize the acidified sample by adding 0.1 ml 2.7 N NaOH/1 M HEPES.
5. Mix well.
6. Prior to the assay, dilute the activated serum sample 10-fold with Calibrator Diluent RD6M.

The concentration read off the standard curve must be multiplied by the dilution factor, 30.

For each new lot of acidification and neutralization reagents, measure the pH of several representative samples was measured after neutralization to ensure that it was within pH 7.2-7.6. The volume and corresponding dilution factor of the neutralization reagent was adjusted as needed.

2.2.7.3 Reagent preparation

All reagents were brought to room temperature before use.

1. Wash Buffer: if crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals completely dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.
2. Substrate Solution: color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µl of the resultant mixture was required per well.
3. TGF-β1 Standard: referred to vial label for reconstitution volume. Reconstitute the TGF-β1 Standard with Calibrator Diluent RD6M. This reconstitution produce a stock solution of 2000 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Polypropylene tubes were used. Pipette 500 µl of the appropriate Calibrator Diluent into each tube. Stock solution was used to produce a dilution series (below). Mixed each tube thoroughly before the next transfer. The undiluted standard served as the high standard (2000 pg/ml). The appropriate Calibrator Diluent served as the zero standard (0 pg/ml). Any unused reconstituted TGF-β1 Standard was discarded after use.

2.2.7.4 Assay procedure

All reagents and samples were brought to room temperature before use. It was recommended that all samples and standards were assayed in duplicate.

1. Prepare all reagents, working standards, and activated samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, resealed.
3. Add 200 µl of Standard or activated sample per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature.
4. Aspirate each well and wash, repeat the process twice for a total of three washes.

Wash by filling each well with Wash Buffer (400 µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step was essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.

5. Add 200 µl of TGF-β1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1.5 hours at room temperature.
6. Repeate the aspiration/wash as in step 4.
7. Add 200 µl of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Protect from light.
8. Add 50 µl of Stop Solution to each well. If color change did not appear uniform, gently tape the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. Wavelength correction was set to 540 nm or 570 nm. Readings made directly at 450 nm without correction may be higher and less accurate.

2.3 Statistical analysis

Data were presented as median (range), or mean ± SEM. In morphometric analysis, to compare the different incidence of intimal hyperplasia in SV and ITA grafts, the Pearson chi square test was performed.

Because of non-normal distribution of the morphometric parameters, the multiple comparisons between the different groups were evaluated with the use of Mann-Whitney U test. For 20 paired SV and ITA grafts, Wilcoxon test was performed to evaluate the morphometric data.

To compare the expression of peptides between the different groups, statistical calculations were performed on the semiquantitative immunostaining data applying student *t*-test for independent samples.

Correlations between the TGF-β1 staining intensity and the indices of intimal

hyperplasia were evaluated using Spearman's correlation coefficients (ρ).

Results were considered significant with a probability level of $p<0.05$. All the statistical calculations were performed using the SPSS (version 8.0) for windows.

3. Results

3.1 Morphologic data

3.1.1 Occurrence of intimal hyperplasia in saphenous vein and internal thoracic artery grafts

Intimal hyperplasia is defined as cellular or stromal tissue existing between the endothelium and the internal elastic lamina. Among 46 SV vessel segments examined, various degrees of pre-existing intimal hyperplasia were detected in 31 grafts (67.4%) (Figure 3.1, Figure 3.2A). In other 15 SV segments (32.6%), no pre-existing intimal hyperplasia was detected. (Figure 3.2B).

Among 27 ITA vessel segments examined, there were 8 (29.6%) grafts with mild to moderate intimal hyperplasia (Figure 3.1, Figure 3.2C), and other 19 (70.4%) grafts without intimal hyperplasia (Figure 3.2D). The incidence of intimal hyperplasia was significantly higher in SV grafts than in ITA grafts ($p<0.05$)(Figure 3.1). The pathological changes that occurred in ITA grafts tended to be focal intimal hyperplasia, while the pathological changes in SV grafts were more often concentric and serious than in ITA grafts.

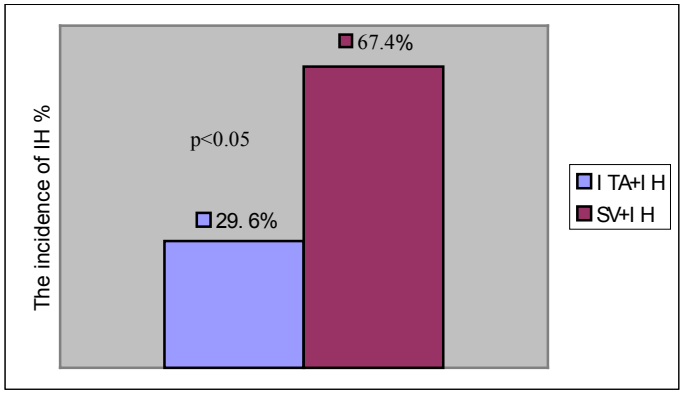

Figure 3.1 The incidence of intimal hyperplasia was significantly higher in SV grafts than in ITA grafts.

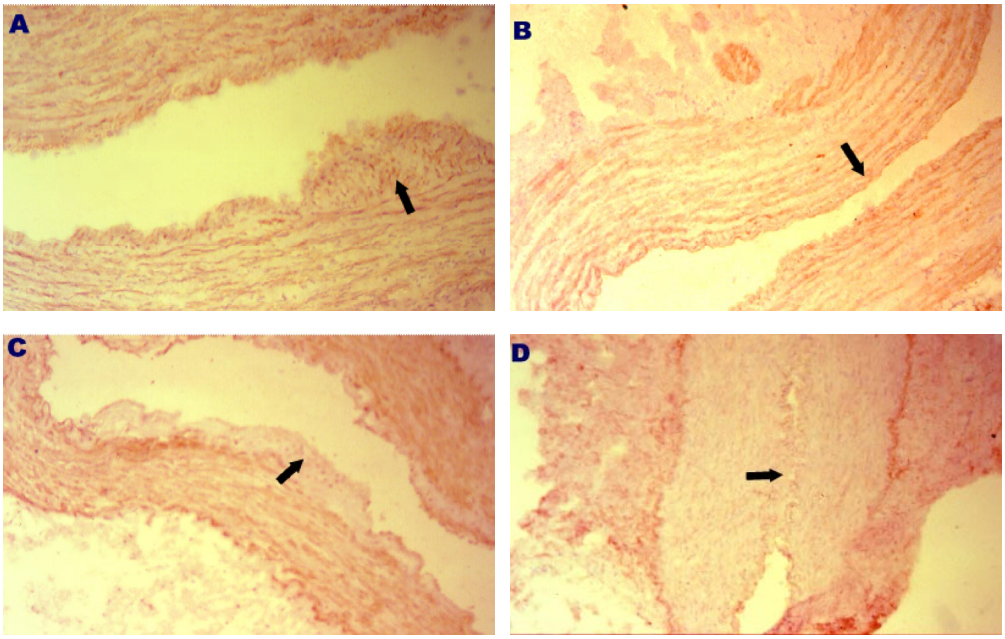

Figure 3.2 Micrographs of SV and ITA grafts with and without intimal hyperplasia. A. SV with intimal hyperplasia (arrow), original magnification, ×10. B. Normal SV without intimal hyperplasia, no cellular or stromal tissue between endothelium and intima (arrow), original magnification, ×10. C. ITA with intimal hyperplasia (arrow), original magnification, ×10. D. Normal ITA with characteristic single IEL. The intima is a very thin layer consisting mainly of endothelium resting on IEL (arrow), original magnification, ×10.

3.1.2 Comparison of morphological data in paired SV and ITA vessel segments

In 20 patients, we have collected both the SV and the ITA grafts. So there were 20 paired SV and ITA segments collected from the same patients. Intimal hyperplasia was detected in 13 (65%) of SV segments and 6 (30%) of corresponding ITA segments. The incidence of intimal hyperplasia was significantly higher in SV grafts than in ITA grafts in the same patient ($p<0.05$) (Figure 3.3). All patients showing IH in ITA grafts also had IH in their SV grafts.

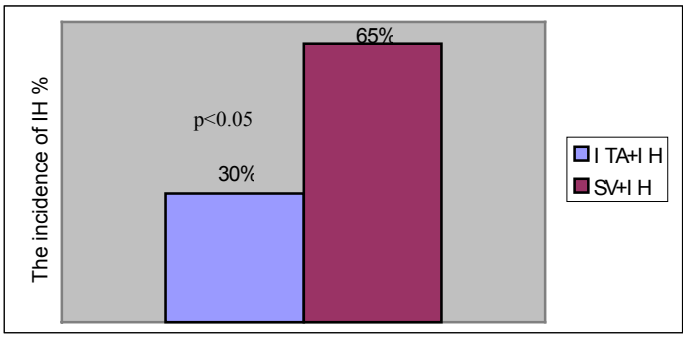

Figure 3.3 Incidence of intimal hyperplasia in 20 paired SV and ITA grafts

3.2 Morphometric analysis

3.2.1 Increased intimal and medial thickness in saphenous vein grafts

We used a Nikon microscope and a color image analysis system to perform the morphometric analysis. The average of intimal area, medial area, width of the intima, and width of the media were calculated.

Morphometric analysis of SV and ITA grafts showed that the intimal area, medial area, width of the intima, and width of the media were significantly greater in SV than in ITA grafts (all p<0.001) (Table 3.1). The SV grafts had a thicker intima and media than those of the ITA grafts.

Table 3.1 Comparative morphometric data of SV and ITA grafts

	intimal area (mm^2)	medial area (mm^2)	intima width (mm)	media width (mm)
SV				
Median	0.78	3.20	0.16	0.41
Range	0.12~7.21	1.24~7.59	0.01~0.90	0.19~0.94
ITA				
Median	0.22	1.01	0.03	0.27
Range	0.06~1.16	0.46~3.32	0.01~0.25	0.15~0.62
p value	<0.001	<0.001	<0.001	<0.001

3.2.2 Comparison of indices of intimal hyperplasia (ITI vs IMR)

According to the morphologic findings, all the vessel segments were divided into four groups. Intimal hyperplasia is defined as any cellular or stromal tissue existing between the endothelium and the IEL. The indices of IH were calculated from the most severely diseased section of the specimens using the following formulas: ITI=imtimal area / medial area, and IMR=width of intima at maximal intimal thickness / width of media at maximal intima thickness.

group 1: SV with IH group (n=31)

group 2: SV without IH group (n=15)

group 3: ITA with IH group (n=8)

group 4: ITA without IH group (n=19)

The ITI and the IMR of group 1 and 3 were significantly greater than those of group 2 and group 4 (all $p<0.05$) (Table 3.2), showing that both ITI and IMR are sensitive methods for grading intimal hyperplasia. The IMR of group 1 was significantly greater than those of all other three groups, while there was no difference of the ITI between group 1 and group 3, indicating that the IMR maybe more sensitive in comparing intimal disease in different vascular beds.

Table 3.2 Comparative morphometric data in four groups

	SV+IH	SV without IH	ITA + IH	ITA without IH
	median (range)	median (range)	median (range)	median (range)
ITI	0.32 [bd]	0.15 [ac]	0.30 [bd]	0.15 [ac]
	(0.11~2.68)	(0.06~0.48)	(0.17~0.38)	(0.06~0.48)
IMR	0.43 [bcd]	0.24 [acd]	0.13 [abd]	0.07 [abc]
	(0.09~2.93)	(0.02~1.34)	(0.09~0.62)	(0.02~0.52)

a $p<0.05$ versus SV+IH group
b $p<0.05$ versus SV without IH group
c $p<0.05$ versus ITA +IH group
d $p<0.05$ versus ITA without IH group

3.2.3 Data analysis in paired saphenous vein and internal thoracic artery grafts

We have compared morphometric data in 20 paired SV and ITA grafts. There was no difference of the ITI between SV and ITA grafts, but the IMR of SV was significantly greater when compared to ITA grafts ($p<0.001$) (Table 3.3). All patients that had an IH in their ITA grafts also showed an IH in their SV grafts.

There was no correlation between the ITI of SV and the ITI of ITA, and the IMR of SV was also not correlated with the IMR of ITA.

Table 3.3 Data analysis in paired saphenous vein and internal thoracic artery

	SV	ITA	p value
Morphometric data	median (range)	median (range)	
ITI	0.22 (0.03~1.80)	0.16 (0.06~0.48)	p>0.05
IMR	0.31 (0.02~2.91)	0.08 (0.03~0.25)	p<0.001

3.3 Immunohistochemistry

3.3.1 Expression of TGF-β1 in saphenous vein and internal thoracic artery grafts

TGF-β1 expression was found to be significantly higher in the endothelium and intimal layers in SV with IH as compared to SV and ITA without IH. The expression of TGF-β1 was also significantly higher in intimal layers in ITA with IH as compared to ITA without IH. There was no difference in TGF-β1 expression in all layers between SV with IH and ITA with IH (Figure 3.4, Table 3.4), indicating that diseased vessels with IH produce more of the proatherogenic growth factor TGF-β.

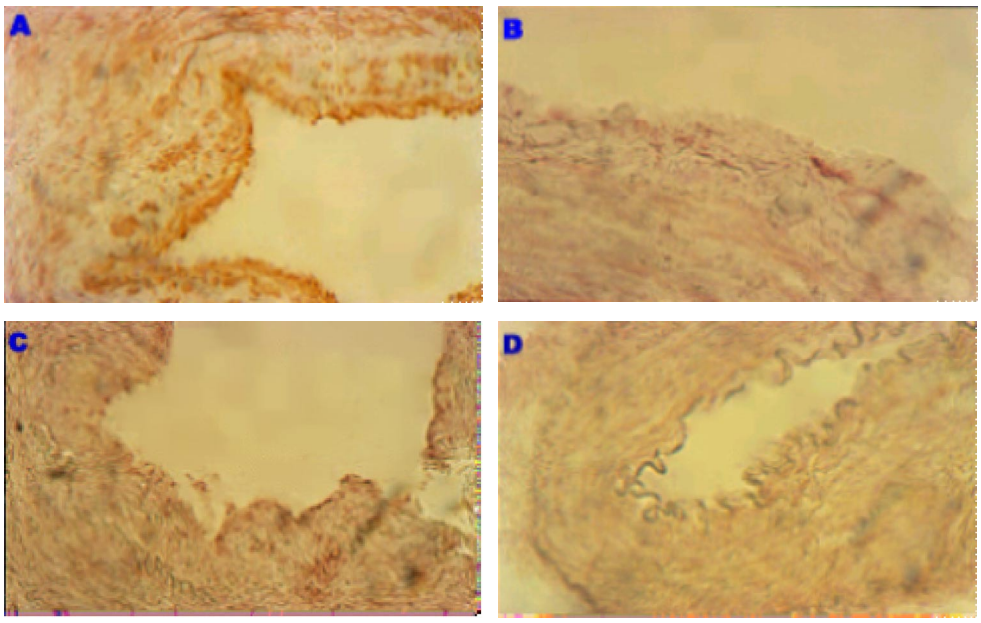

Figure 3.4 Expression of TGF-β1 in SV and ITA grafts with or without IH. A. In SV with IH, TGF-β1 staining intensity was higher in endothelial and intimal layers as compared to SV without IH, original magnification, ×20. B. In SV without IH, the TGF-β1 staining intensity was lower, original magnification, ×20. C. In ITA with IH, TGF-β1 was expressed higher staining intensity in intimal layers as compared to ITA without IH, original magnification, ×20. D. In ITA without IH, the TGF-β1 staining intensity was lower, original magnification, ×20.

3.3.2 Expression of LTBP-1 in saphenous vein and internal thoracic artery grafts

The expression of LTBP-1 was found to be significantly higher in the intimal and medial layers of SV and ITA with IH as compared to SV and ITA without IH. LTBP-1 expression was significantly higher in the media and in vasa vasorum of SV without IH when compared to ITA without IH. There was no difference of LTBP-1 expression in all layers between SV without IH and ITA without IH (Figure 3.5, Table 3.4). Vessels with an intimal hyperplasia show an increased staining intensity against LTBP-1. LTBP-1 expression was generally higher in saphenous veins than in internal thoracic arteries.

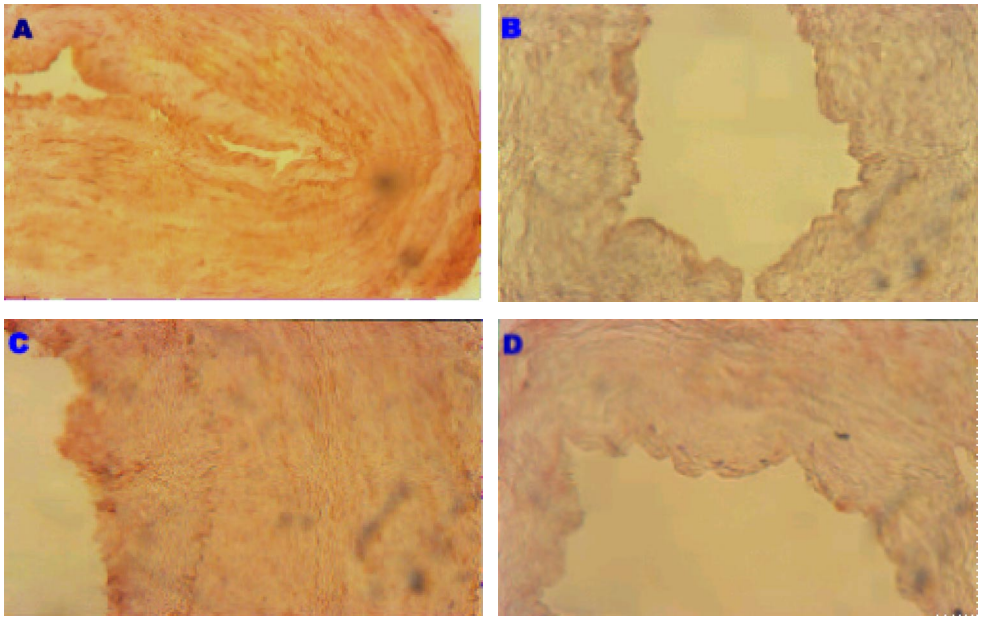

Figure 3.5 Expression of LTBP-1 in SV and ITA grafts with or without IH. A. In SV with IH, staining intensity for LTBP-1 was increased in intimal and medial layers as compared to SV without IH, original magnification, ×20. B. In SV without IH, LTBP-1 expression was lower, original magnification, ×20. C. In ITA with IH, LTBP-1 expression was higher in intimal and medial layers as compared to ITA without IH, original magnification, ×20. D. In ITA without IH, LTBP-1 expression was lower, original magnification, ×20.

3.3.3 Expression of TGF-β type II receptor (RII) in saphenous vein and internal thoracic artery grafts

RII expression was found to be significantly higher in endothelial and intimal layers in SV with IH as compared to SV without IH. The expression of RII was significantly higher in the media and the vasa vasorum in ITA with IH as compared to ITA without IH. RII expression was significantly higher in endothelial and intimal layers in SV with IH as compared to ITA with IH. RII expression was higher in all layers except adventitia in SV without IH than in ITA without IH (Figure 3.6, Table 3.4). Saphenous veins have an overall higher expression level of the TGF-β type II receptor than internal thoracic arteries.

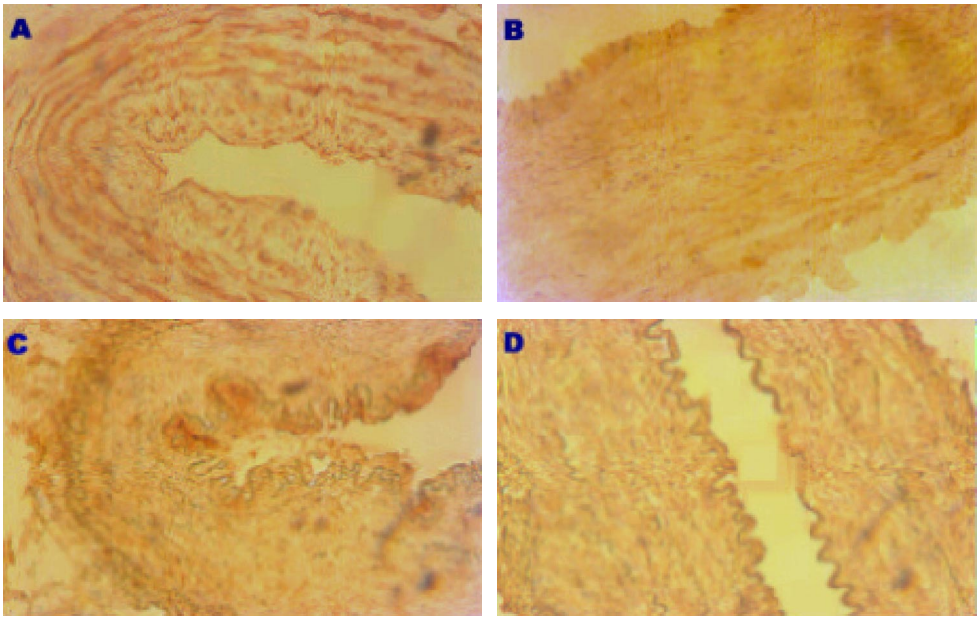

Figure 3.6 Expression of RII in SV and ITA grafts with or without IH. A. In SV with IH, RII staining intensity was higher in endothelial and intimal layers as compared to SV without IH, original magnification, ×20. B. In SV without IH, RII staining intensity was lower, original magnification, ×20. C. In ITA with IH, RII staining intensity was increased in medial and vasa vasorum as compared to ITA without IH, original magnification, ×20. D. In ITA without IH, RII was less expressed, original magnification, ×20.

3.3.4 Expression of fibronectin in saphenous vein and internal thoracic artery grafts

We looked at fibronectin as a marker for the potency of TGF-β to stimulate the extracellular matrix production. Fibronectin staining intensity higher in the media of all four groups. There was no difference of fibronectin expression between SV and ITA with and without IH. Fibronectin expression was higher in vasa vasorum in SV with and without IH as compared to ITA without IH (Figure 3.7, Table 3.4).

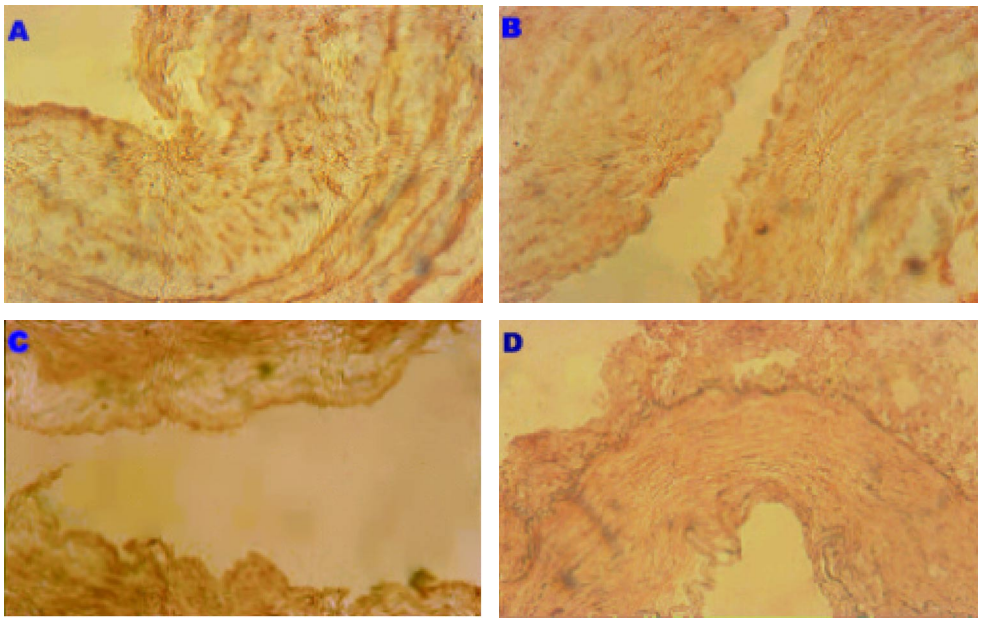

Figure 3.7 Expression of fibronectin in SV and ITA grafts with or without IH. A. In SV with IH, fibronectin expression was higher in the media and in vasa vasorum as compared to ITA with and without IH, original magnification, ×20. B. In SV without IH, fibronectin expression was found to be higher in the vasa vasorum as compared to ITA without IH, original magnification, ×20. C. In ITA with IH, expression of fibronectin was not differet from other groups, original magnification, ×20. D. ITA without IH, original magnification, ×20.

Table 3.4 Immunohistochemical staining scores in SV and ITA grafts

	endothelium	intima	media	adventitia	vasa vasorum
SV+IH					
TGF-β1	1.82±0.12 [bd]	1.97±0.13 [bd]	2.23±0.13 [d]	0.11±0.04 [d]	2.05±0.15 [d]
LTBP-1	2.21±0.10 [d]	1.79±0.13 [bd]	2.02±0.12 [bd]	0.71±0.09 [d]	1.92±0.11 [d]
RII	1.71±0.14 [bcd]	1.87±0.11 [bcd]	2.37±0.10 [d]	0.23±0.06 [d]	2.11±0.13 [d]
Fibronectin	1.50±0.12	1.63±0.15	2.45±0.10	0.26±0.07 [d]	2.34±0.11 [d]
SV without IH					
TGF-β1	1.23±0.13 [a]	1.27±0.13 [a]	2.10±0.18 [d]	0.13±0.06 [d]	1.77±0.19
LTBP-1	2.03±0.16	1.13±0.10 [ac]	1.37±0.15 [ad]	0.80±0.14 [d]	1.80±0.13 [d]
RII	1.20±0.14 [ad]	1.23±0.15 [a]	2.10±0.12 [d]	0.23±0.15 [d]	1.87±0.12 [d]
Fibronectin	1.43±0.14	1.77±0.19	2.37±0.14	0.37±0.16	2.10±0.14 [d]
ITA+IH					
TGF-β1	1.38±0.18	1.63±0.21 [d]	2.19±0.27 [d]	0.06±0.06 [d]	1.75±0.31
LTBP-1	2.06±0.22	1.88±0.16 [bd]	1.81±0.31 [d]	1.19±0.21	1.88±0.26 [d]
RII	0.88±0.16 [a]	1.19±0.25 [a]	2.56±0.15 [d]	0.56±0.18	2.06±0.20 [d]
Fibronectin	1.69±0.15	1.50±0.31	2.06±0.24	0.50±0.28	1.56±0.26
ITA without IH					
TGF-β1	0.95±0.17 [a]	0.63±0.15 [ac]	1.34±0.16 [abc]	0.39±0.10 [abc]	1.32±0.15 [a]
LTBP-1	1.68±0.16 [a]	1.03±0.19 [ac]	0.92±0.16 [abc]	1.18±0.10 [ab]	1.39±0.14 [abc]
RII	0.68±0.15 [ab]	0.76±0.17 [a]	1.37±0.14 [abc]	1.00±0.13 [ab]	1.39±0.15 [abc]
Fibronectin	1.66±0.15	1.71±0.17	2.13±0.17	0.66±0.15 [a]	1.53±0.16 [ab]

a $p<0.05$ versus SV+IH group

b $p<0.05$ versus SV without IH group

c $p<0.05$ versus ITA +IH group

d $p<0.05$ versus ITA without IH group

3.4 The correlation analysis between the indices of intimal hyperplasia and TGF-β1 expression in SV and ITA grafts

We evaluated the correlations between the indices of intimal hyperplasia and the local expression of TGF-β1, LTBP-1, RII and fibronectin in all vessel layers using Spearman's correlational analysis. The TGF-β1 expression correlated with the presence of an intimal hyperplasia in SV and ITA: the TGF-β1 staining intensity in the intima weakly correlated with the ITI in SV grafts ($\rho=0.317$, $p<0.05$), but did not correlate with the IMR. In ITA grafts, the data showed that the TGF-β1 staining intensity in the intima correlated with the IMR ($\rho=0.536$, $p<0.05$) and ITI ($\rho=0.428$, $p<0.05$). There were no other correlations of peptide expression with the indices of intimal hyperplasia.

There was a weak negative correlation between the TGF-β1 staining intensity in the intima of SV grafts and the corresponding TGF-β1 serum levels ($\rho=-0.316$, $p<0.05$). There was no such correlation in ITA grafts.

In our patients, there was no correlation of the presence of an intimal hyperplasia with the classical risk factors, nor with sex, age or TGF-β serum levels.

4. Discussion

4.1 Aortocoronary saphenous vein graft disease and morphologic findings

Aortocoronary saphenous vein graft disease, with its increasing clinical sequelae, presents an important and unresolved dilemma in clinical bypass surgery practice [Motwani et al. 1998]. Vein graft atherosclerosis is a distinct pathological entity that differs from native atherosclerosis in several aspects. Vein graft atherosclerosis is a rapidly progressive disease and tends to be diffuse throughout the length of the vein graft, concentric, and friable with a poorly developed fibrous cap and little evidence of calcification. In contrast, native vessel atheroma is proximal, focal, eccentric and non-friable with a fibrous cap and frequent calcification [Motwani et al. 1998, Kalan et al. 1990, Ratliff et al. 1989]. Histologically, vein graft atheroma show more foam cells and inflammatory cells than native atheroma [Ratliff et al. 1989]. Intimal hyperplasia is the foundation for later development of graft atherosclerosis and is the major disease process in venous grafts between 1 month and 1 year post-operatively. Intimal hyperplasia follows a pathogenic sequence: initially, medial smooth muscle cells proliferate in response to a number of cytokines and growth factors released from platelets and macrophages including TGF-β. This is followed by migration of smooth muscle cells into the intima with subsequent further proliferation. Later, synthesis of extracellular matrix by activated smooth muscle cells leads to a progressive increase in intimal fibrosis [Motwani et al. 1998, Dilley et al. 1992]. Risk factors predisposing to vein graft disease are different from the classical risk factors for atherosclerosis and comprise a diameter <1.5 mm of the grafted vessel, a proximal stenosis less than 70% and time after bypass-surgery. The classical cardiovascular risk factors as there are cigarette smoking, hyperlipidemia, arterial hypertension and diabetes mellitus are also risk factors for vein graft disease [Motwani et al. 1998].

Early vein graft failures may be attributed to technical reasons, and very often to graft thrombosis caused by a combination of alterations in the vessel wall, changes in

blood rheology and altered flow dynamics [Adcock et al. 1984, Motwani et al. 1998], whereas late failures are thought to result from the development of intimal hyperplasia and subsequent atheroma and luminal narrowing [Veith et al. 1990]. The fate of human saphenous vein as an arterial conduit has been extensively studied in post mortem analyses of occluded grafts [Motwani et al. 1998, Kalan et al. 1990]. Post mortem changes show a high incidence of intimal hyperplasia and vein graft disease.

In our series, the incidence of intimal hyperplasia before arterialisation in SV was 67.4% as compared to 29.6% in ITA. Consistently, in the 20 paired SV and ITA vessel segments from 20 patients, the incidence of intimal hyperplasia in SV was 65% compared with 30% in ITA. The intimal hyperplasia that is present before arterialisation is likely to predispose for later development of vein graft disease. The different incidence of pre-existing intimal hyperplasia between SV and ITA grafts can partially explain the definite long-term superiority of the ITA grafts over the SV grafts.

We have now shown that the pathogenic mechanisms that later lead to vein graft disease were present even before bypass surgery as intimal hyperplasia occurred in about 2/3 of saphenous veins, and increased growth factor expression was found in the intimal hyperplastic areas even before arterialisation. Only few morphological studies have noted the presence of pre-existing pathological changes in the human saphenous vein [Panetta et al. 1992, Davies et al. 1993, Marin et al. 1991, Veith et al. 1980]. So far, very few reports have addressed the idea that the severity of these pre-existing changes in venous grafts may be a predictor for the development of vein graft stenosis [Marin et al. 1993, Wilson et al. 1997]. In this study, we investigated the incidence of intimal hyperplasia, its histological and morphometric characteristics, and the expression of TGF-β1 as a potential factor promoting intimal hyperplasia and graft atherosclerosis.

4.2 Morphometric analysis

Our morphometric measurement showed that SV grafts have a thicker intima and

media both in area and width than those of ITA grafts. From our morphological findings, it is evident that the pre-existing pathological changes are more prevalent and serious in SV grafts than in ITA grafts. This can be caused by the different structural and physical properties between SV and ITA grafts. Numerous reports have confirmed that when used as a bypass conduit, ITA grafts have a much higher long-term patency rate when compared with SV grafts. The underlying mechanism is the striking resistance of the ITA to intimal hyperplasia and other pathological changes [Motwani et al. 1998]. Our and others data suggest that the ITA is the conduit of choice for the routine bypass operation.

The ITI and the IMR are sensitive methods of comparing intimal disease in different vascular beds [Kay et al. 1976, Malhotra et al. 1996, Kaufer et al. 1997]. Of these two indices, the ITI should be more accurate because areas of intima and media were used rather than width. It allows accurate evaluation of eccentric or irregular vascular disease. In our study, we confirmed the sensitivity of the ITI and IMR. They were much higher in SV and ITA with IH as compared to SV and ITA without IH. However, some authors [Ruengsakulrach et al. 1999] have already noted the limitations of these two indices. When comparing two different vascular beds, an assumption is made that any thickening of the media (the denominator in both ITI and IMR) occurs to the same degree in both vascular beds, as for example in hypertension. But this hypothesis may not be valid all the time. Our morphometric data showed that the SV have a thicker intima and media than the ITA. Thus, the greater thickness of the media of the SV compared with the ITA may invalidate the use of the ITI and the IMR as comparative indices to compare these two different vascular beds.

In our opinion, the ITI and the IMR are more valid to compare the intimal hyperplasia in the same kind of vessel among the different patients but should not be used in two different types of vessels. Then, in our series we used the ITI and IMR to further grade the intimal hyperplasia of the same kind of vessel. We found that the ITI and the IMR of the SV and ITA with IH were significantly greater than those of the SV and ITA without IH. Thus these indices can facilitate us to divide the SV and ITA grafts into groups with or without IH for evaluating the vessels.

4.3 Immunohistochemistry

Nowadays, bypass surgery with autologous SV grafts is necessary for many patients with diffuse coronary artery disease, but the failure rate of these grafts after 1 year approaches 15% [Fitzgibbon et al. 1996, Campeau et al. 1984, Bourassa et al. 1991]. The development of intimal hyperplasia and atherosclerosis in the grafted vessel may lead to occlusion of the graft vessel. The hallmarks of intimal hyperplasia are mononuclear cell infiltration, smooth muscle cell proliferation, and extracellular matrix deposition. The molecular mechanisms of this process are not fully understood; no successful clinical interventions have been identified.

Accumulated data suggest that TGF-β1 is involved in all the aspects of the intimal hyperplasia forming process (Waltenberger 1997, Porreca et al, 1997, Fortunel et al. 2000). This growth factor has been shown to be produced by vascular endothelial cells, smooth muscle cells, and inflammatory cells. Several *in vitro* studies have successfully linked TGF-β1 to the development of intimal hyperplasia by stimulating smooth muscle cell proliferation and deposition of extracellular matrix [Gibbons et al, 1992, Antonelli-Orlidge et al, 1989].

In the present study, we have compared the differential expressions of TGF-β1, its binding protein, LTBP-1; its receptor, RII; and one of its effector proteins, fibronectin in SV and ITA grafts before bypass operation. The results have demonstrated a significantly increased expression of TGF-β1 and LTBP-1 in SV and ITA grafts with IH when compared to SV and ITA without IH, especially in intimal and medial layers. The expression of RII was also found to be significantly higher in endothelium, intimal and medial layers in SV and ITA with IH as compared to SV and ITA without IH. Fibronectin was expressed strongly in the media in all four groups. Our data also demonstrate a correlation between the TGF-β1 staining intensity in the intima of SV and the ITI of SV grafts, suggest an essential role of TGF-β1 in the development of intimal hyperplasia *in vivo*, and thus may play a causative role in the formation of vein graft disease.

The location of TGF-β1 is not necessarily the site of action of TGF-β1 as this

growth factor has to go through a multi-step procedure of activation and has to leave the cytoplasm of the producer cell. The mechanisms by which latent TGF-β1 is activated *in vivo* are not fully understood yet. Proteases seem to play an important role, particularly plasmin [Odekon et al. 1994]. It has been suggested that LTBP-1 may participate in the activation of TGF-β1 by concentrating the latent TGF-β1 on the cell surface where activation occurs. After secretion, the large latent form of TGF-β1 associates with the extracellular matrix via LTBP-1, and the release of the small latent TGF-β1 complex from the extracellular matrix is a consequence of the proteolytic cleavage of LTBP-1 [Taipale et al. 1997]. Taken together, our current understanding proposes a model of activation in which latent TGF-β1 is released from the extracellular matrix by proteases cleaving LTBP-1, subsequently localized to cell surface by either LAP or LTBP-1, and activated by cell-associated plasmin [Nakajima et al. 1997, Taipale et al. 1997, Munger et al. 1997]. An accumulation of LTBP-1 over time due to a longer half life of LTBP-1 versus TGF-β1 has been suggested earlier [Waltenberger et al. 1993a, Miyazono et al. 1991]. We therefore used LTBP-1 as a marker for TGF-β1 production, which should be more sensitive than the presence of latent TGF-β1 itself. Antibodies against active TGF-β1 are not yet available. Bioassays have to be used to prove the presence of active TGF-β1. Due to the limited availability of the tissue, we could not use these bioassays.

4.4 Inhibiting intimal hyperplasia: future perspectives

A multifaceted strategy aimed at prevention of vein graft disease is emerging, elements of which include: continued improvements in surgical technique; more effective antiplatelet drugs; increasingly intensive risk factor modification, in particular early and aggressive lipid-lowering drug therapy; and a number of evolving therapies, such as gene transfer and nitric oxide donor administration, which target vein graft disease at an early and fundamental level.

We have noticed prevalent pre-existing pathological changes in SV grafts before bypass operation, and TGF-β1 is an important growth factor confirmed to be

influential in the generation of hyperplastic lesion. Initial studies showed when antibodies against TGF-β1 were used in an *in vivo* model, that there is a marked reduction in the formation of intimal hyperplasia [Wolf et al. 1994]. However, novel approaches to this persistent problem are certainly warranted, and the growing area of vascular gene therapy provides one potential paradigm by which to apply these therapies. Recently, many investigators have developed murine genetic models in which genes are over-expressed, deleted, or mutated [Zou et al. 1998]. Such mouse models have considerable advantages over other animal systems in that they overcome the need to administer factors or their inhibitors, which can be problematic and often difficult to quantify. When used with vein autografts in mice subjected to targeted gene deletion, the model could provide a powerful tool for dissecting the relative contributions of each genes, including TGF-β1, low-density lipoprotein receptors, endothelial adhesion molecules and nitric oxide synthase, in the development of intimal hyperplasia.

By using this model, the underlying mechanisms of how TGF-β1 is involved in modulating the formation of the intimal hyperplasia will be clarified, and we believe that significant progress in understanding the pathogenesis of vein graft disease may be seen in the near future. These could be helpful for the design of future therapeutic strategies to inhibit the intimal hyperplasia in human SV grafts used for bypass operation and to improve long-term patency rate.

4.5 Conclusions: Implications in cardiac surgery

Most studies about vein graft disease are done post mortem investigating occluded or stenosed bypass vessels. These studies show intimal hyperplasia in high percentage in the stenosed grafts and also an increased growth factor expression. Saphenous vein grafts after arterialisation were found to express high numbers of receptors for basic fibroblast growth factor [Nguyen et al. 1994]. We have now demonstrated that intimal hyperplasia as the first pathogenic mechanism in the development of vein graft disease even starts in the native saphenous vein. In these

areas of intimal hyperplasia, the expression of the proatherogenic growth factor TGF-β1, its binding protein and its receptor is increased, again already before arterialisation. In contrast, these changes were found in corresponding internal thoracic arteries in a much lower percentage. Therefore, patients with intimal hyperplasia in their saphenous vein may be at a higher risk for the development of vein graft diseae. In this study, we were not able to correlate the presence of an intimal hyperplasia with the classical risk factors nor with age, sex or local overexpression of TGF-β1 which maybe due to the limited number of patients.

Established strategies for preventing vein graft disease are cessation of smoking, lipid-lowering drug therapy, and use of antithrombotic agents. It is a salutary but sobering tenet that the only certain strategy at present for preventing vein graft disease is to avoid the problem by implanting an arterial graft – preferrably the internal thoracic artery - rather than a venous graft as a conduit for choice whenever possible. For patients needed multi-vessel bypass, it is recommended to carefully choose the vein graft using any available methods, histology, intravascular ultrasound and angioscopy.

5. Summary

Aortocoronary saphenous vein graft disease still remains an important and unresolved dilemma in clinical bypass surgery. The knowledge about this disease is derived from post mortem studies in stenosed or occluded bypass grafts. Few studies have investigated native saphenous veins. TGF-β1 is known to act as a proatherogenic, profibrotic growth factor. We have therefore investigated the incidence of intimal hyperplasia in saphenous veins (SV) and internal thoracic arteries (ITA) before coronary artery bypass grafting (CABG) using morphometric analysis. Then we applied immunohistochemistry to assess the expression of TGF-β1, its binding LTBP1, its receptor RII, and fibronectin as a marker for production of extracellular matrix proteins.

We found that SV grafts have a thicker intima and media both in area and width than ITA grafts. The intimal hyperplasia is more prevalent and serious in SV grafts compared to ITA grafts. By evaluating indices for intimal hyperplasia, namely ITI (intimal area / medial area) and IMR (intimal width / medial width), we defined subgroups of SV and ITA grafts with or without intimal hyperplasia. We found a significantly higher expression of TGF-β1, LTBP-1 and RII mainly in intimal and medial layers in SV and ITA grafts with intimal hyperplasia as compared to SV and ITA grafts without intimal hyperplasia. TGF-β1 may therefore be of importance in mediating the development of intimal hyperplasia *in vivo*.

In summary, in this study we demonstrated that the development of intimal hyperplasia occurs in a high degree (67.4%) in SV grafts before arterialisation, and in a lower percentage also in internal thoracic arteries (29.6%). Growth factor expression was increased in areas of intimal hyperplasia. Thus, locally active TGF-β1 might be involved in mediating intimal thickening in SV before arterialisation and might even aggravate this phenomenon after arterialisation contributing to a rapidly progressive vein graft disease.

6. References

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