

BASIC RESEARCH

Transforming growth factor- β in graft vessels: histology and immunohistochemistry

Shi-Min Yuan,¹ Yan-Qing Wang,¹ Yi Shen,¹ Hua Jing¹

¹Department of Cardiothoracic Surgery, Jinling Hospital, School of Clinical Medicine, Nanjing University, No. 305 Zhongshan East Road, Nanjing 210002, Jiangsu Province, People's Republic of China. ²Department of Cardiology, The 81 Hospital of Nanjing, No. 34, Biao 34, Yanggongjing, Nanjing 210002, Jiangsu Province, People's Republic of China.

OBJECTIVES: The biological functions of transforming growth factor- β signaling that involves Smad proteins have not been previously investigated with respect to coronary artery bypass grafts. The aim of the present study was to observe the immunostaining of proteins that are related to this signaling pathway.

METHODS: Fifteen remnants of coronary artery bypass grafts, including nine saphenous veins, three radial arteries and three mammary arteries, were collected from 12 patients who were undergoing coronary artery bypass. Hematoxylin and eosin, Masson's trichrome, and immunohistochemical staining of transforming growth factor- β_1 , type I receptor of transforming growth factor- β , Smad2/3, Smad4, and Smad7 were performed.

RESULTS: The saphenous veins showed more severe intimal degeneration, more severe smooth muscle cell proliferation and more collagen deposition than the arterial grafts, as evidenced by hematoxylin and eosin and Masson's trichrome stainings. Immunohistochemical assays demonstrated that the majority of the transforming growth factor- β_1 signaling cytokines were primarily localized in the cytoplasm in the medial layers of all three types of grafts, whereas ectopic transforming growth factor- β_1 , type I receptor of transforming growth factor- β , and Smad7 overexpressions in the interstices were observed particularly in the saphenous vein and radial arterial grafts.

CONCLUSION: Enhanced transforming growth factor- β_1 signal transduction with medial smooth muscle cell proliferation and ectopic transforming growth factor- β_1 , the presence of the type I receptor of transforming growth factor- β , and Smad7 overexpressions in the extracellular matrix may provide primary evidence for early or late graft failure.

KEYWORDS: Blood Vessels; Coronary Artery Bypass; Immunohistochemistry; Signal Transduction; Transforming Growth Factor- β .

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E-mail: dr.jing@163.com

Tel.: 0086 2580860075

INTRODUCTION

Transforming growth factor (TGF)- β_1 is implicated in the development of intimal hyperplasia subsequent to extracellular matrix accumulation,¹ which increases the thickness of both arteries and veins.² The overexpression of TGF- β_1 is typically present in the diseased grafts,³ including the saphenous vein and internal mammary arterial grafts, suggesting that TGF- β_1 may play a role in the irreversible deposition of extracellular matrix and the further development of intimal hyperplasia.² Moreover, TGF- β_1 overexpression has also been observed in the intimal hyperplasia of stenosed venous fistulas for hemodialysis.⁴

Graft failure is a common issue following coronary artery bypass grafting^{5,6} that puzzles cardiac surgeons and

requires increasingly effective solutions. Even though TGF- β expression has drawn attention to the development of vascular remodeling, the biological functions of the TGF- β signaling pathway, including the Smad proteins, have not been sufficiently investigated with respect to coronary artery bypass grafts. We have hypothesized that the TGF- β signaling pathway may be enhanced so as to drive the fibrotic process that is responsible for the failure of coronary artery bypass grafts. The aim of the present study was to observe the immunostaining of the proteins that are related to this signaling pathway.

MATERIALS AND METHODS

From October 2009 to January 2010, 15 remnants of coronary artery bypass grafts, including nine saphenous veins, three radial arteries and three mammary arteries, were collected from 12 patients who were undergoing coronary artery bypass after their surgeries. Ten males and two females were included in the study, and their ages

ranged from 50 to 83 with a mean of 66.25 ± 10.37 years. The major symptoms were chest/precordial pain in six patients (50%), chest pain and palpitations in two patients (16.67%), chest distress in one patient (8.33%), chest distress and dyspnea in one patient (8.33%), and chest distress and palpitations in two patients (16.67%). The time since the onset of symptoms ranged from 1 day to 20 years (mean 5.41 ± 6.59 years, median 2 years). Hypertension was present in eight patients (66.67%), and type II diabetes was present in three patients (25%). Four patients had a myocardial infarction, two of which were non-ST-segment elevation myocardial infarctions (NSTEMI), and one patient had a left ventricular pseudoaneurysmal formation. Conventional coronary artery bypass was performed in four patients (33.33%), off-pump coronary artery bypass in six patients (50%), beating heart coronary revascularization in one patient (8.33%), and off-pump coronary artery bypass with subsequent coronary artery bypass in one patient (8.33%). A total of 41 grafts were bypassed with a mean of 3.42 ± 0.51 grafts per patient. Thirteen (31.71%) left internal mammary arteries were grafted, as were one (2.44%) right internal mammary artery, two (4.88%) radial arteries, and 25 (60.98%) saphenous veins. The associated procedures included left ventricular pseudoaneurysmectomy, mitral valve replacement, and intra-aortic balloon pump insertion in one patient each.

Fresh specimens of the graft remnants were collected and cut into 1-cm^3 blocks/rings and immersed in a 10% methanol solution in appropriately sized bottles for pathological inspection.

Hematoxylin and eosin (H&E) staining was performed on the $4\text{-}\mu\text{m}$ sections, and collagen fibers were stained

using Masson's trichrome protocol. Immunohistochemical staining was performed on the $4\text{-}\mu\text{m}$ paraffin-embedded sections to detect TGF- β_1 , transforming growth factor- β receptor I (T β RI), Smad2/3, Smad4, and Smad7 using the Envision method. The following primary antibodies were utilized: TGF- β_1 (Y369) (1:150) (Bioworld Technology, Inc., Louis Park, MN, USA), T β RI (E161) (1:100) (Bioworld Technology, Inc., Louis Park, MN, USA), Smad2/3 (S2) (1:100) (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), Smad4 (L43) (1:200) (Bioworld Technology, Inc., Louis Park, MN, USA), and Smad7 (Z8-B): sc-101152 (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

The immunostaining density was characterized as follows: - (background) was defined as 0, + (weak yellow) as 1, ++ (yellow) as 2, and +++ (brown) as 3 in the semi-quantitative analyses. Photographs were acquired at $\times 100\text{--}400$.

This study was approved by the institutional ethical committee and was conducted following the guidelines of the Declaration of Helsinki. Informed consent for the graft vessel remnants that were used for other than therapeutic purposes was obtained from each patient in compliance with the guidelines in "Surgically Removed Human Tissue For Research" that were proposed by Thasler et al.⁷

RESULTS

According to H&E staining, the saphenous veins exhibited proliferative medial smooth muscle cells with focal or diffuse disruption and severe intimal degeneration (Fig. 1A). The radial arteries exhibited smooth muscle

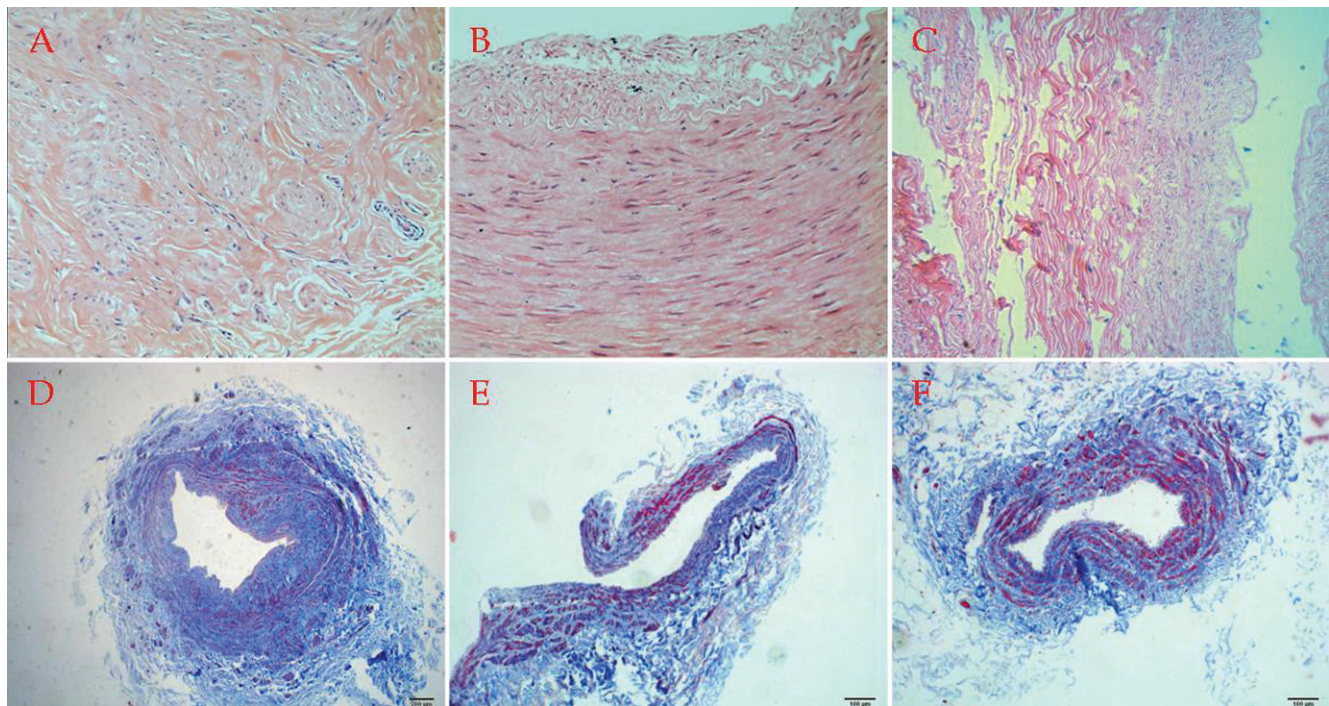


Figure 1 - Hematoxylin and eosin staining showing (A) proliferative medial smooth muscle cells with inflammatory cell infiltration in the saphenous veins, (B) focal intimal degeneration with slight disrupted media in the radial arteries, and (C) roughly normal vascular wall structures of the internal mammary arteries; H&E $\times 200$. Masson's trichrome staining showing that (D) the saphenous veins showed more collagen deposition but less muscular fibers, (E) the radial arteries showed less collagen deposition and more muscular tissues, and (F) the mammary arteries showed the least collagen accumulation in the vascular wall but the most muscular tissue; Masson $\times 100$.

cell proliferation, elastic fibrous degeneration and collagenization (Fig. 1B). Evenly distributed smooth muscle cells of the media with mild degenerative changes in the intima could be observed in the internal mammary arteries (Fig. 1C).

Based on Masson's trichrome staining, the saphenous veins showed more collagen deposition but less muscular fibers (Fig. 1D), the radial arteries had less collagen deposition with more muscular tissues (Fig. 1E), and the

mammary arteries had the least collagen accumulation in the vascular wall but the most muscular tissues (Fig. 1F).

Immunostaining demonstrated that the five tested proteins were positive in the cytoplasm of the medial layers of all three types of grafts. The investigated signaling cytokines were the most intense in the saphenous vein, followed by the radial arterial grafts and then the internal mammary arterial grafts.

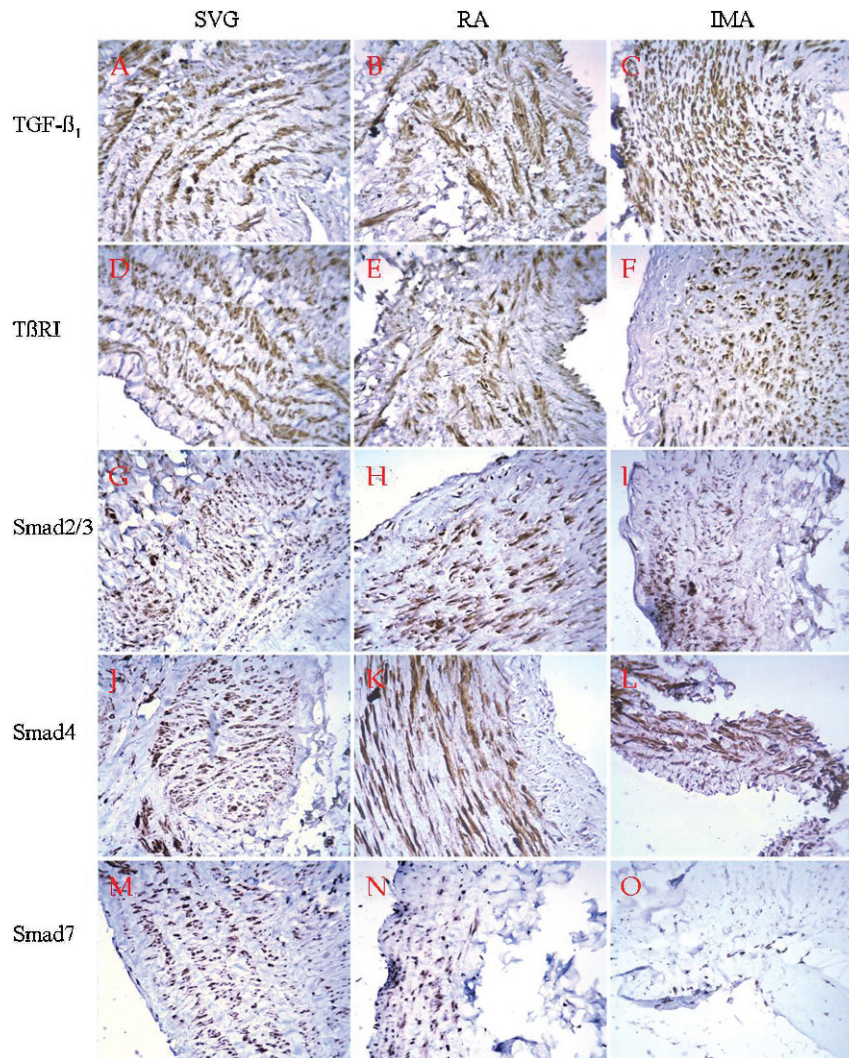


Figure 2 - Immunostaining showed that the five tested proteins (TGF- β_1 , T β RI, Smad2/3, Smad4, and Smad7) were positive, primarily in the cytoplasm of the medial layers, in all three kinds of grafts. (A) (B) In the saphenous vein and radial arterial grafts, TGF- β_1 expression was moderately positive (++) in the cytoplasm and interstices of the intima, intensely positive (+++) in the cytoplasm and interstices of the media, and weakly positive (+) in the cytoplasm and interstices of the adventitia. (C) In the intima (++) , media (+++) and adventitia (+) of the internal mammary arterial grafts, TGF- β_1 staining was only observed in the cytoplasm and not in the interstices. In the saphenous vein grafts, T β RI was moderately positive (++) in the cytoplasm and interstices (++) of the intima, intensely positive (+++) primarily in the cytoplasm but also in the nuclei and interstices of the media, and weakly positive (+) or negative (-) in the cytoplasm or interstices, respectively, of the adventitia (D). This receptor (++) was positive in the cytoplasm and interstices of the intima (+), media (++) and adventitia (+) of the radial arterial grafts (E), and positive in the cytoplasm of the intima (+), media (++) and adventitia (+) of the internal mammary arterial grafts (F). Smad2/3 positivity was more intense in the saphenous vein grafts (G) than in the internal mammary arterial grafts (I), whereas the radial artery (H) showed the least intense Smad2/3 staining. In the saphenous vein grafts, Smad4 was weakly positive (+) in the cytoplasm of the intima, intensely positive (+++) in the cytoplasm and interstices of the media, and weakly positive (++) in the cytoplasm of the adventitia (J). In the radial arteries, it was weakly positive (+) and negative (-) in the intima, moderately positive (++) in the cytoplasm and interstices of the media, and weakly positive in the cytoplasm and interstices of the adventitia (K). In the internal mammary arteries, it was positive in the cytoplasm of the intima (+), media (++) , and adventitia (+) (L). Smad7 was expressed most intensely in the saphenous veins (M), more intense in the radial arteries (N), and it was weaker in scattered nuclei and interstices in the internal mammary arterial grafts (O). IMA = internal mammary artery; RA = radial artery; SVG = saphenous vein graft; TGF- β_1 : transforming growth factor- β_1 ; T β RI: transforming growth factor- β receptor- β receptor I. Envision $\times 200$.

TGF- β_1 expression was moderately positive (++) in the cytoplasm and interstices of the intima, intensely positive (+++) in the cytoplasm and interstices of the media, and weakly positive (+) in the cytoplasm and interstices of the adventitia of the saphenous vein and radial arterial grafts. In the intima (++) , media (+++), and adventitia (+) of the internal mammary arterial grafts, TGF- β_1 staining was seen only in the cytoplasm and not in the interstices. TGF- β_1 staining was the most intense in the internal mammary artery, less intense in the saphenous vein, and the least intense in the radial arterial grafts (Fig. 2A–2C).

T β RI was moderately positive in the cytoplasm and interstices (+++) of the intima of the saphenous vein grafts, intensely positive (+++) primarily in the cytoplasm but also in the nuclei and interstices of the media, and weakly positive (+) or negative (-) in the cytoplasm and interstices of the adventitia. This receptor was positive in the cytoplasm and interstices of the intima (+), media (++) and adventitia (+) of the radial arterial grafts. It was also positive in the cytoplasm of the intima (+), media (++) and adventitia (+) of the internal mammary arterial grafts (Fig. 2D–2F).

Smad2/3 was virtually only present in the cytoplasm. Smad2/3 positivity was more intense in the saphenous vein grafts than in the internal mammary arterial grafts, whereas the radial arteries showed the least intense Smad2/3 staining (Fig. 2G–2I).

In the saphenous vein grafts, Smad4 was weakly positive (+) in the cytoplasm of the intima, intensely positive (+++) in the cytoplasm of the media, and weakly positive (–+) in the cytoplasm of the adventitia, and the positivity rate was 85.71% (6/7). For the radial arteries, Smad4 was weakly positive in the cytoplasm (+) and negative (-) in the intima, moderately positive (++) in the cytoplasm and interstices of the media, and weakly positive in the cytoplasm and interstices of the adventitia. In the internal mammary arteries, it was positive in the cytoplasm of the intima (+), media (++) , and adventitia (+) (Fig. 2J–2L).

Smad7 was expressed in the cytoplasm and nucleus and was also present in the interstices in one of the radial arteries. Smad7 was the most intense in the saphenous vein, more intense in the radial artery, and weaker in scattered nuclei and interstices of the internal mammary arterial grafts. The positive rates were 62.5% (5/8), 75% (6/8) and 62.5% (5/8), respectively for the intima, media, and adventitia of the saphenous vein grafts. In the radial artery, it was negative in 1/3, 1/3 and 2/2 of the intima, media, and adventitia, respectively (Fig. 2M–2O) (Table 1).

DISCUSSION

Masson's trichrome staining is a popular technique for observing collagen deposition between elastin layers, in which the reactivity and integrity of the vascular wall,⁸ vascular regeneration, and graft patency⁹ can all be assessed. This technique stains the extracellular matrix blue and the cellular portion red.¹⁰ Using Masson's trichrome staining, the saphenous veins that were endoscopically harvested with a no-touch technique showed well-preserved collagen fibers, whereas those harvested using conventional techniques showed more subendothelial collagen degradation.¹¹ In comparison, the architectures of the radial arterial grafts were preserved with both endoscopic and conventional techniques,⁸ indicating that the wall structures of the vein grafts were more prone to

being damaged by surgical maneuvers. In addition, the observed higher durability of the left internal mammary arterial grafts may be due to their appropriate elastic tension and internal diameter¹², as well as to the relatively limited atherosclerotic changes.¹³

TGF- β_1 stimulates arteriogenesis, thereby contributing to the occurrence of restenosis after neointimal damage caused by angioplasty or stenting. TGF- β_1 is upregulated rapidly in the restenotic and injured vessels following balloon catheter injury along with associated increases in TGF- β_3 , activin receptor-like kinase 5 (ALK-5), and transforming growth factor- β receptor II (T β RII) immunoreactive peptide levels.¹⁴ Smooth muscle cells and macrophages in the atherosclerotic lesions may be predisposed to the upregulation of T β RII and ALK5.¹⁵ TGF- β antagonists may inhibit fibroblast differentiation and intimal injury following angioplasty,¹⁶ and it may prevent adventitial fibrosis.¹⁷ Both TGF- β_1 and TGF- β_2 upregulate type VII collagen gene expression.¹⁸ They may increase the expression of protease inhibitors, including inhibitors of matrix metalloproteinases and of tissue plasminogen activator-1, and they may crosstalk with proteins of the Smad signaling pathway.¹⁹ Plasminogen activator inhibitor-1 (PAI-1),²⁰ matrix metalloproteinases,²¹ and vascular endothelial growth factor²² have been shown to be modulated by TGF- β_1 and are thus involved in the signal transduction.

In TGF- β signal transduction, Smad2/3 are considered to be the major mediators of TGF- β -induced fibrotic pathogenesis.²³ Smad4 is implicated in the pathology of human vascular disorders, with essential roles in vascular remodeling, maturation, and integrity. Smad4 deficiency may cause failures of remodeling and efficient sprouting *in vivo*.²⁴ Smad7 is an inhibitor of TGF- β signaling, and it is usually expressed in human vascular endothelial cells that have been injured by shear stress.²⁵ The ectopic expression of Smad7 inhibits TGF- β responses in vascular smooth muscle cells, and the biological function of Smad7 can be reversed by Smad2.²⁶ Conversely, Smad7 overexpression reduces Smad2 phosphorylation in response to TGF- β_1 via T β RI.²⁷ Smad7 may induce the ubiquitination, degradation, and endocytosis of T β RI and, hence, play an important role in the crosstalk between different signaling pathways. Moreover, an alternative biological function of Smad7 is to mediate TGF- β -induced apoptosis.²⁸ In addition, it has been reported that a marked Smad7 deficiency may be responsible for TGF- β hyperresponsiveness.²⁹ The overexpression of Smad7 had been shown to counteract TGF- β , activin A, and bone morphogenetic protein-induced growth arrest and apoptosis in tumor B cell lines,³⁰ and the overexpression of Smad7 in the adventitia of the carotid arteries significantly attenuated α -smooth muscle actin expression in the adventitia, media, and neointima, or, in other words, in areas of reduced lumen, after balloon injury.³¹

Increased flow and shear stress can mediate the release of TGF- β_1 in rabbit arteries.³² Stress in the endothelial and smooth muscle cells may alter the synthesis and secretion of collagen, elastin, and connective tissue proteases.³³ Flow supply to the conduits could be a determinant of graft patency as evidenced by a series of observations; <50% or <70% coronary stenosis may be associated with increased internal mammary or radial arterial graft occlusion, respectively, during follow-up.^{34,35} TGF- β_1 may promote monocyte adhesion to the endothelial cells and migration across the endothelium, probably due to interaction with CD44, which may link more to T β RI than to T β RII, thereby increasing

Table 1 - Immunostaining of the coronary artery bypass grafts.

Cytokine	RA				IMA				
	Intima	Media	adventitia	Intima	Media	adventitia	Intima	Media	adventitia
TGF-β ₁	Cytoplasm + - + + (n = 8)/ interstices ++ (n = 1)	Cytoplasm +++ (n = 8)/ interstices ++ (n = 1)	Cytoplasm+ (n = 7)/ interstices ++(n = 1)/ -(n = 1)	Cytoplasm + - + + (n = 3)/ interstices+(n = 1)	Cytoplasm + ++(n = 2)/ interstices (n = 1)	Cytoplasm ++(n = 2)/ interstices (n = 1)	Cytoplasm + - + + (n = 3)	Cytoplasm +++ (n = 3)	Cytoplasm - - + (n = 3)
TβRI	Cytoplasm +(n = 8)/ interstices ++(n = 1)	Cytoplasm ++(n = 5)/ cytoplasm & interstices + (n = 1)/ interstices ++(n = 1)/ nucleus ++(n = 2)/cytoplasm &nucleus ++(n = 1)	Cytoplasm +(n = 3)/ cytoplasm +(n = 5)	Cytoplasm +(n = 2)/ interstices +(n = 1)	Cytoplasm ++(n = 2)/ interstices +(n = 1)	Cytoplasm ++(n = 2)/ interstices+ (n = 1)	Cytoplasm +(n = 3)	Cytoplasm ++(n = 3)	Cytoplasm +(n = 3)
Smad2/3	Cytoplasm + - + + (n = 7)	Cytoplasm + + + + + (n = 7)	Cytoplasm +(n = 6)/ interstices +(n = 1)	Cytoplasm +(n = 3)	Cytoplasm + - + + (n = 3)	Cytoplasm +(n = 1)	Cytoplasm +(n = 3)	Cytoplasm ++(n = 3)	Cytoplasm +(n = 3)
Smad4	-(n = 1)/ cytoplasm +(n = 7)	-(n = 1)/ Cytoplasm ++(n = 7)	-(n = 1)/ Cytoplasm - - + (n = 7)	Cytoplasm +(n = 2)/ -(n = 1)	Cytoplasm + - + + (n = 2)/ cytoplasm & interstices + - + + (n = 1)	Interstices+ (n = 2)/ cytoplasm - - + (n = 1)	Interstices +(n = 1)/ cytoplasm +(n = 1)	Interstices +(n = 1)/ cytoplasm ++(n = 1)	Interstices +(n = 1)/ cytoplasm +(n = 1)
Smad7	-(n = 3)/ nucleus + - + + (n = 1)/ - - + (n = 4)	-(n = 2)/nucleus + - + + (n = 1)/ cytoplasm + - + + + (n = 5)	-(n = 3)/interstices +(n = 1)/nucleus + - + + (n = 1)/ cytoplasm ++(n = 4)	-(n = 1)/ cytoplasm & nucleus +(n = 1)	-(n = 1)/ interstices = (n = 1)/ cytoplasm & interstices ++(n = 1)	-(n = 2)	Cytoplasm +(n = 2)	Cytoplasm +(n = 2)/ nucleus - - + (n = 1)	Cytoplasm +(n = 2)/ nucleus - - + (n = 1)

IMA: internal mammary artery; RA: radial artery; SVG: saphenous vein graft; TGF-β₁: transforming growth factor-β₁; TβRI: transforming growth factor-β receptor I.

Smad2/3 phosphorylation.³⁶ TGF gene expression was found to be increased in arterialized vein grafts from the coronary artery bypasses.^{37,38} Therefore, the ectopic implantation of either venous or arterial grafts into the coronary circulation may place these vessels in a state of increased stress, which may upregulate TGF- β signaling cytokines.

We found that the internal mammary arteries showed a weak Smad7 expression. Therefore, the dual regulatory effects of TGF- β on the activation and phosphorylation of the Smad proteins may lead to the normal transcription of target genes. The most prominent difference in the signaling pathways between the three grafts may lie in the ectopic TGF- β_1 , T β RI, and Smad7 overexpression in the interstices was observed particularly in the saphenous veins and radial arteries relative to the internal mammary arteries. Therefore, the increased TGF- β signaling activity in the extracellular matrix of the saphenous vein and radial arterial grafts may lead to considerable proliferation of the intima and muscular layers of these the grafts.

CONCLUSION

In conclusion, severe vascular wall degeneration and collagen deposition together with overexpressed TGF- β signaling cytokines may provide preliminary evidence for the failure (early or late) of the saphenous vein and radial arterial grafts. Weak Smad7 expression in the internal mammary arterial grafts with well-preserved structures may imply less matrix deposition, which may explain their superior durability. More comprehensive studies of the grafts are required to obtain more accurate information for the prevention of graft disorders.

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