TGF-βシグナリングの役割

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POINT

- TGF-βは，主にSmad依存性シグナル伝達を介して，細胞増殖抑制，マトリックス産生，炎症抑制などの作用を示す多機能分子である．
- TGF-βは，アテローム性動脈硬化プラーク安定化作用と内皮傷害後の新生内膜肥厚増強作用を示す．
- TGF-βの主要シグナル分子であるSmad3の特異的欠損は，新生内膜およびアテローム性動脈硬化病変をともに増強する．
- TGF-βシグナルの修飾を通じて，動脈硬化の新しい治療法を開発できる可能性がある．

はじめに

TGF-β(transforming growth factor-β)は，アクチビンやBMP(bone morphogenic protein)などとともにTGF-βスーパーファミリーを形成する多機能分子であり，細胞増殖抑制，マトリックス産生，炎症抑制などの作用を示す．TGF-βには，TGF-β_1, TGF-β_2, TGF-β_3という3つのアイソフォームが存在する．特にTGF-β_1は血管壁において高い発現を示すことから血管構造の維持や動脈硬化病変形成への関与が示唆されてきた．

本稿では，TGF-βによる細胞内シグナル伝達に着目し，これまでに明らかとなってきた動脈硬化とのかかわりについて述べる．

TGF-βによる細胞内シグナル伝達

ジスルフィド結合した二量体分子であるTGF-β_1(以下TGF-βと呼称)は，潜在型として細胞外へ放出され，プロテアーゼの働きによって活性化，細胞表面の受容体へ結合できるようになる[1]．TGF-β受容体はI型とII型の2種類に分けられ，いずれも細胞内にセリンスレオニンキナーゼ領域をもっている．リガンドであるTGF-βが結合すると，受容体はヘテロ四量体を形成し，II型がI型受容体の細胞内領域をリン酸化する(図1)．I型受容体は特異型Smad(R-Smad)とよばれるSmad2やSmad3を基質としてリン酸化し，R-Smadは共有型Smad(Co-Smad)であるSmad4と結合，ともに核内へと移行する．そしてさまざまな転写因子や転写の共役因子，共役抑制因子などと結合し，標的遺伝子の転写を調節する．一方，抑制型Smad(I-Smad)とよばれるSmad7は活性化されたI型受容体に結合するなどしてその作用を抑制する．このほか，MAPキナーゼ経路もTGF-βによって活性化されるが，主たる作用はSmad経路によるものと認識されている．

TGF-βはアテローム性動脈硬化プラークを安定化する

近年，“プラークの不安定化と破綻”が心血管イベント発生の本質であると考えられるようになった[2]．アテローム性動脈硬化プラークの形成と不安定化を規定する血管壁細胞の要因は，①内皮細胞の活性化，すなわち酸化LDLやサイトカイン刺激による白血球接着分子や遊走因子の発現な

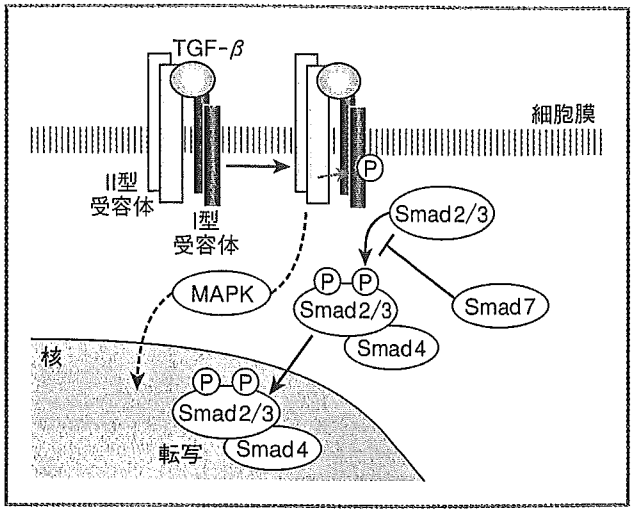

図1　TGF-βによる細胞内シグナル伝達経路
Smad2/3はSmad2またはSmad3を，MAPKはMAPキナーゼを表す．Pはリン酸化されていることを示す．

ど，②内皮下へと侵入した単球由来マクロファージやTリンパ球の集簇と各種サイトカイン・増殖因子・マトリックス分解酵素の産生，③平滑筋細胞（SMC）の不十分な集積とマトリックス沈着の減少，にまとめることができる．

これまでに行われてきた*in vitro*ならびに*in vivo*の検討は，いずれもTGF-βがプラーク安定化に働くことを示している．すなわち，*in vitro*でTGF-βは内皮細胞における接着分子の発現やマクロファージにおけるコレステロールエステルの蓄積，Tリンパ球の機能等を抑制する[3,4]．そしてSMCによるマトリックス産生を増加させ，その分解を阻害することから，TGF-βはアテローム性動脈硬化病変における炎症性細胞の集簇と活性化を阻害し，マトリックスに富む安定なプラークを形成することが期待される．動脈硬化形成の主要ステップとそれぞれに対するTGF-βの作用を図2に示す．

事実，*in vivo*の検討でも，アテローム性動脈硬化モデルとして知られるアポEノックアウトマウス（以下KOと略）にLp(a)を過剰発現させるとTGF-βの活性化が損なわれてアテロームの形成が促進されること[5]，可溶性のII型受容体やTGF-β中和抗体の投与によってTGF-β作用を抑制した場合にも病変部位におけるマクロファージの集積がより高度となることが報告された[6]．逆に，タモキシフェンを投与してTGF-βの血中濃度を上昇させれば病変形成を抑制しうることも，マウスを用いた実験ならびにヒトでの臨床的観察により示されている[7,8]．

さらに，最近，Tリンパ球特異的にTGF-β受容体の機能を失わせたアポE KOにおいて，アテロームが著しく増大し，かつコラーゲン線維が少なく炎症に富む“不安定プラーク”様の病像を呈することが示された[9,10]．Tリンパ球に対する作用のみですべてを説明できるかはさておき，少なくとも複数の動物モデルによる知見からTGF-βがアテローム性動脈硬化病変の抑制と安定化に働くことがコンセンサスとなりつつある．

TGF-βは内皮傷害に伴う新生内膜肥厚を増強する

冒頭にも述べたように，TGF-βは一般に細胞の増殖を抑制し，マトリックス産生を促す作用を

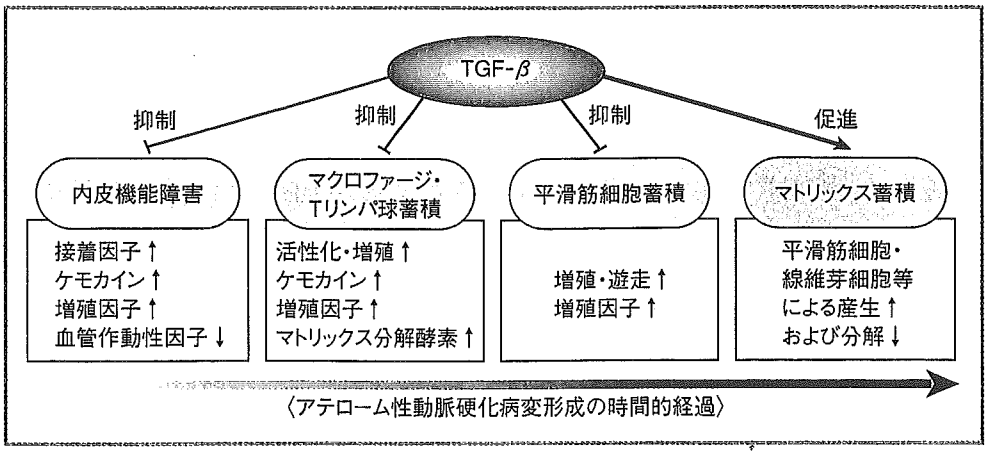

図2　アテローム性動脈硬化病変形成の主要ステップにみられる現象とそれらに対するTGF-βの作用

示す．同様に培養SMCに対しても，基本的にTGF-βはその増殖を抑制し[11]，コラーゲン産生と沈着を促進すると考えられている．培養条件によってTGF-βがSMCの増殖を促進するとの報告もあるが，これはマトリックス沈着を介した二次的作用の可能性がある．

経皮的冠動脈インターベンション（percutaneous coronary intervention ; PCI）後の再狭窄病変組織にはTGF-βが高発現すると報告されている[12]．再狭窄病変やその動物実験モデルとして知られるバルーン傷害後の新生内膜は，主にSMCとマトリックスにより構成されるため，その成り立ちにTGF-βがどのように影響を及ぼすかは興味のもたれるところである．

これまでに行われてきた動物実験の多くは，これら血管傷害後の内膜肥厚の形成をTGF-βが促進することを示唆している．すなわち，TGF-β蛋白の全身投与やウイルスベクターによる動脈壁へのTGF-β遺伝子導入はいずれも血管傷害後の内膜肥厚を増強させる[13, 14]．また，中和抗体，可溶性II型受容体，リボザイムなどを用いたTGF-β作用の抑制はいずれも内膜肥厚による狭窄性変化を軽減させた[15-17]．これらの報告において病理学的な解析結果は必ずしも一定していないが，血管傷害後の内膜肥厚におけるTGF-βの作用は，特にそのマトリックス産生能が反映されているように見受けられる．つまり，SMCの増殖に対する抑制効果を上回ってマトリックス沈着が増強し，内膜の体積が増大するというものである．

動物モデルとは異なり，臨床的に観察される動脈硬化ではアテローム性病変と再狭窄病変とを明確に区別することがしばしば困難となる．すなわち，再狭窄に先立つPCIも，そもそもは不安定プラークに対する治療として行われたはずであり，実際の動脈壁においては両方の性質をもつ病変が混在しうる．そして動脈硬化に対する根本的治療法として，本来，その両者をカバーできるものが望ましいと考えられる．この点，アテローム性動脈硬化に対しては抑制的，再狭窄に対しては促進的に働くTGF-βは功罪相半ばした分子であり，臨床上，最良の治療標的とはなりにくい．

前述のように，TGF-βはいくつかの並行する細胞内シグナル伝達経路を活性化し，その差異が多様な機能の源泉となっている可能性がある．そこで筆者らはTGF-β下流の単一経路（Smad3経路）に着目し，これを特異的に修飾した場合に動脈硬化病変がいかに変容するかを検討した．その結果，Smad3が新たな治療標的となる可能性が示唆されたので，次に紹介する．

TGF-β/Smad3シグナル経路と動脈硬化

R-Smadに分類されるSmad3は，TGF-β受容体の下流でさまざまなマトリックス遺伝子の発現誘導などにかかわる主要シグナル分子の一つである．筆者らはSmad3 KOを用い，血管傷害後の内膜肥厚ならびにアポE KOとの交配によるアテローム性動脈硬化病変について検討した．

光化学的血栓形成により大腿動脈に内皮剥離を惹起し，形成される新生内膜肥厚を形態学的に観察したところ，Smad3 KOでは野生型マウスに比べて著しい肥厚の増強がみられた（図3A）[18]．Smad3 KOの肥厚内膜は単位断面積あたりのコラーゲン沈着の減少とSMC数の著しい増加を特徴としていた．一方，Smad3 KOの骨髄細胞を野生型マウスに移植した後で傷害を加えた場合には内膜肥厚の増強がみられなかったため，この表現型は血管壁に内在するSMCの性質に依存し，骨髄由来の炎症性細胞等に起因するものではないことが示唆された．マウス大動脈由来SMCを用いた*in vitro*の成績と肥厚内膜におけるPCNA（proliferating cell nuclear antigen；増殖細胞核抗原）陽性細胞数の増加から，TGF-βによるSMC増殖抑制作用の減弱が肥厚の増強をもたらすと考えられた．また，Smad3 KO SMCではTGF-βによるI型コラーゲンの誘導が低下し，TGF-βによるMMP活性の抑制作用も失われることがわかった．これが内膜におけるマトリック

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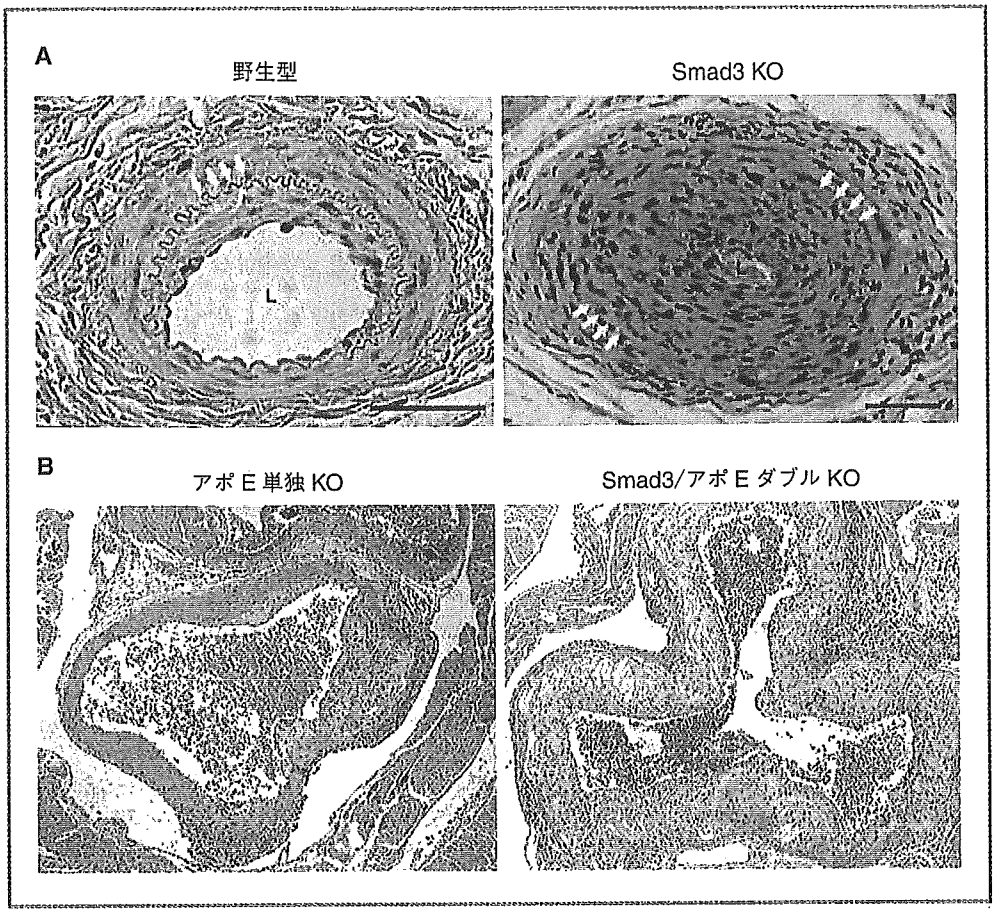

図3 Smad3 ノックアウトマウス（KO）では，内皮傷害後の内膜肥厚ならびに高脂血症によるアテローム性動脈硬化がともに促進される

A：血管傷害後の新生内膜肥厚．光化学的血栓形成による内皮傷害後 3 週の大腿動脈内膜肥厚病変．矢印は内弾性板の位置を表す．Smad3 KO 血管壁では，細胞密度の高い内膜肥厚病変が著しく増強する．スケールは 50 mm を表す．

B：高脂血症によるアテローム性動脈硬化病変．通常餌飼育下 20 週のアポ E KO および Smad3/アポ E ダブル KO の大動脈切片（弁上部）．ダブル KO では著しいアテローム様変化と血管壁リモデリングによる拡大がみられる．

（Kobayashi K, et al. Circ Res 2005；96：904-12[18] より改変）

スの減少と，ひいては中膜から内膜への SMC 遊走を促進したと推察される（図 4）．

前述のように，“TGF-β分子そのもの”の働きを阻害すると血管傷害後の内膜肥厚が軽減されるのに対し，そのシグナル分子である Smad3 の欠損マウスではなぜ逆に病変が増強したのであろうか？ いまだ推測の域を出ないが，Smad3 だけを欠如し，その他のメディエーター，すなわち Smad2 や MAP キナーゼ依存性の経路は保たれていることが特殊な病態を生み出した可能性がある．さらに Smad3 は，TGF-β以外にも，SMC に対して脱分化抑制作用をもつアクチビンの細胞内シグナルを担うことが知られている[19]．SMC に分化型の表現型を維持させるアクチビンのシグナルを Smad3 が担うとすれば，Smad3 KO にみられた著しい内膜肥厚が理解できる．

一方，Smad3/アポ E ダブル KO は，アポ E 単独 KO と比べ，マクロファージ蓄積を特徴とするアテローム様動脈病変の著しい増強を呈した（図 3B，小林ら未発表データ）．このように，Smad3 欠損は再狭窄・アテローム性動脈硬化いずれのモデルにおいても病変形成を促進することから，逆に Smad3 シグナルのみを特異的に増強することができれば，アテローム性動脈硬化と再狭窄の両者に有効な治療法を創出できるはずであり，現在

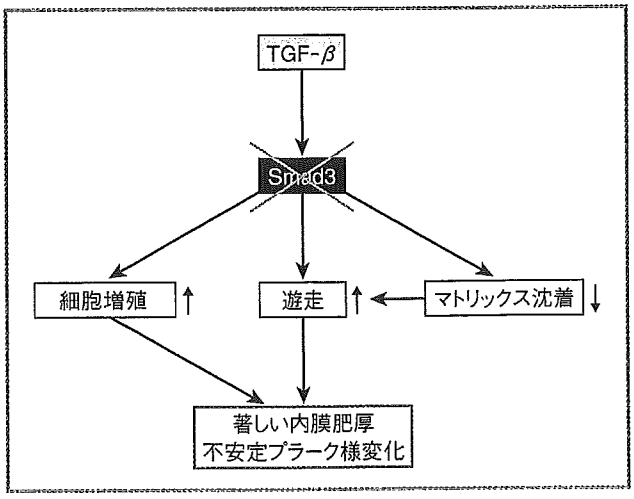

図4 Smad3 欠損の特異的欠損に伴う動脈硬化の促進機序

鋭意検討を進めている．

おわりに

以上述べてきたように，TGF-βはアテローム性動脈硬化病変に対してはプラークの安定化に働く一方，PCI後の再狭窄病変のように血管傷害に伴う内膜肥厚病変に対してはこれを促進する方向に働くとの見方が一般的となっている．これに対してSmad3シグナルの特異的欠損は，両タイプの血管病変の形成をいずれも著しく促進した．古くから知られるTGF-βの多彩な機能を，新たに個々のシグナルのレベルでとらえなおすことによって動脈硬化病変の新しい側面ならびに新しい治療アプローチの方法が浮かび上がることを期待する．

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Targeted Disruption of TGF-β–Smad3 Signaling Leads to Enhanced Neointimal Hyperplasia With Diminished Matrix Deposition in Response to Vascular Injury

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Abstract—The role of transforming growth factor (TGF)-β and its signal in atherogenesis is not fully understood. Here, we examined mice lacking Smad3, a major downstream mediator of TGF-β, to clarify the precise role of Smad3-dependent signaling in vascular response to injury. Femoral arteries were injured in wild-type and Smad3-null (null) male mice on C57Bl/6 background. Histopathological evaluation of the arteries 1 to 3 weeks after the injury revealed significant enhancement of neointimal hyperplasia in null compared with wild-type mice. Transplantation of null bone marrow to wild-type mice did not enhance neointimal thickening, suggesting that vascular cells in situ play a major role in the response. Null intima contained more proliferating smooth muscle cells (SMC) with less amount of collagen compared with wild-type intima. TGF-β caused significant inhibition of cellular proliferation in wild-type aortic SMC, whereas the growth of null SMC was only weakly inhibited by TGF-β in vitro, indicating a crucial role of Smad3 in the growth inhibitory function. On the other hand, Smad3-deficiency did not attenuate chemotaxis of SMC toward TGF-β. TGF-β increased transcript level of α2 type I collagen and tissue inhibitor of metalloproteinases-1, and suppressed expression and activity of matrix metalloproteinases in wild-type SMC. However, these effects of TGF-β were diminished in null SMC. Our findings altogether show that the loss of Smad3 pathway causes enhanced neointimal hyperplasia on injury through modulation of growth and matrix regulation in vascular SMC. These results indicate a vasculoprotective role of endogenous Smad3 in response to injury. (***Circ Res.* 2005;96:904-912.**)

Key Words: transforming growth factor-β ■ Smad3 ■ atherosclerosis ■ neointimal hyperplasia ■ smooth muscle cells

Transforming growth factor (TGF)-β is a prototypic member of the TGF-β superfamily that exerts a wide range of biological effects on various cell types.[1] Well described functions of TGF-β including growth inhibition, cell migration, differentiation, extracellular matrix production, and immunomodulation. Abnormality in TGF-β signaling may cause pathological conditions such as tumorigenesis, fibrotic disorders, and vascular diseases.[2] At present, however, the role of TGF-β and its signaling molecules in atherogenesis is not fully understood.

TGF-β is often regarded to have proatherosclerotic effect on arteries. For example, TGF-β expression is increased in human restenotic lesions as well as in neointimal hyperplasia after balloon injury in animals.[3] TGF-β facilitates extracellular matrix deposition by stimulating production of procollagen and fibronectin, downregulating the expression of proteases, and upregulating protease inhibitors, such as plasminogen activator inhibitor type I (PAI-I) and tissue inhibitor of metalloproteinase-1 (TIMP-1).[4–8] TGF-β transgene into vascular wall causes fibroproliferative intimal thickening in animal models in the presence or absence of vascular injury.[9,10] Moreover, TGF-β antagonism by antibody, soluble receptor, or ribozyme reduces constrictive remodeling after balloon injury in animals.[11–13]

On the other hand, considerable evidence implies antiatherosclerotic effects of TGF-β. TGF-β has been shown to inhibit proliferation and migration of vascular smooth muscle cells (SMCs) in vitro.[14,15] Inhibition of TGF-β signal systemically by use of neutralizing antibody and soluble TGF-β receptor type (TβR)-II or in T-cells by expressing a dominant-negative TβR-II results in an unstable plaque phenotype in mouse models of atherosclerosis.[16–18] SMCs

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obtained from human atherosclerotic plaques were shown to be defective in the TGF-β signal pathway and were resistant to TGF-β–mediated growth suppression and apoptosis.[19,20] Furthermore, low blood levels of active TGF-β were associated with severity of vascular disease in a manner consistent with an antiatherosclerotic effect of TGF-β.[21]

TGF-β elicits its effects via signaling through tetramerization of two different receptor serine/threonine kinases, TβR-I and TβR-II.[22,23] Activation of the receptors leads to phosphorylation of cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smad). The activated R-Smad heteroligomerizes with Smad4, a common mediator Smad, and the complex is transported to the nucleus where it regulates gene expression. In addition, pathways independent of Smads, which involve MAP kinases have also been described.[23] In mice lacking TGF-β signaling molecules, ie, TβR-I and TβR-II, Smad2 and Smad4 turned out to be embryonic lethal.[24–26] However, it was recently found that the mice null for Smad3 survive into adulthood.[27]

We undertook the present study examining Smad3-null mice in vivo and in vitro to elucidate the precise role of Smad3-dependent TGF-β signaling in the vascular response to injury.

Materials and Methods

Reagents

Reagents are described in an expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

Mice

The generation of $Smad3^{ex8/ex8}$ null mice by homologous recombination was described previously.[27] See expanded Materials and Methods section for details.

Femoral Artery Injury

Mice femoral arteries were injured by use of photochemically-induced thrombosis method.[28] See expanded Materials and Methods section for details.

Histological Evaluation

Fixed femoral artery segments were embedded in paraffin and cut into 5-μm–thick serial sections. Six sections per one irradiated segment at 1-mm intervals were stained with hematoxylin and eosin. Neointima was defined as the region between the lumen and the internal elastic lamina. The media was defined as the region between the internal and external elastic lamina. The cross-sectional areas of intima and media were measured using NIH image version 1.62f (National Institutes of Health, USA). The intima-to-media (I/M) ratio was then calculated, and the mean I/M of all 6 sections per one irradiated segments was determined. The sections with intimal hyperplasia were also subjected to Masson's trichrome staining and immunohistochemistry. Masson's trichrome-positive intimal area was analyzed using Photoshop version 7.0 (Adobe). All the measurements were made in blinded manner.

Immunohistochemistry

Immunohistochemistry is described in the expanded Materials and Methods section.

Bone Marrow Transplantation

Bone marrow transplantation (BMT) was performed principally as described previously.[29] Briefly, bone marrow cell suspensions obtained from either Smad3-null or wild-type mice thigh bone were treated with ACK lysis buffer (0.155 mol/L ammonium chloride, 0.1 mol/L disodium EDTA, and 0.01 mol/L potassium bicarbonate) to lyse erythrocytes. The cells were intravenously injected to recipient Smad3-null or wild-type mice (1×10^6 per body) between the age of 6 and 9 weeks, 3 hours after lethal irradiation (8.5 Gy). Engraftment of the transferred bone marrow was confirmed by polymerase chain reaction (PCR) on peripheral blood DNA according to the protocol by Yang et al.[26] Femoral artery injury was performed 6 weeks after the bone marrow transfer.

Cell Culture

Mouse aortic SMCs were obtained and cultured as described by Ohmi et al[30] (see expanded Materials and Methods section). Experiments were performed on cells after 5 to 10 passages from the primary culture.

Immunocytochemistry

Immunocytochemical staining using anti–α-smooth muscle actin (SMA) and smooth muscle myosin (SMM) antibodies was performed as described by Hasegawa et al[31] with some modification (see expanded Materials and Methods section).

Immunoblotting

Immunoblotting was essentially performed as previously described[32] (see expanded Materials and Methods section).

Growth Inhibition Assay

Growth inhibition assay was performed as described by Datto et al[33] (see expanded Materials and Methods section).

Cell Migration Assay

SMC migration was evaluated by modified Boyden chamber method[34] (see expanded Materials and Methods section).

Real-Time Quantitative PCR

Real-time quantitative PCR is described in expanded Materials and Methods section.

Gelatin Zymography

Gelatin zymography is described in the expanded Materials and Methods section.

Statistical Analysis

Results were presented as mean±SEM. Statistical analyses used two-tailed, unpaired student *t* test.

Results

Mice Lacking Smad3 Show Enhanced Neointimal Hyperplasia in Response to Injury

To evaluate a role of Smad3 in the pathogenesis of neointimal hyperplasia, femoral arteries of wild-type (n=12) and Smad3-null (n=10) male mice were injured by use of the photochemically-induced thrombosis method.[28] Histopathological examination of the arteries 1 to 3 weeks after the injury revealed markedly enhanced neointimal thickening in Smad3-null mice compared with wild-type mice (Figure 1A and 1B). As shown in Figure 1C, mean I/M ratios evaluated at 1 and 3 weeks after the injury were significantly higher in Smad3-null arteries (0.193±0.034 at 1 week and 0.541±0.093 at 3 weeks) than those of wild-type arteries (0.059±0.018 at 1 week and 0.115±0.060 at 3 weeks, $P<0.01$ at each time point).

Immunohistochemical examination showed that both neointimal and medial cells were positive for α-SMA (Figure 2A and 2B) but negative for pan-leukocyte marker CD45 (Figure

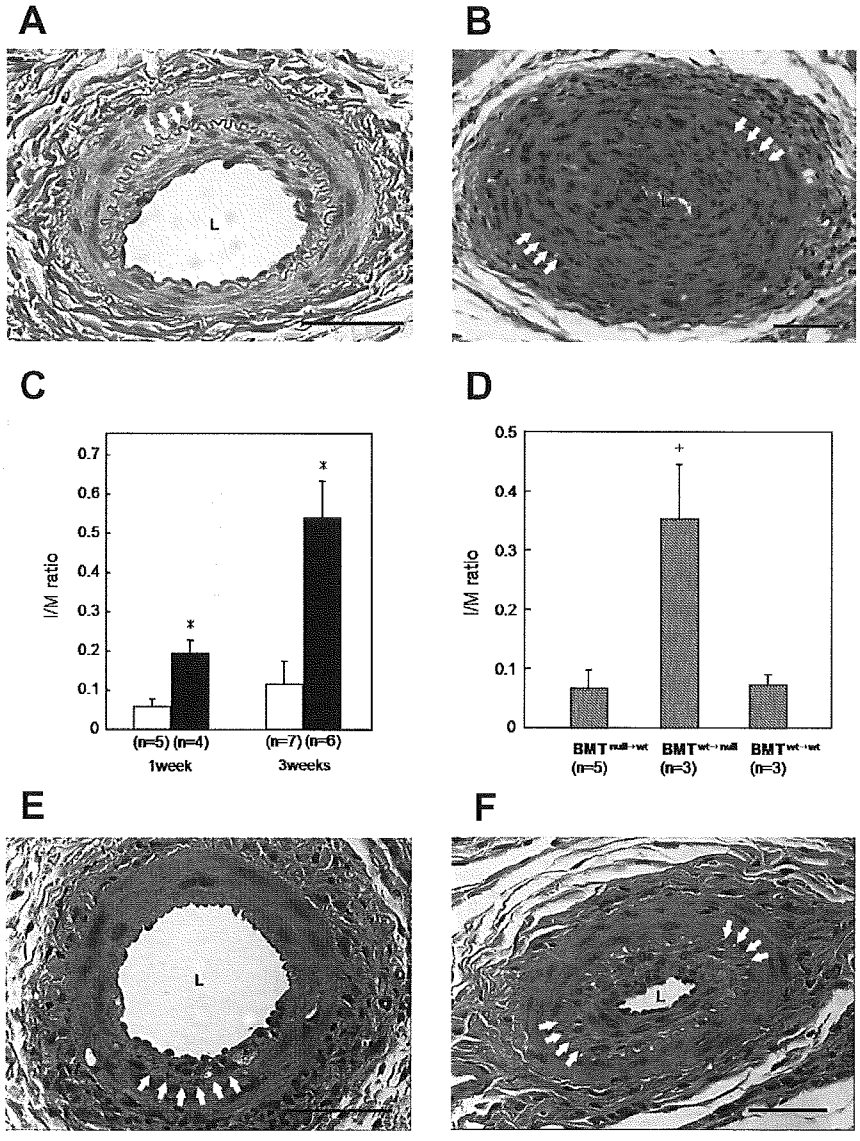

Figure 1. Neointimal thickening in injured femoral arteries of wild-type and Smad3-null mice. Photomicrographs showing representative cross sections of hematoxylin and eosin–stained femoral arteries from wild-type (A) and Smad3-null (B) and $BMT^{null\rightarrow wild}$ (E) and $BMT^{wild\rightarrow null}$ (F) mice 3 weeks after endothelial injury. L indicates vascular lumen. Arrows indicate the positions of the internal elastic lamina. Original magnification ×200; bar=50 μm. Intima-to-media (I/M) ratios at 1 and 3 weeks in wild-type and Smad3-null mice (C) and in $BMT^{null\rightarrow wild}$, $BMT^{wild\rightarrow null}$, and $BMT^{wild\rightarrow wild}$ at 3 weeks (D) were calculated from cross sectional areas morphometrically measured using an image analyzer. Open and closed columns indicate wild-type and Smad3-null mice, respectively. *$P<0.01$ compared with wild type at each time point; †$P<0.05$ compared with $BMT^{null\rightarrow wild}$.

2C and 2D), indicating that the intima was exclusively composed of SMCs. The same anti-CD45 antibody recognized leukocytes in vasa vasorum (Figure 2D) as well as lymphocytes in the mouse spleen (Figure 2E).

TGF-β is well known for its antiinflammatory effect.[1,2] To determine whether systemic inflammation due to Smad3 deficiency contributes to enhanced neointimal formation, we injured femoral artery of wild-type and Smad3-null mice after bone marrow transplantation (BMT). Lethally irradiated Smad3-null mice received 1×10^6 bone marrow cells from a wild-type mouse ($BMT^{wild\rightarrow null}$ mice). At the same time, irradiated wild-type mice were given bone marrow either from Smad3-null or wild-type mice ($BMT^{null\rightarrow wild}$ and $BMT^{wild\rightarrow wild}$ mice). Photochemical injury was performed 6 weeks after the bone marrow transfer, and the arterial cross section was analyzed 3 weeks later. As shown in Figure 1D, mean I/M ratio was significantly higher in $BMT^{wild\rightarrow null}$ arteries (0.353±0.091) than those of $BMT^{null\rightarrow wild}$ (0.067±0.031, $P=0.011$) or $BMT^{wild\rightarrow wild}$ (0.073±0.018, $P=0.039$) arteries. I/M ratios in $BMT^{wild\rightarrow null}$ and $BMT^{null\rightarrow wild}$ mice tended to be lower than those of Smad3-null and wild-type mice, respectively, presumably due to the effect of vascular irradiation.[35,36] Representative cross sections of $BMT^{null\rightarrow wild}$ and $BMT^{wild\rightarrow null}$ femoral arteries are shown in Figure 1E and 1F.

Smad3-Null Intima Is Rich in Proliferating Cells but Contains Low Amounts of Collagen Fibers

Intimal cell proliferation was assessed by immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in the femoral artery sections 1 week after the injury (Figure 3A and 3B). The ratio of the PCNA-positive nuclei to total cell nuclei was higher by 1.8-fold in Smad3-null intima compared with wild-type intima (Figure 3C). The result shows an increased proliferative activity of SMCs in Smad3-null artery at the early stage after injury.

We next evaluated intimal cell density in hematoxylin and eosin–stained arterial sections 3 weeks after the injury. As shown in Figure 4A, the ratio of intimal cell number to total intimal area was 1.6-fold higher in Smad3-null artery

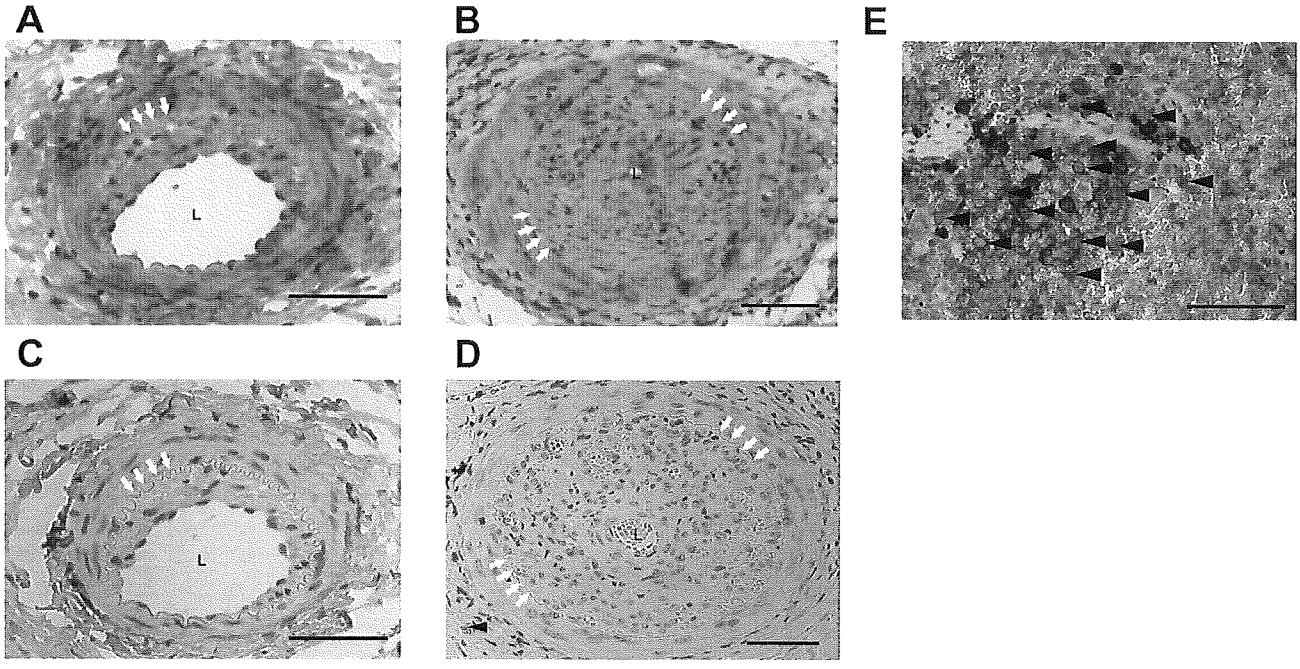

Figure 2. Immunohistochemical analysis of neointimal components. Cross sections of femoral arteries from wild-type (A and C) and Smad3-null (B and D) mice 3 weeks after endothelial injury and of mouse spleen (E). Immunostaining was performed using specific antibodies for α-SMA (A and B) and CD45 (C, D, and E). L indicates vascular lumen. Arrows indicate the positions of the internal elastic lamina. Arrowheads indicate the positions of representative CD45-positive leukocytes. Original magnification ×200; bar=50 μm.

(133±8.6) compared with wild-type artery (85.3±7.7, $P<0.01$), indicating higher cell density relative to extracellular area in Smad3-null intima. Because TGF-β/Smad3 signal is implicated in extracellular matrix (ECM) deposition, Masson's trichrome staining was also performed on a 3-week artery specimen to evaluate the amount of extracellular collagen fibers (Figures 4C and 4D). As summarized in Figure 4B, Smad3-null neointima showed 60% reduction in the ratio of Masson's trichrome-positive area to total intimal area compared with that of wild-type intima. These results suggest that Smad3 deficiency caused increased SMC number with less collagen deposition in neointima.

Growth Inhibition by TGF-β Is Attenuated in SMCs Lacking Smad3

To identify the mechanisms by which Smad3 deficiency caused exaggerated intimal hyperplasia, biological responses of the aortic SMCs obtained from wild-type and Smad3-null mice were examined in vitro. The cells were positive for both α-SMA and SMM (Figure 5A and 5B) as examined by immunocytochemistry. They also exhibited the classic "hills and valley" appearance, a feature characteristic of confluent cultured vascular SMCs. No morphological differences were observed between wild-type and Smad3-null SMCs (data not shown). It was confirmed by immunoblotting that SMCs derived from Smad3-null mice lacked expression of Smad3, whereas Smad2 level was similar in both cells (Figure 5C).

The SMCs were first tested for proliferation. As shown in Figure 6A, TGF-β dose-dependently inhibited FBS-stimulated DNA synthesis in wild-type SMCs with the maximal inhibition of 70% at 1 ng/mL and higher doses. In contrast, growth of Smad3-null SMCs was only weakly (<30%) inhibited by TGF-β. In addition, the basal growth rate of the null cells was ≈1.4-fold higher than that of the wild-type. Similar results were obtained for two additional cell lines of each genotype. The results firmly establish an essential role for Smad3 in TGF-β–mediated inhibition of cellular proliferation in vascular SMCs.

Smad3 Deficiency Does Not Attenuate TGF-β–Mediated Migratory Response in SMCs

The cells were next examined for migration, another function crucial to neointimal formation. Aschcroft et al[37] previously reported that Smad3-null monocytes and neutrophils were unable to migrate toward TGF-β, suggesting Smad3 is required for migration signal downstream of TGF-β. As shown in Figure 6B, Smad3-null SMCs dose-dependently migrated toward TGF-β at least to a similar extent as wild-type SMCs in a modified Boyden chamber assay. Moreover, Smad3-null cells showed a higher migratory capacity ($P<0.05$) than wild-type cells at 10 ng/mL TGF-β. The result suggests that Smad3-dependent signal is not essential for TGF-β–induced chemotaxis in murine vascular SMCs.

SMCs Require Smad3 for the Regulation of Type I Collagen, Matrix Metalloproteinases, and TIMP-1 by TGF-β

Previous studies suggested that migration of medial SMCs to intima involves extracellular matrix degradation.[38,39] Because TGF-β is implicated in extracellular matrix metabolism through transcriptional regulation of collagens, matrix metal-

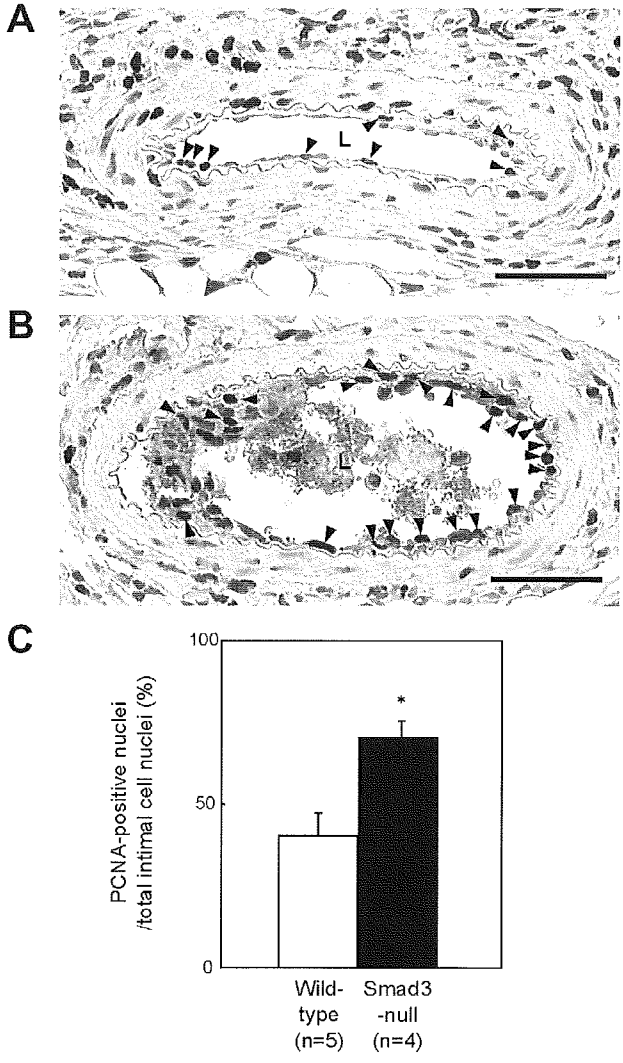

Figure 3. In vivo evaluation of cell proliferation in neointima. Representative anti-PCNA–stained cross sections of femoral arteries from wild-type (A) and Smad3-null (B) mice obtained 1 week after the injury. Arrowheads indicate PCNA-positive cells in intima. C, Ratios of PCNA-positive intimal cell number to total intimal cell number. L indicates vascular lumen. Original magnification ×200; bar=50 μm. *$P<0.05$ compared with the wild type.

loproteinases (MMPs), and TIMP-1,[7,8] we examined the ability of TGF-β to regulate mRNA expression of these components in wild-type and Smad3-null SMC. Transcript levels of COL1A2, membrane-type matrix metalloproteinase 1 (MT1-MMP), and TIMP-1 were evaluated by real-time quantitative PCR. As shown in Figure 7A, TGF-β time-dependently upregulated mRNA level of COL1A2 in wild-type SMCs with a maximal increase of 3-fold. Induction of COL1A2 by TGF-β was significantly less in Smad3-null SMCs compared with wild-type cells at all time points. TGF-β suppressed mRNA expression of MT1-MMP, an activator of pro–MMP-2,[40] to 64% of the basal level in wild-type SMCs (Figure 7B). However, MT1-MMP level was not affected by TGF-β in Smad3-null SMCs. Moreover, TGF-β increased TIMP-1 expression by 5-fold over the basal level in wild-type SMCs (Figure 7C), whereas no significant induction was observed in Smad3-null SMCs. Finally, the effect of TGF-β on MMP activity in SMC culture media was examined by gelatin zymography (Figure 7D). The basal gelatinolytic activity of MMP-2 in a serum-free conditioned media was similar for wild-type and Smad3-null SMCs. TGF-β time-dependently suppressed MMP-2 activity in wild-type cells with the maximal suppression of 29% at 24 hours, but it did not show significant effect in Smad3-null SMCs. These results suggest that Smad3 plays an essential role in TGF-β–mediated regulation of type I collagen, MMPs, and TIMP-1 in vascular SMCs.

Discussion

We report six novel findings in this article. First, mice lacking Smad3 showed a significant enhancement of neointimal hyperplasia on endothelial injury compared with corresponding wild-type mice. Second, neointima of Smad3-null mouse after injury contained a larger number of PCNA-positive cells compared with wild-type, indicating an increased proliferative activity of Smad3-null SMCs in vivo. Third, Smad3-null neointima showed higher cell density with reduced collagen area. Fourth, TGF-β–induced growth inhibition was diminished in Smad3-null SMCs in vitro. Fifth, Smad3-null SMCs retained migratory activity toward TGF-β. And finally, Smad3-null SMCs were impaired in induction of type I collagen and TIMP-1 as well as in suppression of MMPs by TGF-β. These results confirm a regulatory role of endogenous Smad3 in vascular remodeling in response to injury.

Enhanced neointimal hyperplasia in Smad3-null mice (Figure 1) lend support to previous reports describing the association of low TGF-β activity either at the ligand or receptor levels with intimal lesion formation. Grainger et al[41] showed that transgenic expression of apolipoprotein(a) promoted SMC proliferation and subsequent development of early vascular lesions by inhibiting proteolytic activation of TGF-β. Conversely, treatment with the antiestrogen tamoxifen increased serum TGF-β_1 levels and suppressed the formation of aortic lesions in mice[42]; a similar effect was also observed in human subjects.[43] McCaffrey et al[19] reported that reduced TβR-II activity due to genomic mutations led to SMC expansion in human atherosclerosis. Moreover, inhibition of TGF-β by use of a soluble type II receptor or a neutralizing antibody accelerated atherosclerosis and induced an unstable plaque phenotype in apoE-deficient mice.[17,18] And our present findings, for the first time, demonstrate a direct evidence that attenuation of TGF-β signal at the postreceptor level results in enhanced neointimal formation on injury.

Increased PCNA-positive intimal cells in vivo (Figure 3) and defect in TGF-β–induced growth suppression in vitro (Figure 6A) suggest that increased proliferative activity of SMCs contributes to the prominent neointimal formation in Smad3-null mice. Importance of Smad3 in TGF-β–mediated growth inhibition has well been described in other cell types such as αCD-stimulated primary splenocytes and embryonic fibroblasts.[33] Our results verify that Smad3, also in vascular SMCs, plays a major role in growth inhibitory function of

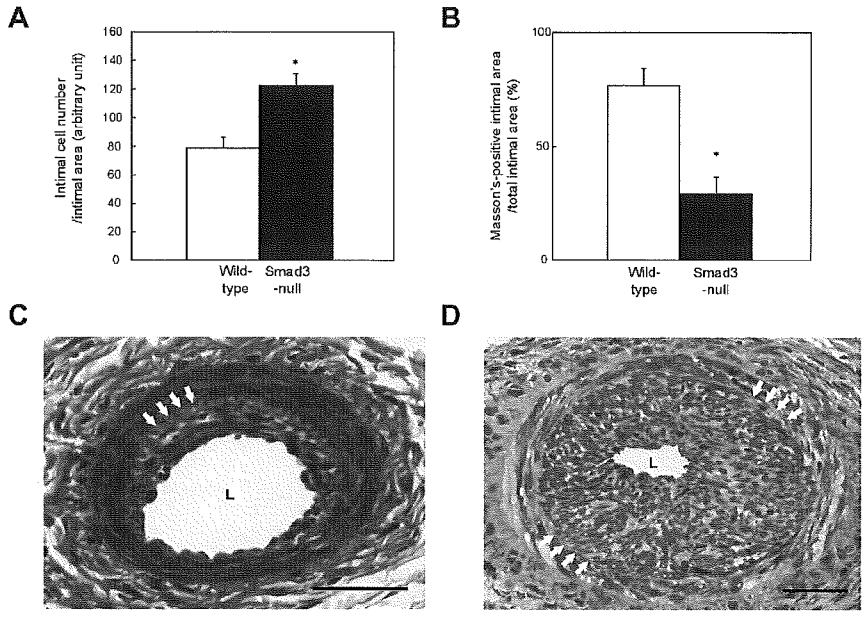

Figure 4. Evaluation of cell density and matrix deposition in neointima. A, Ratios of intimal cell number to total intimal area evaluated on hematoxylin and eosin–stained femoral arterial sections from wild-type (n=7) and Smad3-null (n=6) mice obtained 3 weeks after the injury. B, Ratios of Masson's trichrome–positive intimal area to total intimal area in femoral arterial sections from wild-type (n=7) and Smad3-null (n=6) mice 3 weeks after the injury. C and D, Photomicrographs showing the representative Masson's trichrome–stained sections of wild-type (C) and Smad3-null (D) femoral arteries. Arrows indicate the positions of the internal elastic lamina. L indicates vascular lumen. Original magnification ×200; bar=50 μm. *$P<0.01$ compared with the wild type.

TGF-β. It is to be noted that lack of Smad3 did not eliminate TGF-β–induced growth suppression in SMCs (Figure 6A). The residual growth inhibitory activity is likely to depend on another mediator downstream of TGF-β receptors, possibly Smad2.

Ashcroft et al[37] reported that Smad3 is required for TGF-β–induced migration of monocytes, leukocytes, and keratinocytes. Unexpectedly, Smad3-null SMCs were able to migrate toward TGF-β (Figure 6B). The finding suggests that, in contrast to the growth inhibitory function, Smad3-dependent signal is not essential for chemotaxis by TGF-β in murine vascular SMCs. It is therefore likely that the ability of medial SMCs to migrate into intima is preserved in Smad3-null arteries. The signaling pathway responsible for TGF-β–induced SMC motility remains to be elucidated.

TGF-β is known as a potent inducer of ECM deposition. It has been demonstrated that overexpression and intravenous administration of TGF-β caused arterial intimal thickening largely consisted of increased ECM.[10,44] TGF-β exerts fibrogenic activity through enhancement of ECM synthesis as well as inhibition of ECM degradation by downregulating MMP expression and upregulating MMP inhibitors.[6–8] Previous studies, mainly performed on dermal fibroblasts, showed that TGF-β–mediated regulation of many ECM-related genes, such as type I, III, V, and VI collagens, TIMP-1 and MMP-1 was Smad3-dependent.[45–47] In this study, we reported that Smad3-null neointima was rich in SMCs with relatively less matrix-deposition compared with wild-type intima, as evaluated by intimal cell density and Masson's trichrome staining (Figure 4), confirming a crucial role of Smad3-dependent signals in vascular ECM regulation. Moreover, TGF-β was unable to enhance mRNA expression of COL1A2 and TIMP-1 or suppress MT1-MMP expression in Smad3-null SMCs (Figure 7), establishing Smad3-dependency of these genes in vascular SMCs. Regulation of MMP-2 or gelatinase also seems to depend on Smad3-pathway in SMCs, because TGF-β attenuated MMP-2 activity in the culture media of wild-type but not in Smad3-null SMCs. Because degradation of matrix scaffold by MMPs enables cell movement and general tissue reorganization,[38,39] inability of TGF-β to suppress MMPs in Smad3-null SMCs may facilitate cell migration from media to intima in vivo.[48] Our in vitro finding that Smad3-null SMCs show a higher migration than wild-type at 10 ng/mL TGF-β (Figure 6B) may support this idea. MMP activity uninhibited by TGF-β as well as decreased matrix deposition might also have contributed to enhancement of intimal thickening in Smad3-null mice.

There have been reports on injury models suggesting that TGF-β promotes intimal thickening.[3,9–13,49] The present result that Smad3 deficiency accelerates intimal response to injury appears inconsistent with these results. However, we do not think that our findings contradict other reports on TGF-β transgene or antagonism. Our model differs from any other previous models in the point it specifically lacks Smad3 signal but not other TGF-β signal components, eg, Smad2 and MAP kinases. Smad3 not only transduces signal downstream of TGF-β, but also plays a major role in signaling of activins,[22,23] other members of the TGF-β superfamily. Activin A is expressed in atherosclerotic lesion[50] and promotes the contractile or nonproliferative phenotype of SMCs,[51] playing a role in stabilization of atherosclerotic plaque. Adenovirus-mediated overexpression of activin A suppresses neointimal formation.[51] Although we have not examined the involvement of activin A in the present study, it is assumable that the defect in activin A signal in addition to TGF-β accounts for the drastic neointimal hyperplasia in Smad3-null mice. It is of interest to determine whether specific activation of Smad3 in arterial SMCs in vivo attenuates neointimal hyperplasia. As another possibility, proinflammatory status caused by systemic Smad3 deficiency[27] might have influenced neointimal response. Although our BMT results (Figure 2D through 2F) show that the degree of intimal hyper-

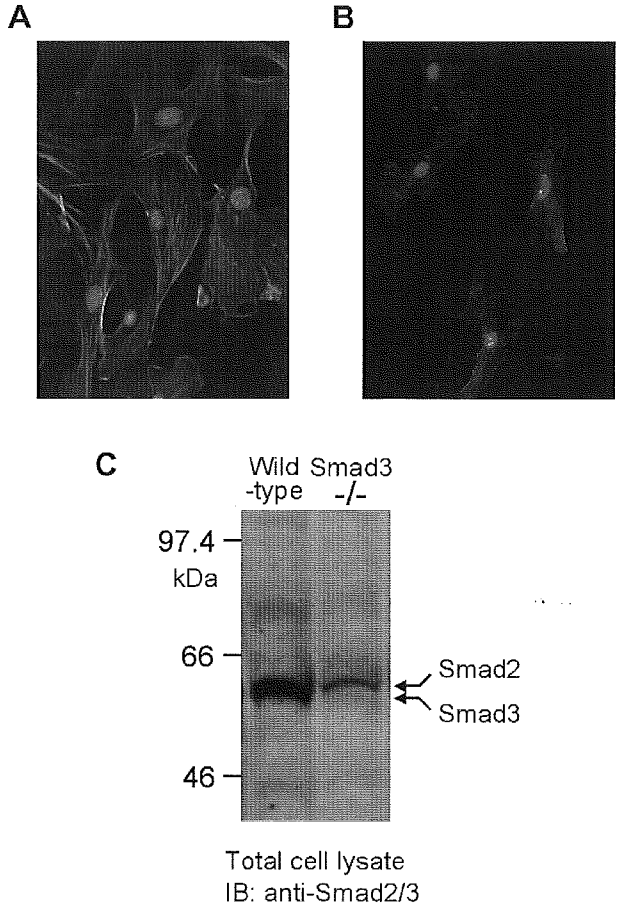

Figure 5. Characterization of cultured mice aortic SMCs. SMCs enzymatically isolated from the aorta of wild-type mice were immunocytochemically stained using anti-SMA (A, green) and anti-SMM (B, red) antibodies, counterstained with DAPI (blue, for nuclei), and subjected to fluorescent microscopy. Original magnification ×200. C, Total cell lysates of wild-type and Smad3-null SMCs were analyzed by SDS-PAGE and subjected to immunoblotting with an anti-Smad2/3 antibody. Migration positions of Smad2 and Smad3 are indicated.

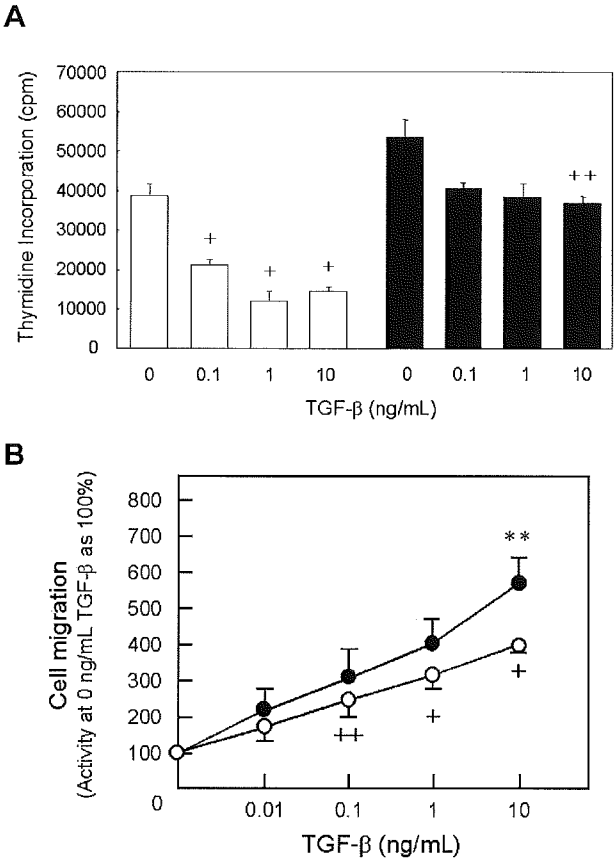

Figure 6. TGF-β–induced growth inhibition and migration of wild-type and Smad3-null SMCs. A, Wild-type (open columns) and Smad3-null (closed columns) SMCs were assayed for TGF-β–induced growth inhibition using ^{3}H-thymidine incorporation. Data are expressed as the means of three separate experiments, each performed in quadruplicate. $+P<0.01$, $++P<0.05$, compared with the value of 0 ng/mL TGF-β. B, Migration of wild-type (open circles) and Smad3-null (closed circles) SMCs toward various doses of TGF-β was measured by use of modified Boyden chamber method. Data represent the percentage of cell numbers relative to those in the absence of TGF-β and are expressed as the means of 5 separate experiments, each performed in triplicate. $+P<0.01$, $++P<0.05$, compared with the value of 0 ng/mL TGF-β. $**P<0.05$, compared with the value of wild-type at 10 ng/mL TGF-β.

plasia mainly depends on the origin of blood vessels and not of bone marrow cells, further investigation is needed to elucidate the entire role of inflammation in Smad3-null vascular response.

Finally, overactivation of TGF-β–Smad3 pathway is implicated in various fibrotic diseases involving organs such as skin, lung, liver, and kidney. Molecular agents that block Smad3-dependent TGF-β signal are anticipated as an ideal therapeutic option for these disorders.[46] However, our present results lead us to surmise that systemic suppression of Smad3 signaling can cause undesirable effects in the arteries by facilitating proliferative intimal lesions. Therefore, selective drug-delivery to the affected organs as well as careful monitoring of possible vascular lesions should be considered on clinical application of Smad3 inhibitors for fibrotic diseases.

In conclusion, mice lacking Smad3 developed marked neointimal hyperplasia on injury accompanying modulation of growth and matrix regulation in vascular SMCs. This study documents direct evidence and novel information on the functional significance: a vasculoprotective role of Smad3-dependent TGF-β signaling in response to injury.

Acknowledgments

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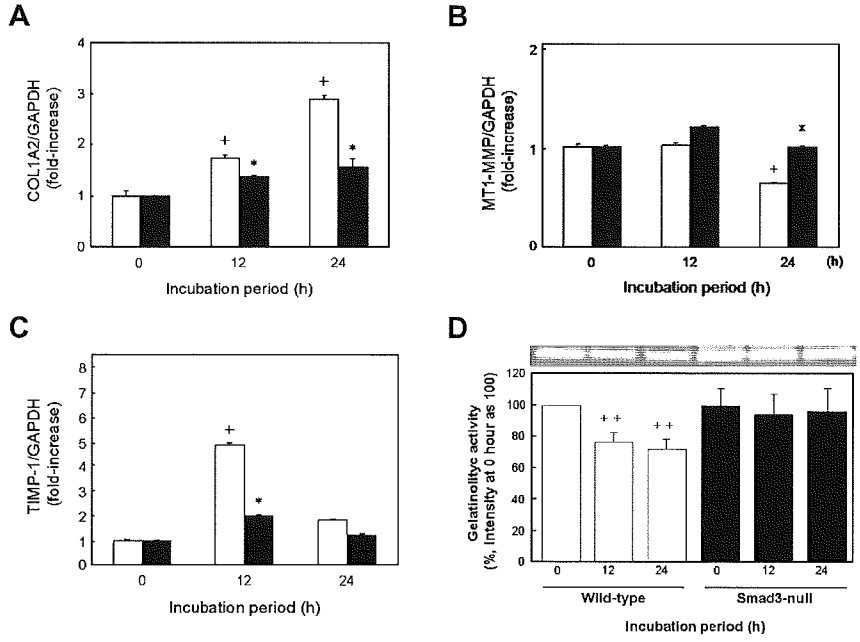

Figure 7. Effect of TGF-β on expression of type I collagen, MMPs, and TIMP-1 in wild-type and Smad3-null SMCs. Transcript levels of COL1A2 (A), MT1-MMP (B), and TIMP-1 (C) in wild-type and Smad3-null SMCs treated with TGF-β. Wild-type (open columns) and Smad3-null (closed columns) SMC were incubated with 10 ng/mL TGF-β for the indicated periods, the total RNA was isolated and used for cDNA synthesis. Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix and analyzed on an ABI PRISM 7000 Sequence Detector System. Data were calculated relative to the value for the cells without TGF-β and are expressed as the means of 3 separate experiments, each performed in triplicate. $+P<0.01$, compared with the value of 0 hour; $*P<0.01$, compared with the wild type at the same time point. D, MMP-2 gelatinolytic activity in the culture media of wild-type and Smad3-null SMCs treated with TGF-β. Culture media of SMCs incubated with 10 ng/mL TGF-β for the indicated periods was analyzed by gelatin zymogram. Proteolytic degradation of gelatin by MMP was visualized as a translucent band on the dark background. Graph shows the gelatinolytic activity, evaluated by densitometrical scanning of the bands, relative to those of wild-type SMCs at 0 hour. Data were expressed as the means of 4 separate experiments. $++P<0.05$, compared with the value of 0 hour.

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The role of Smad3-dependent TGF-β signal in vascular response to injury

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Abstract

Transforming growth factor (TGF)- β is a multifunctional cytokine involved in the regulation of proliferation, differentiation, migration, and survival of many different cell types. The role of TGF-β in atherosclerosis has been intensively studied, but the precise function of the downstream signals in this disease entity remains unclear. We recently discovered that mice lacking Smad3, a major downstream mediator of TGF-β, show enhanced neointimal hyperplasia with decreased matrix deposition in response to vascular injury. This review summarizes the current view on involvement of TGF-β in atherosclerotic vascular disease, and discusses the role of Smad3-dependent TGF-β signal in vascular response to injury.

Introduction

Transforming growth factor (TGF)- β is a prototypic member of the TGF- β superfamily which exerts wide range of biological effects on many different cell types (Roberts and Sporn, 1990). TGF- β is involved in growth inhibition, extracellular matrix production, immunomodulation, differentiation and cell migration. Aberrant activation of TGF- β signaling is implicated in various pathological conditions, such as cancer and fibrotic disorders (Blobe, 2000). The role of TGF-β in atherosclerotic vascular disease has also been the subject of intensive study. In this review, we will summarize the current view on the involvement of TGF-β in atherosclerotic vascular disease, and discuss the potential implications of Smad3-dependent signal in vascular response to injury.

Intracellular signal transduction by TGF-β

TGF- β is a dimer of polypeptides, secreted as latent form and become activated through proteolytic cleavage. Three isoforms, TGF-β1, -β2 and -β3, have been identified, among which TGF-β1 being best-studied. Figure 1 shows a schematic illustration of intracellular signal transduction by TGF-β. TGF-β elicits the effects via signaling through tetramerization of two different receptor serine/threonine kinases, TGF- β receptor type (TβR)-I and T βR-II (Heldin et al. 1997, Massagué J and Chen Y-G 2000). Receptor activation leads to phosphorylation of cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smad). The activated R-Smad heteroligomerizes with Smad4, a common mediator Smad, and the complex is transported to the nucleus where it regulates expression of target

genes. Smad7, an inhibitory Smad, binds to TβR-I, interferes with the phosphorylation of R-Smad, and results in suppression of the signaling. In addition, pathways independent of Smads, *e.g.* those involving MAP kinases, have also been described (Massagué J and Chen Y-G 2000).

The two R-Smads may have distinct functions downstream of TGF-β receptors, as judged from their structure and the patterns of gene induction (Roberts et al. 2001). Mice in which the Smad2 or Smad3 genes have been deleted by homologous recombination also show dramatically different phenotypes. Target deletion of the Smad2 gene results in early embryonic lethality (Waldrip et al. 1998). In contrast, the mice lacking Smad3 are viable and show various phenotypes including impaired mucosal immunity (Yang et al.1999), accelerated wound healing (Ashcroft et al. 1999), protection against diabetic glomerular changes (Fujimoto et al. 2003), attenuation of fibrotic response (Flanders et al. 2004) and tumorigenesis (Waldrip et al. 1998, Wolfraim et al. 2004). These findings indicate the biological importance of Smad3-dependent signal particularly after birth.

TGF-β promotes restenotic vascular lesions

More than a decade ago, it was reported that human vascular restenosis lesions as well as neointimal segment in injured rat carotid arteries express TGF-β1 mRNA and protein (Majesky et al. 1991, Nikol et al. 1992), suggesting the involvement of TGF-β in the lesion formation. As a matter of fact, direct application of TGF-β to the arterial wall in animal models either by production *in situ* through gene transfer or by intraluminal administration of recombinant protein resulted in enhanced neointimal hyperplasia composed of smooth

muscle cells (SMCs) and extracellular matrix, in the presence or absence of vascular injury (Nabel et al. 1993, Kanzaki et al. 1995, Schulick et al. 1998). TGF- β activity to stimulate procollagen and fibronectin production, downregulate matrix metalloproteinases (MMPs), and upregulate protease inhibitors (Ignotz RA and Massagué J 1986, Uría et al. 1998, Westerhausen 1991) may play a critical role in the promotion of neointimal hyperplasia. It was also shown that TGF-β antagonism either by antibody, soluble receptor or ribozyme oligonucleotides effectively reduced neointimal formation and constrictive remodeling after balloon-injury in animals (Wolf et al. 1994, Yamamoto et al. 2000, Kingston et al. 2001).

TGF-β stablizes atherosclerotic plaques

Recent progress in vascular research underscores the importance of inflammatory process in formation of atherosclerotic vascular diseases (Libby 2002). According to this concept, rupture or erosion of vulnerable atheromatous plaque plays a central role in the onset of cardiovascular events. It is now widely recognized that such lipid-rich atheromatous plaque is distinct from SMC/matrix-rich post-angioplasty restenotic lesions in their biological characteristics.

Figure 2 illustrates the major steps in atherosclerotic lesion formation and the putative effects of TGF-β on the each step. *In vitro*, TGF-β has been shown to attenuate endothelial activation through downregulation of adhesion molecules (Gamble et al. 1993) and upregulation of endothelial nitric oxide synthase (Inoue et al. 1995). It also inhibits cholesteryl ester accumulation in macrophages and deactivates T-lymphocytes (Argmann et al. 2001, Gamble et al.