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Ⅲ　研究成果の刊行に関する別冊一式

clinical trials or meta-analysis. The epidemiological literature did not need a clinical trial to conclude that smoking causes lung cancer. In the case of propoxyphene, clinical trials have already indicated its lack of efficacy advantages over other alternatives, and epidemiological studies have shown its potential to increase risk of fractures in addition to other known side effects. We appreciate Dr. Morton's clinical experience of treating many patients with propoxyphene and achieving acceptable analgesic efficacy with only rare adverse experiences over the last 25 years. But, again, this by itself is not rigorous scientific evidence of the efficacy and safety of propoxyphene in elderly patients; in fact, in epidemiological terms, Dr. Morton's own clinical experience can be considered an example of only a case series study design.

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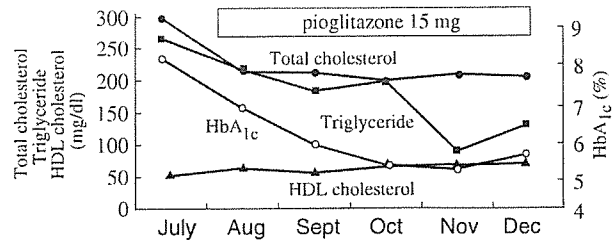
METABOLIC IMPROVEMENT AND ABDOMINAL FAT REDISTRIBUTION IN WERNER SYNDROME BY PIOGLITAZONE

To the Editor: Werner syndrome is a rare autosomal recessive disorder known for its premature aging phenotype including loss of hair, cataracts, atrophy of peripheral soft tissue, diabetes mellitus, and atherosclerosis. Mutations in the deoxyribonucleic acid (DNA) helicase gene have been identified as the cause of this disease.[1] One common feature of Werner syndrome is insulin resistance, but the mechanism by which insulin resistance occurs in this syndrome is unknown. We have previously described that visceral fat accumulation is strongly associated with insulin resistance in Werner syndrome.[2] We report a case of Werner syndrome in which administration of pioglitazone, a thiazolidinedione derivative, improved insulin sensitivity, glucose tolerance, lipid metabolism, and abdominal fat distribution.



Fasting serum insulin (μU/ml)	45.5	13.0
Insulin sensitivity index	19.5	24.9
Visceral fat area (cm^2)	111.6	104.3
Subcutaneous fat area (cm^2)	162.5	218.5

Fig. 1 Metabolic parameters and abdominal fat areas before and during pioglitazone treatment. HDL = high-density lipoprotein; Hb = hemoglobin.

A 46-year old woman with Werner syndrome came to our hospital for glycemic control. After obtaining written informed consent, we analyzed genomic DNA from peripheral leukocytes, which revealed that the patient was homozygote for type 4 mutation in the Werner helicase gene.[3] She was thin (body mass index = 16.5 kg/m^2) but had accumulated visceral fat in excess, as determined using a computed tomography scan at the umbilical level (visceral fat area = 111.6 cm^2, normal range for Japanese women <90).[4] She also had type IIb hyperlipidemia according to World Health Organization classification. She had significant insulin resistance, as determined using an insulin sensitivity index calculated from the value of steady state plasma glucose (19.4, normal range 55–162).[5] After 1 week of treatment with diet, pioglitazone 15 mg daily was initiated. After 16 weeks of pioglitazone treatment, the patient's fasting plasma glucose had decreased from 198 mg/dL to 115 mg/dL, glycated hemoglobin A1c from 8.4% to 5.9% (normal = 5.9% or less), serum total cholesterol from 270 mg/dL to 209 mg/dL (normal = 130–220 mg/dL), serum triglyceride from 301 mg/dL to 90 mg/dL (normal = 80–150 mg/dL), and serum high-density lipoprotein-cholesterol increased from 52 mg/dL to 64 mg/dL (normal ≥ 40 mg/dL). Fasting serum insulin decreased from 45.5 μU/mL to 13.0 μU/mL (normal = 6–26 μU/mL), and insulin sensitivity index had improved to 24.9 (Figure 1, July to November). Although the patient gained weight, from 35.9 kg to 39.0 kg, during the period, her visceral fat area (V) decreased to 104.3 cm^2. In contrast, abdominal subcutaneous fat area (S) increased from 162.5 cm^2 to 218.5 cm^2. As a result, her V/S ratio decreased from 0.69 to 0.48 (normal range for Japanese

<0.4).[4] Liver function monitored using serum transaminase level did not show abnormality throughout the period.

These results suggest that pioglitazone was effective in ameliorating impaired insulin sensitivity, glycemic control, and hyperlipidemia in the patient. Human and animal studies have shown that a possible mechanism for thiazolidinedione to improve insulin sensitivity is through the specific promotion of subcutaneous adipocyte differentiation through the activation of peroxisome proliferator-activated receptor-γ.[6] It has also been reported that troglitazone-treatment of type 2 diabetic patients resulted in subcutaneous fat increase in accordance with improvement of glucose tolerance.[7] It was also proven experimentally that, in lipoatrophic diabetes mellitus, lack of fat is directly associated with insulin resistance and hyperglycemia.[8] Marked atrophy of soft tissues in the extremities, a characteristic feature of Werner syndrome, may at least in part account for the insulin resistance. Leptin administration was recently reported to ameliorate severe insulin resistance in leptin-deficient lipodystrophic patients,[9] but in our patient, serum leptin levels were in the normal range before and during the pioglitazone treatment (data not shown). Therefore, in this case, induction of subcutaneous fat using pioglitazone would have accompanied production of another mediator than leptin to improve insulin sensitivity.

Recently, accumulating evidence suggests that thiazolidinedione has direct antiatherosclerotic effects on vascular cells.[10] Because atherosclerotic vascular disease is a leading cause of middle-age mortality in Werner syndrome, pioglitazone may provide an ideal choice for the treatment of metabolic disorders to improve prognosis of this syndrome.

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TREATMENT OF FRACTURES OF THE ATROPHIC MANDIBLE IN THE ELDERLY

To the Editor: The surgical treatment of facial injuries in elderly patients becomes increasingly important in geriatric medicine.[1] The number of injuries increases in the geriatric population due to a longer life expectation of the people in our society, in combination with a higher frequency of leisure activities and mobility of older people. The therapeutic concept of injuries in elderly patients has some special aspects; often, the general condition of the patients and some local factors complicate surgical management and increase comorbidity. The preferably fast reconstruction of function with as little invasiveness as possible is the most important aim of surgical care of the aged.[2]

The surgical management of maxillofacial fractures in elderly patients has the same problems. Whereas fractures of the midface can be treated surgically using conventional miniplates, mandibular fracture treatment, being biomechanically more complex and challenging, imposes some unresolved problems. The mandible in the elderly is often edentulous and atrophic. The reduced cross section and small contact area of the fractured ends produce minor primary stability, and the correct anatomical reposition is often difficult.[3] Moreover, increasing atrophy frequently represents a risk during bone healing because of the sclerotic bone and the lack of blood circulation.[4] These unfavorable conditions contribute to the high morbidity of atrophic mandibular fractures.[5]

In the literature, various therapeutic concepts are described. Although some authors favor noninvasive, conservative management, plate osteosynthesis of the fractured atrophic mandible is the most commonly preferred treatment option in trauma centers.[6] Surgeons who use bicortical plate osteosynthesis systems report difficulties in inserting the thick screws into the thin bone fragments. Additionally, the exact reposition with direct contact between the thin bone fragments is often impossible with the strong metal plates. A major disadvantage of thick plates is the inability of patients to wear full dentures during the time of plate incorporation (Figure 1, top right), but the application of conventional miniplates does not generally result in enough stability of the bone fragments[7] (Figure 1, top left).

Therefore, none of the therapeutic concepts discussed in the literature has gained general acceptance. Whereas some controversy existed in the literature on the stability of osteosynthetic fracture treatment, recent research indicates a range of optimized micromovements (500–2,000 μstrain) in the fracture gap.[8] A unique plate system fulfilling the individual biological, biomechanical, and clinical requirements

Our report is, to the best of our knowledge, the first one describing the effects of obesity surgery in type 1 diabetes. In our opinion, gastric bypass surgery, which is being performed increasingly often (~100,000 operations in the U.S. annually [10]) in obese individuals, also with type 2 diabetes (4–8), is a feasible, safe, and effective method of weight reduction in young type 1 diabetic patients with severe obesity and comorbidities leading to metabolic syndrome (e.g., hypertension, hyperlipidemia) (11). In our patients, surgery-induced weight loss was also associated with a decrease in insulin requirement per kilogram of body weight (0.60 to 0.53 IU/kg in the first patient and from 0.95 to 0.83 IU/kg in the second patient). This observation may suggest the presence of clinically significant insulin resistance in severely obese type 1 diabetic subjects (12), which was subsequently reduced once weight loss occurred. Importantly, neither of the patients had any significant hypoglycemic episodes after the surgery, despite considerable reduction in HbA_{1c} level and apparent increase in insulin sensitivity.

In conclusion, gastric bypass surgery not only leads to a significant and maintained weight loss in type 1 diabetic patients, but also results in remarkable improvement in metabolic control (absolute reduction in HbA_{1c} of 3–4%) and concomitant disorders. Interestingly, the need for constant intensive insulin therapy in these patients had no detrimental influence on weight loss as an effect of obesity surgery. Both patients lost 50–60% of their excessive body weight during the follow-up period, which is also the rate reported in nondiabetic subjects (4,5,7).

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Dysadipocytokinemia in Werner Syndrome and Its Recovery by Treatment With Pioglitazone

Werner syndrome (WS) (Mendelian Inheritance in Man no. 277700) is an autosomal recessive disorder known for progeroid phenotypes including graying and loss of hair, juvenile cataracts, insulin-resistant diabetes, skin atrophy, premature atherosclerosis, and cancer (1). Mutations in *WRN*, a RECQ family DNA/RNA helicase gene, have been identified to cause this disease. The mechanism for insulin resistance in WS remains to be elucidated.

Adipocytes secrete a number of hormones (or adipocytokines), such as tumor necrosis factor-α (TNF-α), leptin, adiponectin, and resistin, thereby regulating insulin sensitivity (2). WS patients typically show the lipoatrophic skinny extremities with an obese trunk (1). The accumulated intra-abdominal visceral fat (3) suggests an altered production of adipocytokines.

To investigate the role of adipocytokines in the pathophysiology of WS, we examined the serum levels of TNF-α and adiponectin in WS. Sera sampled from 24 WS patients (14 men and 10 women; 16 with and 8 without diabetes) proven to be homozygous for *WRN* mutations, and 40 age- and sex-matched normoglycemic healthy volunteers were assayed after informed consent was obtained. Age (43 ± 8.1 vs. 41.6 ± 7.5 years) and BMI (19.4 ± 1.9 vs. 18.8 ± 2.0 kg/m^2) were similar for diabetic and nondiabetic WS patients.

The serum level of TNF-α, a mediator of insulin resistance, was significantly elevated in WS regardless of having diabetes (21.8 ± 8.7 pg/ml, $P < 0.0001$ by Mann-Whitney test) or not having diabetes (14.0 ± 3.2 pg/ml, $P = 0.002$) compared with the healthy control group (6.05 ± 3.0 pg/ml). Adiponectin levels in diabetic WS patients (3.1 ± 2.9 μg/ml) was significantly lower than in nondiabetic WS patients (11.6 ± 9.2 μg/ml, $P = 0.006$) or control subjects (14.4 ± 8.8 μg/ml, $P < 0.0001$). The growing evidence indicates insulin sensitizing as well as antiatherogenic actions of adiponectin and the association of decreased serum adiponectin with insulin resistance, obe-

sity, and type 2 diabetes (2,4). Although WS patients are usually not obese by the definition of BMI, the visceral fat specifically accumulated by an unknown mechanism (3) might cause high TNF-α and low adiponectin levels, characteristics similar to morbid obesity.

We recently reported the successful improvement of glycemic control and insulin sensitivity by pioglitazone in diabetic WS patients (5). Therefore, we next assessed adipocytokines before and after 16 weeks on pioglitazone (15 mg/day) in three diabetic WS patients. The treatment significantly elevated adiponectin levels from 2.57 ± 1.36 to 7.07 ± 2.48 μg/ml ($P = 0.03$ by paired t test). TNF-α and HbA_{1c} levels showed a tendency to decline from 16.1 ± 4.75 to 3.53 ± 0.58 pg/ml ($P = 0.052$) and from 7.7 ± 0.6 to 6.4 ± 0.5% ($P = 0.17$), respectively.

To our knowledge, this is the first study to examine serum adipocytokine levels in WS patients. Reduced insulin sensitivity with increased visceral adiposity is the hallmark of both WS and normal aging. Because pioglitazone achieved improvement of glycemic control as well as correction of adiponectin and TNF-α levels, these cytokines are likely to be at least in part responsible for insulin resistance in WS. Adipocyte function may be a key element linking *WRN* mutation and the metabolic abnormalities observed in WS. It is also of our interest to know whether pioglitazone and other thiazolidinediones can prevent or delay the onset of diabetes in WS by modulating adipocytokines. Our present findings raise a possibility that pioglitazone could extend the lifespan of WS patients by improving metabolism and preventing early cardiovascular death.

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Effect of α-Linolenic Acid–Containing Linseed Oil on Coagulation in Type 2 Diabetes

Blood coagulation in diabetes is known to be increased (1,2). Because levels of n-3 and n-6 polyunsaturated fatty acids (PUFAs) influence the parameters of blood coagulation, the aim of this study was to determine the effects of n-3 PUFA supplementation on coagulation and fibrinolytic factors in type 2 diabetic subjects. While it is not clear what the appropriate intake ratio of n-6 to n-3 PUFAs should be for diabetic subjects, it is known that the dietary intake ratio of n-6 to n-3 PUFAs is roughly 4:1 in Japanese subjects (3).

Ten subjects (six women and four men, average age 59.6 years) with type 2 diabetes participated in this study as inpatients. Their average BMI and HbA_{1c} values were 20.9 ± 3.8 kg/m^2 and 10.8 ± 1.1%, respectively. Their daily energy intake during the course of the study was 1,490 ± 166 kcal. After 2 weeks on the control diet, our subjects were placed on a diet in which 5 g linseed oil was added (in salads, miso soup, etc., without heating) in exchange for 5 g cooking oil. The ratio of PUFAs to saturated fatty acids in the subjects' prestudy and study diets were 1.2 and 1.6, respectively, while the ratios of n-6 to n-3 PUFAs in their prestudy and study diets were 3.6 and 1.5, respectively. Blood samples were collected before and 14 days after initiation of the study. Plasmin α2-plasmin inhibitor complex (PPI) level and plasminogen activator inhibitor-1 (PAI-1) activity in plasma was measured using a latex photometric immunoassay, while thrombin antithrombin III complex (TAT) level was measured using an enzyme-linked immunoassay. Differences in these parameters obtained at the start and end of the study were analyzed using a paired t test; values were considered to be significant if the P value was <0.05. Values are expressed as the mean ± SD.

After 2 weeks on a linseed oil–supplemented diet, PPI level, PAI-1 activity, and TAT level fell significantly (0.72 ± 0.19 vs. 0.47 ± 0.14 μg/ml, $P = 0.0009$; 73.3 ± 37.5 vs. 51.6 ± 25.0 ng/ml, $P = 0.02$; and 9.6 ± 9.1 vs. 2.5 ± 1.1 ng/ml, $P = 0.04$; respectively).

Boberg et al. (4) reported that PAI-1 activity was increased in type 2 diabetic subjects after supplementation of their diet with 10 g eicosapentaenoic acid. Kelly et al. (5) reported that a diet containing flaxseed oil (60% α-linolenic acid) did not alter indexes of blood coagulation, i.e., bleeding time, prothrombin time, and partial prothrombin time. Chan et al. (6) showed that altering the dietary n-6–to–n-3 PUFA ratio had no effect on

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 Neontimal 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

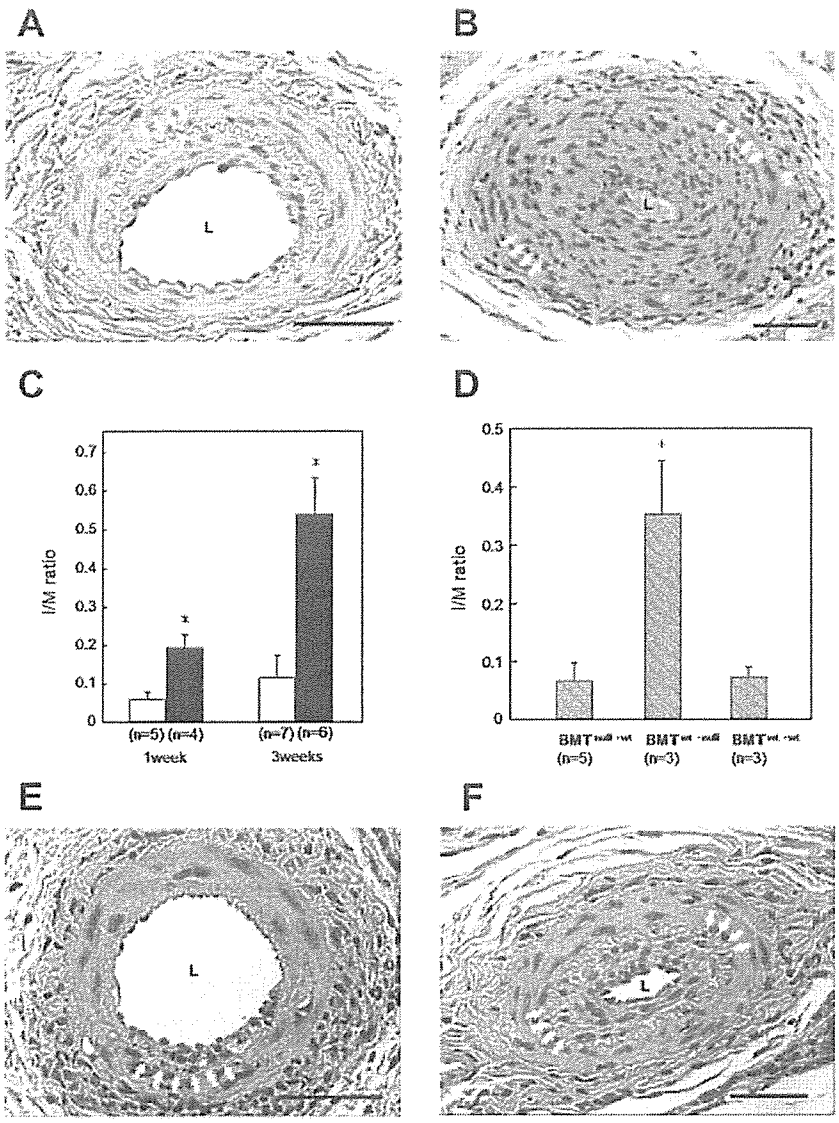

Figure 1. Neointimal thickening in injured femoral arteries of wild-type and Smad3-null mice. Photomicrographs showing representative cross sections of hematoxylin and eosin–stained femoral arteries from wild-type (A) and Smad3-null (B) and $BMT^{null \rightarrow wild}$ (E) and $BMT^{wild \rightarrow null}$ (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

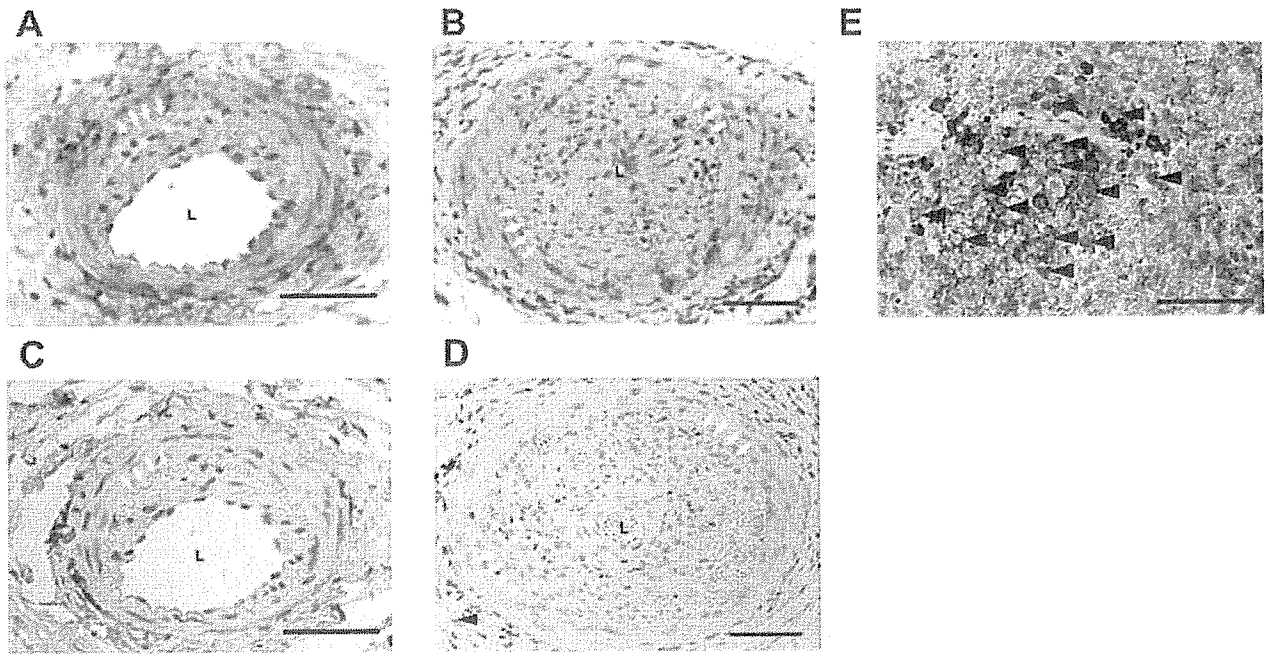

Figure 2. Immunohistochemical analysis of neointimal components. Cross sections of femoral arteries from wild-type (A and C) and Smad3-null (B and D) mice 3 weeks after endothelial injury and of mouse spleen (E). Immunostaining was performed using specific antibodies for α-SMA (A and B) and CD45 (C, 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-

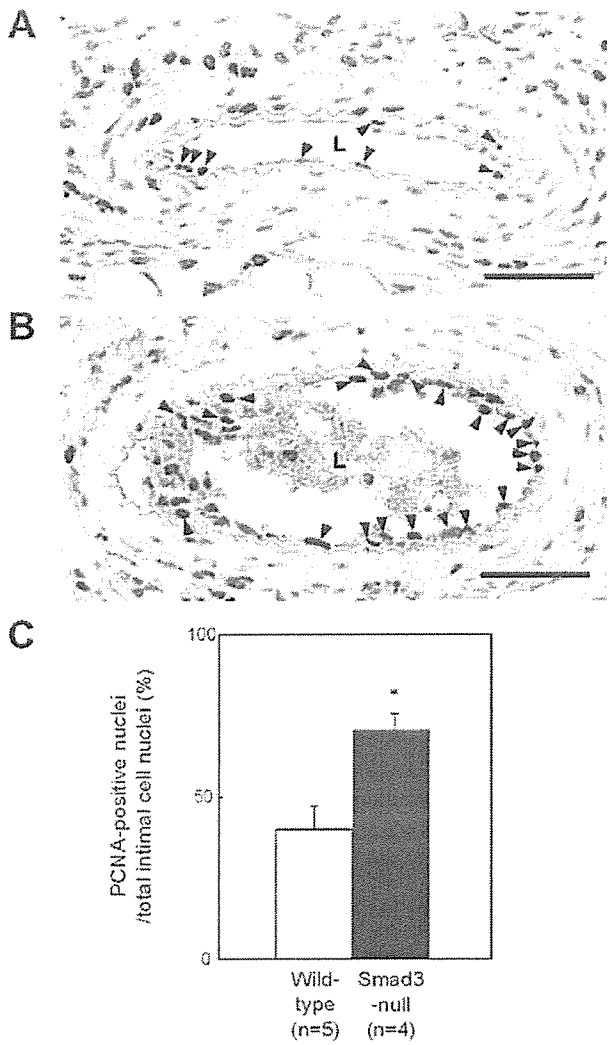

Figure 3. In vivo evaluation of cell proliferation in neointima. Representative anti-PCNA–stained cross sections of femoral arteries from wild-type (A) and Smad3-null (B) mice obtained 1 week after the injury. Arrowheads indicate PCNA-positive cells in intima. C, Ratios of PCNA-positive intimal cell 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

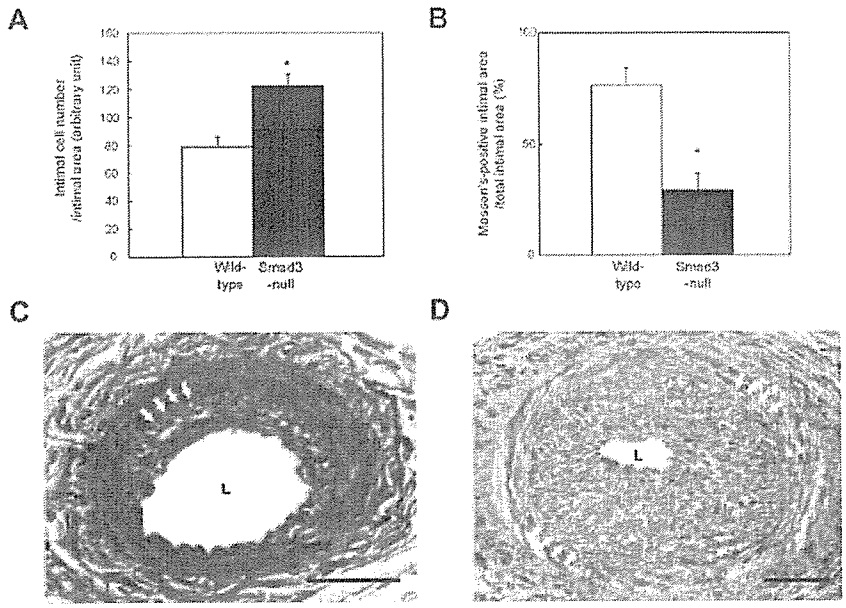

Figure 4. Evaluation of cell density and matrix deposition in neointima. A, Ratios of intimal cell number to total intimal area evaluated on hematoxylin and eosin–stained femoral arterial sections from wild-type (n=7) and Smad3-null (n=6) mice obtained 3 weeks after the injury. B, Ratios of Masson's 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-

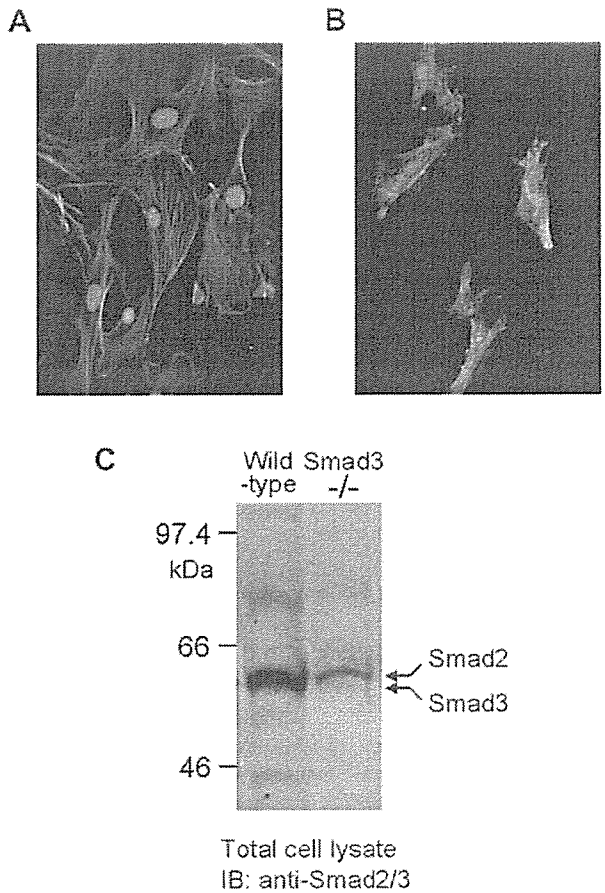

Figure 5. Characterization of cultured mice aortic SMCs. SMCs enzymatically isolated from the aorta of wild-type mice were immunocytochemically stained using anti-SMA (A, green) and anti-SMM (B, red) antibodies, counterstained with DAPI (blue, for nuclei), and subjected to fluorescent microscopy. Original 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.

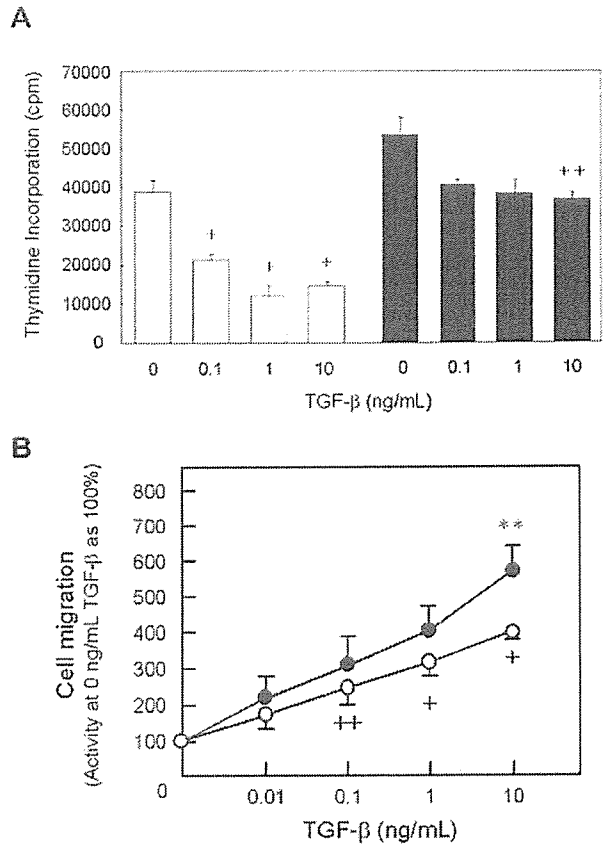

Figure 6. TGF-β–induced growth inhibition and migration of wild-type and Smad3-null SMCs. A, Wild-type (open columns) and Smad3-null (closed columns) SMCs were assayed for TGF-β–induced growth inhibition using ^{3}H-thymidine incorporation. Data are expressed as the means of three separate experiments, 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.

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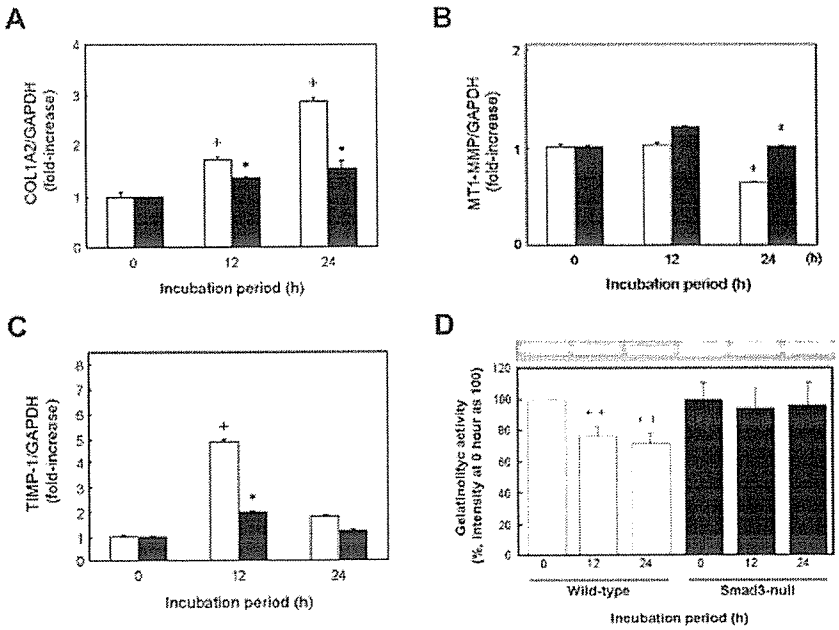

Figure 7. Effect of TGF-β on expression of type I collagen, MMPs, and TIMP-1 in wild-type and Smad3-null SMCs. Transcript levels of COL1A2 (A), MT1-MMP (B), and TIMP-1 (C) in wild-type and Smad3-null SMCs treated with TGF-β. Wild-type (open columns) and Smad3-null (closed columns) SMC were incubated 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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implications of the change of each of these two parameters in older subjects without cardiovascular disease, it can be hypothesized that exercise training improves functional cardiovascular capacity and vagal/sympathetic balance and that this effect is proportional to an improvement in lung ventilation. Future researches should investigate the significance of this correlation.

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ETIDRONATE AMELIORATES PAINFUL SOFT-TISSUE CALCIFICATION IN WERNER SYNDROME

To the Editor: Bisphosphonates, chemical compounds widely used as antiosteoporotic agents, were originally brought into clinical practice to treat ectopic calcification.[1] Their application for this purpose has been almost forgotten because the therapeutic dose may also affect normal bone formation. Here, we report that etidronate, a first-generation bisphosphonate, ameliorated soft-tissue calcification and improved performance in a patient with progeroid Werner syndrome without apparent adverse effects.

A 47-year-old woman visited our hospital because of intolerable pain in the left knuckle, bilateral elbows, and ankles. She had graying and loss of hair, peripheral soft tissue atrophy, a skin ulcer on the right ankle, marked insulin resistance, and a history of cataract at the age of 30. Werner syndrome was suspected; peripheral blood deoxyribonucleic acid (DNA) analysis confirmed homozygous type 4 mutations in the causative WRN helicase gene.[2]

Pain in the left knuckle was due to a hard subcutaneous nodule (Figure 1, left panel), which turned out to be an ectopic calcification (Figure 1, middle panel). Similar calcification was also found in the elbows and Achilles tendons; all of them coincided with the positions of pain. Her hands, elbows, and left ankle were free of ulcers. X-rays of the lumbar and thoracic spines showed no sign of osteoporosis. Serum calcium, phosphorus, alkaline phosphatase, and parathyroid hormone were in the normal range.

The patient could hardly clench her left fist or walk more than 1 m because of pain in the knuckle and ankles. Etidronate at a dose of 20 mg/kg per day was started orally in an attempt to suppress the ectopic calcification.

Clinical symptoms improved dramatically after 3 months of treatment. She was now able to walk for more than 6 m, was free of pain in the elbows, and felt remarkably less pain in her knuckle. The size of the nodule became smaller (Figure 1, right panel), indicating the effectiveness of etidronate in reversing calcification. No adverse effects were described at this point, but etidronate was stopped to avoid possible inhibition of bone formation.

Bisphosphonates, first synthesized in the 1860s, was originally used in industry to prevent scaling or precipitation of calcium carbonate.[1] Their biological effect of inhibiting ectopic calcification in vivo, as inspired by the structural similarity to inorganic pyrophosphate, was initially reported in 1968,[3] but clinical use of bisphosphonates for this purpose has not developed further, because they also interfere with mineralization of normal bone. Instead, they are now established as drugs against osteoporosis because of their property of preventing bone resorption when given at lower doses.

Werner syndrome, an autosomal recessively inherited progeroid disorder caused by homologous mutations in a RecQ family DNA helicase, often accompanies soft-tissue calcification for unknown reasons.[4,5] It can be asymptomatic but often results in severe pain and may promote skin ulcer formation. These symptoms limit patients' daily activity, threaten their quality of life, and facilitate development of overt diabetes mellitus due to inactivity on the base of insulin resistance.

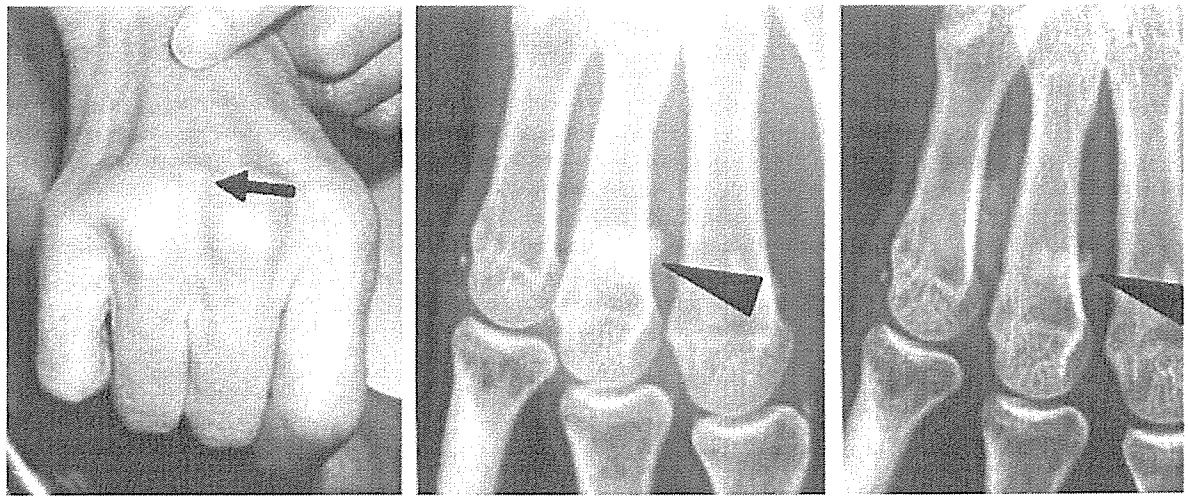

Figure 1. Left panel, subcutaneous nodule with pain (arrow). Radiographic examination before (middle panel) and after 3 months on etidronate (right panel). Arrowheads indicate the positions of nodular calcification.

Our present clinical experience demonstrates a novel therapeutic option for an otherwise incurable complication of Werner syndrome. Moreover, it rediscovers the usefulness of bisphosphonate for ectopic calcification.

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HYPOADIPONECTINEMIA IN BEDRIDDEN FEMALE PATIENTS YOUNGER THAN 75

To the Editor: Older people have several hormonal alterations, but the effect on the endocrine function of adipose tissue in older bedridden patients has not been fully elucidated. Adiponectin is a newly discovered antiinflammatory protein, secreted exclusively by adipocytes, that plays a protective role against atherosclerosis.[1] Hypoadiponectinemia plays a crucial role in atherosclerosis in men, but there have been no studies of plasma adiponectin in bedridden women. The aim of the present study was to estimate plasma adiponectin concentration in bedridden elderly female patients in comparison with age-matched healthy volunteers.

Seventy-four bedridden female patients admitted to geriatric wards and nursing homes in Osaka, Japan, and age-matched volunteers were studied. Clinical diagnoses were defined using detailed physical examination and routine biochemical analyses of blood and urine, as well as clinical tools including computed tomography. Their mean bedridden period ± standard deviation was 49.4 ± 37.4 months. All plasma analyses were performed on samples from fasting subjects. Adiponectin was measured using high-sensitive radioimmunoassay (Linco Research, St. Louis, MO). Bedridden subjects and healthy volunteers were divided into two groups: younger than 75 and aged 75 and older. All statistical analyses were performed using the SPSS (SPSS Inc., Chicago, IL). The statistical differences in the variables were compared using the Mann-Whitney *U* test, and the association between any two parameters was assessed using Spearman correlation.

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PII S1050-1738(06)00070-3 **TCM**

The Role of Smad3-Dependent TGF-β Signal in Vascular Response to Injury

Koutaro Yokote*, Kazuki Kobayashi, and Yasushi Saito

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. (Trends Cardiovasc Med 2006;16:240–245) © *2006, Elsevier Inc.*

• Introduction

Transforming growth factor (TGF)-β is a prototypic member of the TGF-β superfamily, which exerts wide range of biologic effects on many different cell types (Roberts and Sporn 1990). Transforming growth factor-β is involved in growth inhibition, extracellular matrix production, immunomodulation, differentiation, and cell migration. Aberrant activation of TGF-β signaling is implicated in various pathologic conditions, such as cancer and fibrotic disorders (Blobe et al. 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

Transforming growth factor-β is a dimer of polypeptides, secreted as latent form, and become activated through proteolytic cleavage. Three isoforms, TGF-β1, TGF-β2, and TGF-β3, have been identified, among which TGF-β1 is best studied. Figure 1 shows a schematic illustration of intracellular signal transduction by TGF-β. Transforming growth factor-β elicits the effects via signaling through tetramerization of two different receptor serine/threonine kinases, TGF-β receptor type (TβR)-I and TβR-II (Heldin et al. 1997, Massagué and Chen 2000). Receptor activation leads to phosphorylation of cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smads). 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, for example, those involving mitogen-activated pro-

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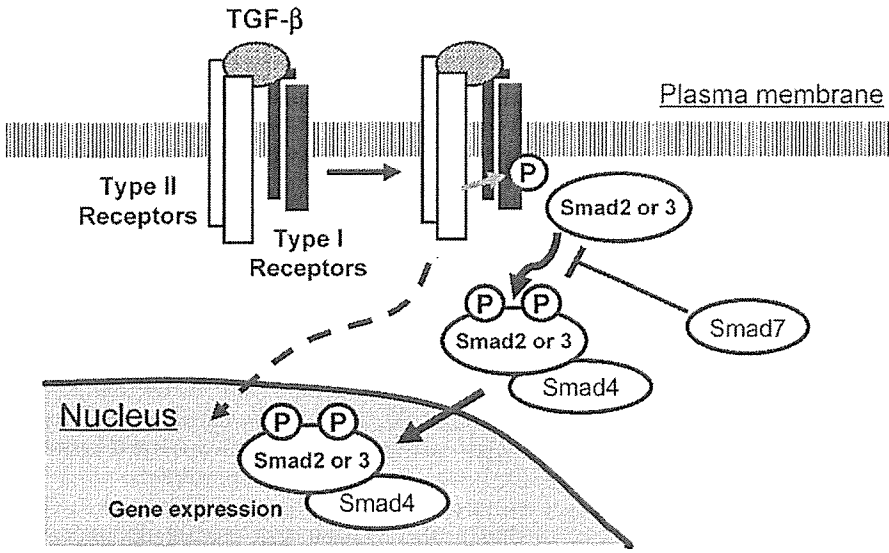

Figure 1. Schematic illustration of intracellular signal transduction pathways by TGF-β. Upon ligand-induced heteromeric complex formation and activation of type-I and type-II receptors, cytoplasmic signal transducers Smad2 and Smad3, classified as so-called R-Smads, are phosphorylated and heteroligomerized with Smad4, a common mediator Smad. The complex then translocates into the nucleus, where it regulates expression of target genes. Smad7 binds to type-I receptor, interferes with the phosphorylation of R-Smad, and results in suppression of the signaling. Non-Smad signaling pathways, indicated as a *broken arrow*, are also reported. P, phosphorylated serine/threonine residues.

tein kinases, have also been described (Moustakas and Heldin 2005).

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 (Nomura and Li 1998, 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 2004), and tumorigenesis (Waldrip et al. 1998, Wolfraim et al. 2004). These findings indicate the biologic importance of Smad3-dependent signal, particularly after birth.

• Transforming Growth Factor-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). Transforming growth factor-β activity to stimulate procollagen and fibronectin production down-regulates matrix metalloproteinases (MMPs) and up-regulate protease inhibitors (Ignotz and Massagué 1986, Uría et al. 1998, Westerhausen et al. 1991) and may play a critical role in the promotion of neointimal hyperplasia. It was also shown that TGF-β antagonism 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).

• Transforming Growth Factor-β Stabilizes 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 atherom-

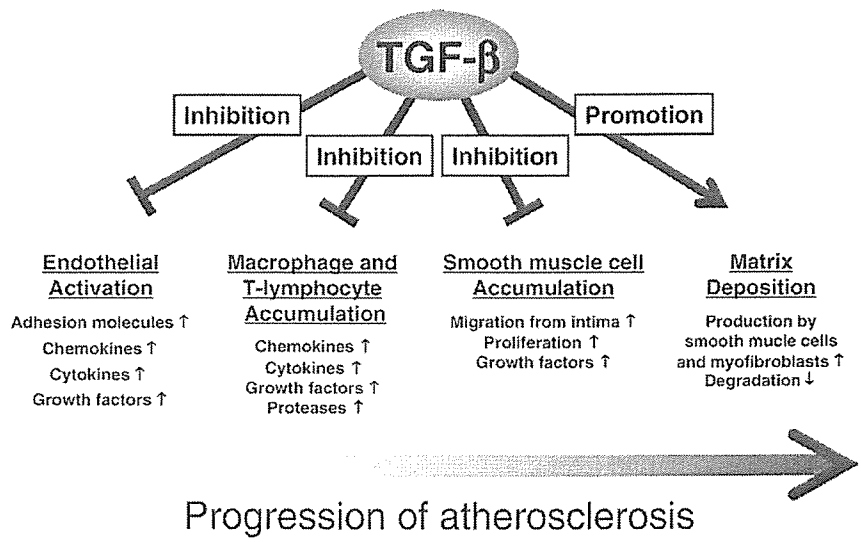

Figure 2. Major steps in atherosclerotic lesion formation and the putative effects of TGF-β on the each step. Both in vitro and in vivo evidence suggests that TGF-β inhibits activation of endothelial cells and intimal accumulation of inflammatory cells and smooth muscle cells. On the other hand, TGF-β promotes deposition of extracellular matrix.