も薄くなる．確固たるエビデンスがない領域だからこそ，症例ごとに，その治療の有益性を十分に検証することが大切である．

謝辞：ご校閲賜りました齋藤　康教授に感謝申し上げます．

文　献

1) 横手幸太郎，齋藤　康：メタボリックシンドローム概念と今後の展開．Mebio **23**(1)：66-71，2006.
2) メタボリックシンドローム診断基準検討委員会．日本内科学会雑誌 **94**：794-809，2005.
3) Stamler J et al：Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care **16**(2)：434-444，1993.
4) 厚生労働省ホームページ「平成15年国民栄養調査結果の概況」.
5) Roessner S：Obesity in the elderly—a future matter of concern? Obes Rev **2**(3)：183-188，2001.
6) Beaufrere B and Morio B：Fat and protein redistribution with aging：metabolic considerations. Eur J Clin Nutr **54**(Suppl)：S48-53，2000.
7) 日本肥満学会肥満症診断基準検討委員会：新しい肥満の判定と肥満症との診断基準．肥満研究 **6**：18-28，2000.
8) Lillioja S：Skeletal muscle capillary density and fiber type are possible determinants of *in vivo* insulin resistance in man. J Clin Invest **80**：415-424，1987.
9) Paolisso G et al：Advancing age and insulin resistance. Eur J Clin Invest **29**：758-769，1999.
10) Dey DK and Lissner L：Obesity in 70-year-old subjects as a risk factor for 15-year coronary heart disease incidence. Obes Res **11**：817-827，2003.
11) Folsom AR et al：Associations of general and abdominal obesity with multiple health outcomes in older women：the Iowa Women's Health Study. Arch Intern Med **160**：2117-2128，2000.
12) Newman AB et al：Weight change in old age and its association with mortality. J Am Geriatr Soc **49**：1309-1318，2001.
13) Dey DK et al：Body mass index, weight change and mortality in the elderly. A 15 y longitudinal population study of 70 y olds. Eur J Clin Nutr **55**：482-492，2001.
14) Calle EE et al：Body-mass index and mortality in a prospective cohort of U.S. adults. N Engl J Med **341**：1097-1105，1999.
15) Stevens J et al：The effect of age on the association between body-mass index and mortality. N Engl J Med **338**：1-7，1998.
16) Peeters A et al：Obesity in adulthood and its consequences for life expectancy：a life-table analysis. Ann Intern Med **138**：24-32，2003.
17) 横手幸太郎，齋藤　康：高齢者肥満への対応と指導．日本醫事新報 **4257**：39-44，2005.
18) Villareal DT et al：American Society for Nutrition：NAASO, The Obesity Society. Obesity in older adults：technical review and position statement of the American Society for Nutrition and NAASO, The Obesity Society. Obes Res **13**(11)：1849-1863，2005.

（執筆者連絡先）　曽根崎桐子　〒260-8670　千葉県千葉市中央区亥鼻1-8-1　千葉大学医学部付属病院糖尿病・代謝・内分泌内科

内分泌・糖尿病科，22(1)：22-28，2006

特集 糖尿病と動脈硬化―その機序から診断と治療へ

糖尿病と血管平滑筋細胞機能*

小林一貴**
横手幸太郎**
齋藤　康**

Key Words：vascular smooth muscle cell, growth factor, insulin resistance, oxidized LDL

はじめに

糖尿病は動脈硬化症の重要な危険因子であるが，近年，糖尿病における動脈硬化の進展は，細小血管障害と異なり血糖コントロールだけでは十分に抑制できないことが明らかとなってきた(詳しくは他項に譲る)．このことは，糖尿病における動脈硬化促進要因として高血糖のみならず，併存するインスリン抵抗性/高インスリン血症，脂質代謝異常，高血圧など複数の危険因子が関与していることを示しており，これらの因子がそれぞれ血管壁にどのような作用を及ぼすかが重要となってくる．

動脈硬化とVSMC

動脈硬化病変の形成過程において，血管平滑筋細胞(vascular smooth muscle cell；VSMC)が重要な役割を果たすことについては，1976年にRossらが提唱した「傷害反応仮説」に始まり，その後の修正を経て現在の動脈硬化炎症説にいたるまで広く認知されている．すなわち，高血糖など前述のさまざまな要因による血管内皮障害を契機としたTcellやマクロファージの血管壁への集簇に伴い，VSMCが中膜より内膜へ遊走する．内膜中のVSMCは脂質コアの周囲で増殖し，またコラーゲン，フィブロネクチン，プロテオグリカンなどの細胞外マトリクス(extracellular matrix；ECM)を産生・蓄積して内膜肥厚病変を形成していく．病変部のVSMCおよびECMは，脂質コアを覆う線維性被膜を構成し，その性状は急性冠症候群の病因とされる「プラークの不安定化」を規定するなど，病変の量および質の両面にわたって深く関与すると考えられている[1](図1)．

正常中膜のVSMCと比べ，肥厚内膜中のVSMCはその形態および機能においてさまざまな相違を呈している[2]．前者を収縮型と呼ぶのに対し後者は合成型と呼ばれ，遊走・増殖能，ECM産生能，さまざまな因子の発現・産生およびそれらに対する反応などの各種機能が，動脈硬化促進的に変化している．このようなVSMCの機能変化を促す因子は，すなわち動脈硬化促進因子と考えられ，糖尿病はさまざまな機序でこの変化に関与している．

本稿では，血管壁/動脈硬化病変構成細胞の中でもとくにVSMCにスポットをあて，糖尿病および前述の個々の危険因子などがVSMCにいかに働きかけ，その機能をどのように変化させて動脈硬化の発症・進展にかかわっていくかについて論じる．

* Alteration of vascular smooth muscle cell function related to diabetes mellitus.

** Kazuki KOBAYASHI, M.D., Ph.D., Kotaro YOKOTE, M.D., Ph.D. & Yasushi SAITO, M.D., Ph.D.: 千葉大学大学院医学研究院細胞治療学〔〒260-8670 千葉市中央区亥鼻1-8-1〕; Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, Chiba 260-8670, JAPAN

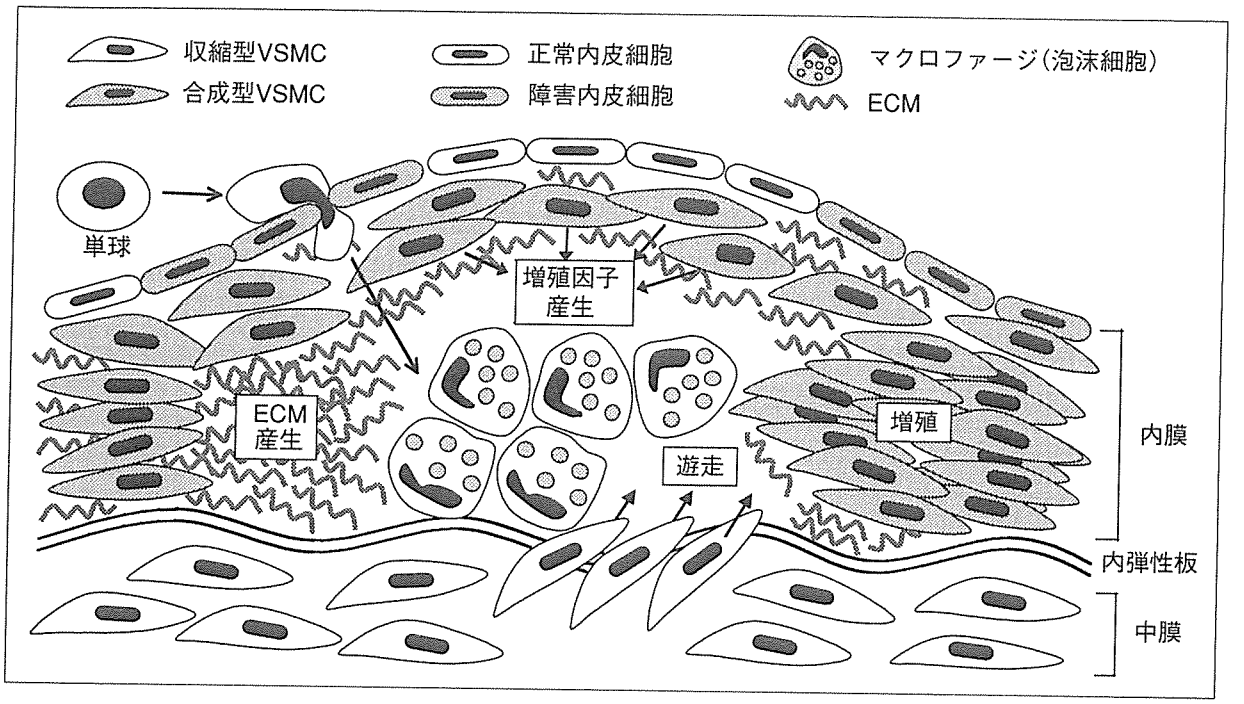

図1　動脈硬化病変形成におけるVSMCの役割

糖尿病VSMCの性質

まず，糖尿病状態におかれたVSMCの各種機能はどうなっているか．糖尿病動物中膜由来培養VSMC(DM-VSMC)や糖尿病動脈の血管壁を材料とした研究から，両者は前述のような種々の機能変化を呈することが報告されている．

DM-VSMCは正常動物中膜由来培養VSMC(normo-VSMC)と比較して前述の合成型の形態を有し，代表的な増殖因子であるplatelet-derived growth factor(PDGF)への反応性が増加しているという報告がある．1型および2型糖尿病のモデル動物を用いた検討によれば，DM-VSMCや糖尿病動脈壁中膜ではPDGF受容体の発現増加が認められ，PDGFによるDM-VSMCの遊走・増殖能も亢進していた．またこの糖尿病動脈壁にバルーンカテーテルによる内皮傷害を与えると，非糖尿病群に比べ強い内膜肥厚が生じた[3)~5)]．これらのことから，糖尿病状態におかれたVSMCのPDGF受容体発現増加は，VSMCの遊走・増殖能亢進をきたして糖尿病の動脈硬化形成過程に重要な役割を果たしていると考えられる．

また，DM-VSMCにおけるtransforming growth factor(TGF)-βの作用変化についても報告されている．TGF-βは主に細胞増殖抑制作用やECM産生作用が知られ，濃度その他の条件により相反する作用を示すbifunctionalな因子であるが，一般に内膜肥厚病変においてはVSMCによる種々のECM産生・蓄積をもたらすと言われている[6)7)]．糖尿病患者の動脈壁でも，タイプⅣコラーゲンやフィブロネクチンの蓄積量が増加していることなどが報告されている[8)9)]．DM-VSMCや糖尿病動脈壁中膜では，対照と比べてTGF-β受容体の発現が増加しており，TGF-β添加によるDM-VSMCのフィブロネクチン産生も亢進していた[4)10)]．これらのことより，糖尿病状態におかれたVSMCではTGF-βの作用が増強し，ECM蓄積が亢進するという機序が想定される．各種ECMの蓄積は病変容積の増大につながると考えられる．また，なかでもフィブロネクチンはVSMCの収縮型から合成型へのフェノタイプ変換を促し，またVSMCのPDGF受容体発現を増加させるというように直接的にVSMCの機能変化をもたらし，PDGFによるVSMCの遊走・増殖能亢進にも寄与している可能性がある[11)12)]．

このように糖尿病状態にさらされたVSMCは，さまざまな機序を介して遊走・増殖能亢進やECM産生増加といった動脈硬化促進的な性質を獲得している．

糖尿病における個々の病態因子がVSMCにおよぼす作用

では，これまで述べてきたようなDM-VSMCの機能変化は，糖尿病状態におけるどのような因子の影響によりもたらされるのであろうか．糖尿病においては，代表的な因子である高グルコース状態をはじめとして，さまざまな糖尿病付随因子の作用がそれぞれVSMCの機能変化に関与していることが明らかとなってきている．

1．高グルコース状態

高グルコース(20～30mM)条件下では，VSMCの遊走・増殖能が正グルコース(5.5mM)条件下と比べて増加することが報告されている[13]．高グルコースによるこのような反応の原因として，グルコース過剰状態により誘導される種々の因子の働きが研究されている．高グルコース条件下の細胞では，protein kinase C(PKC)の活性化，ポリオール経路やヘキソサミン経路の亢進，酸化ストレスの発生など種々の代謝異常が生じることが知られている．VSMCにおいても，高グルコース刺激がこれらの経路を介して，さまざまな増殖因子およびその受容体の発現を変化させるという報告がある．

前述のPDGFに関しては，高グルコースがPKC活性化などを介してVSMCのPDGFβ受容体発現を増加させ，PDGF作用の増強が高グルコースによる遊走能の亢進をもたらすことが示されている[14][15]．

また，同じく前述のTGF-βについても，高グルコース条件下のVSMCにおいて，PKC経路を介してTGF-βおよびその受容体の発現が増加し，TGF-β-induced gene-h3(βig-h3)発現の増強などによる遊走・増殖能亢進をもたらすという報告がある[16]～[18]．

われわれもこのような因子のひとつとして，糖尿病患者や糖尿病モデル動物の血管壁中膜VSMCに高発現しているオステオポンチン(osteopontin；OPN)の検討を行っている．これまでの検討により，OPNは高グルコース条件下のVSMCにおいて，PKC経路やヘキソサミン経路を介して発現が亢進し，mitogen activated protein kinase(MAPK)系の活性化を経て遊走能およびPDGFによる増殖能を増強することが明らかとなっている[19][20]．

このほか，やはりVSMCに作用する増殖因子heparin-binding epidermal growth factor-like growth factor(HB-EGF)によるVSMCの遊走・増殖能が，高グルコース刺激で亢進することも報告されている．これは，高グルコース条件下でヘパラン硫酸の代謝がPKC非依存性に変化し，性質の変わったヘパラン硫酸の作用によりHB-EGFに対する反応性が亢進したもので，PKC非依存性かつECM代謝を介した高グルコースの影響を示すものである[21]．

以上のような，高グルコース状態におけるVSMCの，各種増殖因子作用を介した遊走・増殖能亢進などの性質変化は，前述のDM-VSMCの形質として糖尿病の動脈硬化形成に深くかかわると考えられる(図2)．

2．Advanced glycation end product(AGE)

AGEは，慢性的な高血糖状態において生成された種々の糖化蛋白質が，長期間の反応により不可逆性の終末産物となったものである(詳しくは他項に譲る)．生成されたAGEは糖尿病の動脈硬化病変に蓄積され，“hypreglycemic memory”として短期的な血糖の変動によらず病変の形成・進展に寄与すると考えられている[22]．

VSMCに対する作用としては，AGEがVSMCの遊走やフィブロネクチン合成を促進させるという報告がある．それらの促進作用はTGF-βの抗体で抑制されることから，TGF-βを介した作用であることが示唆されている[23][24]．これらは，糖尿病における動脈硬化進展過程にAGEによるVSMCの性質変化が関与している可能性を示すものである．

3．インスリン抵抗性/高インスリン血症

2型糖尿病は，病態としてインスリン抵抗性を伴うことが多い．これには内臓脂肪蓄積を背景として，脂肪細胞由来の炎症性サイトカインなどが重要な役割を果たしていると考えられている(詳しくは他項に譲る)．

インスリン抵抗性はしばしば代償性高インスリン血症を惹起するが，過剰なインスリンはVSMCに対して種々の動脈硬化促進的な作用を示すことが報告されている．これまでに，インスリン

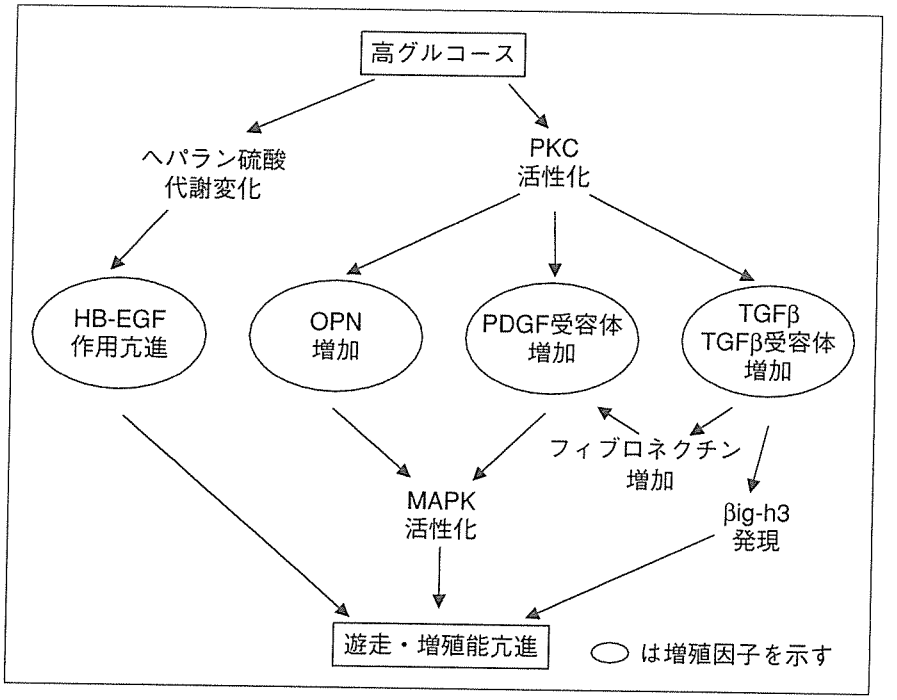

図2　高グルコース状態におけるVSMCの遊走・増殖能亢進機序

がVSMCのMAPK系を活性化してPDGFなどの作用を増強し，遊走・増殖能を亢進させることや，VSMCによるECMの産生を促進させることなどが明らかとなっている[25)26)]．また，インスリンはVSMCにおいて，炎症性サイトカインの一種であるmonocyte chemoattractant protein (MCP)-1や，angiotensinogenの発現を増加させることも示されている[27)28)]．血管壁での炎症機転や組織renin-angiotensin (RA)系の亢進は，VSMCにおける各種増殖因子の産生増加，遊走・増殖能およびECM産生の亢進，細胞肥大，酸化ストレスの上昇，インスリンシグナル阻害などをもたらし[29)30)]，動脈硬化促進とインスリン抵抗性のさらなる増悪，という悪循環につながると考えられる．このようにインスリン抵抗性/高インスリン血症は，VSMCへのさまざまな作用を介して糖尿病の動脈硬化促進に寄与すると推定される．

4．脂質代謝異常/酸化LDL

糖尿病における動脈硬化進展を予防する上で，強力な脂質低下療法が有効であることは論を待たない(詳しくは他項に譲る)．とくに2型糖尿病においては，インスリン抵抗性を背景として高LDL, 高VLDLおよび低HDLといった脂質代謝異常が高率に合併し，動脈硬化発症に重要な役割をはたしている．なかでも動脈硬化発症に重要とされる酸化LDLが，高血糖状態がひき起こす種々の酸化ストレス産生を背景として増加しており，糖尿病における動脈硬化促進の一因となっている[1)31)32)]．

酸化LDLは内皮障害やマクロファージの泡沫化を惹起する主要な動脈硬化促進因子であるが，VSMCにおいてもスカベンジャーレセプターを介して取り込まれ，遊走・増殖能を亢進させたり[33)]，アポトーシスを誘導するという報告がある[34)]．また筆者らの検討においても，酸化LDLがVSMC内の酸化ストレスを上昇させ，前述の増殖因子OPNの発現を増加させることを観察している(Tokuyamaら未発表データ)．このように酸化LDLは糖尿病で増加し，VSMCに対する作用も介して動脈硬化を促進していると考えられる．

5．高血圧

主にインスリン抵抗性を有する2型糖尿病においては，脂質代謝異常とならび高血圧も高率に合併することが知られ，動脈硬化の危険因子と考えられている．高血圧が存在すると，動脈壁はずり応力や壁張力といったメカニカルストレスを受ける．血流方向のずり応力は主に内皮細胞に，血管内圧により壁内腔面が垂直に受け

表1　糖尿病および個々の病態因子とVSMCの性質変化

	細胞機能変化(作用仲介因子)	増殖因子などに関する変化
・糖尿病(DM-VSMC)	遊走・増殖↑(PDGF) フィブロネクチン産生↑(TGFβ)	PDGF受容体発現↑ TGFβ受容体発現↑
・高グルコース	遊走↑(PDGF) 遊走・増殖↑(TGFβ) 遊走↑(OPN)・増殖↑(PDGF, OPN) 遊走・増殖↑(HB-EGF)	PDGF受容体発現↑ TGFβ発現・受容体発現↑ OPN発現↑ HB-EGF作用↑
AGE	遊走・フィブロネクチン産生↑(TGFβ)	
高インスリン血症	遊走・増殖↑(PDGF) ECM産生↑	PDGF作用↑ MCP-1発現↑ Angiotensinogen発現↑
酸化LDL	遊走・増殖↑ アポトーシス↑ 細胞内取り込み↑	OPN発現↑
高血圧	増殖↑(壁張力) フィブロネクチン産生↑(壁張力)	Angiotensin II発現↑

る壁張力は，進展刺激として中膜のVSMCに影響を及ぼすと考えられている．

培養血管壁中膜やVSMCに進展刺激を加えると，増殖能やフィブロネクチン産生の亢進およびangiotensin IIの発現増加が認められるという報告がある[35)36)]．前述のように，血管壁RA系の亢進はさまざまな動脈硬化促進作用を呈するため[29)30)]，高血圧による動脈硬化進展機序としても重要であると考えられる．このように糖尿病では，合併する高血圧がVSMCに対する作用を介して動脈硬化を促進する機序も存在している．

おわりに

糖尿病における動脈硬化進展機序について，糖尿病のさまざまな病態がVSMCの機能に及ぼす作用を中心に述べた表1．すでに述べたように，糖尿病の大血管障害を抑制するには危険因子をそれぞれコントロールしなければならないが，その原因はこのような多岐にわたる病変促進機序にあると思われる．今後，病変の発症メカニズムを各病態ごとに，かつVSMCを含めた病変構成細胞ごとに詳細に解明していくことで，包括的にも特異的にもターゲットがより明確な治療が可能になると考えられる．

文　献

1) Ross R. Atherosclerosis－an inflammatory disease. N Engl J Med 1999 ; 340 : 115, 126.
2) Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004 ; 84 : 767, 801.
3) Kawano M, Koshikawa T, Kanzaki T, et al. Diabetes mellitus induces accelerated growth of aortic smooth muscle cells : association with overexpression of PDGFβ-receptors. Eur J Clin Invest 1993 ; 23 : 84.
4) Tamura K, Kanzaki T, Tashiro J, et al. Increased atherogenesis in Otsuka Long-Evans Tokushima fatty rats before the onset of diabetes mellitus : association with overexpression of PDGFβ-receptors in aortic smooth muscle cells. Atherosclerosis 2000 ; 149 : 351.
5) Kanzaki T, Shinomiya M, Ueda S, et al. Enhanced arterial intimal thickening after balloon catheter injury in diabetic animals accompanied by PDGFβ-receptor overexpression of aortic media. Eur J Clin Invest 1994 ; 24 : 377.
6) McCaffrey TA. TGF-βs and TGF-β receptors in atherosclerosis. Cytokine Growth Factor Rev 2000 ; 11 : 103.
7) Agrotis A, Kalinina N, Bobik A. Transforming growth factor-β, cell signaling and cardiovascular disorders. Curr Vasc Pharmacol 2005 ; 3 : 55.
8) Rasmussen LM, Heickendorff L. Accumulation of fibronectin in aortas from diabetic patients. A quan-

titative immuno histichemical and biochemical study. Lab Invest 1989 ; 61 : 440.

9) Rasmussen LM, Ledet T. Aortic collagen alterations in human diabetes mellitus. Changes in basement membrane collagen content and in the susceptibility of total collagen to cyanogen bromide solubilization. Diabetologia 1993 ; 36 : 445.

10) Kanzaki T, Shiina R, Saito Y, et al. Transforming growth factor-beta receptor and fibronectin expressions in aortic smooth muscle cells in diabetic rats. Diabetologia 1997 ; 40 : 383.

11) Hedin U, Thyberg J. Plasma fibronectin promotes modulation of arterial smooth muscle cells from contractile to synthetic phenotype. Differentiation 1987 ; 33 : 440.

12) Tamura K, Yokote K, Takemoto M, et al. Fibronectin stimulates transcription of the platelet-derived growth factor β-receptor in cultured rat aortic smooth muscle cells. Biochem Biophys Res Commun 1998 ; 251 : 677.

13) Yasunari K, Kohno M, Kano H, et al. Mechanisms of action of troglitazone in the prevention of high glucose-induced migration and proliferation of cultured coronary smooth muscle cells. Circ Res 1997 ; 81 : 953.

14) Inaba T, Ishibashi S, Gotoda T, et al. Enhanced expression of platelet-derived growth factor-beta receptor by high glucose. Involvement of platelet-derived growth factor in diabetic angiopathy. Diabetes. 1996 ; 45 : 507.

15) Campbell M, Allen WE, Silversides JA, et al. Glucose-induced phosphatidylinositol 3-kinase and mitogen-activated protein kinase-dependent upregulation of the platelet-derived growth factor-beta receptor potentiates vascular smooth muscle cell chemotaxis. Diabetes 2003 ; 52 : 519.

16) Yasuda Y, Nakamura J, Hamada Y, et al. Role of PKC and TGF-β receptor in glucose-induced proliferation of smooth muscle cells. Biochem Biophys Res Commun 2001 ; 281 : 71.

17) Ha SW, Bae JS, Yeo HJ, et al. TGF-β-induced protein β ig-h3 is upregulated by high glucose in vascular smooth muscle cells. J Cell Biochem 2003 ; 88 : 774.

18) Lindschau C, Quass P, Menne J, et al. Glucose-induced TGF-β1 and TGF-β receptor-1 expression in vascular smooth muscle cells is mediated by protein kinase C-α. Hypertension 2003 ; 42 : 335 .

19) Takemoto M, Yokote K, Nishimura M, et al. Enhanced expression of osteopontin by high glucose in cultured rat aortic smooth muscle cells. Arterioscler Thromb Vasc Biol 2000 ; 20 : 624.

20) Takemoto M, Yokote K, Yamazaki M, et al. Enhanced expression of osteopontin by high glucose in cultured rat aortic smooth muscle cells. Biochem Biophys Res Commun 1999 ; 258 : 722.

21) Fukuda K, Kawata S, Inui Y, et al. High concentration of glucose increases mitogenic responsiveness to heparin-binding epidermal growth factor-like growth factor in rat vascular smooth muscle cells. Arterioscler Thromb Vase Biol 1997 ; 17 : 1962.

22) Yamagishi S, Imaizumi T. Diabetic vascular complications : pathophysiology, biochemical basis and potential therapeutic strategy. Curr Pharm Des 2005 ; 11 : 2279.

23) Higashi T, Sano H, Saishoji T, et al. The receptor for advanced glycation end products mediates the chemotaxis of rabbit smooth muscle cells. Diabetes 1997 ; 46 : 463.

24) Sakata N, Meng J, Takebayashi S. Effects of advanced glycation end products on the proliferation and fibronectin production of smooth muscle cells. J Atheroscler Thromb. 2000 ; 7 : 169.

25) King GL, Wakasaki H. Theoretical mechanisms by which hyperglycemia and insulin resistance could cause cardiovascular diseases in diabetes. Diabetes Care 1999 ; 22 Suppl 3 : C31.

26) Thogersen VB, Heickendorff L, Ledit T, et al. Effect of insulin and growth hormone on the synthesis of radiolabelled proteoglycans from cultured human arterial smooth muscle cells. Eur J Endocrmol 1996 ; 134 : 326.

27) Sekine O, Nishio Y, Egawa K, et al. Insulin activates CCAAT/enhancer binding proteins and proinflammatory gene expression through the phosphatidylinositol 3-kinase pathway in vascular

smooth muscle cells. J Biol Chem 2002 ; 277 : 36631.
28) Kamide K, Hori MT, Zhu JH, et al. Insulin-mediated growth in aortic smooth muscle and the vascular renin-angiotensin system. Hypertension 1998 ; 32 : 482.
29) Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II -mediated cardiovascular and renal diseases. Pharmacol Rev 2000 ; 52 : 11.
30) Folli F, Alvaro D, Gigliozzi A, et al. Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk. J Clin Invest 1997 ; 100 : 2158.
31) Nishikawa T, Edelstein D, Du XL, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000 ; 404 : 787.
32) Toshima S, Hasegawa A, Kurabayashi M, et al. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. Arterioscler Thromb Vasc Biol 2000 ; 20 : 2243.
33) Tirziu D, Jinga VV, Serban G, et al. The effects of low density lipoproteins modified by incubation with chondroitin 6-sulfate on human aortic smooth muscle cells. Atherosclerosis 1999 ; 147 : 155.
34) Kashiwakura Y, Watanabe M, Kusumi N, et al. Dynamin-2 regulates oxidized low-density lipoprotein-induced apoptosis of vascular smooth muscle cell. Circulation 2004 ; 110 : 3329.
35) Bardy N, Merval R, Benessiano J, et al. Pressure and angiotensin II synergistically induce aortic fibronectin expression in organ culture model of rabbit aorta. Evidence for a pressure-induced tissue renin-angiotensin system. Circ Res 1996 ; 79 : 70.
36) Hishikawa K, Nakaki T, Marumo T, et al. Pressure promotes DNA synthesis in rat cultured vascular smooth muscle cells. J Clin Invest 1994 ; 93 : 1975.

*　　*　　*

日病薬誌 第42巻11号（1433-1435）2006年

③血管石灰化の予防と治療

千葉大学大学院医学研究院細胞治療学教室
本城 聡 Satoshi HONJO
横手幸太郎 Koutaro YOKOTE
斎藤 康 Yasushi SAITO

はじめに

骨以外の軟部組織へのカルシウム（以下，Ca）が非生理的に沈着することを異所性石灰化と称するが，なかでも血管への異所性石灰化は，高血圧，心肥大，心不全発症などに大きく相関し，患者の予後を左右し得る病変である。

血管石灰化が見られるのは高齢者，糖尿病患者などだが，最も深刻な石灰化を来すのは腎不全患者，なかでも人工透析中の患者である。石灰化の程度は透析期間と比例し，10年以上の経過を持つ透析患者では，小児でも血管石灰化を来し得る。

透析患者の死亡原因の約41％は心不全・脳血管障害など，心血管病変に起因する疾患で占められ[1]，血管石灰化に対する予防と治療は緊要の課題と言えよう。本稿では，主として腎不全患者における血管石灰化の予防と治療について述べる。

Ca×P積抑制について

血管石灰化を促進する重要な原因は血清Caおよびリン（以下，P）の高値であると考えられている。まずPに関しては，透析患者ではPの排泄障害のため高P血症になりやすい。Caについては，当初はVitD低下などの要因により低Ca血症を示すが，やがて①持続する低Ca・高P・低VitD刺激を受けて副甲状腺が腫大し，副甲状腺ホルモン（以下，PTH：parathyroid hormone）の過剰分泌を来したり（これを2次性副甲状腺機能亢進症という），あるいは②投与薬剤（Caを含有するリン吸着剤，活性型VitD製剤など）の影響により高Ca血症に転ずることが多い。

生体内でCa，P値が上昇するとリン酸化Ca結晶が形成され，安定したハイドロキシアパタイトに構造が変化して石灰化が形成される。これが血管石灰化発症の大まかなメカニズムである。リン酸化Ca結晶は血清のCa×P値の積が60～70（mg/dL）^{2}を超えると飽和状態となり析出しやすくなるとされ，CaとPの積，すなわちCa×P積が治療の指標として重要視されている。

2003年，米国腎臓財団（National Kidney Foundation：NKF）からK/DOQI（kidney disease outcomes quality initiative）ガイドラインが発表された。同ガイドラインでは，ステージ3以上の腎不全患者でのCa×P積の目標値を55（mg/dL）^{2}以下と定めている。各ステージ別の治療目標値を**表1**に示す。また同ガイドラインでは，総Ca摂取量制限，血清Ca，P，Ca×P値高値での活性型VitD製剤の投与を避けるように明記しており，さらに低Ca透析液（2.5mEq/L）の使用を推奨している[2]。

表1 K/DOQIガイドライン[2]より一部改変

ステージ	GFR (mL/min/1.73m^2)	目標血清 i-PTH (pg/mL)	目標血清 P (mg/dL)	目標血清 Ca (mg/dL)	目標 Ca×P (mg/dL)^2
1	≧90				
2	60～89				
3	30～59	35～70	2.7～4.6	8.4～9.5	＜55
4	15～29	70～110	2.7～4.6	8.4～9.5	＜55
5	＜15 または透析中	150～300	3.5～5.5	8.4～9.5＜10.2 （いずれも下限を目指す）	＜55

以上を踏まえ，腎不全における血管石灰化を予防・治療する薬剤について説明していきたい。

リン吸着薬

高P血症それ自体は自覚症状に乏しいが，副甲状腺を刺激し2次性副甲状腺機能亢進症を発症させて骨代謝異常をもたらす。さらに最近では，石灰化の主犯は前述のCa×P積ではなく，過剰なPそのものが血管平滑筋細胞の骨芽細胞様に形質転換させ血管石灰化を誘導するという学説もみられるようになった。

血清P値を抑制する方法としては食事中のP制限（蛋白制限食），透析でのP除去の増加が挙げられるが，多くの場合，P吸着剤の投与を必要とする。以下にP吸着剤について述べる。

1．Ca製剤（沈降炭酸カルシウム：カルタンなど）

2004年末現在，血液透析患者で使われるリン吸着薬の75％を占め，最も使用されるP吸着剤である[1]。かつてはアルミニウム（以下，Al）製剤がP吸着薬として汎用されたが，Al骨症やAl脳症などの危険性があり，1992年に我が国では透析患者への使用は中止された。

このため，一時期はCa製剤が唯一のP吸着剤として使われた。P吸着能ではAl製剤に劣ったが，臨床試験上は約70％の患者でAl製剤に匹敵する血清Pのコントロールを得られるとされた。しかし，腸管からのCa吸収により高Ca血症を来しやすく，大量に使用すると逆に血管石灰

化を促進してしまうという成績[3]もあるので，血中Ca濃度をモニタリングしつつ注意して用いる必要がある。

2．塩酸セベラマー（sevelamer hydrochloride：レナジェル，フォスブロック）

塩酸セベラマーは米国で開発された陽性荷電基をもつポリマーで，食物中のPを吸着し，糞便中に排泄させる。本剤はAlやCaを含まないうえに腸管で分解も吸収もされず，Al製剤やCa製剤のような腸管吸収による副作用の問題点がない。本剤での加療群とCa製剤での加療群とを比較すると，同等の血清Pのコントロールを得た場合，塩酸セベラマー投与群での高Ca血症，高PTH血症の発症頻度，さらに血管石灰化の進展度はいずれもCa製剤投与群を下回ることが米国の臨床試験で示された[4]。食物中のPの吸収目的であるため，食直前に服用するのが特徴である。

2003年に我が国でも認可された。ただし1錠あたりのP吸着量はCa製剤に劣る（約3分の1）とされ，Ca製剤との併用も試みられている。

3．ニコチン酸誘導体（niceritol：ペリシット）

細胞膜におけるPの輸送主要経路はNa-P共輸送体（以下，NPC：sodium-phosphate cotransporter）である。NPCのうちtype IIbは小腸壁に存在し，消化管からのP吸収を担う。

ニコチン酸のプロドラッグであるniceritolは本来高脂血症薬であるが，NPCtype IIbの遺伝子発現を低下させる作用をもち，高P血症の治療薬として注目されている（従って厳密にはP“吸着”剤ではない）。しかし，腎不全患者では血小板減少などの副作用も報告され，保険適応は認められていない。

他に，鉄製剤や高脂血症薬であるコレスチミドでP吸着効果が報告されているが，まだ臨床データの蓄積が不十分であり，保険適応外である。また，炭酸ランタンが非Al非Ca性のP吸着薬として注目されており，米国FDAを2004年に通過，我が国でも現在治験が進行中である。

2次性副甲状腺機能亢進症治療薬

腎不全により2次性副甲状腺機能亢進症を来すと高PTH血症が続くため，骨粗鬆症・線維性骨炎などを発症する。また，血清Caの上昇ももたらすため，PTHの抑制はCa×P積を抑制するうえでも重要である。

1．新規活性型VitD製剤

現在，2次性副甲状腺機能亢進症の治療の中心はVitD製剤である。しかしPTHを十分に抑制する程度のVitD製剤を投与すると，逆にVitDによる高Ca血症を誘発する危険があった。このためVitD製剤の改良が行われ，2000年に認可されたmaxacalcitol（オキサロール）や2001年に認可されたfalecalcitriol（フルスタン，ホーネル）はPTH抑制作用に比してCa上昇作用が少ないように設計され，血中のPTHを抑制しつつ異所性石灰化も抑制できることが利点である。

2．Ca感受性受容体作動薬

副甲状腺細胞のCa感受性受容体（Ca sensing receptor：CaSR）に作用し，PTHの分泌を抑制する薬剤が2004年にFDAで認可された。calcimimeticsと総称されている。代表的な薬剤は塩酸シナカルセト（cinacalcet HCL：Sensipar）で，すでに臨床試験では血清PTH抑制，Ca抑制，P抑制，Ca×P積抑制のすべてで良好な成績が報告されている[5]。我が国でも近く承認申請の予定であり，今後の2次性副甲状腺機能亢進症治療の主力となることが期待されている。

しかし，進行性の2次性副甲状腺機能亢進症で，上記治療でもCaの上昇が抑えきれない場合は経皮的にエタノールを副甲状腺に注入（PEIT）したり，外科的に副甲状腺を摘出する等の方法も取られる。

その他の治療薬

他の機序で，血管石灰化を抑制する治療薬について述べる。まだ治療法として確立していないものが多い。

1．ビスホスホネート（以下，BP：bisphosphonate）

BPはハイドロキシアパタイトに強い親和性を有し，体内でこれらの結晶や非晶性前駆物質の凝集・成長を効果的に阻害するとされる。第一世代のビスホスホネートであるダイドロネル（以下，EHDP：etidronate）は高用量投与で異所性石灰化の抑制作用を示し，脊髄損傷後や股関節形成術後に関節部に生じる異所性石灰化を抑制する目的で用いられる。この使用法は保険適応が認められている。

透析患者の腹部大動脈や冠動脈石灰化に対するEHDPの石灰化抑制作用の報告が見られる。しかし腎不全患者に対しての安全性は未確立であり，保険適応は認められていない。

2．スタチン

高脂血症治療薬であるスタチンは，抗炎症作用やメバロン酸カスケードに対する作用などを介して異所性石灰化を抑制するとされる。非透析者であっても，高齢者では大動脈や心臓の弁組織などの石灰化の頻度が増加するが，スタチン内服者は非内服者に比してこの加齢に伴う大動脈弁の石灰化の進行が遅いことが臨床試験で報告されている[6]。

透析患者での血管石灰化抑制についてはまだデータの

蓄積が不十分である。石灰化抑制目的での投与は保険適応外である。

その他，ビタミンK製剤で血管石灰化が抑制されたとする報告や，n-3系多価不飽和脂肪酸であるエイコサペンタエン酸（EPA）の投与により動物レベルで石灰化が抑制されたという報告もある。

まとめ

主として透析患者における血管石灰化の予防と治療について述べた。石灰化予防は腎不全治療の重要な一角を占める。予防にあたっては現在Ca×P積が重要視され，その抑制を目標として研究が進められてきた。まだ完全な克服への道は遠いが，新規薬剤の開発も急速に進んでおり，今後さらなる研究の発展が期待される。

参考文献

1）日本透析医学会統計調査委員会：わが国の慢性透析療法の現状（2004年12月31日現在）.

2）National Kidney Foundation : K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease, *Am. J. Kidney. Dis.*, **42**(4 Suppl 3), S1-201 (2003).

3）Guerin A.P., London G.M., Marchais S.J., Metivier F. : Arterial stiffening and vascular calcifications in end-stage renal disease, *Nephrol. Dial. Transplant.*, **15**, 1014-1021 (2000).

4）Chertow G.M., Burke S.K., Raggi P. ; Treat to Goal Working Group : Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients, *Kidney. Int.*, **62**, 245-252 (2002).

5）Moe S.M., Chertow G.M., Coburn J.W., Quarles L.D., Goodman W.G., Block G.A., Drueke T.B., Cunningham J., Sherrard D.J., McCary L.C., Olson K.A., Turner S.A., Martin K.J. : Achieving NKF-K/DOQI bone metabolism and disease treatment goals with cinacalcet HCL, *Kidney. Int.*, **67**, 760-771 (2005).

6）Shavelle D.M., Takasu J., Budoff M.J., Mao S., Zhao X.Q., O'Brien K.D. : HMG CoA reductase inhibitor (statin) and aortic valve calcium, *Lancet.*, **359**(9,312), 1125-1126 (2002).

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.*

Koutaro Yokote, Kazuki Kobayashi and Yasushi Saito are at the Division of Diabetes, Metabolism and Endocrinology, Department of Internal Medicine, Chiba, Japan.

* Address correspondence to: Koutaro Yokote, MD, PhD, DMSci, Division of Diabetes, Metabolism and Endocrinology, Department of Internal Medicine, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Tel.: (+81) 43 222 7171x5257; fax: +81 43 226 2095;
e-mail: kyokote@faculty.chiba-u.jp.

© 2006, Elsevier Inc. All rights reserved.
1050-1738/06/$-see front matter

• 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-

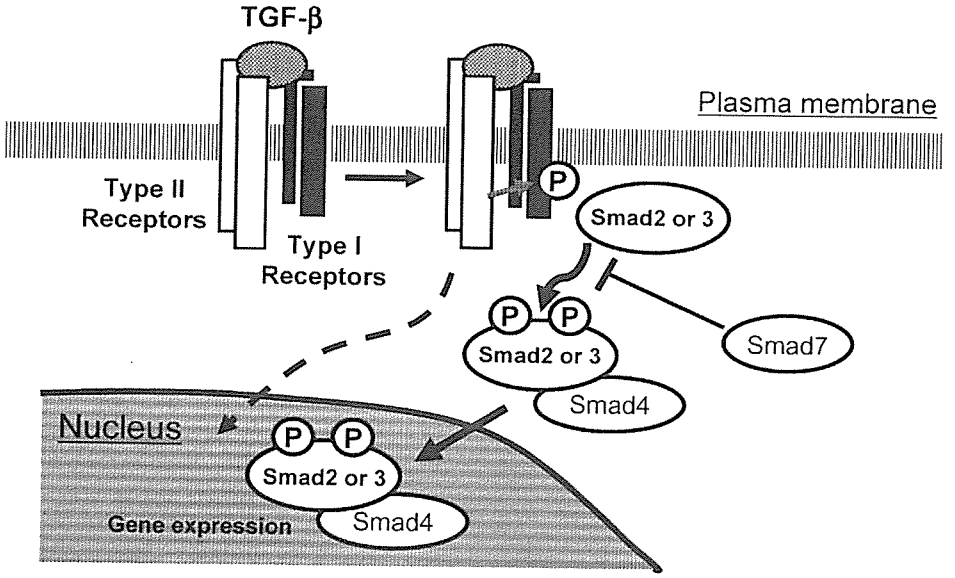

Figure 1. Schematic illustration of intracellular signal transduction pathways by TGF-β. Upon ligand-induced heteromeric complex formation and activation of type-I and type-II receptors, cytoplasmic signal transducers Smad2 and Smad3, classified as so-called R-Smads, are phosphorylated and heteroligomerized 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-

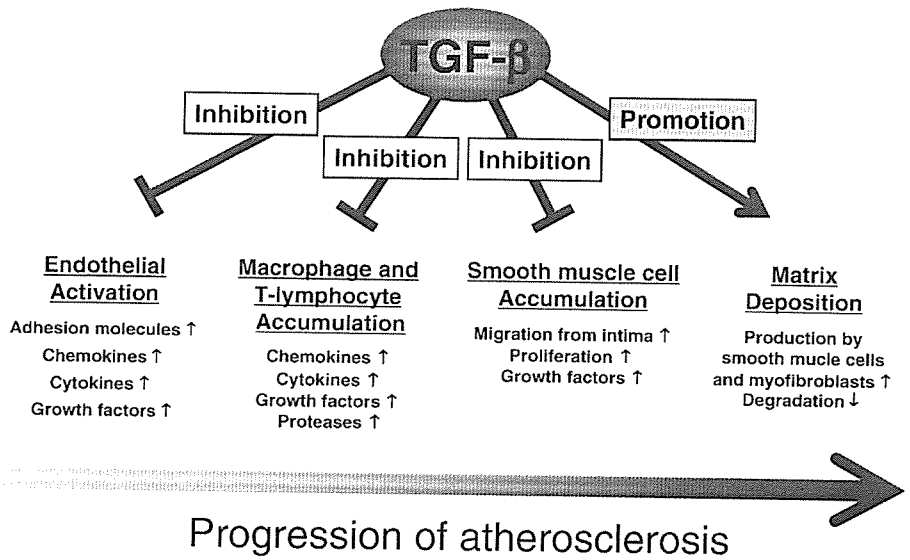

Figure 2. Major steps in atherosclerotic lesion formation and the putative effects of TGF-β on the each step. Both in vitro and in vivo evidence suggests that TGF-β inhibits activation of endothelial cells and intimal accumulation of inflammatory cells and smooth muscle cells. On the other hand, TGF-β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

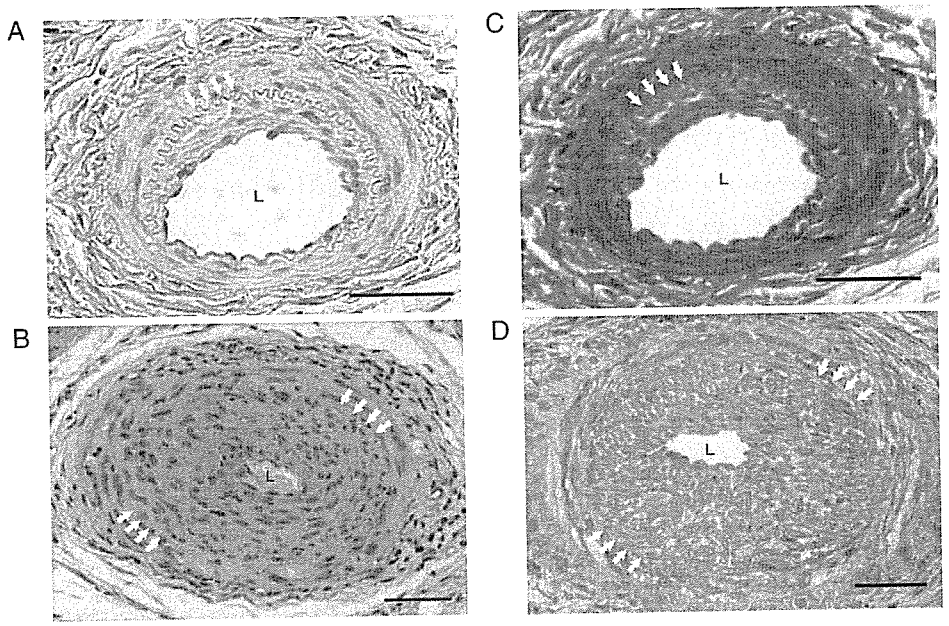

Figure 3. Enhanced neointimal hyperplasia and reduced matrix deposition in the arteries of Smad3-null mice upon injury. Photomicrographs showing representative cross sections of hematoxylin/eosin-stained (**A** and **B**) and Masson's trichrome-stained (**C** and **D**) femoral arteries from wild-type (**A** and **C**) and Smad3-null (**B** and **D**) mice 3 weeks after endothelial injury by photochemically induced thrombosis method. *Arrows* indicate the positions of the internal elastic lamina (original magnification ×200; *bar* = 50 μm). L, vascular lumen. Reproduced with permission from *Circ Res* 2005;96:904–912.

atous plaques are distinct from SMC/matrix-rich postangioplasty restenotic lesions in their biologic 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 down-regulation of adhesion molecules (Gamble et al. 1993) and up-regulation 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. 1993). In SMCs, TGF-β is known to inhibit proliferation induced by growth factors (Owens et al. 1988). As described above, TGF-β also shows a potent matrix-depositing activity in the vascular wall. These biologic properties of TGF-β seem to affect each step of atherogenesis in a favorable manner, yielding a stable plaque phenotype rich in matrix, with limited accumulation of inflammatory cells.

In vivo experimental results support the antiatherogenic or plaque-stabilizing function of TGF-β. Mice heterozygous for TGF-β1 gene showed endothelial activation and lipid lesion formation when fed on high-fat diet (Grainger et al. 2000). Inactivation of TGF-β signal by systemic administration of neutralizing antibody or soluble TβR-II in atherosclerosis-prone apoE-knockout mice resulted in vascular lesions with a higher ratio of inflammatory cells and reduced fibrosis, compared with the controls (Lutgens et al. 2002, Mallat et al. 2001). Conversely, treatment with the antiestrogen tamoxifen increased serum TGF-β levels and suppressed the formation of aortic lesions in mice (Grainger et al. 1995b). More recently, it was shown that dominant-negative TβR-II specifically expressed in T cells to abrogate TGF-β signaling gave "unstable-like" plaque phenotype in both apoE- and low-density lipoprotein receptor-knockout mice (Gojova et al. 2003, Robertson et al. 2003). The results indicate the prominent role of TGF-β function in T cells for its antiatherosclerotic activity.

These findings are in line with clinical observations that low blood levels of active TGF-β associates with the severity of vascular disease, suggesting a protective effect of TGF-β against atherosclerosis in humans (Grainger et al. 1995a, Stefoni et al. 2002). A TGF-β1 gene polymorphism resulting in a low-circulating TGF-β1 level was shown to associate with increased risk of myocardial infarction (Cambien et al. 1996, Arkhurst 2004). Altogether, TGF-β seems to have plaque-stabilizing potential in general. But when excessively activated, such as upon vascular injury, it may facilitate

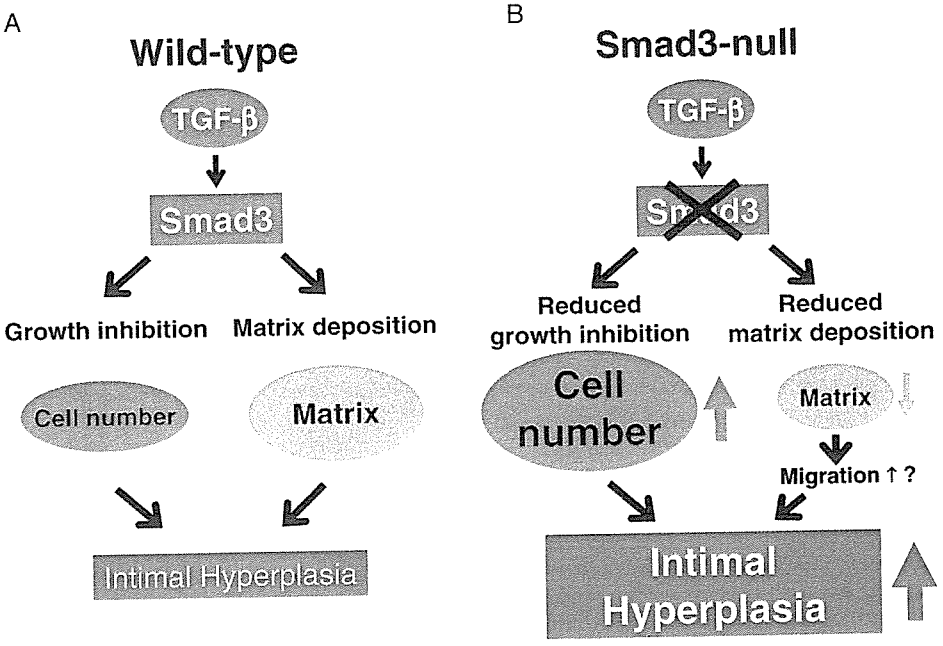

Figure 4. Possible mechanism of enhanced intimal hyperplasia in the artery of Smad3-null mice after injury. In the presence of endogenous Smad3 (**A**), TGF-β inhibits intimal smooth muscle cell growth and induces deposition of matrix, modestly limiting the intimal thickening. In lack of Smad3 (**B**), intimal smooth muscle cells are resistant to growth inhibition by TGF-β. Increased proliferation of smooth muscle cells leads to enhanced intimal hyperplasia. Reduced matrix scaffold may also facilitate migration of smooth muscle cells from the medial layer.

restenosis through accumulation of matrix and constrictive remodeling.

- **Disruption of Smad3-Dependent TGF-β Signal Enhances Neointimal Hyperplasia with Reduced Matrix Deposition Upon Vascular Injury**

In contrast to the accumulating knowledge on the role of TGF-β in restenosis and atherosclerosis, the precise function of individual signaling molecules for TGF-β in vascular disease remains unclear. Therefore, we examined the mice null for Smad3 in vivo and in vitro to clarify the function of Smad3-dependent signaling in vascular response to injury (Kobayashi et al. 2005).

Femoral arteries of Smad3-null mice showed significant enhancement of neointimal hyperplasia, compared with those of wild-type mice (Figure 3A, B) upon endothelial injury by the photochemically induced thrombosis method. Immunohistochemical examination revealed that neointima was exclusively composed of SMCs. Transplantation of Smad3-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. Smad3-null neointima compared with wild-type showed a higher cell density with increased proliferative activity of SMCs. On the other hand, Masson's trichrome staining revealed significantly reduced extracellular collagen accumulation relative to total intimal area in Smad3-null artery (Figure 3C, D). These findings suggest that Smad3 deficiency causes neointimal lesions rich in SMC but scarce in matrix upon vascular injury.

In vitro, TGF-β inhibited serum-stimulated DNA synthesis of wild-type aortic SMCs, with the maximal inhibition of 70%. However, growth of Smad3-null SMCs was only weakly inhibited by TGF-β, indicating an essential role of Smad3 in TGF-β-mediated growth inhibition of vascular SMCs as reported in other cell types (Ashcroft et al. 1999, Datto et al. 1999). Unexpectedly, Smad3-null SMCs dose-dependently migrated toward TGF-β at least to a similar extent as wild-type cells, suggesting that non-Smad3 signal mediates TGF-β-induced chemotaxis in murine vascular SMCs. The finding differs from the previous report demonstrating an indispensable role of Smad3 in migration of monocytes and neutrophils toward TGF-β by (Ashcroft et al. 1999). In terms of matrix regulation, TGF-β increased the transcript levels of α2 type-I collagen and tissue inhibitor of metalloproteinases-1 but suppressed expression and activity of MMPs in wild-type SMCs. In Smad3-null SMCs, TGF-β was inefficient in inducing collagen or suppressing MMPs, giving a possible explanation for reduced extracellular matrix in Smad3-null neointima in vivo.

- **Role of Endogenous Smad3 in Vascular Homeostasis: Limiting Neointimal Hyperplasia?**

Figure 4 illustrates the possible mechanism underlying the enhanced neointimal hyperplasia in Smad3-null mice. Upon endothelial injury in the wild-type artery, endogenous Smad3 allows TGF-β to elicit growth inhibitory effect on intimal SMCs and to promote extracellular matrix accumulation, resulting in "healing" of vascular lesions with modest intimal thickening (Figure 4A). When Smad3 is absent (Figure 4B), SMCs are largely resistant to growth inhibitory control by TGF-β and, thus, undergo increased proliferation. On the other hand, impaired collagen synthesis as well as overall up-regulation of matrix-degrading activity by TGF-β leads to reduced amount of matrix in the intima of Smad3-null arteries. Degradation of matrix scaffold by MMPs enables cell movement and tissue reorganization (Lijnen et al. 1999, Galis and Khatri 2002). As mentioned earlier, the migratory capacity toward TGF-β is preserved in Smad3-null SMCs. Therefore, inability of TGF-β to suppress MMPs in null cells may facilitate migration from media to intima in vivo, allowing further accumulation of SMCs in intima. Moreover, because Smad3 is known to mediate anti-inflammatory activity of TGF-β in SMCs (Feinberg et al. 2004), activation of inflammatory genes in Smad3-null SMCs may also contribute to the accelerated neointimal formation.

The results taken together, endogenous Smad3 is likely to have an effect to limit the extent of neointimal hyperplasia through modulation of SMC functions in the process of restenotic vascular response.

- **Future Perspectives**

As described earlier, TGF-β ligand itself promotes intimal thickening in balloon injury models. Our findings that targeted deletion of Smad3, a major signal mediator of TGF-β, accelerates neointimal

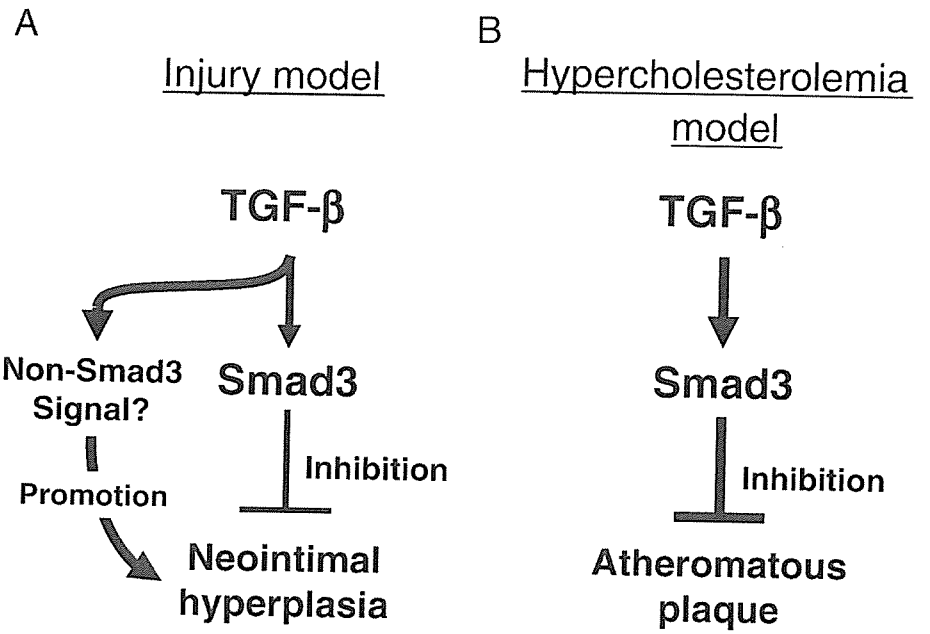

Figure 5. Putative role of Smad3 in two distinct models of vascular disease. (**A**) In the vascular injury model, Smad3 has an inhibitory effect on the formation of neointimal hyperplasia. The effect of TGF-β to promote neointimal hyperplasia is likely to be mediated by non-Smad3 signal. (**B**) In the hypercholesterolemia model, Smad3, at least in part, mediates the antiatherogenic function of TGF-β.

formation appear inconsistent with those observed on TGF-β. A possible explanation would be that our model differs from any other previous ones in the point it that lacks Smad3 but not other TGF-β signal components. Non-Smad3 signals, such as mitogen-activated protein kinases, may act promotive on the lesion formation in vascular injury model (Figure 5A). Although unlikely, difference in the method of endothelial injury, either balloon or thrombotic, should also be considered.

Finally, mice in which both the Smad3 and apoE genes have been deleted show marked enhancement of atheromatous lesion resembling "unstable plaque" compared with single apoE knockout mice (Kobayashi et al., unpublished observation). Therefore, in this hypercholesterolemia model of atherosclerosis, Smad3 seems to, at least in part, mediate protective function of TGF-β (Figure 5B). As judged from the results of Smad3-null mice, Smad3 plays a protective role in both injury/restenosis and hypercholesterolemia/atherosclerosis models. From a therapeutic point of view, it is of interest to know whether specific potentiation of Smad3 activity in the vascular wall leads to amelioration of neointimal hyperplasia and atheromatous lesions.

• Acknowledgments

This study is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology; the Ministry of Health, Labour and Welfare; and the Japan Heart Foundation; grants from NOVARTIS Foundation for Gerontological Research and Takeda Science Foundation (KY); and a Grant from Japan Foundation of Cardiovascular Research (KK). The authors thank Dr Anita B Roberts for the critical reading of the manuscript.

References

Argmann CA, Van Den Diepstraten CH, Sawyez CG, et al.: 2001. Transforming growth factor-β1 inhibits macrophage cholesteryl ester accumulation induced by native and oxidized VLDL remnants. Arterioscler Thromb Vasc Biol 21:2011–2018.

Arkhurst RJ: 2004. TGFβ signaling in health and disease. Nat Genet 36:790–792.

Ashcroft GS, Yang X, Glick AB, et al.: 1999. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol 1: 260–266.

Blobe GC, Schiemann WP, Lodish HF: 2000. Role of transforming growth factor β in human disease. N Engl J Med 342: 1350–1358.

Cambien F, Ricard S, Troesch A, et al.: 1996. Polymorphisms of the transforming growth factor-b1 gene in relation to myocardial infarction and blood pressure. Hypertension 28:881–887.

Datto MB, Frederick JP, Pan L, et al.: 1999. Targeted disruption of Smad3 reveals an essential role in transforming growth factor β-mediated signal transduction. Mol Cell Biol 19:2495–2504.

Feinberg MW, et al.: 2004. Transforming growth factor-β1 inhibition of vascular smooth muscle cell activation is mediated via Smad3. J Biol Chem 279:16388–16393.

Flanders KC: 2004. Smad3 as a mediator of the fibrotic response. Int J Exp Pathol 85: 47–64.

Fujimoto M, Maezawa Y, Yokote K, et al.: 2003. Mice lacking Smad3 are protected against streptozotocin-induced diabetic glomerulopathy. Biochem Biophys Res Commun 305:1002–1007.

Galis ZS, Khatri JJ: 2002. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 90:251–262.

Gamble JR, Khew-Goodall Y, Vadas MA: 1993. Transforming growth factor-β inhibits E-selectin expression on human endothelial cells. J Immunol 150:4494–4503.

Gojova A, Brun V, Esposito B, et al.: 2003. Specific abrogation of transforming growth factor-β signaling in T cells alters atherosclerotic lesion size and composition in mice. Blood 102:4052–4058.

Grainger DJ, Kemp PR, Metcalfe JC, et al.: 1995a. The serum concentration of active transforming growth factor-β is severely depressed in advanced atherosclerosis. Nat Med 1:74–79.

Grainger DJ, Witchell CM, Metcalfe JC: 1995b. Tamoxifen elevates transforming growth factor-β and suppresses diet-induced formation of lipid lesions in mouse aorta. Nat Med 1:1067–1073.

Grainger DJ, Mosedale DE, Mecalfe JC, Bottinger EP: 2000. Dietary fat and reduced level of TGF-β1 acts synergistically to promote activation of the vascular endothelium and formation of lipid lesions. J Cell Sci 113:2355–2361.

Heldin C-H, Miyazono K, ten Dijke P: 1997. TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471.

Ignotz RA, Massagué J: 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345.

Inoue N, Venema RC, Sayegh HS, et al.: 1995. Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-β1. Arterioscler Thromb Vasc Biol 15:1255–1261.

Kanzaki T, Tamura K, Takahashi K, et al.: 1995. In vivo effect of TGF-β1. Enhanced intimal thickening by administration of TGF-β1 in rabbit arteries injured with a balloon catheter. Arterioscler Thromb Vasc Biol 15:1951–1957.

Kingston PA, Sinha S, David A, et al.: 2001. Adenovirus-mediated gene transfer of a secreted transforming growth factor-β type II receptor inhibits luminal loss and constrictive remodeling after coronary angioplasty and enhances adventitial collagen deposition. Circulation 104:2595–2601.

Kobayashi K, Yokote K, Fujimoto M, et al.: 2005. Targeted disruption of TGF-β-Smad3 signaling leads to enhanced neointimal hyperplasia with diminished matrix deposition in response to vascular injury. Circ Res 96:904–912.

Libby P: 2002. Inflammation in atherosclerosis. Nature 420:868–874.

Lijnen HR, Soloway P, Collen D: 1999. Tissue inhibitor of matrix metalloproteinases-1 impairs arterial neointima formation after vascular injury in mice. Circ Res 85: 1186–1191.

Lutgens E, Gijbels M, Smook M, et al.: 2002. Transforming growth factor-β mediates balance between inflammation and fibrosis during plaque progression. Arterioscler Thromb Vasc Biol 22:975–982.

Majesky MW, Lindner V, Twardzik DR, et al.: 1991. Production of transforming growth factor β1 during repair of arterial injury. J Clin Invest 88:904–910.

Mallat Z, Gojova A, Marchiol-Fournigault C, et al.: 2001. Inhibition of transforming growth factor-β signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. Circ Res 89:930–934.

Massagué J, Chen Y-G: 2000. Controlling TGF-β signaling. Genes Dev 14:627–644.

Moustakas A, Heldin C-H: 2005. Non-Smad TGF-b signals. J Cell Sci 118:3573–3584.

Nabel EG, Shum L, Pompili VJ: 1993. Direct transfer of transforming growth factor β1 gene into arteries stimulates fibrocellular hyperplasia. Proc Natl Acad Sci U S A 90:10759–10763.

Nikol S, Isner JM, Pickering JG, et al.: 1992. Expression of transforming growth factor-β1 is increased in human vascular restenosis lesions. J Clin Invest 90:1582–1592.

Nomura M, Li E: 1998. Smad2 role in mesoderm formation, left-right patterning and craniofacial development. Nature 393:786–790.

Owens GK, Geisterfer AA, Yang YW, Komoriya A: 1988. Transforming growth factor-β-

induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. J Cell Biol 107:770–774.

Roberts AB, Sporn MB: 1990. The transforming growth factor-betas. Sporn MB , Roberts AB ds., Peptide growth factors and their receptors, ISpringer-Verlag, Heidelberg. 419–472.

Roberts AB, Piek E, Boettinger EP, et al.: 2001. Is Smad3 a major player in signal transduction pathways leading to fibrosis? Chest 120:43S–47S.

Robertson AK, Rudling M, Zhou X, et al.: 2003. Disruption of TGF-β signaling in T cells accelerates atherosclerosis. J Clin Invest 112:1342–1350.

Schulick AH, Taylor AJ, Zuo W, et al.: 1998. Overexpression of transforming growth factor β1 in arterial endothelium causes hyperplasia, apoptosis, and cartilaginous metaplasia. Proc Natl Acad Sci U S A 95:6983–6988.

Stefoni S, Cianciolo G, Donati G, et al.: 2002. Low TGF-β1 serum levels are a risk factor for atherosclerosis disease in ESRD patients. Kidney Int 61:324–335.

Uría JA, Jiménez MG, Balbín M, et al.: 1998. Differential effects of transforming growth factor-β on the expression of collagenase-1 and collagenase-3 in human fibroblasts. J Biol Chem 273:9769–9777.

Waldrip WR, Bikoff EK, Hoodless PA, et al.: 1998. Smad2 signaling in extraembryonic tissues determines anterior–posterior polarity of the early mouse embryo. Cell 92:797–808.

Westerhausen Jr DR, Hopkins WE, Billadello JJ: 1991. Multiple transforming growth factor-β-inducible elements regulate expression of the plasminogen activator inhibitor type-1 gene in Hep G2 cells. J Biol Chem 266:1092–1100.

Wolf YG, Rasmussen LM, Rouslahti E: 1994. Antibodies against transforming growth factor-β1 suppresses intimal hyperplasia in a rat model. J Clin Invest 93:1172–1178.

Wolfraim LA, Fernandez TM, Mamura M, et al.: 2004. Loss of Smad3 in acute T-cell lymphoblastic leukemia. N Engl J Med 351: 552–559.

Yamamoto K, Morishita R, Tomita N, et al.: 2000. Ribozyme oligonucleotides against transforming growth factor-β inhibited neointimal formation after vascular injury in rat model: potential application of ribozyme strategy to treat cardiovascular disease. Circulation 102:1308–1314.

Yang X, Letterio JJ, Lechleider RJ, et al.: 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. EMBO J 18:1280–1291.

PII S1050-1738(06)00071-5 **TCM**

DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2006; **22**: 313–322.
Published online 3 January 2006 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/dmrr.612

RESEARCH ARTICLE

Influence of C-peptide on early glomerular changes in diabetic mice

Yoshiro Maezawa[1], Koutaro Yokote[1]*, Kiriko Sonezaki[1], Masaki Fujimoto[1], Kazuki Kobayashi[1], Harukiyo Kawamura[1], Takahiko Tokuyama[1], Minoru Takemoto[2], Shiro Ueda[3], Tomoyuki Kuwaki[4], Seijiro Mori[1], John Wahren[5], Yasushi Saito[1]

[1]*Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan*

[2]*Laboratory of Vascular Biology, Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden*

[3]*Department of Drug Information and Communication, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan*

[4]*Department of Autonomic Physiology, Chiba University Graduate School of Medicine, Chiba, Japan*

[5]*Section of Clinical Physiology, Department of Surgical Sciences, Karolinska Institute, Stockholm, Sweden*

*Correspondence to: Koutaro Yokote, Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chiba 260-8670, Japan. E-mail: kyokote-cib@umin.ac.jp

Received: 8 August 2005
Revised: 3 October 2005
Accepted: 11 November 2005

Abstract

Background C-peptide has been shown to ameliorate diabetes-induced functional and structural renal changes in animal models as well as in patients with type 1 diabetes. This study aims to examine the molecular effects of C-peptide on early glomerular changes in a mouse model of type 1 diabetes.

Methods Fourteen days after induction of diabetes by streptozotocin (STZ), the animals received rat C-peptide for either 24 h or 7 days. Urinary albumin excretion was measured by ELISA. Glomerular mRNA expression of the transforming growth factor (TGF)-β_1 and type IV collagen was quantified by real-time PCR. The effect of C-peptide on type IV collagen gene expression in cultured murine podocytes was also examined.

Results C-peptide decreased urinary albumin excretion from 0.29 to 0.18 μg/min (−40.7%, $P < 0.01$). The transcript level of (α3)IV collagen in glomeruli was up-regulated 2.2-fold in diabetic mice and was inhibited by 45–70% ($P < 0.05$) upon C-peptide treatment. C-peptide suppressed glomerular expression of TGF-β_1 by 36.6% after 7 days ($P < 0.05$) but not 24 h after injection. *In vitro* studies using cultured podocytes revealed that C-peptide dose-dependently inhibited TGF-β-induced up-regulation of type IV collagen. Moreover, both pertussis toxin (PTX) and a specific inhibitor for extracellular signal-regulated kinase (ERK) pathway reversed the inhibitory effect of C-peptide on TGF-β. Finally, C-peptide was shown to up-regulate the activity of ERK in podocytes.

Conclusions These findings indicate that C-peptide suppresses specific aspects of early glomerular changes in a mouse model of diabetes and that the effect is at least in part mediated via interaction with the TGF-β signal in glomerular podocytes. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords C-peptide; diabetic nephropathy; type IV collagen; TGF-β; ERK; streptozotocin

Introduction

Diabetic nephropathy, a major microvascular complication of diabetes mellitus, affects approximately one-third of all diabetic patients. Clinically, it is characterized by albuminuria and gradually diminished glomerular filtration rate. It often shows a deteriorating course that may lead to end-stage renal failure [1]. Histopathological hallmarks for diabetic nephropathy include mesangial expansion, thickening of the glomerular basement membrane (GBM) and the subsequent glomerulopathy.

Copyright © 2006 John Wiley & Sons, Ltd.

It has recently been established that pharmaceutical interventions including intensified glycemic control and aggressive blood pressure lowering, especially involving agents that block the renin–angiotensin–aldosterone system, can successfully retard the development of diabetic nephropathy. However, such treatments have not so far been able to arrest the progression to end-stage renal failure [2]. Therefore, new therapeutic strategies in the management of diabetic nephropathy are required.

Proinsulin C-peptide, a cleavage product of proinsulin, is co-released with insulin in equimolar amounts from the pancreatic beta cells into the circulation. C-peptide has long been considered to have no biological function of its own. However, it has recently been demonstrated that C-peptide elicits several physiological effects both *in vivo* and *in vitro*. Administration of C-peptide to type 1 diabetic patients with microalbuminuria is reported to diminish both glomerular hyperfiltration and urinary albumin excretion [3–5]. Similar renal effects along with a marked reduction in the degree of glomerular hypertrophy and mesangial matrix expansion have been observed in streptozotocin-diabetic rats that were given C-peptide in replacement dose [6–9].

In several cell-types including renal tubular epithelial cells, it has been shown that C-peptide binds specifically to cell membranes, probably to a G-protein coupling receptor, Ca^{2+}, and mitogen-activated protein kinase (MAPK)-dependent signaling pathways are activated, resulting in the stimulation of Na,K-ATPase [10–15]. C-peptide is also reported to stimulate endothelial nitric oxide synthase [16,17], and information on the cellular mechanisms of C-peptide action is now accumulating [18,19]. However, the mechanism by which C-peptide exerts its renoprotective effects has not been clarified.

The aim of the present study is to examine the molecular effects of C-peptide on glomerular changes in the early stages of diabetes. In particular, we focused on the effect of C-peptide on extracellular matrix accumulation, the main cause of GBM thickening and mesangial expansion [20]. To this end, we examined the effect of C-peptide on urinary albumin excretion and glomerular expression of TGF-β and type IV collagen in a mouse model of type 1 diabetes. In addition, we investigated the effects of C-peptide on the expression of type IV collagen using cultured murine podocytes.

Materials and methods

Reagents

Rat C-peptide II and scrambled C-peptide, both with a purity greater than 96% by high performance liquid chromatography, were obtained from Sigma-Genosys (Cambridge, UK). Pertussis toxin (PTX) was purchased from Sigma Aldrich (St Louis, MO, USA). PD98059, SB203580, and SP600125 were purchased from Calbiochem (Nottingham, UK). TGF-β_1 was from R&D Systems (Minneapolis, MN, USA).

Animals, induction of diabetes, and C-peptide administration

All the experiments in this study were performed in accordance with the Guidelines of the Animal Care and Use of Chiba University, Japan, which follows the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). Eight-week-old male C57/Bl6J mice were housed in the animal facilities and fed standard laboratory chow. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ; Sigma Aldrich) freshly dissolved in an ice-cold citrate buffer at a dose of 100 mg/kg once a day for 3 days. Control mice received the citrate buffer only. Diabetes was verified 7 days later by the demonstration of hyperglycemia after 12 h fasting and glycosuria.

After 2 weeks of untreated diabetes, mice were anesthetized by an intraperitoneal injection of 60 mg/kg sodium pentobarbiturate (Nembutal; Dainippon Chemicals, Tokyo, Japan) and received a subcutaneously implanted osmotic minipump (Alzet micro-osmotic pump, DURECT corporation, Cupertino, CA, USA) filled with rat C-peptide II dissolved in saline, for either 24 h or 7 days. Mice were treated with C-peptide with a dose of 290 pmol/kg/min to achieve a physiological serum level [7]. Diabetic control mice were given saline or scrambled C-peptide (a peptide with the same amino acid composition as C-peptide but with residues arranged in random order). Blood pressure was measured by the tail cuff method. Serum C-peptide concentrations were determined using a rat C-peptide EIA kit (Yanaihara Institute, Shizuoka, Japan), which does not cross-react with murine C-peptide.

Mice urine

Mice were placed in metabolic cages individually for 12 h to collect urine. Urinary albumin concentrations were determined with a murine microalbuminuria ELISA kit (Albuwell M; Exocell, Philadelphia, PA, USA). The plasma- and urinary creatinine levels were measured by a standard enzymatic method using an autoanalyzer. Creatinine clearance (mL/hr) was calculated as creatinine excretion divided by plasma creatinine concentration.

Isolation of glomeruli

The isolation of glomeruli from mice was performed as described previously [21]. Briefly, magnet microbeads (Dynabeads M-450 Tosylactivated, DYNAL, Compiègne, France) of 4.5 μm diameter resuspended in phosphate-buffered saline were infused into the left ventricle of anesthetized mice to enter systemic circulation. Kidneys were excised, minced and enzymatically digested through incubation with collagenase and DNase. The homogenate was then filtrated through a nylon mesh and the glomeruli

Copyright © 2006 John Wiley & Sons, Ltd.

embolized with microbeads were selectively collected by use of a magnetic apparatus.

Cell culture

Mouse podocytes, conditionally immortalized with a temperature-sensitive variant of the SV40 large T-antigen, were kindly provided by Dr Peter Mundel (Albert Einstein College of Medicine, NY, USA). The preparation and characterization of these cells have been described elsewhere [22]. Podocytes were maintained in RPMI 1640 (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich). To propagate podocytes, cells were cultivated at 33 °C and treated with 10 U/mL of murine recombinant γ-interferon (Pepro Tech EC Ltd, London, UK) to enhance the expression of T-antigen (permissive conditions). To induce differentiation, podocytes were cultured at 38 °C without γ-interferon in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) with 5.5 mM glucose (non-permissive conditions). Cells were cultured in non-permissive condition for at least 7 days to induce full differentiation before being subjected to all the experiments. Cells at passages 12 to 18 were used for the present experiments.

Real-time PCR

The expression of mRNA in glomeruli or podocytes was analyzed by real-time PCR. Total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. After treatment with DNase to avoid genomic contamination, 5 μg of total RNA was reversely transcribed using oligo dt primer, pd(T)12–18 (Amersham Biosciences, Piscataway, NJ, USA) and Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). Quantitative real-time PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) and was analyzed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). For quantification, a standard curve was generated with four different amounts of cDNAs. The sense and antisense primers for α3 chain of type IV collagen (α3(IV) collagen; Gene Bank Accession No. AF169387, 5190–5274 bp) were 5′-CCCAGCCAGTCCATTTATAGAATG-3′ and 5′-CAGCGAAGCCAGCCAGAA-3′, the primers for TGF-β1 (Gene Bank Accession No. N13177, 847–952 bp) were 5′-GCAACATGTGGAACTCTACCAGAA-3′ and 5′-GACGTCAAAAGACAGCCACTCA-3′, and those for plasminogen activator inhibitor-1 (PAI-1; Gene Bank Accession No. M33960, 1550–1660 bp) were 5′-CCACAAAGGTCTCATGGACCAT-3′ and 5′-TGAAAGTGTTGTGCCCTCCAC-3′. The relative mRNA levels of each molecule were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Gene Bank Accession No. M32599, 757–855 bp) and were amplified using the primers 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′.

Measurement of ERK, SAPK/JNK and p38 MAPK activities

Cells subconfluent in 100-mm dishes were serum-starved for 12 h and then incubated with various concentrations of C-peptide for 5 min. Activities of ERK, stress-activating protein kinase/c-Jun *N*-terminal kinase (SAPK/JNK), and p38 in the cell lysates were measured using commercially available kits (Cell Signaling Technology, Beverly, MA, USA) according to manufacturer's instructions. Briefly, cell extracts were immunoprecipitated with an immobilized phospho-p44/42 MAPK antibody, and *in vitro* kinase assay was performed using Elk-1 as substrate to determine ERK activation. For SAPK/JNK activity assay, c-Jun fusion protein was used both for immunoprecipitation and also as the substrate. P38 kinase activity was measured using an immobilized phospho-p38 MAPK antibody for immunoprecipitation and ATF-2 as substrate. Phosphorylation of Elk-1, c-Jun, or ATF-2 was visualized by western blotting using a phospho-Elk-1 antibody, phospho-c-Jun antibody, or phospho-ATF-2 antibody, respectively.

Western blotting

Western blotting was performed essentially as described previously [23]. Samples were dissolved in SDS sample buffer and boiled for 5 min, and the proteins were separated by SDS-PAGE on 5–10% (wt/vol) polyacrylamide-resolving gels and electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences). After blocking non-specific binding of the protein, the membranes were probed with the type IV collagen antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1 : 200), the phospho-Elk-1 antibody (Cell Signaling Technology, dilution 1 : 1000), the phospho-c-Jun antibody (Cell Signaling Technology, dilution 1 : 1000), or the phospho-ATF-2 antibody (Cell Signaling Technology, dilution 1 : 1000) at 4 °C overnight. Then, the membranes were incubated with an anti-goat IgG horseradish peroxidase-linked (HRP) antibody (Santa Cruz Biotech, dilution 1 : 2500) or anti-rabbit IgG-HRP antibody (Amersham Biosciences, dilution 1 : 2000) at room temperature for 1 h. After washing, detection was achieved by an enhanced chemiluminescence system (ECL Western blotting detection reagents; Amersham Biosciences). The intensity of chemiluminescence for the corresponding bands was analyzed by the NIH Image, public-domain image processing and analysis program (US NIH, available on the Internet at http://rsb.info.nih.gov/nih-image/).

Copyright © 2006 John Wiley & Sons, Ltd.