ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 325 (2004) 80–84

BBRC

www.elsevier.com/locate/ybbrc

Adrenomedullin induces matrix metalloproteinase-2 activity in rat aortic adventitial fibroblasts

Toshihiro Tsuruda[a,b,*], Johji Kato[a], Yuan-Ning Cao[a], Kinta Hatakeyama[c], Hiroyuki Masuyama[a], Takuroh Imamura[a], Kazuo Kitamura[a], Yujiro Asada[c], Tanenao Eto[a]

[a] *First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki, Japan*
[b] *Department of Nutrition Management, Faculty of Health and Nutrition, Minami-Kyushu University, Japan*
[c] *First Department of Pathology, Miyazaki Medical College, University of Miyazaki, Japan*

Received 27 September 2004
Available online 22 October 2004

Abstract

Background. The delicate balance of the extracellular matrix (ECM) determines the stiffness of the vascular wall, and adventitial fibroblasts are involved in ECM formation by synthesizing and degrading matrix proteins. In the present study, we examined the effect of the bioactive peptide adrenomedullin (AM) on activity and expression of matrix metalloproteinases (MMPs) in cultured aortic adventitial fibroblasts.
Methods and results. In cultured adventitial fibroblasts isolated from aorta of adult Wistar rats, 10^{-6} mol/L angiotensin II (Ang II) significantly ($p < 0.05$) down-regulated MMP-2 activity as determined by in vitro gelatin zymography. In contrast, 10^{-7} mol/L synthetic rat AM significantly ($p < 0.05$) stimulated zymographic MMP-2 activity by 23%, increasing intracellular cAMP, and AM abolished the action of Ang II, augmenting the MMP-2 activity. Similarly, Ang II down-regulated MMP-2 protein expression assessed by Western blotting, whereas AM increased it. Furthermore, 8-bromo-cAMP, an analogue of cAMP, mimicked the effect of AM, and H-89, an inhibitor for protein kinase A (PKA), significantly decreased the basal and AM-induced MMP-2 activity.
Conclusion. This study provides a new insight into the biological action of AM and its intracellular signaling system of cAMP/PKA stimulating the matrix degrading enzyme MMP-2, suggesting an important role for this molecule in modulating ECM deposition in the adventitial layer.
© 2004 Elsevier Inc. All rights reserved.

Keywords: Adrenomedullin; Vascular remodeling; Collagen; Adventitia; Matrix metalloproteinase

Vascular remodeling, defined as a change of vessel size, is initially a physiological adaptation to physical stimuli, such as blood flow, shear stress, and wall tension, to preserve the luminal size of the vessels [1,2]. However, the remodeling process becomes maladaptative in diseased situations such as atherosclerosis or vascular injury, leading to inappropriate extracellular matrix (ECM) reorganization. The delicate balance of ECM synthesis and degradation determines the level of ECM deposition in the vascular wall. A group of Ca^{2+}- and Zn^{2+}-dependent endopeptidases, matrix metalloproteinases (MMPs), contribute to the vascular remodeling by degrading several ECM proteins [3]. MMPs are present in the vasculature: MMPs-1, -14 in endothelial cells, MMPs-1, 2, 3, 9, 14 in smooth muscle cells [3], and MMPs-2, -9 in adventitial fibroblasts [4]. The adventitial layer of vascular wall has recently been recognized to have a significant role in the process of vascular remodeling [5], and either the systemic or local

* Corresponding author. Fax: +81 985 85 6596.
E-mail address: ttsuruda@med.miyazaki-u.ac.jp (T. Tsuruda).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.014

renin–angiotensin system plays a pivotal role in vascular fibrosis by stimulating proliferation and collagen production of adventitial fibroblasts [6,7].

Enhanced ECM deposition makes the vascular wall stiffer, subsequently increasing peripheral resistance [2], therefore pharmacological intervention to ameliorate ECM deposition might be attractive in maintaining a distensibility of the vasculature. The bioactive peptide adrenomedullin (AM), initially isolated from human pheochromocytoma [8], has been shown to be produced by the cardiovascular tissues where it exerts multiple actions mainly via the intracellular cyclic AMP (cAMP) [9]. For example, AM has been found to inhibit cellular proliferation and collagen synthesis of cardiac fibroblasts in vitro [10,11]. Based upon the action of AM on the fibroblasts in the heart, we hypothesized that AM plays an important role in the vascular remodeling by affecting the ECM turnover. In the present study, we conducted in vitro experiments to examine whether AM affects expression and activity of the matrix degrading enzyme MMPs in cultured fibroblasts isolated from rat aortic adventitia.

Methods

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2003-023).

Materials. Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum were from Gibco-BRL. Synthetic rat AM was purchased from Peptide Institute (Osaka, Japan), H-89 was from Seikagaku (Tokyo, Japan), and other reagents were from Sigma or otherwise indicated in the text.

Cell culture. Adventitial fibroblasts of aorta were prepared according to the method of Gu and Brecher with our modification [12]. Aortic tissues resected aseptically from 10-week-old male Wistar rats were placed in ice-cold DMEM/F-12 containing 10% fetal bovine serum and antibiotics. Loosely adhering connective tissue was rapidly removed from the aorta, and the luminal surface was opened by a longitudinal cut. Endothelial cells were removed by gently rubbing the lumen with the blunt side of dissecting scissors, and the medial layer was peeled off with the use of two forceps. The remaining tissues, predominantly adventitia, were cut into segments $\sim$2 mm^3 and placed in DMEM/F-12 solution for subsequent enzymatic digestion with 0.12% trypsin and 0.03% collagenase. The cells were then incubated in 10 cm collagen type 1-coated culture plates. After achieving confluence, they were harvested with trypsin and used for experiments at passage 3–5.

The cultured cells were incubated in DMEM/F12 medium with 10% fetal bovine serum, followed by the incubation with serum-free medium containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite for 24 h. Thereafter, the cells were incubated with fresh serum-free medium described above in the presence or absence of angiotensin II (Ang II), synthetic rat AM or 8-bromo-cAMP (8-Br-cAMP). In another set of experiments, H-89, a specific protein kinase A (PKA) inhibitor, was added 30 min prior to AM administration. The isolated cells were positive for vimentin, α-smooth muscle actin, but negative for von-Willebrand factor, desmin, smooth muscle-myosin heavy chain and caldesmon, indicating that they are activated adventitial fibroblasts.

In vitro gelatin zymography. Conditioned media of adventitial fibroblasts (2 μg protein) were concentrated and used for the zymographic gelatinase activity assay as described before [13]. In brief, samples mixed with Laemmli sample buffer were loaded onto 10% Tris–glycine gels with 0.1% gelatin (Invitrogen) in Tris–glycine sodium dodecyl sulfate running buffer. The gels were then washed with renaturing buffer, followed by incubation with zymogen developing buffer for 24 h. They were stained with 0.5% (wt/vol) Coomassie brilliant blue overnight and then with destaining buffer. Lytic bands corresponding to the pro- and active-forms of MMP-2 were analyzed as a total MMP-2 activity with NIH image software v. 1.63.

Western blot. Denatured protein extracts (30 or 60 μg) from cultured adventitial fibroblasts were subjected to sodium dodecyl sulfate–polyacrylamide gel as previously described [13]. The separated proteins were electrically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Equal protein loading was verified by staining the gels with Coomassie brilliant blue. After blocking the non-specific background with 5% skim milk, polyvinylidene difluoride membranes were incubated with anti-MMP-2 polyclonal antibody (AB809, Chemicon, 1:1000), anti-tissue inhibitor of MMP-2 (TIMP-2) polyclonal antibody (AB801, Chemicon, 1:1000) or anti-MMP-14 monoclonal antibody (Daiichi Fine Chemical F-84, 10 μg/mL) followed by incubation with horseradish peroxidase-coupled secondary antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and the intensity of the bands was analyzed densitometrically (Chemi Doc Documentation System, Bio-Rad).

Assays for cAMP and AM. Intracellular cAMP concentration in the adventitial fibroblasts was measured as previously described [10]. In brief, the fibroblasts were incubated in Hanks' balanced salt solution containing 20 mmol/L *N*-[2-hydroxyethyl]piperazine-3-isobutyl-1-methylxanthine for 10 min at 37 °C. AM was then added at the indicated concentrations. The reaction was terminated by aspirating the medium and adding cold 6% trichloroacetic acid. Cells were extracted with water-saturated ethyl ether, and cAMP was measured by radioimmunoassay. To evaluate AM secretion from the adventitial fibroblasts, conditioned media were collected at indicated time points, and rat AM immunoreactivity in the media was measured with commercially available immunoradiometric assay kits (Shionogi, Japan).

Statistical analysis. All data are expressed as means ± SEM. Results of the Western blot and zymography are expressed as ratios relative to the respective control. Comparisons between groups were assessed with one-way ANOVA followed by Fisher's test. A statistical significance was accepted at $p < 0.05$.

Results

Effects of Ang II and AM on MMP-2 activity and protein

Fig. 1A illustrates the effects of Ang II and AM on zymographic MMP-2 activity in the cultured aortic adventitial fibroblasts. One μmol/L of Ang II significantly ($p < 0.05$) decreased zymographic MMP-2 activity in these cells. Conversely, 10^{-7} mol/L synthetic rat AM significantly increased MMP-2 activity by 23% ($p < 0.05$), and AM abolished the action of Ang II, augmenting MMP-2 activity, at 10^{-7} and 10^{-8} mol/L. MMP-9 is another metalloproteinase having an important role in the adventitia [4], but the band corresponding to MMP-9 activity of 92 kDa was too faint to be quantified in control fibroblasts and those stimulated by either Ang II or AM. Fig. 1B illustrates the effects of Ang II and AM on MMP-2 protein expression. Similar to the zymographic finding, 10^{-6} mol/L Ang II down-regulated protein expression of MMP-2, while 10^{-7} mol/L AM increased the MMP-2 expression whether or not Ang II was added.

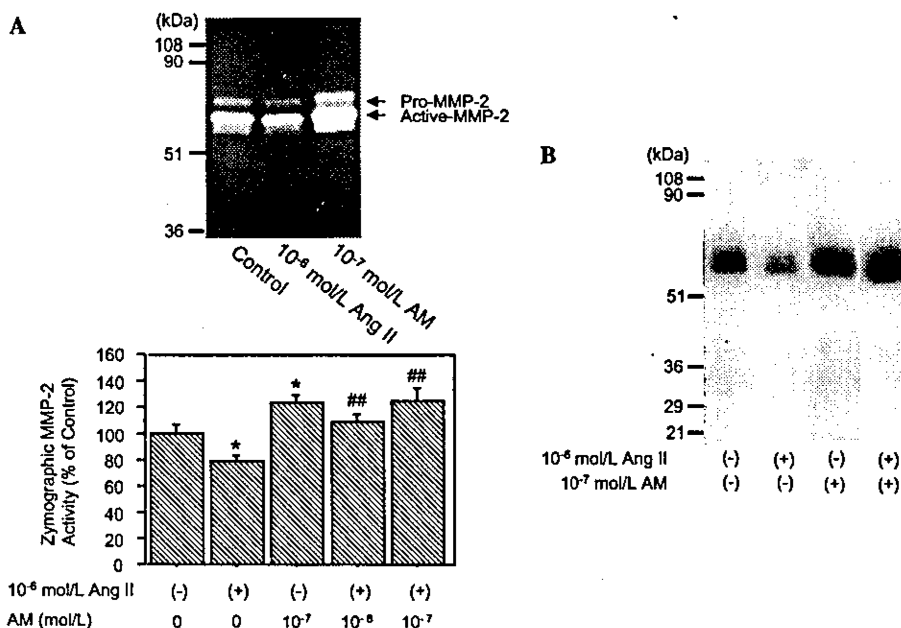

Fig. 1. (A) Effects of Ang II and AM on zymographic MMP-2 activity in cultured adventitial fibroblasts. Adventitial fibroblasts were incubated with or without 10^{-6} mol/L Ang II in the absence or presence of indicated concentration of synthetic rat AM for 24 h. Values are shown as means ± SEM ($n = 7$–8). The upper panel shows a representative zymographic picture. $^{*}p < 0.05$, vs. control cells; $^{\#\#}p < 0.01$, vs. cells incubated with Ang II. (B) Effects of Ang II and AM on protein expression of MMP-2 in adventitial fibroblasts. Identical results were obtained in three independent experiments.

Roles of cAMP/PKA signaling on zymographic MMP-2 activity

Figs. 2A and B illustrate the effect of AM on intracellular cAMP content in cultured adventitial fibroblasts. One μmol/L AM significantly stimulated cAMP production, peaking at 10 min (Fig. 2A), and this effect was dose-dependent (Fig. 2B). Figs. 2C and D illustrate the effects of the cAMP analogue 8-Br-cAMP and the specific PKA inhibitor H-89 on zymographic MMP-2 activity. 8-Br-cAMP mimicked the effect of AM stimulating MMP-2 activity (Fig. 2C), while H-89 significantly decreased the MMP-2 activity of the cells incubated with or without 10^{-7} mol/L AM (Fig. 2D).

AM secretion from adventitial fibroblasts

Adventitial fibroblasts time-dependently secreted AM into the media up to 48 h under the serum-free conditions. Rat AM concentrations in the conditioned media were: 6 h, 1.4 ± 0.1; 24 h, 2.0 ± 0.3; and 48 h, 5.5 ± 0.3 fmol/10^5 cells ($n = 6$).

Discussion

Balance between ECM synthesis and degradation determines its abundance in the vasculature. Although hemodynamic stimuli largely regulate vascular remodeling in normal arteries, humoral factors such as Ang II, inappropriately stimulating collagen synthesis, seem to be of importance in the pathological situation [1]. MMPs reorganize the vessel structure by degrading the ECM proteins and a significant role for MMPs in vascular remodeling has been suggested [3]. For example, the plasma level of MMP-1 was reduced in hypertensive patients [14], and MMPs-1, 3, and 9 were up-regulated in the atherosclerotic lesion [15,16]. Constitutively produced by vascular endothelial and smooth muscle cells, MMP-2 degrades gelatin, type I, IV, V, VII collagen, and elastin [17,18]. Here we report for the first time that the bioactive peptide AM increased protein level and enzymatic activity of MMP-2 in cultured aortic adventitial fibroblasts of rats. Hernandez-Barrantes et al. [19] showed that MMP-2 is regulated by MMP-14, a membranous type of MMP, and by the tissue inhibitor of MMP (TIMP)-2. We examined expressions of these proteins by Western blotting, but neither AM nor Ang II affected the MMP-14 protein expression and TIMP-2 was undetectable in the fibroblasts (data not shown).

Renin–angiotensin system activation has been shown to be involved in the process of vascular remodeling. Angiotensinogen is expressed in the adventitia [20], and angiotensin converting enzyme was found to be induced in the injured artery [21]. Further, mast cell mainly distributed in the adventitia has been reported to be an additional source of renin [22]. These findings suggest an existence of the local renin–angiotensin system in vascular adventitia. Recent reports have shown that MMP-2 activity is down-regulated in the

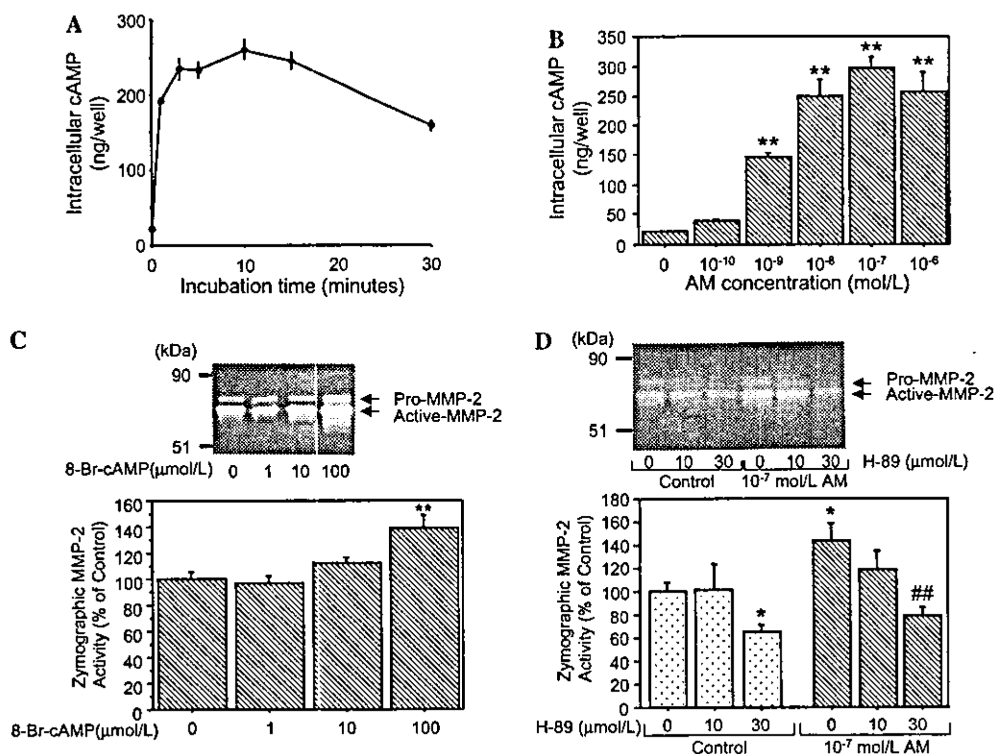

Fig. 2. (A,B) Time course of cAMP elevation by 10^{-6} mol/L rat synthetic AM (A) and dose-dependent effect of AM on cAMP elevation at 10 min (B) in adventitial fibroblasts. (C,D) Effects of 8-Br-cAMP (C) and H-89 (D) on zymographic MMP-2 activity in adventitial fibroblasts. The upper panels show representative zymographic pictures. Values are shown as means ± SEM, and the sample numbers were four in (A) and (B) and six in (C) and (D), respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control fibroblasts; $^{\#\#}p < 0.01$, vs. AM-treated cells without H-89.

vasculature of rats and humans with diabetes mellitus [23,24], where the renin–angiotensin system has been shown to be activated; though, the role of AM in the vascular adventitia remains unknown. In the present study, we examined the effects of Ang II and AM on the ECM metabolism in the cultured adventitial fibroblasts. When assessed by [^{3}H]proline incorporation into the cells, AM failed to inhibit the Ang II-induced de novo collagen synthesis (data not shown). However, AM increased not only basal levels of MMP-2 protein expression and its enzymatic activity but also those reduced by Ang II.

AM was initially isolated during experiments monitoring cAMP elevation in rat platelets [8], and many of the actions of AM have been shown to be mediated by intracellular cAMP [9–11]. In the present study, the cAMP analogue 8-Br-cAMP mimicked the effect of AM, stimulating zymographic MMP-2 activity, and PKA inhibition with H-89 attenuated its activity. These results suggest that the cAMP/PKA signaling system is involved in the AM-induced activation and up-regulation of MMP-2 in the aortic adventitial fibroblasts. This is comparable with the finding by Maioli et al. [25] who reported that parathyroid hormone-related peptide stimulated MMP-2 activity by intracellular cAMP accumulation without affecting collagen synthesis in human skin fibroblasts.

It has been well documented that AM is produced by the vascular wall, particularly in endothelial and smooth muscle cells [26]. Immunohistochemical staining for AM was also observed in the adventitia of rat femoral artery [27], and consistent with this, we found that cultured aortic adventitial fibroblasts also secreted AM in this study. According to our unpublished observation, intravenous AM infusion attenuated collagen accumulation in the adventitia following ballooning injury in rat carotid artery, suggesting an important role of AM as an anti-fibrotic factor in the vascular remodeling. Taken together, renin–angiotensin system activation increases ECM formation by stimulating collagen synthesis and by decreasing MMP-2 activity in the adventitia, making the vascular wall stiffer; while AM may reduce vascular stiffness by enhancing action of the matrix degradation enzyme MMP-2, thus antagonizing the action of Ang II in the adventitial layer. However, this hypothesis should be tested further by in vivo studies.

In summary, this study provides a new insight into the biological action of AM in vascular remodeling, suggesting that AM may modulate ECM metabolism by augmenting the MMP-2 action in the adventitia.

Acknowledgments

This study was supported by the Grants-in-Aid for Scientific Research and for the 21st Century Centers of

Excellence Program (Life Science) from the Ministry of Education, Culture, Sport, Science and Technology, Japan, and by a Grant-in-Aid from AstraZeneca Research 2003.

References

[1] M.R. Ward, G. Pasterkamp, A.C. Yeung, C. Borst, Arterial remodeling. Mechanisms and clinical implications, Circulation 102 (2000) 1186–1191.

[2] H.D. Intengan, E.L. Schiffrin, Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis, Hypertension 38 (2001) 581–587.

[3] Z.S. Galis, J.J. Khatri, Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly, Circ. Res. 90 (2002) 251–262.

[4] W.J. Cai, S. Koltai, E. Kocsis, D. Scholz, S. Kostin, X. Luo, W. Schaper, J. Schaper, Remodeling of the adventitia during coronary arteriogenesis, Am. J. Physiol. Heart Circ. Physiol. 284 (2003) H31–H40.

[5] S. Sartore, A. Chiavegato, E. Faggin, R. Franch, M. Puato, S. Ausoni, P. Pauletto, Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant, Circ. Res. 89 (2001) 1111–1121.

[6] K.T. Weber, Y. Sun, E. Guarda, Structural remodeling in hypertensive heart disease and the role of hormones, Hypertension 23 (1994) 869–877.

[7] D.K. Kim, J.E. Huh, S.H. Lee, K.P. Hong, J.E. Park, J.D. Seo, R.W. Lee, Angiotensin II stimulates proliferation of adventitial fibroblasts cultured from rat aortic explants, J. Korean Med. Sci. 14 (1999) 487–496.

[8] K. Kitamura, K. Kangawa, M. Kawamoto, Y. Ichiki, S. Nakamura, H. Matsuo, T. Eto, Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma, Biochem. Biophys. Res. Commun. 192 (1993) 553–560.

[9] K. Kitamura, K. Kangawa, T. Eto, Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions, Microsc. Res. Tech. 57 (2002) 3–13.

[10] T. Tsuruda, J. Kato, K. Kitamura, M. Kawamoto, K. Kuwasako, T. Imamura, Y. Koiwaya, T. Tsuji, K. Kangawa, T. Eto, An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth, Cardiovasc. Res. 43 (1999) 958–967.

[11] T. Horio, T. Nishikimi, F. Yoshihara, H. Matsuo, S. Takishita, K. Kangawa, Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts, Eur. J. Pharmacol. 382 (1999) 1–9.

[12] M. Gu, P. Brecher, Nitric oxide-induced increase in p21 (Sdi1/Cip1/Waf1) expression during the cell cycle in aortic adventitial fibroblasts, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 27–34.

[13] T. Tsuruda, G. Boerrigter, B.K. Huntley, J.A. Noser, A. Cataliotti, L.C. Costello-Boerrigter, H.H. Chen, J.C. Burnett Jr., Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases, Circ. Res. 91 (2002) 1127–1134.

[14] C. Laviades, N. Varo, J. Fernández, G. Mayor, M.J. Gil, I. Monreal, J. Díez, Abnormalities of the extracellular degradation of collagen type I in essential hypertension, Circulation 98 (1998) 535–540.

[15] Z.S. Galis, G.K. Sukhova, M.W. Lark, P. Libby, Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques, J. Clin. Invest. 94 (1994) 2493–2503.

[16] S.T. Nikkari, K.D. O'Brien, M. Ferguson, T. Hatsukami, H.G. Welgus, C.E. Alpers, A.W. Clowes, Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis, Circulation 92 (1995) 1393–1398.

[17] F.G. Spinale, Matrix metalloproteinases: regulation and dysregulation in the failing heart, Circ. Res. 90 (2002) 520–530.

[18] R.M. Senior, G.L. Griffin, C.J. Fliszar, S.D. Shapiro, G.I. Goldberg, H.G. Welgus, Human 92- and 72-kilodalton type IV collagenases are elastases, J. Biol. Chem. 266 (1991) 7870–7875.

[19] S. Hernandez-Barrantes, M. Toth, M.M. Bernardo, M. Yurkova, D.C. Gervasi, Y. Raz, Q.A. Sang, R. Fridman, Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation, J. Biol. Chem. 275 (2000) 12080–12089.

[20] L.A. Cassis, K.R. Lynch, M.J. Peach, Localization of angiotensinogen messenger RNA in rat aorta, Circ. Res. 62 (1988) 1259–1262.

[21] J.L. Zhuo, P. Froomes, D. Casley, J.J. Liu, C. Murone, Y.S. Chai, B. Buxton, F.A. Mendelsohn, Perindopril chronically inhibits angiotensin-converting enzyme in both the endothelium and adventitia of the internal mammary artery in patients with ischemic heart disease, Circulation 96 (1997) 174–182.

[22] R.B. Silver, A.C. Reid, C.J. Mackins, T. Askwith, U. Schaefer, D. Herzlinger, R. Levi, Mast cells: a unique source of renin, Proc. Natl. Acad. Sci. USA 101 (2004) 13607–13612.

[23] S. Jesmin, I. Sakuma, Y. Hattori, A. Kitabatake, Role of angiotensin II in altered expression of molecules responsible for coronary matrix remodeling in insulin-resistant diabetic rats, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 2021–2026.

[24] V. Portik-Dobos, M.P. Anstadt, J. Hutchinson, M. Bannan, A. Ergul, Evidence for a matrix metalloproteinase induction/activation system in arterial vasculature and decreased synthesis and activity in diabetes, Diabetes 51 (2002) 3063–3068.

[25] E. Maioli, V. Fortino, C. Torricelli, B. Arezzini, C. Gardi, Effect of parathyroid hormone-related protein on fibroblast proliferation and collagen metabolism in human skin, Exp. Dermatol. 11 (2002) 302–310.

[26] T. Eto, J. Kato, K. Kitamura, Regulation of production and secretion of adrenomedullin in the cardiovascular system, Regul. Pept. 112 (2003) 61–69.

[27] M. Yamasaki, J. Kawai, T. Nakaoka, T. Ogita, A. Tojo, T. Fujita, Adrenomedullin overexpression to inhibit cuff-induced arterial intimal formation, Hypertension 41 (2003) 302–307.

ELSEVIER

Cardiovascular Research 65 (2005) 921–929

Cardiovascular Research

www.elsevier.com/locate/cardiores

Antifibrotic effect of adrenomedullin on coronary adventitia in angiotensin II-induced hypertensive rats

Toshihiro Tsuruda[a,b,*], Johji Kato[a], Kinta Hatakeyama[c], Hiroyuki Masuyama[a], Yuan-Ning Cao[a], Takuroh Imamura[a], Kazuo Kitamura[a], Yujiro Asada[c], Tanenao Eto[a]

[a]*First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki, 5200 Kihara Kiyotake, Miyazaki 889-1692, Japan*
[b]*Department of Nutrition Management, Faculty of Health and Nutrition, Minami-Kyushu University, Japan*
[c]*First Department of Pathology, Miyazaki Medical College, University of Miyazaki, Japan*

Received 2 September 2004; received in revised form 29 October 2004; accepted 3 November 2004
Available online 24 November 2004
Time for primary review 26 days

Abstract

Objective: The extracellular matrix (ECM) determines the structural integrity of the heart and vasculature, participating in cardiovascular remodeling. We previously reported that adrenomedullin (AM) inhibited cellular proliferation and protein synthesis of cardiac fibroblasts; however, the precise mechanisms of AM actions as an antifibrotic factor remain unknown. The purpose of this study was to examine the biological actions of AM against the profibrotic factor angiotensin II (Ang II) in coronary adventitia.
Methods and results: Rats with hypertension induced by Ang II infusion were administered 0.06 μg/kg/min recombinant human AM subcutaneously for 14 days. The AM infusion significantly (p<0.05) reduced the Ang II-induced increase of coronary adventitial fibroblasts expressing Ki-67 and α-smooth muscle actin (α-SMA) in the left ventricle, by 65%, and 62%, respectively, without affecting systolic blood pressure, left ventricle/body weight, or cross-sectional area of myocardial fibers. Collagen deposition of coronary arteries was reduced by the AM infusion (−24%, p<0.01), and these effects of AM were accompanied by significant reductions in gene expression of type 1 collagen (−49%, p<0.05) and transforming growth factor-β1 (TGF-β1) (−55%, p<0.01). In cultured cardiac fibroblasts, 10^{-7} mol/L AM exerted an inhibitory effect on TGF-β1-induced α-SMA expression (p<0.01) that was mimicked by 8-bromo-cAMP and attenuated by the protein kinase A inhibitor H-89.
Conclusion: AM decreased Ang II-induced collagen deposition surrounding the coronary arteries, inhibiting myofibroblast differentiation and expressions of ECM-related genes in rats. The present findings further support the biological action of AM as an antifibrotic factor in vascular remodeling.
© 2004 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Extracellular matrix; Fibrosis; Hypertension; Peptide hormone; Remodeling

1. Introduction

Cardiac fibrosis is an important clinical disorder leading to deleterious consequences for myocardial function such as systolic and diastolic heart failure [1]. Particularly, thickening of the adventitia surrounding intramyocardial coronary arteries, where extracellular matrix (ECM) first accumulates in response to systemic hypertension, has been thought to reduce oxygen and nutrient supply to the *myocardium*, resulting in deterioration of ventricular function [2]. Emerging concepts of vascular remodeling underline the importance of the ECM scaffold in the vessel wall. The activated adventitial fibroblasts, known as myofibroblasts characterized by α-smooth muscle actin (α-SMA) expression, play important roles in the pathological vascular remodeling [3,4]. Therefore, both understanding of the regulation of fibroblast activation and the

* Corresponding author. First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki, 5200 Kihara Kiyotake, Miyazaki 889-1692, Japan. Tel.: +81 985 85 0872; fax: +81 985 85 6596.
E-mail address: ttsuruda@med.miyazaki-u.ac.jp (T. Tsuruda).

0008-6363/$ - see front matter © 2004 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2004.11.004

development of effective pharmacological intervention to manipulate fibroblast function are necessary to attenuate adverse remodeling.

A body of evidence suggests that the renin–angiotensin–aldosterone system is an important factor in progression of myocardial and vascular fibrosis accompanied by upregulation of transforming growth factor-β1 (TGF-β1) [5–7]. TGF-β1 induces a phenotypic change of fibroblasts to myofibroblasts in hypertensive heart disease, coronary restenosis following angioplasty, and in the healing process after myocardial infarction [8,9]. Blockage of TGF-β1 signaling was indeed reported to prevent fibroblast proliferation and diastolic cardiac dysfunction [10].

Adrenomedullin (AM), initially isolated from human pheochromocytoma [11], has been reported to have multiple functions in the cardiovascular system [12]. We and others have previously reported that AM inhibited proliferation and collagen synthesis induced by angiotensin II (Ang II) in cardiac fibroblasts of neonatal rats in vitro [13,14], suggesting a possible role of AM in attenuating cardiovascular remodeling. However, the precise mechanism by which AM acts as an antifibrotic factor in vivo remains to be elucidated.

Based upon previous studies, we hypothesized that activation of adventitial fibroblasts would result in coronary matrix remodeling in rats infused with Ang II and that pharmacological intervention with AM would lead to attenuation of perivascular fibrosis by modulating fibroblast function. Our aim in this study was to examine the biological action of AM against the profibrotic factor Ang II in coronary adventitia of rats.

2. Methods

2.1. Animals experiments

Eight-week-old male Wistar rats (Charles River Japan) weighing 200 to 250 g were housed in a temperature- and light-controlled room (25±1 °C; 12/12-h light/dark cycle) with normal rat chow and water given ad libitum. The rats were divided into three groups: control group (n=5) and two Ang II-infused groups with (n=11) or without (n=15) AM treatment. They were implanted with miniosmotic pumps (Alzet model 2002) under pentobarbital sodium anesthesia, that released either saline or 250 ng/kg/min Ang II for 14 days. In the Ang II-infused groups, another pump was implanted to infuse saline or 0.06 μg/kg/min of recombinant human AM (Shionogi & Co., Japan). The dose of AM used in this study was determined by referring to our previous observation, in which cardiac remodeling after myocardial infarction was significantly inhibited without affecting systemic blood pressure [15]. Blood pressure was measured while awake at least 9 times by tail-cuff plethysmography (Softron, BP-98A), and the mean value was recorded. At day 14, the rats were killed by decapitation and trunk blood was collected for measuring AM concentration. Plasma levels of human and rat AM were determined with commercially available immunoradiometric assay kits (Shionogi & Co., Japan). After removing atria and right ventricle of the heart, left ventricle was frozen in liquid nitrogen or fixed in 10% formalin and was embedded in paraffin wax.

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2003-023). This investigation confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Histology and immunohistochemistry

Ventricular tissues, fixed in 10% formalin and embedded in paraffin, were sectioned at 2 μm thickness. After deparaffinization with xylene and graded alcohol, slides were immersed in 3% H_2O_2 in methanol to block endogenous peroxidase activities, thereafter incubated with 5% skim milk to reduce the nonspecific background. The section slides were then stained with either anti-monoclonal α-SMA antibody (Clone 1A4, DAKO) at a dilution of 1:200, or antipolyclonal TGF-β1 antibody (sc-146, Santa Cruze) at 1:100 at 4 °C. After the overnight reaction with antibodies, the slide sections were incubated with EnVision+ (DAKO) for 30 min, visualized with 0.05% 3, 3′-diaminobenzidine containing hydrogen peroxide, and counterstained with hematoxylin. For detection of Ki-67 antigen, a nuclear protein expressed in proliferating cells, tissue sections were autoclaved at 121 °C for 10 min in 10 mmol/L citrate buffer (pH 6.0) prior to incubation with primary antibody (Clone MIB-5, DAKO) at a dilution of 1:25. For the detection of collagen, slides were incubated with 0.1% picrosirius red (Direct Red 80, Sigma) dissolved in saturated picric acid for 10 min as described before [15]. The specificity of the antibody for TGF-β1 was confirmed by substitution of nonimmune rabbit serum and the absorption test as described before [16].

2.3. Morphology and cell counting

Morphological evaluation and cell counting of coronary arteries sectioned at the middle portion in the left ventricle were performed by a single observer in a blind manner. Each section immunostained with the antibody against either Ki-67 or α-SMA was scanned at a magnification of ×200, and the number of positive cells surrounding the coronary artery was determined. At least five images of fibrosis areas surrounding the blood vessel were randomly selected from each slide, and examined using an image

analysis system (Axio Vison 2.05 Carl ZEISS, Munchen, Germany) to calculate ratios of the perivascular fibrosis area to the total vascular area. To evaluate the interstitial fibrosis, collagen volume fraction in the interstitial space between myocardial fibers was determined by calculating the ratio of collagen area to the selected myocardial tissue area as previously described [15]. To measure the *cardiocyte* size, cross-sectional area of *myocardial fiber* was measured at the level of nuclei in at least 10 *cardiocytes* as described before [16]. Longitudinal- or oblique-sectioned *cardiocytes* were excluded for the analysis.

2.4. Gene expression

Gene expressions for TGF-β1 and type 1 collagen in total RNA isolated from left ventricle were measured by using real time-quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems) as previously described [17]. cDNA reverse transcribed from total RNA was amplified with the following oligonucleotide probes labeled with 6-carboxy-fluorescencein as reporter fluorescence and 6-carboxy tetramethyl-rhodamine as quencher fluorescence: TGF-β1 [18], TACGCCTGAGTGGCTGTCTTTTGA (nucleotide 985–1008); type 1 collagen [19], ACTGGAGACAGAGGA-CCGCGTGGAC (nucleotide 103–127); 18*S* ribosomal RNA [20], TGCCGACGGGCGCTGACC (nucleotide 176–193) and with the following pairs of oligonucleotides: TGF-β1 [18], TTCCTGGCGTTACCTTGGT (nucleotide 943–961, forward primer) and GCCACTGCCGGACAACT (nucleotide 1018–1034, reverse primer); type 1 collagen [19], TGCTGCTTGCAGTAACGTCG (nucleotide 32–51, forward primer) and TCAACACCATCTCTGCCTCG (nucleotide 148–167, reverse primer); 18*S* rRNA [20], CTTTGGTCGCTCGCTCCTC (nucleotide 118–136, forward primer) and CTGACCGGGTTGGTTTTGAT (nucleotide 229–248, reverse primer). The PCR products electrophoresed were observed at the expected molecular sizes, and the gene expression levels were normalized relative to that of 18*S* rRNA.

2.5. Cell culture

Cultured cardiac fibroblasts of neonatal rats were prepared as previously described [13]. After achieving confluence in the DMEM/F12 medium with 10% FBS, the cells were incubated with serum-free medium containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite for 24 h. The medium was then exchanged for fresh serum-free medium described above and incubated with or without synthetic rat AM (Peptide Institute, Osaka, Japan), recombinant human TGF-β1 or 8-bromo-cAMP (Sigma, MO, USA). In another series of experiment, H-89 (Seikagaku, Tokyo, Japan), a specific protein kinase A inhibitor, was added to culture medium at least 30 min before the incubation with AM or TGF-β1.

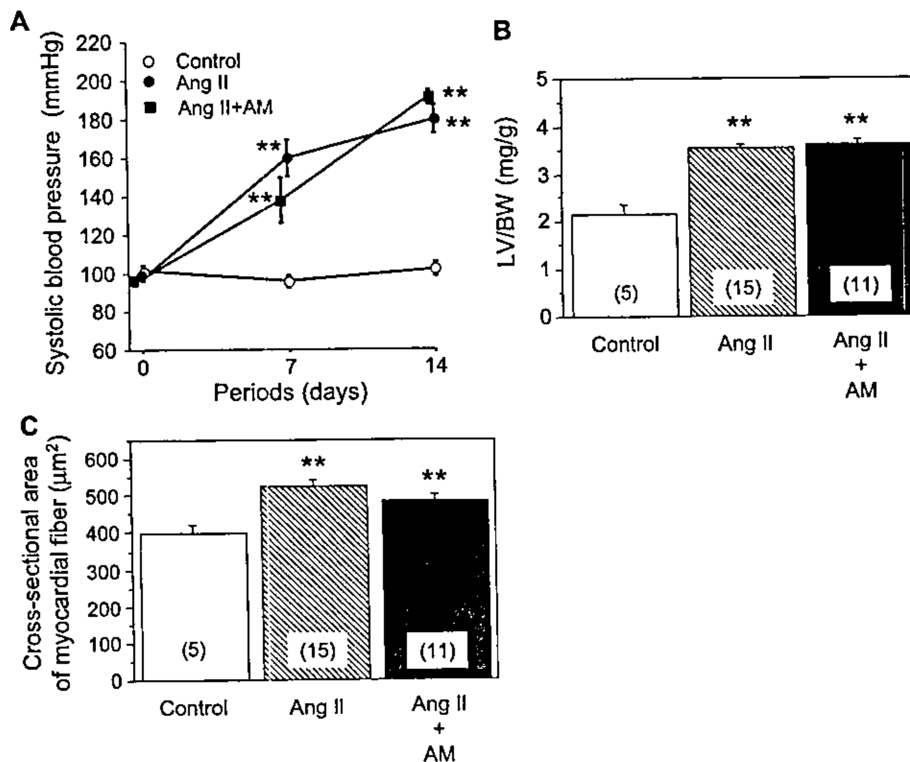

Fig. 1. Effects of Ang II and co-administration of AM on systolic blood pressure (A), left ventricular weight/body weight (LV/BW) (B), and *cross-sectional area of myocardial fiber* (C). Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined. $^{**}p<0.01$, compared to controls.

2.6. Western blot

Denatured protein extract (5 μg) from the cultured cardiac fibroblasts was subjected to sodium dodecyl sulfate-polyacrylamide gel as previously described [21]. The separated proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (BIO-RAD). Equal protein loading was verified by staining the gels with Coomassie brilliant blue. After blocking the non-specific background with 5% skim milk, PVDF membranes were incubated with the anti-α-SMA monoclonal antibody at a dilution of 1:1000, followed by incubation with horseradish peroxidase-coupled second antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and intensities of the bands were analyzed densitometrically (Chemi Doc™ Documentation System, BIO-RAD).

2.7. Statistical analysis

All data are expressed as means±S.E.M. Comparisons between groups were assessed with one-way ANOVA followed by the Fisher's test. A statistical significance was accepted at $p<0.05$.

3. Results

3.1. Systolic blood pressure, left ventricle/body weight, and cardiocyte size

Fig. 1A illustrates the effects of Ang II and AM on systolic blood pressure. Continuous, subcutaneous Ang II infusion significantly ($p<0.01$) increased systolic blood pressure at days 7 and 14, and the co-administration of AM

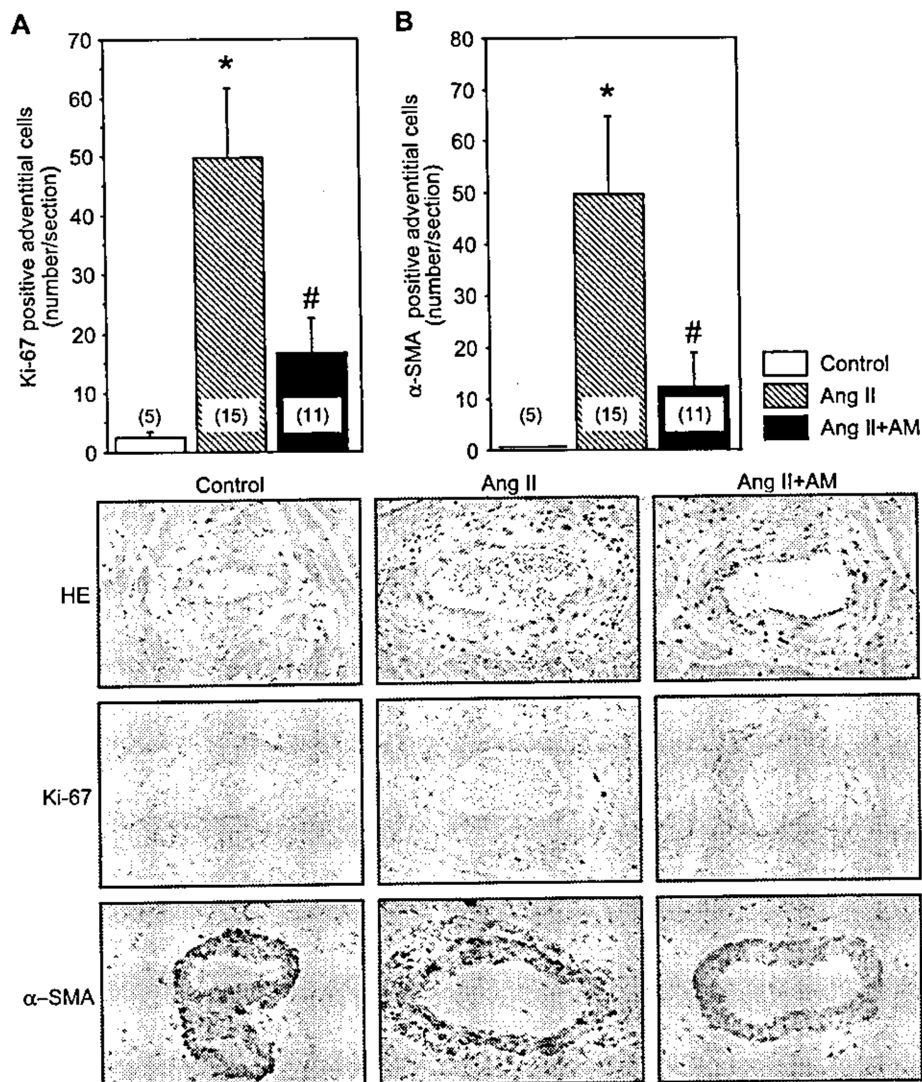

Fig. 2. Effects of Ang II and AM on the number of adventitial fibroblasts expressing Ki-67 antigen (A) and of those positive for α-SMA (B). The bottom panels show the representative histological sections stained with hematoxylin eosin (HE), anti-Ki-67, and α-SMA antibodies. Values are shown as means±S.E.M. Parentheses indicate the number of rats examined. *$p<0.05$, compared to controls; #$p<0.05$, compared to Ang II group.

with Ang II did not affect systolic blood pressure significantly. In addition, Ang II significantly ($p<0.01$) increased the left ventricle/body weight (LW/BW) and *cross-sectional area of myocardial fiber*, compared to control at day 14, without a significant difference in LV/BW or *cardiocyte* size between the Ang II and Ang II+AM groups (Fig. 1B and C).

3.2. Fibroblast proliferation and myofibroblast differentiation

Fig. 2A and B illustrate the effects of Ang II and AM on staining for Ki-67 antigen and α-SMA in the perivascular area of coronary arteries. Ang II significantly ($p<0.01$) increased the number of fibroblasts expressing Ki-67 antigen, a marker for proliferating fibroblasts, and this increase was significantly ($p<0.05$) inhibited by the co-administration of AM at day 14 (Fig. 2A). Similarly, the Ang II-induced increase in number of the fibroblasts expressing α-SMA, a marker for myofibroblast differentiation, was significantly ($p<0.05$) reduced by AM (Fig. 2B).

3.3. Type 1 collagen gene expression and adventitial area

Fig. 3A illustrates the effects of Ang II and AM on type 1 collagen mRNA expression. The Ang II infusion significantly ($p<0.05$) increased type 1 collagen expression in the left ventricle, and the co-administration of AM significantly ($p<0.05$) attenuated its expression by 49% at day 14. The effects of Ang II and AM on the adventitial area surrounding the coronary arteries are shown in Fig. 3B as composite data and in Fig. 3C as representative pictures. Ang II significantly ($p<0.01$) increased perivascular fibrosis at day 14, and the co-administration of AM significantly ($p<0.01$) decreased it. Similarly, the Ang II infusion significantly increased interstitial fibrosis of the left ventricular myocardium (+130%, $p<0.01$), while AM inhibited this Ang II effect (−54%, $p<0.01$).

3.4. TGF-β1 expression

As shown in Fig. 4A, Ang II significantly ($p<0.01$) increased TGF-β1 gene expression in the left ventricle, while the co-administration of AM significantly ($p<0.01$) attenuated its expression by 55%. Fig. 4B illustrates the distribution of TGF-β1 immunoreactivity in the coronary arteries. TGF-β1 immunoreactivity was intensely stained in the adventitial fibroblasts, as well as in vascular smooth muscle cells and *myocardial fibers* of the Ang II-treated rats, while those cells were faintly stained in the control and AM-treated rats.

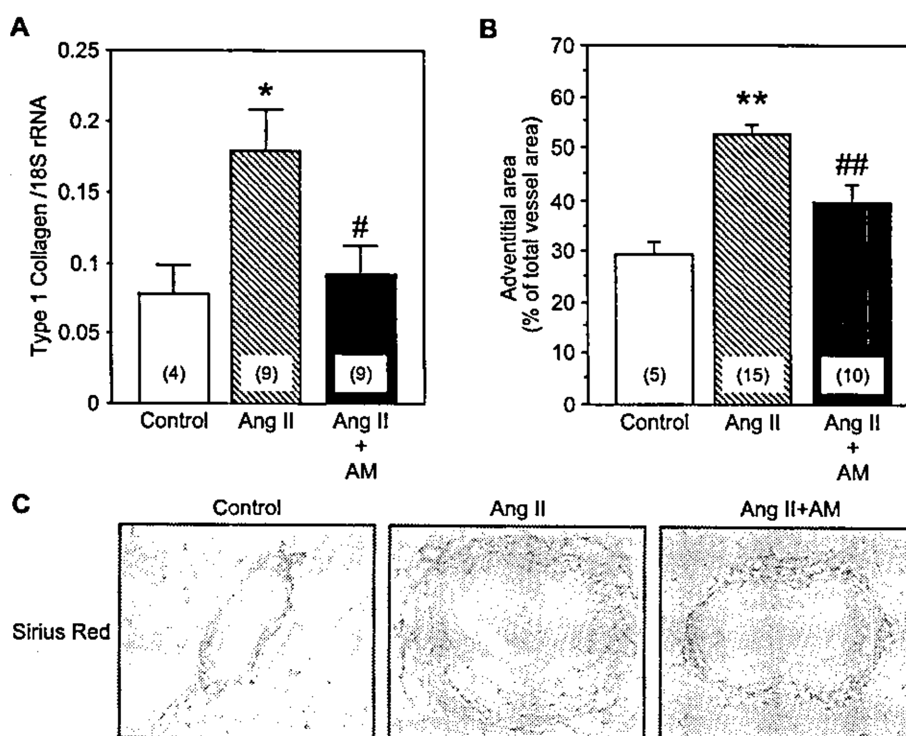

Fig. 3. Effects of Ang II and AM on gene expression of type 1 collagen (A) and on adventitial area determined by sirius red staining (B). The bottom panels (C) show the representative pictures for sirius red staining. Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined. *$p<0.05$, **$p<0.01$, compared to controls; #$p<0.05$, ##$p<0.01$, compared to Ang II.

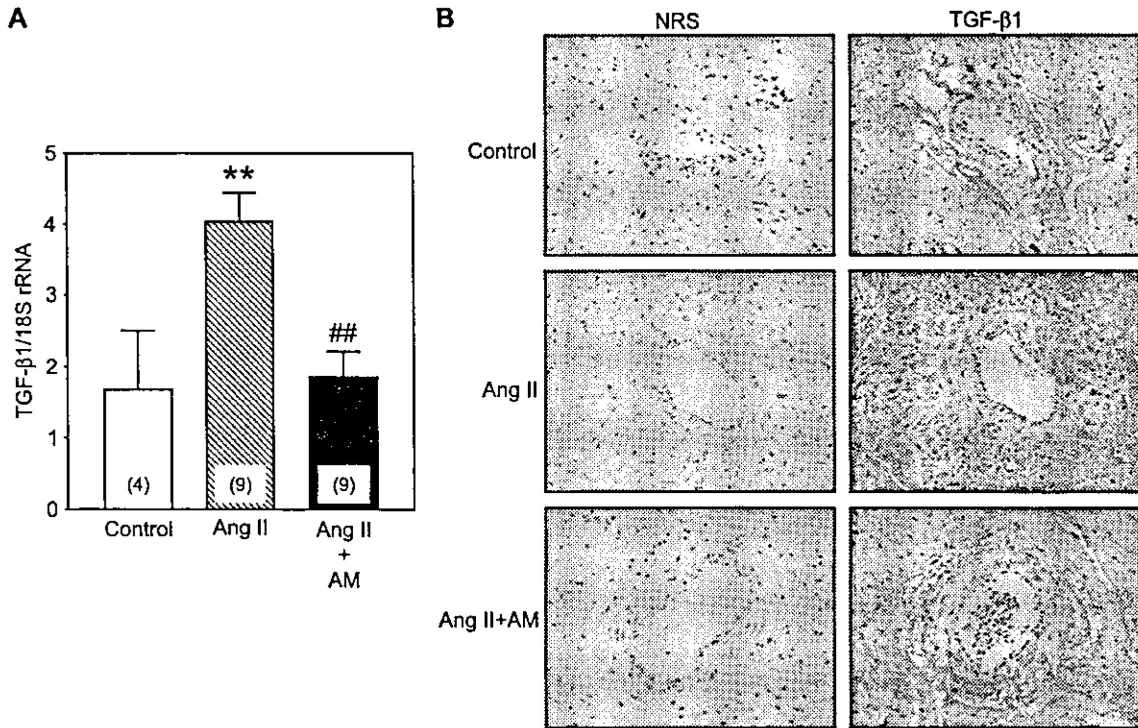

Fig. 4. Effects of Ang II and AM on the gene expression of TGF-β1 in LV (A) and representative pictures for the distribution of TGF-β1 immunoreactivity (B). Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined and NRS denotes nonimmune rabbit serum. $^{**}p<0.01$, compared to controls; $^{\#\#}p<0.01$, compared to Ang II.

3.5. Plasma levels of rat and human AM

The Ang II infusion had no significant effect on the plasma levels of endogenous rat AM at day 14 (control, 4.7±0.5; Ang II, 5.0±0.3 fmol/mL). Human AM immunoreactivity was detectable only in the plasma of recombinant AM-treated rats at 0.7±0.4 fmol/mL at day 14.

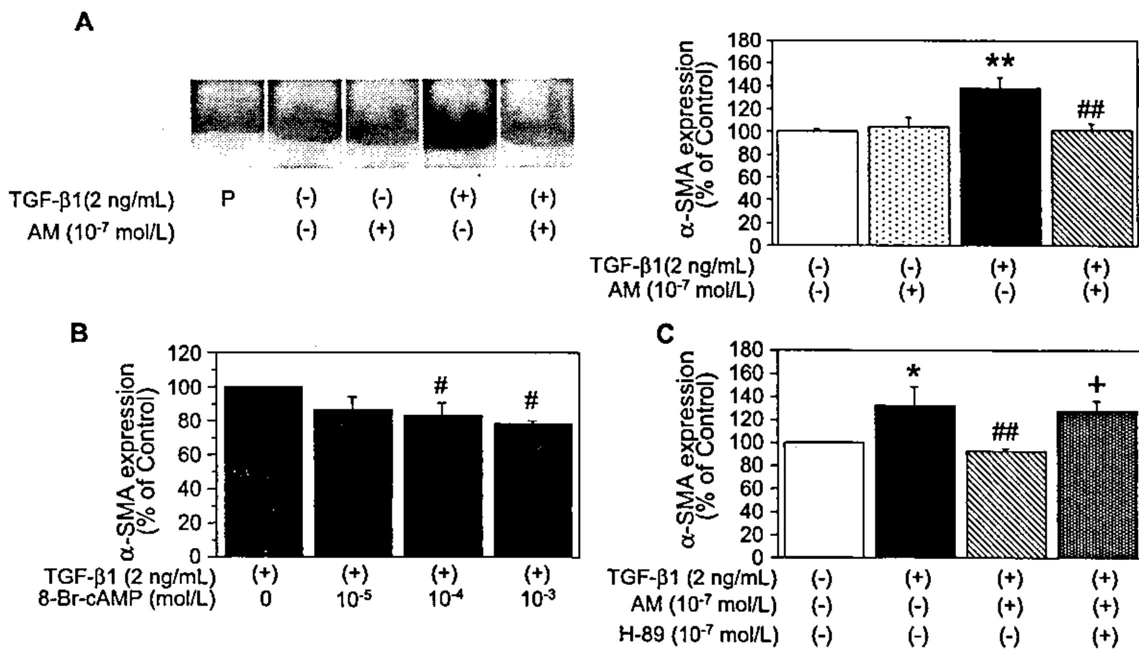

Fig. 5. Effects of AM (A), 8-bromo-cAMP (8-Br-cAMP; B), and H-89, a protein kinase A inhibitor (C) on the α-SMA expression stimulated by TGF-β1 in cultured cardiac fibroblasts. Values are shown as the means±S.E.M. of 5 to 7 (A), 3 (B), and 5 (C) samples examined. $^{*}p<0.05$, $^{**}p<0.01$, compared to controls; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to 2 ng/mL TGF-β1; $^{+}p<0.05$, compared to TGF-β1 plus AM. P: human aorta.

3.6. α-SMA expression in vitro

To further clarify the direct action of AM on myofibroblast phenotypic change, cultured cardiac fibroblasts were treated with TGF-β1 and/or AM to look at the expression level of α-SMA. Fig. 5A illustrates a representative Western blot and the composite data. Two ng/mL TGF-β1 significantly ($p<0.01$) increased the α-SMA expression by 38% in these cells. Treatment with 10^{-7} mol/L AM significantly ($p<0.01$) inhibited the TGF-β1-induced α-SMA expression by 27%. Similarly, 8-bromo-cAMP, an analogue of cyclic AMP (cAMP), inhibited the α-SMA expression induced by TGF-β1 (Fig. 5B); while pretreatment with 10^{-7} mol/L H-89, a specific protein kinase A inhibitor, significantly ($p<0.05$) attenuated the action of AM (Fig. 5C).

4. Discussion

In this study, we report that AM attenuates the Ang II-induced perivascular fibrosis of coronary arteries, suppressing myofibroblast differentiation and expressions of TGF-β1 and type 1 collagen, without affecting blood pressure, left ventricular weight, and cross-sectional area of myocardial fiber. Ventricular remodeling characterized by *myocardial* hypertrophy and fibrosis results in serious consequences for cardiac function. Remodeling of the myocardium involves alteration of the function of fibroblasts, the major cells making up two-thirds of the total cell number in the heart [22]. Fibroblasts change their phenotype to myofibroblasts capable of producing ECM proteins, and this was reported to be a critical step for progression of the fibrosis [3]. The ECM initially accumulates around coronary arteries in response to systemic hypertension and then expands into the interstitial space between *myocardial fibers* [2], therefore suppressing the activation of perivascular fibroblasts might be important to attenuate the adverse remodeling. Using an Ang II-induced hypertensive model, our study supports the previous report by Campbell et al. [7] that Ang II temporally induces the phenotypic change of fibroblasts in the rat heart.

We previously showed that synthetic AM inhibited the Ang II-induced cellular proliferation and growth of cultured cardiac fibroblasts [13]. Consistent with our previous in vitro study [13], we observed in the present study that AM exerted an antiproliferative effect on fibroblasts as determined by the number of Ki-67-positive cells, counteracting the effect of Ang II. In addition, we demonstrated for the first time that the number of adventitial fibroblasts expressing α-SMA, a marker for fibroblast activation, significantly decreased following the AM administration. It should be noted that these AM effects were observed with little change in blood pressure and in left ventricle/body weight and *size of myocardial fiber*. Accordant with the in vitro study by Tomoda et al. [23], cardiac fibroblasts may be more sensitive to AM than *cardiocytes*. Meanwhile, we recently reported using a rat model of myocardial infarction, that AM infusion in an acute phase of the infarction inhibited not only chronic progression of interstitial fibrosis but also of *myocardial* hypertrophy [15]. This seems inconsistent with the present study in terms of alleviation of cardiac hypertrophy; however, the model differs from each other and left ventricular end-diastolic pressure was lowered by the AM infusion in our myocardial infarction experiment. This difference may support the hypothesis for differential regulation of *myocardial* hypertrophy and fibrosis; inappropriate humoral activations stimulate myocardial fibrosis, while hemodynamic factors regulate *growth of myocardial fibers* [2,10,24]. Another possible explanation for the inconsistency may be a difference in the experimental periods of 2 vs. 9 weeks. Because humoral factors including endothelin-1 and TGF-β1 produced by cardiac fibroblasts have been reported to be involved in the *cardiocyte* growth in vitro [25,26], AM treatment for longer periods of time would reduce *growth of myocardial fibers* by modulating fibroblast function.

TGF-β1 plays an important role in myocardial and vascular fibrosis by stimulating the phenotypic change of fibroblasts to myofibroblasts [3] capable of producing matrix proteins. Indeed, blockage of the TGF-β1 action produced the beneficial effect on fibrosis in pressure-overloaded heart [10]. This is comparable with the report by Jesmin et al. [27] showing that the TGF-β1 immunoreactivity is intensely stained in the perivascular area as well as in the vascular wall, concomitantly with TGF-β1 gene up-regulation, in the process of vascular remodeling. In the present study, the reductions of TGF-β1 and type 1 collagen expression with reduced collagen deposition were observed in the AM-treated rats. Both TGF-β1 and AM have been reported to be expressed in a similar pattern during the development of embryonic mouse heart [28] and, in addition, von der Hardt et al. [29] reported that aerosolized AM inhibited TGF-β1 gene expression in the porcine lung. Thus, there seems to be interaction between these two growth-regulatory factors in the process of vascular remodeling.

Many of the AM actions have been shown to be mediated by accumulation of intracellular cyclic AMP (cAMP) [12] and consistent with this, significance of cAMP signaling in attenuating the myofibroblastic change was reported in lung fibroblasts [30] and in hepatic stellate cells [31]. Our in vitro experiments of this study showed that both AM and the cAMP analogue inhibited protein expression of α-SMA induced by TGF-β1 in cultured cardiac fibroblasts; while the protein kinase A inhibition reversed the action of AM. In comparison with the in vivo experiments, the much higher concentration of AM was required to see the clear suppression of α-SMA levels in cultured cardiac fibroblasts; although the present findings suggest possible involvement of the cAMP-protein kinase A pathway in attenuation of the myofibroblast differentiation by AM.

According to the recent reports, heterozygotes of AM knockout mice have shown augmented responses of interstitial or perivascular fibrosis in the myocardium of pressure overload [32] and Ang II/salt-loading hypertension [33] and of intimal hyperplasia in cuff-induced vascular injury [34], compared to their littermates, suggesting cardiovascular protective effects of AM. The proposed mechanisms for such AM effects protective against cardiovascular remodeling are suppression of the renin–angiotensin–aldosterone system and reductions of oxidative stress and protein kinase C activity [15,32–34]. Our present study suggests the profile of AM as an antifibrotic factor counteracting TGF-β1 action by modulating myofibroblast differentiation in the process of vascular remodeling. Meanwhile, Ang II was used to induce hypertension and coronary perivascular fibrosis in the present study, but we are unable to attribute the beneficial effects of AM to specific inhibition of the action of Ang II. These effects may be expected in other forms of hypertension; although further studies are necessary to clarify this point.

In summary, AM infusion for 2 weeks attenuated the Ang II-induced coronary matrix remodeling, suppressing fibroblast activation and expression of TGF-β1 in rats. Because AM is produced in the myocardium and vascular wall, these findings further support the notion that AM is a modulator of cardiovascular remodeling via modulation of fibroblast function.

Acknowledgements

This study was supported by the grants-in-aid for Scientific Research on Priority Areas and for the 21st Century Centers of Excellence Program (Life Science) from the Ministry of Education, Culture, Sport, Science and Technology, Japan, and by a grant-in-aid from AstraZeneca Research 2003. We gratefully thank Ms. Ritsuko Sotomura and Mariko Tokashiki for their technical assistance.

References

[1] Weber KT. Targeting pathological remodeling: concepts of cardioprotection and reparation. Circulation 2000;102:1342–5.
[2] Nicoletti A, Michel JB. Cardiac fibrosis and inflammation: interaction with hemodynamic and hormonal factors. Cardiovasc Res 1999; 41:532–43.
[3] Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts: I. Paracrine cells important in health and disease. Am J Physiol 1999;277(Cell Physiol 46):C1–9.
[4] Shi Y, O'Brien JE Jr, Fard A, Zalewski A. Transforming growth factor-β1 expression and myofibroblast formation during arterial repair. Arterioscler Thromb Vasc Biol 1996;16:1298–305.
[5] Campbell SE, Katwa LC. Angiotensin II stimulated expression of transforming growth factor-β1 in cardiac fibroblasts and myofibroblasts. J Mol Cell Cardiol 1997;29:1947–58.
[6] McEwan PE, Gray GA, Sherry L, Webb DJ, Kenyon CJ. Differential effects of angiotensin II on cardiac cell proliferation and intramyocardial perivascular fibrosis in vivo. Circulation 1998; 98:2765–73.
[7] Campbell SE, Janicki JS, Weber KT. Temporal differences in fibroblast proliferation and phenotype expression in response to chronic administration of angiotensin II or aldosterone. J Mol Cell Cardiol 1995;27:1545–60.
[8] Lijnen PJ, Petrov VV, Fagard RH. Association between transforming growth factor-β and hypertension. Am J Hypertens 2003;16:604–11.
[9] Wilcox JN, Okamoto EI, Nakahara KI, Vinten-Johansen J. Perivascular responses after angioplasty which may contribute to postangioplasty restenosis: a role for circulating myofibroblast precursors? Ann N Y Acad Sci 2001;947:68–90.
[10] Kuwahara F, Kai H, Tokuda K, Kai M, Takeshita A, Egashira K, et al. Transforming growth factor-β function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. Circulation 2002;106:130–5.
[11] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.
[12] Kitamura K, Kangawa K, Eto T. Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions. Microsc Res Tech 2002;57:3–13.
[13] Tsuruda T, Kato J, Kitamura K, Kawamoto M, Kuwasako K, Imamura T, et al. An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth. Cardiovasc Res 1999;43:958–67.
[14] Horio T, Nishikimi T, Yoshihara F, Matsuo H, Takishita S, Kangawa K. Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts. Eur J Pharmacol 1999;382:1–9.
[15] Nakamura R, Kato J, Kitamura K, Onitsuka H, Imamura T, Cao Y, et al. Adrenomedullin administration immediately after myocardial infarction ameliorates progression of heart failure in rats. Circulation 2004;110:426–31.
[16] Tsuruda T, Jougasaki M, Boerrigter G, Costello-Boerrigter LC, Cataliotti A, Lee SC, et al. Ventricular adrenomedullin is associated with myocyte hypertrophy in human transplanted heart. Regul Pept 2003;112:161–6.
[17] Tsuruda T, Kato J, Kitamura K, Imamura T, Koiwaya Y, Kangawa K, et al. Enhanced adrenomedullin production by mechanical stretching in cultured rat cardiomyocytes. Hypertension 2000;35:1210–4.
[18] Ota T, Takamura T, Ando H, Nohara E, Yamashita H, Kobayashi K. Preventive effect of cerivastatin on diabetic nephropathy through suppression of glomerular macrophage recruitment in a rat model. Diabetologia 2003;46:843–51.
[19] Nishikawa N, Yamamoto K, Sakata Y, Mano T, Yoshida J, Miwa T, et al. Differential activation of matrix metalloproteinases in heart failure with and without ventricular dilatation. Cardiovasc Res 2003;57:766–74.
[20] Proudnikov D, Yuferov V, Zhou Y, LaForge KS, Ho A, Kreek MJ. Optimizing primer-probe design for fluorescent PCR. J Neurosci Methods 2003;123:31–45.
[21] Tsuruda T, Boerrigter G, Huntley BK, Noser JA, Cataliotti A, Costello-Boerrigter LC, et al. Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases. Circ Res 2002;91:1127–34.
[22] Zak R. Cell proliferation during cardiac growth. Am J Cardiol 1973;31:211–9.
[23] Tomoda Y, Kikumoto K, Isumi Y, Katafuchi T, Tanaka A, Kangawa K, et al. Cardiac fibroblasts are major production and target cells of adrenomedullin in the heart in vitro. Cardiovasc Res 2001;49:721–30.
[24] Burlew BS, Weber KT. Connective tissue and the heart. Functional significance and regulatory mechanisms. Cardiol Clin 2000; 18:435–42.
[25] Gray MO, Long CS, Kalinyak JE, Li HT, Karliner JS. Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of

TGF-β1 and endothelin-1 from fibroblasts. Cardiovasc Res 1998;40: 352–63.
[26] Harada M, Saito Y, Nakagawa O, Miyamoto Y, Ishikawa M, Kuwahara K, et al. Role of cardiac nonmyocytes in cyclic mechanical stretch-induced myocyte hypertrophy. Heart Vessels 1997;12:198–200.
[27] Jesmin S, Sakuma I, Hattori Y, Kitabatake A. Role of angiotensin II in altered expression of molecules responsible for coronary matrix remodeling in insulin-resistant diabetic rats. Arterioscler Thromb Vasc Biol 2003;23:2021–6.
[28] Montuenga LM, Mariano JM, Prentice MA, Cuttitta F, Jakowlew SB. Coordinate expression of transforming growth factor-β1 and adrenomedullin in rodent embryogenesis. Endocrinology 1998;139:3946–57.
[29] von der Hardt K, Kandler MA, Popp K, Schoof E, Chada M, Rascher W, et al. Aerosolized adrenomedullin suppresses pulmonary transforming growth factor-β1 and interleukin-1β gene expression in vivo. Eur J Pharmacol 2002;457:71–6.
[30] Kolodsick JE, Peters-Golden M, Larios J, Toews GB, Thannickal VJ, Moore BB. Prostaglandin E_2 inhibits fibroblast to myofibroblast transition via E. prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation. Am J Respir Cell Mol Biol 2003;29:537–44.
[31] Mallat A, Préaux AM, Serradeil-Le Gal C, Raufaste D, Gallois C, Brenner DA, et al. Growth inhibitory properties of endothelin-1 in activated human hepatic stellate cells: a cyclic adenosine monophosphate-mediated pathway. Inhibition of both extracellular signal-regulated kinase and c-Jun kinase and upregulation of endothelin B receptors. J Clin Invest 1996;98:2771–8.
[32] Niu P, Shindo T, Iwata H, Iimuro S, Takeda N, Zhang Y, et al. Protective effects of endogenous adrenomedullin on cardiac hypertrophy, fibrosis, and renal damage. Circulation 2004;109:1789–94.
[33] Shimosawa T, Shibagaki Y, Ishibashi K, Kitamura K, Kangawa K, Kato S, et al. Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. Circulation 2002;105:106–11.
[34] Kawai J, Ando K, Tojo A, Shimosawa T, Takahashi K, Onozato ML, et al. Endogenous adrenomedullin protects against vascular response to injury in mice. Circulation 2004;109:1147–53.

Available online at www.sciencedirect.com

SCIENCE DIRECT

ELSEVIER

European Journal of Pharmacology 508 (2005) 201–204

ejp

www.elsevier.com/locate/ejphar

Adrenomedullin alleviates not only neointimal formation but also perivascular hyperplasia following arterial injury in rats

Toshihiro Tsuruda[a,b,*], Johji Kato[a], Eizaburo Matsui[a], Kinta Hatakeyama[c], Hiroyuki Masuyama[a], Takuroh Imamura[a], Kazuo Kitamura[a], Yujiro Asada[c], Tanenao Eto[a]

[a]*First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki, Japan*
[b]*Department of Nutrition Management, Faculty of Health and Nutrition, Minami-Kyushu University, Japan*
[c]*First Department of Pathology, Miyazaki Medical College, University of Miyazaki, Japan*

Received 19 August 2004; received in revised form 11 November 2004; accepted 10 December 2004
Available online 7 January 2005

Abstract

Producing components of the extracellular matrix, the vascular adventitia has been recognized as an important modulator of the vascular remodeling process, which determines the vessel architecture. In this study, we examined the effect of the vasodilator peptide adrenomedullin on vascular remodeling induced by balloon injury of rat carotid arteries. Endothelial denudation with wall stretch by ballooning not only induced neointimal formation accompanied with a reduced ratio of the lumen to vessel area, but also increased the fibroblast number and collagen deposition in the adventitial layer. When compared with the saline infusion, intravenous adrenomedullin infusion at 200 ng/h for 14 days suppressed the neointimal formation (−33%, P=0.033), reversing the ratio of lumen to vessel ratio (P=0.030), without affecting systolic blood pressure. Moreover, the adrenomedullin infusion decreased the number of adventitial fibroblasts (−41%, P<0.001) and the collagen deposition (−36%, P=0.006) in the adventitial layer of the injured artery. In conclusion, the intravenous adrenomedullin infusion effectively attenuates vascular remodeling following the arterial injury via suppression of hyperplasia in the intima and adventitia, suggesting a potential of adrenomedullin as a therapeutic tool against vascular remodeling.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Remodeling; Extracellular matrix; Adventitia

1. Introduction

Arterial remodeling is a physiological and pathological reaction in response to hemodynamic, immunologic, and biochemical stimuli (Pasterkamp et al., 2004). Medial hypertrophy and neointimal lesion were focused on as important features; however recent studies have concentrated on reorganization of the entire vessel architecture as vascular remodeling (Strauss and Rabinovitch, 2000; Ward et al., 2000). Accumulating evidence suggests an importance for the adventitial layer, which modulate the remodeling process through regulation of the extracellular matrix formation (Sartore et al., 2001; Strauss and Rabinovitch, 2000). A rodent model of arterial balloon injury is widely used to examine the remodeling process due to its similarity to restenotic vascular lesions seen after angioplasty in humans (De Meyer and Bult, 1997). In this model, the vascular injuries cause proliferation and migration of vascular smooth muscle cells (VSMC) into the intima, and fibroblasts increase in cell number, along with an increase in extracellular matrix deposition in the adventitial layer, further aggravating vascular remodeling (Sartore et al., 2001; Ryan et al., 2003). Various humoral interactions between growth factors, inflammatory cytokines or vasoactive peptides have been reported to be involved in the remodeling process (Sartore et al., 2001). Adrenomedullin, initially isolated from human pheochromocytoma (Kitamura et al., 1993), has been shown to have multiple functions in the cardiovascular system (Kitamura

* Corresponding author. First Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki, 5200 Kihara Kiyotake, Miyazaki 889-1692, Japan. Tel.: +81 985 85 0872; fax: +81 985 85 6596.
E-mail address: ttsuruda@med.miyazaki-u.ac.jp (T. Tsuruda).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.12.019

et al., 2002). Adrenomedullin was shown to inhibit the migration and proliferation of VSMC in vitro (Kano et al., 1996; Kohno et al., 1997), and Agata et al. (2003) reported that adrenomedullin gene delivery produced an inhibitory action on neointima formation after balloon injury, suggesting an important role for this bioactive peptide in vascular remodeling. However, it remains unknown whether the adrenomedullin actions are observed only in the vascular intimal layer or in the whole vascular structure in the remodeling process. The aim of the present study was to examine the biological actions of adrenomedullin on vascular remodeling, which includes not only the neointima formation but also the adventitia hyperplasia in balloon-injured carotid arteries of rats.

2. Materials and methods

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2003-023).

2.1. Experimental protocol

Ten- to eleven-week-old male Sprague-Dawley rats (CLEA, Japan, Inc.) weighing 350–400 g were housed in a temperature- and light-controlled room (25±1 °C; 12/12-h light/dark cycle) with normal rat chow and water given ad libitum. After the rats were anesthetized with 40 mg/kg i.p. of pentobarbital sodium, endothelial denudation and wall stretch of the left common carotid artery were carried out by three passages of a Fogarty 2F balloon catheter (Baxter International, Deerfield, IL, USA). Then, the rats were randomly divided into two groups infused with saline (n=9) or with synthetic rat adrenomedullin (Peptide Institute, Osaka, Japan) at 200 ng/h (n=6) over 14 days. Immediately after the balloon injury, miniosmotic pumps (Alzet model 2002) were implanted subcutaneously to release either saline or adrenomedullin into the right external jugular vein. Blood pressure was monitored by tail-cuff plethysmography during the experimental period. At day 14, the rats were anesthetized with 40 mg/kg i.p. of pentobarbital sodium and blood samples were collected from the inferior vena cava. Both the injured left common carotid artery and non-injured contralateral were perfused via the left ventricle with phosphate buffer-saline, followed by perfusion fixation with 4% paraformaldehyde, at the physiological constant pressure of about 100 mm Hg, and were then immediately excised.

2.2. Histology and morphological evaluation

The carotid arteries embedded in paraffin were sectioned at 2 μm thickness. After deparaffinization with xylene and graded alcohol, slides were incubated with 0.1% picrosirius red (Direct Red 80, Sigma) dissolved in saturated picric acid for 10 min. Morphological evaluation of the injured and contralateral uninjured carotid arteries was performed at the middle portion of the artery by a single observer in a blind manner. Two samples were too disfigured to be precisely quantified: one was an injured artery of the control and the other was an intact artery of the adrenomedullin group. Therefore, these two samples were excluded from the analysis. The cross-sectional areas of the lumen and those circumscribed by the internal or external elastic lamina were determined by computerized measurement (Axio Vison 2.05 Carl ZEISS, Munchen, Germany), and the areas of the media and intima were calculated by subtraction. The vessel area was defined as the area surrounded by the external elastic lamina. The number of fibroblasts showing a typical spindle shape in the adventitia was determined at a magnification of ×400. To quantify collagen deposition in the vascular wall, sections stained with picrosirius red were scanned by Mac Scope (v. 2.3.2) software under polarized light. The tightly packed collagen surrounding the carotid artery was defined as the collagen deposition in this study.

2.3. Assay for adrenomedullin

Plasma concentrations of rat adrenomedullin were measured with a specific radioimmunoassay, which detects the C-terminal amide structure of adrenomedullin, an essential portion for the biological activity, as previously described (Tsuruda et al., 1999).

2.4. Statistical analysis

All data are expressed as means±S.E.M. Comparisons between groups were made with one-way analysis of variance followed by the Fisher's test, and statistical significance was accepted at $P<0.05$.

3. Results

3.1. Plasma level of rat adrenomedullin and blood pressure

The adrenomedullin-supplemented rats showed significantly higher rat adrenomedullin levels in the plasma compared with those administered with saline at day 14 (adrenomedullin group, 4.9±0.5; saline group, 3.3±0.2 fmol/ml; P=0.004). Meanwhile, no significant difference in systolic blood pressure was noted before and during the experiment period (data not shown).

3.2. Effects adrenomedullin on neointimal formation and adventitia hyperplasia

Fig. 1 illustrates the hematoxylin-eosin stainings of the intact and balloon-injured carotid arteries at day 14. In the injured artery (B), neointima formation occurred and the

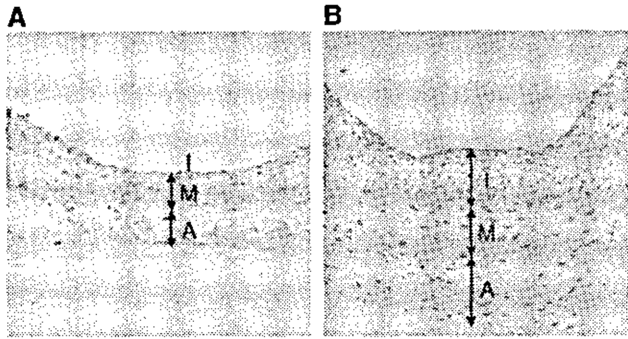

Fig. 1. Histological findings of the intact (A) and injured (B) arteries. I, intima; M, media; A, adventitia. Original magnification, ×200.

adventitial layer thickened with high cellularity, compared with the intact artery (A).

In the quantitative analysis (Fig. 2), the injured arteries showed significant neointimal formation ($P<0.001$) with little influence on the medial area (A) resulting in a significant increase of the intima to media ratio (B). As shown, the adrenomedullin infusion for 14 days significantly attenuated the neointimal formation by 33% ($P=0.033$) and the intima to media ratio by 34% ($P=0.025$), respectively, compared with the saline infusion, while adrenomedullin had no effect on these parameters in the contralateral, intact artery.

Fig. 3A illustrates the effect of adrenomedullin on cell number of fibroblasts in the adventitial layer. The arterial injury increased the number of fibroblasts ($P<0.001$), but this increase was suppressed by the adrenomedullin infusion by 41% ($P<0.001$). Fig. 3B shows the effect of adrenomedullin on the ratio of collagen deposition to the medial areas in the intact and injured arteries. The balloon injury enlarged the collagen deposition area mainly in the adventitia ($P<0.001$); however, the adrenomedullin infusion reduced it by 38% ($P=0.006$).

3.3. *Effect of adrenomedullin on geometrical changes in the carotid arteries*

Fig. 4A and B illustrate the effect of adrenomedullin on the lumen and vessel areas, respectively. The balloon injury slightly reduced the lumen area of rats infused with saline,

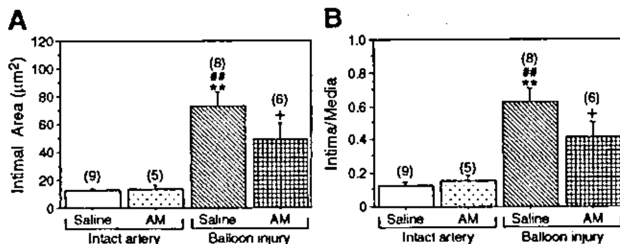

Fig. 2. Effect of adrenomedullin on intimal area (A) and ratio of intima to media (B) in the intact and injured arteries. Values are means±S.E.M., (*n*). **$P<0.01$ vs. intact artery with saline infusion; ##$P<0.01$ vs. intact artery with adrenomedullin infusion; +$P<0.05$ vs. injured artery with saline infusion.

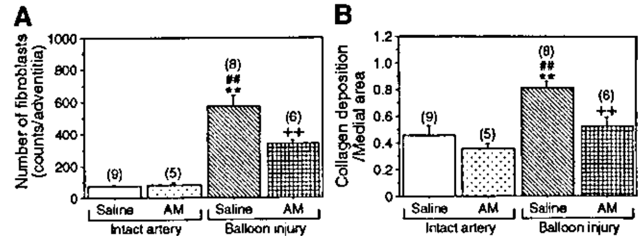

Fig. 3. Effect of adrenomedullin on cell number in the adventitia (A) and collagen deposition/medial area (B). Values are means±S.E.M., (*n*). **$P<0.01$ vs. intact artery with saline infusion; ##$P<0.01$ vs. intact artery with adrenomedullin infusion; ++$P<0.01$ vs. injured artery with saline infusion.

but this reduction was statistically insignificant (Fig. 4A), and no significant differences were noted in the vessel area of four study groups (Fig. 4B). As shown in Fig. 4C, the ratio of the lumen to vessel area was significantly ($P<0.001$) reduced by the balloon injury in the saline group, compared with those of the intact arteries. The adrenomedullin supplement significantly ($P=0.030$) reversed this geometrical change toward those of the intact arteries.

4. Discussion

We report here that intravenous adrenomedullin infusion not only attenuated neointima formation but also inhibited fibroblast proliferation and collagen deposition of the adventitia, reducing the ratio of lumen to vessel area, in the balloon-injured carotid arteries of rats. The three layers of the vascular wall, intima, media and adventitia, contribute to inward or outward remodeling which occurs following arterial injury (Ward et al., 2000). Although neointimal formation and medial hypertrophy have been focused on as targets in preventing adverse remodeling, recent reports have

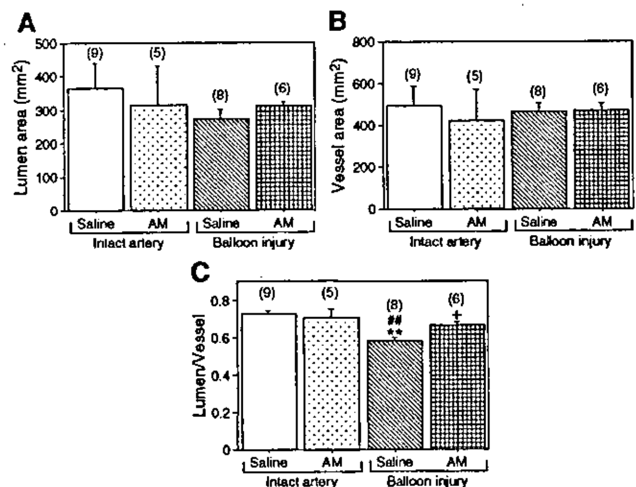

Fig. 4. Effect of adrenomedullin on lumen area (A), vessel area (B) and ratio of lumen to vessel area (C). Values are means±S.E.M., (*n*). **$P<0.01$ vs. intact artery with saline infusion; ##$P<0.01$ vs. intact artery with adrenomedullin infusion; +$P<0.05$ vs. injured artery with saline infusion.

referred more to the role of the adventitial layer (Ryan et al., 2003; Sartore et al., 2001; Strauss and Rabinovitch, 2000).

In a model of arterial injury, endothelial denudation induces VSMC proliferation and migration, making up the neointima formation. Our present data supports previous studies showing that adrenomedullin attenuated the neointima formation induced by arterial injuries in rats (Agata et al., 2003; Yamasaki et al., 2003) and in mice (Imai et al., 2002; Kawai et al., 2004). On the other hand, extracellular matrix deposition in the adventitia with cellular hyperplasia appears to be the major phenomena responsible for adventitial thickening that would subsequently increase stiffness of the vascular walls and peripheral arterial resistance (Intengan and Schiffrin, 2001; Sartore et al., 2001). Importantly, we found that the adrenomedullin administration significantly decreased the number of fibroblasts in the adventitia following the arterial injury in this study. In addition, the adrenomedullin-treated rats showed a significant reduction of collagen deposition in the entire vessel wall, mainly in the adventitia. Considering the importance of extracellular matrix formation in determining stiffness of the vascular wall (Intengan and Schiffrin, 2001), adrenomedullin may exert a beneficial action alleviating vascular stiffness.

In this study, the beneficial effects of adrenomedullin following arterial injury were observed without a significant effect on blood pressure, suggesting a direct action of adrenomedullin on the vascular remodeling. Adrenomedullin has been shown to directly inhibit proliferation and migration of cultured VSMC (Kano et al., 1996; Kohno et al., 1997), and according to our previous report (Tsuruda et al., 1999), adrenomedullin inhibited proliferation of cultured fibroblasts isolated from rat cardiac ventricle. Recently, we reported that adrenomedullin induced matrix metalloproteinase-2 activity in cultured adventitial fibroblasts isolated from rat aorta (Tsuruda et al., 2004). Collagen accumulation is responsible for constrictive remodeling following balloon injury (Ryan et al., 2003). Proteolytic activity induced by adrenomedullin may have contributed to attenuating collagen deposition, however these hypotheses for possible, direct actions of adrenomedullin should be tested in vivo by future experiments.

In summary, the intravenous adrenomedullin infusion effectively improves the vascular geometry of the balloon-injured rat carotid artery, suppressing neointima formation, adventitial fibroblast proliferation and collagen deposition. This study implies a possible utility of adrenomedullin for inhibition of vascular remodeling, where both neointimal formation and adventitial hyperplasia are targeted.

Acknowledgements

This study was supported by the grants-in-aid for Scientific Research and for the 21st Century Centers of Excellence Program (Life Science) from the Ministry of Education, Culture, Sport, Science and Technology, Japan, and by a grant -in-aid from AstraZeneca Research 2003. We gratefully thank Ms. Ritsuko Sotomura for her technical assistance.

References

Agata, J., Zhang, J.J., Chao, J., Chao, L., 2003. Adrenomedullin gene delivery inhibits neointima formation in rat artery after balloon angioplasty. Regul. Pept. 112, 115–120.

De Meyer, G.R., Bult, H., 1997. Mechanisms of neointima formation—lessons from experimental models. Vasc. Med. 2, 179–189.

Imai, Y., Shindo, T., Maemura, K., Sata, M., Saito, Y., Kurihara, Y., Akishita, M., Osuga, J., Ishibashi, S., Tobe, K., Morita, H., Oh-hashi, Y., Suzuki, T., Maekawa, H., Kangawa, K., Minamino, N., Yazaki, Y., Nagai, R., Kurihara, H., 2002. Resistance to neointimal hyperplasia and fatty streak formation in mice with adrenomedullin overexpression. Arterioscler. Thromb. Vasc. Biol. 22, 1310–1315.

Intengan, H.D., Schiffrin, E.L., 2001. Vascular remodeling in hypertension. Roles of apoptosis, inflammation, and fibrosis. Hypertension 38, 581–587.

Kano, H., Kohno, M., Yasunari, K., Yokokawa, K., Horio, T., Ikeda, M., Minami, M., Hanehira, T., Takeda, T., Yoshikawa, J., 1996. Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. J. Hypertens. 14, 209–213.

Kawai, J., Ando, K., Tojo, A., Shimosawa, T., Takahashi, K., Onozato, M.L., Yamasaki, M., Ogita, T., Nakaoka, T., Fujita, T., 2004. Endogenous adrenomedullin protects against vascular response to injury in mice. Circulation 109, 1147–1153.

Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., Eto, T., 1993. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. 192, 553–560.

Kitamura, K., Kangawa, K., Eto, T., 2002. Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions. Microsc. Res. Tech. 57, 3–13.

Kohno, M., Yokokawa, K., Kano, H., Yasunari, K., Minami, M., Hanehira, T., Yoshikawa, J., 1997. Adrenomedullin is a potent inhibitor of angiotensin II-induced migration of human coronary artery smooth muscle cells. Hypertension 29, 1309–1313.

Pasterkamp, G., Galis, Z.S., de Kleijn, D.P., 2004. Expansive arterial remodeling: location, location, location. Arterioscler. Thromb. Vasc. Biol. 24, 650–657.

Ryan, S.T., Koteliansky, V.E., Gotwals, P.J., Lindner, V., 2003. Transforming growth factor-beta-dependent events in vascular remodeling following arterial injury. J. Vasc. Res. 40, 37–46.

Sartore, S., Chiavegato, A., Faggin, E., Franch, R., Puato, M., Ausoni, S., Pauletto, P., 2001. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. Circ. Res. 89, 1111–1121.

Strauss, B.H., Rabinovitch, M., 2000. Adventitial fibroblasts: defining a role in vessel wall remodeling. Am. J. Respir. Cell Mol. Biol. 22, 1–3.

Tsuruda, T., Kato, J., Kitamura, K., Kawamoto, M., Kuwasako, K., Imamura, T., Koiwaya, Y., Tsuji, T., Kangawa, K., Eto, T., 1999. An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth. Cardiovasc. Res. 43, 958–967.

Tsuruda, T., Kato, J., Cao, Y.N., Hatakeyama, K., Masuyama, H., Imamura, T., Kitamura, K., Asada, Y., Eto, T., 2004. Adrenomedullin induces matrix metalloproteinase-2 activity in rat aortic adventitial fibroblasts. Biochem. Biophys. Res. Commun. 325, 80–84.

Yamasaki, M., Kawai, J., Nakaoka, T., Ogita, T., Tojo, A., Fujita, T., 2003. Adrenomedullin overexpression to inhibit cuff-induced arterial intimal formation. Hypertension 41, 302–307.

Ward, M.R., Pasterkamp, G., Yeung, A.C., Borst, C., 2000. Arterial remodeling. Mechanisms and clinical implications. Circulation 102, 1186–1191.

nature medicine

Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway

© 2004 Nature Publishing Group http://www.nature.com/naturemedicine

Akihiro Yasoda[1], Yasato Komatsu[1], Hideki Chusho[1], Takashi Miyazawa[1], Ami Ozasa[1], Masako Miura[1], Tatsuya Kurihara[2], Tomohiro Rogi[2], Shoji Tanaka[2], Michio Suda[1], Naohisa Tamura[1], Yoshihiro Ogawa[1] & Kazuwa Nakao[1]

Achondroplasia is the most common genetic form of human dwarfism, for which there is presently no effective therapy. C-type natriuretic peptide (CNP) is a newly identified molecule that regulates endochondral bone growth through GC-B, a subtype of particulate guanylyl cyclase. Here we show that targeted overexpression of CNP in chondrocytes counteracts dwarfism in a mouse model of achondroplasia with activated fibroblast growth factor receptor 3 (FGFR-3) in the cartilage. CNP prevented the shortening of achondroplastic bones by correcting the decreased extracellular matrix synthesis in the growth plate through inhibition of the MAPK pathway of FGF signaling. CNP had no effect on the STAT-1 pathway of FGF signaling that mediates the decreased proliferation and the delayed differentiation of achondroplastic chondrocytes. These results demonstrate that activation of the CNP–GC-B system in endochondral bone formation constitutes a new therapeutic strategy for human achondroplasia.

Achondroplasia is the most common genetic form of human dwarfism, with a prevalence at birth of about 1/26,000 (ref. 1). Effective therapy for this condition has not as yet been established. Recent advances in molecular genetics have shown that constitutively active mutations in the gene encoding FGFR-3 are responsible for human achondroplasia[2–4]. Constitutive activation of FGFR-3 stimulates various intracellular signaling pathways, including the MAPK and STAT-1 pathways[5]. It has been reported that the STAT-1 pathway of FGFR-3 signaling inhibits endochondral bone growth[6,7]; the contribution of the MAPK pathway, another major component of FGFR-3 signaling, remains unclear.

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and CNP[8]. They exert biological actions by accumulation of intracellular cGMP mediated by two subtypes of particulate guanylyl cyclase: guanylyl cyclase-A (GC-A) for ANP and BNP and guanylyl cyclase-B (GC-B) for CNP[9,10]. Although the natriuretic peptide system has mainly been implicated in regulating the cardiovascular system, we have shown that the CNP–GC-B system is an important regulator of endochondral bone growth[11–14]. Activation of the CNP–GC-B system in transgenic mice with elevated plasma concentrations of BNP[11] or in mice depleted of the clearance receptor for natriuretic peptides[15,16] results in very similar skeletal overgrowth. By contrast, inactivation of the CNP–GC-B pathway in mice depleted of CNP[13] or cGMP-dependent protein kinase II[14], a downstream mediator of the CNP–GC-B system, results in dwarfism caused by defects in endochondral ossification.

Growth plates stimulated by the CNP–GC-B system have widened proliferative and hypertrophic chondrocyte layers, much like those in FGFR-3-depleted bones[12,17,18]. CNP depletion, by contrast, leads to impaired bone growth resembling that of achondroplastic bones, with a similar histological picture of decreased width in both the proliferative and hypertrophic chondrocyte layers of the growth plate[13,19]. These observations raise the possibility that the CNP–GC-B system in endochondral ossification reverses the inhibitory effect of the FGFs–FGFR-3 system in skeletogenesis. As for the interaction of these systems, marked elevation of cGMP induced by CNP has been reported to block activation of the MAPK cascade induced by FGFs in fibroblasts[20] and mesangeal cells[21].

Here, we developed transgenic mice (*Nppc* mice) with targeted overexpression of CNP in growth-plate cartilage using a transgene containing *Col2a1*, a cartilage-specific promoter[22], and *Nppc*, the gene encoding CNP. Using a mouse model of achondroplasia with activated FGFR-3 in cartilage (*Fgfr3*^{ach} mice)[19], we generated and analyzed doubly transgenic *Nppc Fgfr3*^{ach} mice that overexpress CNP in achondroplastic growth-plate chondrocytes to assess the growth-promoting effect of the CNP–GC-B system on achondroplastic bones and the molecular mechanisms by which this occurs.

RESULTS

Generation of *Nppc* mice

We obtained two strains of *Nppc*-transgenic founders of similar phenotype, and one of them, carrying ten copies of the transgene (*Col2a1*-

[1]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho Sakyo-ku, Kyoto 606-8507, Japan. [2]Daiichi Suntory Institute for Biomedical Research, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan. Correspondence should be addressed to K.N. (nakao@kuhp.kyoto-u.ac.jp).

Published online 14 December 2003; doi:10.1038/nm971

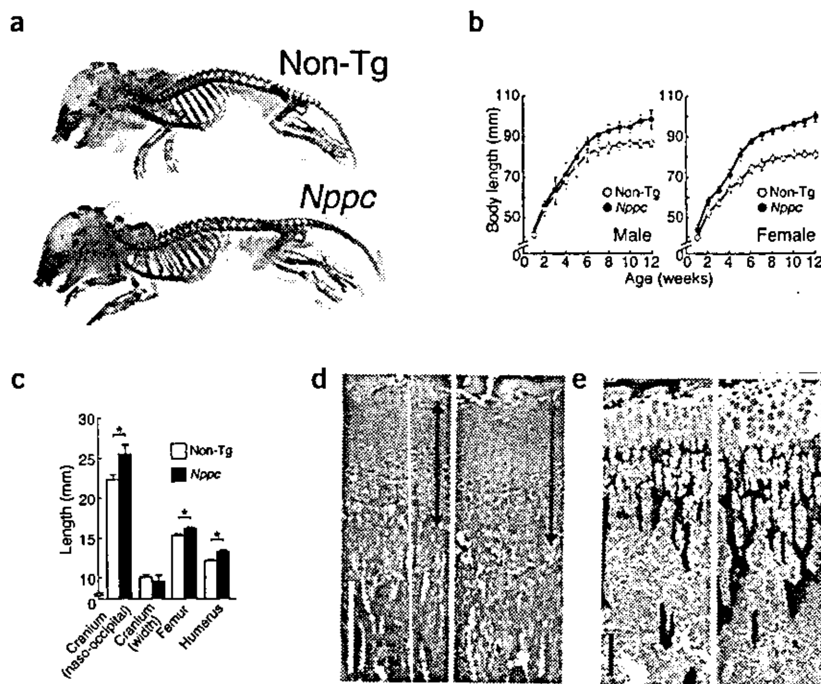

Figure 1 Generation of *Nppc* mice. (**a**) Whole skeletons of nontransgenic (Non-Tg) and *Nppc* mice at birth. (**b**) Growth curves of male (left) and female (right) nontransgenic and *Nppc* mice. (**c**) Bone lengths of 3-month-old female mice. *, $P < 0.05$, $n = 4$–5. (**d**,**e**) Histological analysis of the tibial growth plate of 2-week-old mice. Nontransgenic (left) and *Nppc* (right) in each panel. Alcian blue and H&E staining (**d**) and von Kossa staining (**e**). Arrows indicate growth-plate cartilage in **d**. Scale bar, 200 μm (**d**) or 100 μm (**e**).

Nppc), was used for further analysis. Targeted overexpression of CNP in the cartilage of these *Nppc* mice was confirmed by RT-PCR (see Supplementary Fig. 1 online). The production of cGMP, the second messenger of CNP, was about nine times higher in the cartilage of *Nppc* mice than in that of their nontransgenic littermates (18.5 ± 1.5 versus 2.1 ± 0.7 fmol/mg protein, $P < 0.01$).

At birth, the whole skeleton of *Nppc* mice showed a substantial longitudinal overgrowth of bones, including long bones of limbs, vertebrae and skull (Fig. 1a). No delay in ossification was observed in the periphery of limbs at this stage. Longitudinal overgrowth of *Nppc* mice became prominent in adulthood. The growth curve shows that the naso-anal lengths of 10-week-old male and female *Nppc* mice were 10% and 19% larger, respectively, than those of their nontransgenic littermates (Fig. 1b). The growth-promoting effect in *Nppc* mice was more prominant in females than in males, so we used female mice in later experiments. The length of bones formed through endochondral ossification (cranium, femur and humerus) were significantly larger in *Nppc* mice than in nontransgenic mice, whereas the width of the cranium, which is determined by membranous ossification, did not differ between the two genotypes (Fig. 1c).

© 2004 Nature Publishing Group http://www.nature.com/naturemedicine

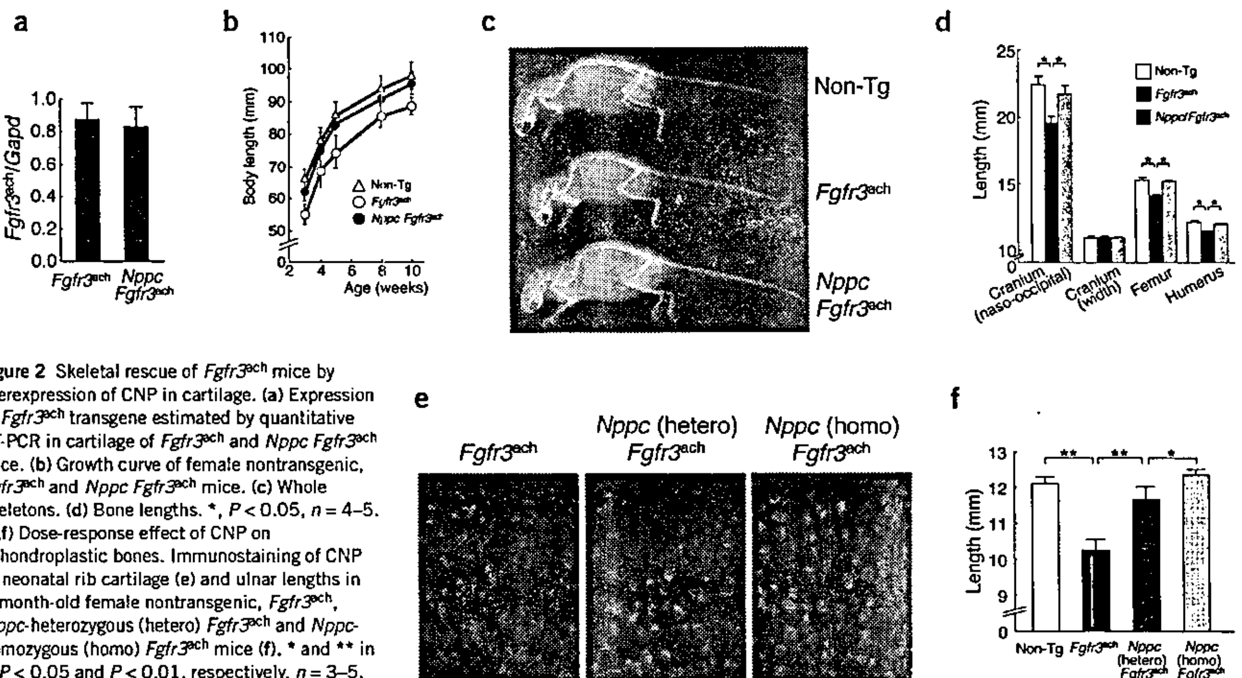

Figure 2 Skeletal rescue of *Fgfr3*^{ach} mice by overexpression of CNP in cartilage. (**a**) Expression of *Fgfr3*^{ach} transgene estimated by quantitative RT-PCR in cartilage of *Fgfr3*^{ach} and *Nppc Fgfr3*^{ach} mice. (**b**) Growth curve of female nontransgenic, *Fgfr3*^{ach} and *Nppc Fgfr3*^{ach} mice. (**c**) Whole skeletons. (**d**) Bone lengths. *, $P < 0.05$, $n = 4$–5. (**e**,**f**) Dose-response effect of CNP on achondroplastic bones. Immunostaining of CNP in neonatal rib cartilage (**e**) and ulnar lengths in 2-month-old female nontransgenic, *Fgfr3*^{ach}, *Nppc*-heterozygous (hetero) *Fgfr3*^{ach} and *Nppc*-homozygous (homo) *Fgfr3*^{ach} mice (**f**). * and ** in **f**, $P < 0.05$ and $P < 0.01$, respectively, $n = 3$–5.