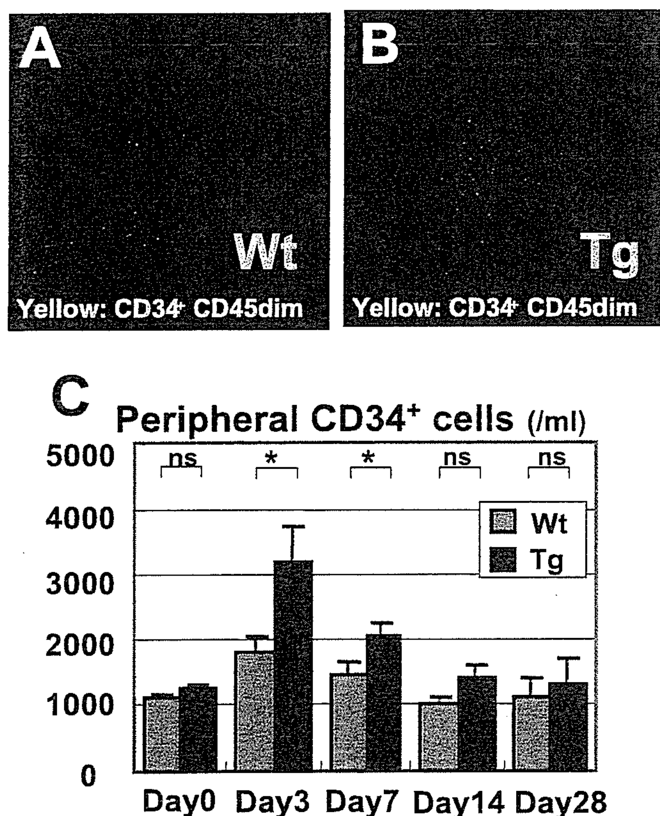

FIG. 5. Effects of AM on mobilization of CD34$^+$ mononuclear cells into peripheral blood after 20m-MCAO. A–C, Quantification of CD34$^+$ mononuclear cells after 20m-MCAO. Scatter plots for fluorescence-activated cell sorting analysis of the CD34$^+$ cells in peripheral blood of Wt (A) and AM-Tg (B) on postoperative d 3. *Yellow*, CD34$^+$-CD45dim mononuclear cells. Comparison of the time course for mobilization of CD34$^+$ cells into peripheral blood between Wt and AM-Tg (C). *, $P < 0.05$; ns, not significant; n = 6.

treatment. The result showed comparable levels of neuroprotection and vascular regeneration between the low-concentration line and the high-concentration line (Table 3). We further analyzed BP-matched mice by administration of low-dose hydralazine (0.1 mM in drinking water) to exclude the possibility that lower BP observed in AM-Tg mice caused beneficial effects after 20m-MCAO. As shown in Table 3, lower BP alone did not reduce the infarct area nor promote vascular regeneration, although hydralazine administration caused BP reduction comparable to that in AM-Tg mice.

Brain edema was reduced in AM-Tg mice at 24 h after 2 h MCAO

The survival rate of mice after the fatal stroke, 2 h-MCAO, was 0% on d 7. We observed no significant difference in the rate between Wt and AM-Tg mice. The edema volume was reduced in AM-Tg mice 24 h after 2 h-MCAO; although the infarct volume showed no significant difference between them. Edema volume (% volume of contralateral hemisphere) was 13.5 ± 1.2 in Wt *vs.* 9.7 ± 0.9 in AM-Tg ($P < 0.05$; n = 9, Fig. 7C), whereas infarct volume (% volume of contralateral hemisphere) was 39.0 ± 4.9 in Wt *vs.* 44.5 ± 7.3 in AM-Tg (not significant; n = 9; Fig. 7, A and B). As shown in Fig. 7D, we found that Evans Blue leakage into the ischemic

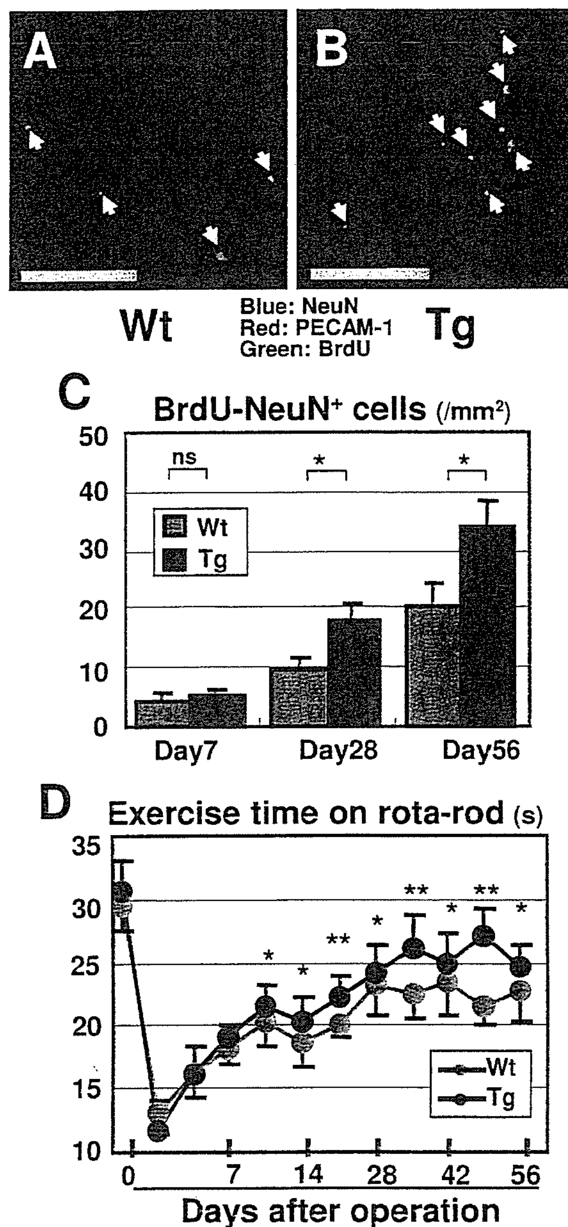

FIG. 6. Effects of AM on neurogenesis and recovery of impaired motor function after 20m-MCAO. A and B, Detection of regenerated neurons on postoperative d 56 by immunostaining for BrdU and NeuN. *Arrows*, BrdU-NeuN double-positive cells in the ischemic core of Wt (A) and AM-Tg (B). *Scale bar*, 100 μm. C, Quantitative analysis of regenerated neurons. *, $P < 0.05$; ns, not significant; n = 12. D, Recovery of impaired motor function after 20m-MCAO, quantified as the exercise time on an accelerating rota-rod from the start to collapse down. *, $P < 0.05$; **, $P < 0.01$ for Wt *vs.* AM-Tg by ANCOVA; n = 14.

core was significantly reduced in AM-Tg mice. The content of Evans Blue (ng/g tissue) in the ischemic brain at 24 h after 2 h-MCAO was 239.4 ± 37.3 in Wt *vs.* 133.9 ± 9.4 in AM-Tg ($P < 0.01$; n = 4; Fig. 7E).

AM exerted direct antiapoptotic and neurodifferentiating effects on neuronal cells in vitro

After 48 h incubation of NHNP under serum-free apoptotic conditions, in which the number of the cells had decreased

TABLE 2. Significant correlation between the regenerative elements and apoptosis, neurogenesis, and functional recovery after 20m-MCAO

X	Y	Regression line	*P*
Capillary density (% field)	Apoptotic cells (/mm^2)	Y = −2.3X+37	0.01
Capillary density (% field)	Regenerated neuron (/mm^2)	Y = 3.2X-21	0.003
Capillary density (% field)	Rota-rod result (sec)	Y = 1.3X+9	0.005
Regenerated neuron (/mm^2)	Rota-rod result (sec)	Y = 0.3X+19	0.08

n = 12–24.

to half, the viable cell number was increased in the AM 10^{-8} mol/liter-treated group to 38.8 ± 7.1% over the control ($P < 0.01$; n = 4; Fig. 8C). The ratio of ssDNA$^+$ cells to total cells (%) was 9.8 ± 1.9 in Wt *vs.* 4.0 ± 0.6 in the AM 10^{-8} mol/liter-treated group ($P < 0.05$; n = 4; Fig. 8, A, B, and D).

After 7-d incubation of PC12 cells under differentiation condition, both the cell number and the length of neuronal process increased dose dependently as a result of AM treatment ($P < 0.01$; n = 6; Fig. 8, E and I). Coculture with endothelial cells also increased the cell number and the length of neuronal process. The effect of AM was canceled by AM blockers, PKA inhibitors, and PI3K inhibitors (Table 4).

Exogenous administration of AM reduced infarct area, promoted vascular regeneration, and improved neurological function after 20m-MCAO

We further examined the effects of exogenous infusion of mature AM by means of an osmotic pump in the amount reported to achieve a plasma concentration of 2 fmol/ml. Implantation of the pump just after the operation resulted in increase in the blood flow and reduction of the infarct area on postoperative d 7 to a comparable level to those in AM-Tg mice. Moreover, the treatment started at 24 h after the operation (d 1) showed almost the same therapeutic effect. However, the implantation at 72 h after the operation (d 3) failed to reveal any significant effect (Fig. 9, A and B). The rota-rod exercise time was significantly improved in the AM-treated group. The exercise time (second) on d 7 was 17.0 ± 1.5 in vehicle group *vs.* 18.1 ± 2.0 in AM-treated group (n = 6 for vehicle group and 12 for AM-treated group; $P < 0.05$ by ANCOVA).

Discussion

In the present study, we generated novel transgenic mice that overproduce AM in their liver without overproduction of mature PAMP and investigated the roles of AM in degeneration or regeneration processes after brain ischemia, which can be defined as brain remodeling, as summarized in Fig. 10. Brain edema in acute phase, neuronal loss and gliosis in subacute to chronic phase after 20m-MCAO were reduced in AM-Tg mice. Furthermore, vascular regeneration, mobilization of CD34$^+$ mononuclear cells and subsequent neurogenesis were enhanced in them. These effects resulted in improved recovery of motor function after the nonfatal stroke. AM was also found to exert direct antiapoptotic and neuro-differentiating effects on neuronal cells *in vitro*. Exogenous administration of AM in mice after 20m-MCAO also

TABLE 3. Comparison of the effects on neuroprotection and vascular regeneration after 20m-MCAO between Wt control mice, hydralazine-administrated mice, and the low and high concentration lines of AM-Tg

Mice	Infarct area (mm^2/field)	Brain blood flow (% Contralateral)	Systolic BP (mm Hg)
Control	0.90 ± 0.09	80.8 ± 2.3	120.1 ± 2.2
Hydralazine	0.94 ± 0.17^{ns}	79.6 ± 2.6^{ns}	101.0 ± 3.9^{a}
Low-conc. AM-Tg	0.58 ± 0.12^{b}	88.4 ± 2.9^{b}	105.1 ± 1.8^{a}
High-conc. AM-Tg	0.67 ± 0.09^{b}	86.3 ± 2.0^{b}	106.4 ± 3.5^{a}

conc., Concentration.
[a] $P < 0.01$; [b] $P < 0.05$; ns, not significant *vs.* control; n = 6.

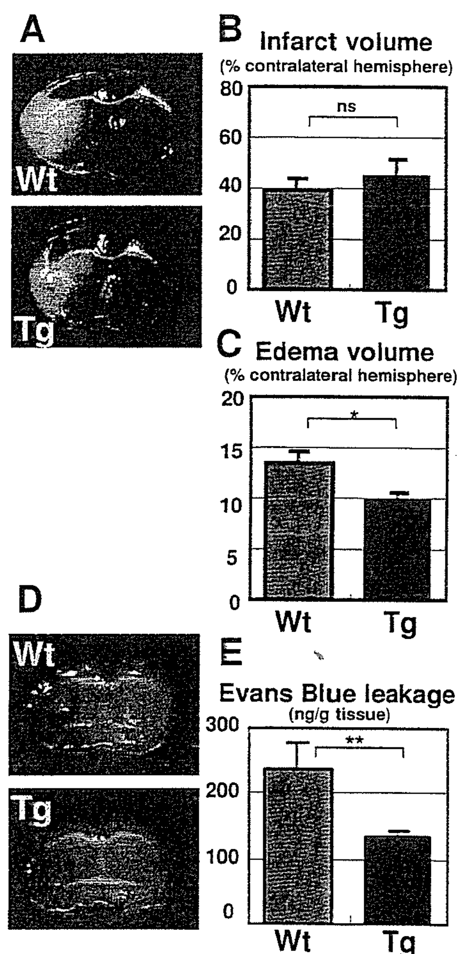

FIG. 7. Effects of AM on infarct size and brain edema in the fatal stroke, 2 h-MCAO. A, Comparison of infarct size between Wt and AM-Tg with 2,3,5-triphenyltetrazolium chloride staining at 4.0 mm from the frontal pole. *White area* represents infarction. B and C, Infarct (B) and edema (C) volumes quantified 24 h after the operation of 2 h-MCAO. *, $P < 0.05$; ns, not significant for Wt and AM-Tg; n = 9. D: Representative image of *in situ* Evans Blue leakage into the ischemic core at 24 h after 2 h-MCAO. E, Quantification of Evans Blue in the ischemic brain. **, $P < 0.01$; n = 4.

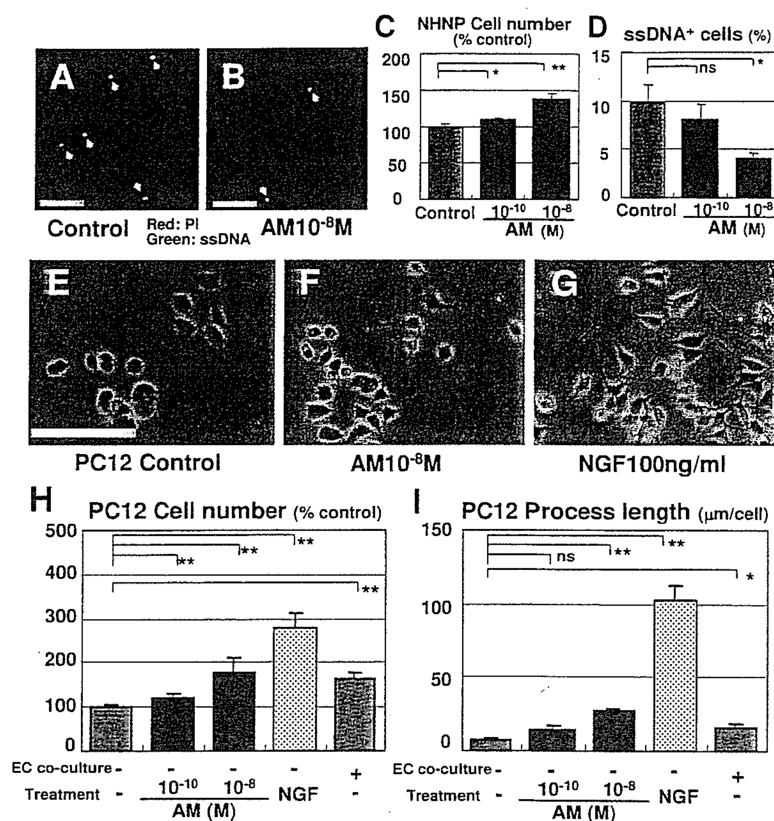

FIG. 8. Effects of AM *in vitro* on apoptosis of NHNP neuronal progenitor cells and neuronal differentiation of PC12 cells. A–D, *In vitro* analysis of apoptotic NHNP after incubation with (B) or without (A) AM. NHNP cell number (C) and the ratio of ssDNA$^+$ cells to total cells (D) after 48 h incubation. *, $P < 0.05$; **, $P < 0.01$; ns, not significant *vs.* control; n = 4; *scale bar*, 100 µm. E–G, Effects of AM on neuronal differentiation of PC12 cells evaluated by the length of neuronal process. Microscopic examination of PC12 cells after incubation for 7 d (E). AM (F) or nerve growth factor (G) was added to the culture medium. Quantification of cell number (H) and the length of neuronal process (I). *, $P < 0.05$; **, $P < 0.01$; ns, not significant; n = 6; *scale bar*, 100 µm.

reduced the infarct area, and promoted vascular regeneration and functional recovery.

Stroke causes two different types of neuronal death: necrosis and apoptosis. Acute neuronal loss, which is completed within a few days after ischemic damage, is necrotic, whereas delayed neuronal loss, which may start several days after transient ischemia, is considered to be apoptotic (27, 28). Many studies have found that treatments that reduce inflammation or oxidative stress are beneficial for the prevention of apoptotic neuronal loss (29, 30).

In this study, we demonstrated that AM exerts neuroprotective actions in the ischemic brain. A significant reduction in neuronal loss in AM-Tg mice after 20m-MCAO became obvious after postoperative d 7, but was not obvious before d 3. A significant decrease in ssDNA-positive cells inside and on the border of the ischemic area was observed in AM-Tg mice in association with a reduction in CD45$^+$ cells and *in situ* ROS production in the subacute phase. AM is therefore assumed to reduce delayed neuronal loss through suppression of the apoptotic process. Furthermore, we confirmed that AM directly suppresses apoptosis of neuronal progenitor cells *in vitro*. These findings suggest that AM exerts neuroprotective effects on the ischemic brain by reducing apoptotic neuronal loss through both its direct antiapoptotic action on neurons and indirect effect via antiinflammation and anti-ROS production. Consistent with the findings in this study, several recent reports have provided evidences for the organ-protective effects of AM against inflammation and oxidative stress (31–33). In addition, we found significant negative correlation between capillary density and apoptotic cells in the same section on postoperative d 7 after 20m-MCAO. Moreover, the infarct area kept expanding between d 7–28 in Wt mice, whereas AM-Tg mice did not show the increase in size in this period. These findings suggest that the increased blood flow in AM-Tg mice was one of the causes of neuroprotection after 20m-MCAO, although we suppose that multiple actions of AM, as described above, could also contribute for neuroprotection.

Increased vascularity is reported to be associated with improved neurological recovery in human patients with stroke (34). This implies that physiological vascular regeneration in the ischemic brain constitutes a beneficial response for the recovery of impaired neurological function. Moreover, neurogenesis after stroke even in adulthood has been demonstrated to occur in a place surrounded by the vascu-

TABLE 4. Effects of AM-antagonists, PKA inhibitors, and PI3K inhibitors on AM-induced neural differentiation of PC12 cells

	Process length (µm/cell)
PC12	6.8 ± 1.7
+AM (10^{-8} mol/liter)	23.6 ± 4.0[a]
+AM+AM(22–52) (10^{-5} mol/liter)	11.8 ± 3.4[b]
+AM+CGRP(8–37) (10^{-5} mol/liter)	14.8 ± 1.9[c]
+AM+Rp-cAMP (10^{-5} mol/liter)	10.2 ± 2.7[b]
+AM+PKA Inh (10^{-6} mol/liter)	7.2 ± 2.3[b]
+AM+LY294002 (10^{-5} mol/liter)	4.6 ± 1.6[b]
+AM+wortmannin (10^{-7} mol/liter)	5.4 ± 1.1[b]
PC12-EC coculture	20.7 ± 2.1[a]

EC, Endothelial cell.
[a] $P < 0.01$ *vs.* PC12 without AM; [b] $P < 0.01$ *vs.* PC12 with AM (10^{-8} mol/liter); [c] $P < 0.05$; n = 8.

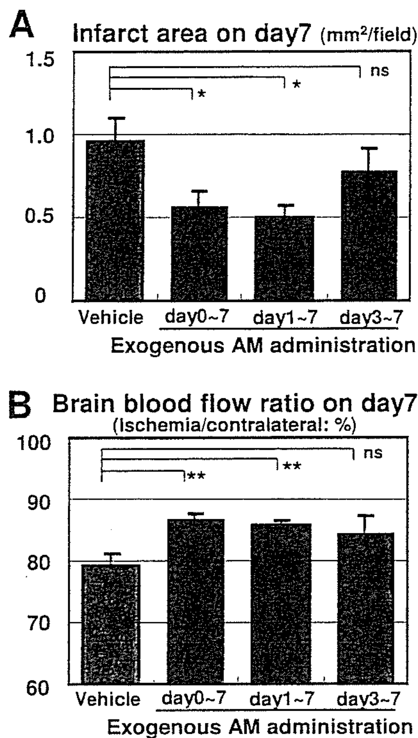

FIG. 9. Effects of exogenously administrated AM on neuroprotection and vascular regeneration after 20m-MCAO. 50 ng/h AM was administrated to mice with an ip implanted osmotic pump. Infarct area (A) and blood flow (B) on postoperative d 7 with different starting points for AM administration. *, $P < 0.05$; **, $P < 0.01$; ns, not significant *vs.* vehicle; n = 6.

lature, the so-called "vascular niche" (35), where endothelial cells secrete neurogenic factors, including basic fibroblast growth factor, vascular endothelial growth factor, and brain-derived neurotrophic factor, and create conditions conducive to neurogenesis (36). Therefore, vascular regeneration is assumed to rescue ischemic brain via not only supply of oxygen and nutrition but also promotion of neurogenesis. We confirmed in this study that neurogenesis occurred adjacent to neovessels in the ischemic core and the number of regenerated neurons was correlated with vascular density.

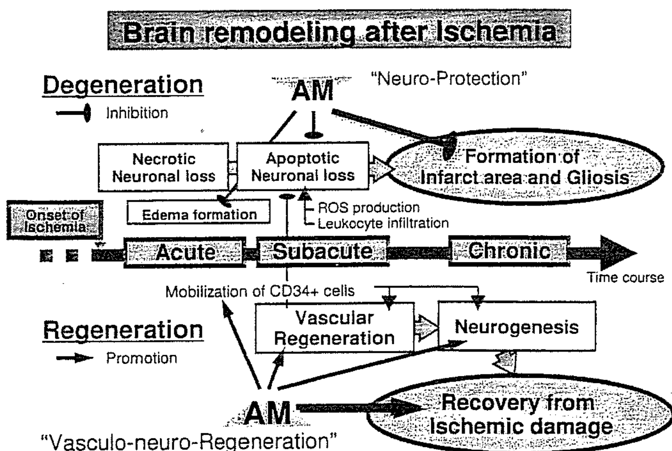

FIG. 10. Summary of brain remodeling after ischemia and effects of AM on the ischemic brain observed in this study.

We have assigned the term "vasculo-neuro-regeneration" to the entire process of enhancement of vasculogenesis and subsequent neurogenesis.

We demonstrated that AM promotes vasculo-neuro-regeneration in the ischemic brain. Blood flow and capillary density in the ischemic brain after 20m-MCAO was significantly enhanced in AM-Tg mice after postoperative d 7 with subsequent promotion of neurogenesis after d 28. The promoted vasculogenesis and neurogenesis observed in AM-Tg mice was significantly correlated with the functional recovery after 20m-MCAO. This result suggests that these two regenerative elements might contribute to the functional recovery after 20m-MCAO. The neovascularization was preceded by augmented mobilization of $CD34^+$ mononuclear cells, which are known to differentiate into endothelial cells and contribute to vasculogenesis (37). Recently, iv infusion of $CD34^+$ cells has reported to promote not only neovascularization but also neurogenesis (38). Furthermore, we observed the direct promoting action of AM on neural differentiation of PC12 cells via cAMP/PKA- and PI3K/Akt-dependent pathways. The totality of these findings suggests that the neurogenic action of AM *in vivo* comprises at least two different mechanisms: a direct action on neuronal cells through activation of PKA and Akt and an indirect action on neurogenesis after enhanced neovascularization.

Judging from the ratio of mature AM to total AM as shown in Table 1, the mature AM concentration in the ischemic brain of AM-Tg mice was expected to be 1~4 fmol/g tissue. The concentration seems to be comparable to the reported effective concentration of mature AM *in vivo* (25, 39). The *in vivo* concentration of human mature AM in the whole brain (1 fmol/g tissue level) and in the plasma (10 fmol/ml level) might be lower than the minimal concentration required for its *in vitro* action (100 fmol/ml) observed in this study. The actual effective concentration *in vitro*, however, might be lower because the administrated peptide is rapidly degradated *in vitro*. In addition, it is demonstrated in previous reports including ours (40, 41), that peptides could exert their significant actions at the stably maintained concentration, that is, by 2 orders of magnitude lower than that of bolus administration. In AM-Tg mice, the AM concentration was maintained at the same level due to the constitutive overproduction by the human serum amyloid P component promoter. Thus, we suppose that the direct neuronal action of AM *in vivo* could be possible in this stroke model.

In view of clinical application, we also tried exogenous administration of AM by ip implanted osmotic pump to determine appropriate amount and timing of AM administration after 20m-MCAO. Previous reports on AM administration for rodents or human set the therapeutic dose at 2~25 fmol/ml (25, 39). For our experiments, therefore, we used two lines of transgenic mice with a plasma concentration of mature AM of 24.9 ± 4.2 and 2.6 ± 0.6 fmol/ml. The results showed comparable effects of AM in these two lines on neuroprotection and vascular regeneration. This led us to conclude that a plasma level of 2~3 fmol/ml of mature AM, 3~5 times higher than its physiological concentration, was sufficient to attain therapeutic effects for the mice after 20m-MCAO. We next tried exogenous infusion of AM with an osmotic pump in the amount reported to achieve a plasma

concentration of 2~3 fmol/ml. The exogenous AM treatment which started just after the induction of 20m-MCAO or at 24 h after produced significant effects that were comparable to those seen in the two lines of AM-Tg mice. However, that from 72 h postoperatively failed to reveal significant effects. These results showed that appropriate timing to start AM administration after stroke is less than 72 h after the event.

We performed two different stroke models, nonfatal 20m-MCAO and fatal 2 h-MCAO. In 2 h-MCAO, we observed significant reduction of brain edema in AM-Tg mice through reduction of vascular permeability, which is compatible with previous report (42). However, infarct size was not reduced on postoperative d 1 after 2 h-MCAO. The result suggests that AM exerts more significant therapeutic effect on the brain tissue after nonfatal ischemia. The therapeutic potential for brain edema after fatal stroke is further to be elucidated.

Cerebral ischemia, including stroke, vascular Parkinson's disease and vascular dementia, is one of the most serious medical problems because it causes critical impairment of activity and quality of daily life. Regenerative medicine is now in the spotlight as a promising therapy to treat ischemic brain which has been considered to be irreversible and indicated for no active treatment. Various humoral factors are anticipated for their therapeutic potential for ischemic brain through neurogenic (*e.g.* basic fibroblast growth factor and epidermal growth factor) and angiogenic (*e.g.* vascular endothelial growth factor and hepatocyte growth factor) effects (43–47). Among them, we believe that the vascular hormone AM has several advantages as a therapeutic agent for ischemic brain. We can expect multiple effects of AM through its neuroprotective and vasculo-neuro-regenerative actions as shown in this study. In addition, AM has already been safely used for human patients with heart failure or pulmonary hypertension without any mention of critical adverse effects resulting from iv administration (39).

Thus, we are prompted to propose a new strategy to rescue ischemic brain by using vascular hormone AM for the combined neuroprotective and vasculo-neuro-regenerative therapy to improve impaired neurological function.

Acknowledgments

This work was supported by grants from Japanese ministry of Education, Culture, Sports, Science and Technology; ministry of Health, Labor and Welfare; and University of Kyoto 21st Century Centers of Excellence program. We thank Dr. Seiichi Hashida (Department of Biochemistry, University of Miyazaki) for measuring mature PAMP; and Dr. Kazuhiko Nozaki and Masaki Nishimura, (Department of Neurosurgery, University of Kyoto) for technical assistance.

Received August 15, 2005. Accepted December 19, 2005.

Address all correspondence and requests for reprints to: Hiroshi Itoh, M.D., Ph.D., Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine; 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: hiito@kuhp.kyoto-u.ac.jp.

This work was supported by Japanese ministry of Education, Culture, Sports, Science and Technology; ministry of Health, Labor and Welfare; and University of Kyoto 21st Century Centers of Excellence program.

References

1. 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 92:553–560
2. Nagaya N, Mori H, Murakami S, Kangawa K, Kitamura S 2005 Adrenomedullin: angiogenesis and gene therapy. Am J Physiol Regul Integr Comp Physiol 288:R1432–R1437
3. Shindo T, Kurihara Y, Nishimatsu H, Moriyama N, Kakoki M, Wang Y, Imai Y, Ebihara A, Kuwaki T, Ju KH, Minamino N, Kangawa K, Ishikawa T, Fukuda M, Akimoto Y, Kawakami H, Imai T, Morita H, Yazaki Y, Nagai R, Hirata Y, Kurihara H 2001 Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. Circulation 104:1964–1971
4. Shimosawa T, Shibagaki Y, Ishibashi K, Kitamura K, Kangawa K, Kato S, Ando K, Fujita T 2002 Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. Circulation 105:106–111
5. 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
6. Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, Nakao K 2003 Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo. FEBS Lett 544:86–92
7. Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi T, Nakao K 2003 Adrenomedullin promotes proliferation and migration of cultured endothelial cells. Hypertens Res 26:S93–S98
8. Abe M, Sata M, Nishimatsu H, Nagata D, Suzuki E, Terauchi Y, Kadowaki T, Minamino N, Kangawa K, Matsuo H, Hirata Y, Nagai R 2003 Adrenomedullin augments collateral development in response to acute ischemia. Biochem Biophys Res Commun 306:10–15
9. Kim W, Moon SO, Sung MJ, Kim SH, Lee S, So JN, Park SK 2003 Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. FASEB J 17:1937–1939
10. Tokunaga N, Nagaya N, Shirai M, Tanaka E, Ishibashi-Ueda H, Harada-Shiba M, Kanda M, Ito T, Shimizu W, Tabata Y, Uematsu M, Nishigami K, Sano S, Kangawa K, Mori H 2004 Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector, gelatin. Circulation 109:526–531
11. Iwase T, Nagaya N, Fujii T, Itoh T, Ishibashi-Ueda H, Yamagishi M, Miyatake K, Matsumoto T, Kitamura S, Kangawa K 2005 Adrenomedullin enhances angiogenic potency of bone marrow transplantation in a rat model of hindlimb ischemia. Circulation 111:356–362
12. Eto T 2001 A review of the biological properties and clinical implications of adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP), hypotensive and vasodilating peptides. Peptides 22:1693–1711
13. Serrano J, Alonso D, Fernandez AP, Encinas JM, Lopez JC, Castro-Blanco S, Fernandez-Vizarra P, Richart A, Santacana M, Uttenthal LO, Bentura ML, Martinez-Murillo R, Martinez A, Cuttitta F, Rodrigo J 2002 Adrenomedullin in the central nervous system. Microsc Res Tech 57:76–90
14. Wang X, Yue TL, Barone FC, White RF, Clark RK, Willette RN, Sulpizio AC, Aiyar NV, Ruffolo Jr RR, Feuerstein GZ 1995 Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage. Proc Natl Acad Sci USA 92:11480–11484
15. Dogan A, Suzuki Y, Koketsu N, Osuka K, Saito K, Takayasu M, Shibuya M, Yoshida J 1997 Intravenous infusion of adrenomedullin and increase in regional cerebral blood flow and prevention of ischemic brain injury after middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab 17:19–25
16. Watanabe K, Takayasu M, Noda A, Hara M, Takagi T, Suzuki Y, Yoshia J 2001 Adrenomedullin reduces ischemic brain injury after transient middle cerebral artery occlusion in rats. Acta Neurochir (Wien) 143:1157–1161
17. Xia CF, Yin H, Borlongan CV, Chao J, Chao L 2004 Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival. Hum Gene Ther 15:1243–1254
18. Hashida S, Kitamura K, Nagatomo Y, Shibata Y, Imamura T, Yamada K, Fujimoto S, Kato J, Morishita K, Eto T 2004 Development of an ultrasensitive enzyme immunoassay for human proadrenomedullin N-terminal 20 peptide and direct measurement of two molecular forms of PAMP in plasma from healthy subjects and patients with cardiovascular disease. Clin Biochem 37:14–21
19. Longa EZ, Weinstein PR, Carlson S, Cummins R 1989 Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84–91
20. Teramoto T, Qiu J, Plumier JC, Moskowitz MA 2003 EGF amplifies the replacement of parvalbumin-expressing striatal interneurons after ischemia. J Clin Invest 111:1125–1132
21. Venditti A, Battaglia A, Del Poeta G, Buccisano F, Maurillo L, Tamburini A, Del Moro B, Epiceno AM, Martiradonna M, Caravita T, Santinelli S, Adorno G, Picardi A, Zinno F, Lanti A, Bruno A, Suppo G, Franchi A, Franconi G, Amadori S 1999 Enumeration of CD34+ hematopoietic progenitor cells for clinical transplantation: comparison of three different methods. Bone Marrow Transplant 24:1019–1027
22. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR 1990 A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290–293
23. Zhang ZG, Zhang L, Croll SD, Chopp M 2002 Angiopoietin-1 reduces cerebral blood vessel leakage and ischemic lesion volume after focal cerebral embolic ischemia in mice. Neuroscience 2002 113:683–687

24. Hayashi H, Ishisaki A, Imamura T 2003 Smad mediates BMP-2-induced upregulation of FGF-evoked PC12 cell differentiation. FEBS Lett 536:30–34
25. Iimuro S, Shindo T, Moriyama N, Amaki T, Niu P, Takeda N, Iwata H, Zhang Y, Ebihara A, Nagai R 2004 Angiogenic effects of adrenomedullin in ischemia and tumor growth. Circ Res 95:415–423
26. Nakano S, Kogure K, Fujikura H 1990 Ischemia-induced slowly progressive neuronal damage in the rat brain. Neuroscience 38:115–124
27. Graham SH, Chen J 2001 Programmed cell death in cerebral ischemia. J Cereb Blood Flow Metab 21:99–109
28. Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ 2001 Early neurodegeneration after hypoxia-ischemia in neonatal rat is necrosis while delayed neuronal death is apoptosis. Neurobiol Dis 8:207–219
29. Stoll G, Jander S, Schroeter M 1998 Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol 56:149–171
30. Gilgun-Sherki Y, Rosenbaum Z, Melamed E, Offen D 2002 Antioxidant therapy in acute central nervous system injury: current state. Pharmacol Rev 54:271–284
31. Kim W, Moon SO, Lee S, Sung MJ, Kim SH, Park SK 2003 Adrenomedullin reduces VEGF-induced endothelial adhesion molecules and adhesiveness through a phosphatidylinositol 3′-kinase pathway. Arterioscler Thromb Vasc Biol 23:1377–1383
32. Kawai J, Ando K, Tojo A, Shimosawa T, Takahashi K, Onozato ML, Yamasaki M, Ogita T, Nakaoka T, Fujita T 2004 Endogenous adrenomedullin protects against vascular response to injury in mice. Circulation 109:1147–1153
33. Niu P, Shindo T, Iwata H, Iimuro S, Takeda N, Zhang Y, Ebihara A, Suematsu Y, Kangawa K, Hirata Y, Nagai R 2004 Protective effects of endogenous adrenomedullin on cardiac hypertrophy, fibrosis, and renal damage. Circulation 109:1789–1794
34. Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM 1994 Role of angiogenesis in patients with cerebral ischemic stroke. Stroke 25:1794–1798
35. Palmer TD, Willhoite AR, Gage FH 2000 Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479–494
36. Louissaint Jr A, Rao S, Leventhal C, Goldman SA 2002 Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34:945–960
37. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM 1997 Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967
38. Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, Tsukamoto Y, Iso H, Fujimori Y, Stern DM, Naritomi H, Matsuyama T 2004 Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest 114:330–338
39. Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, Oya H, Kyotani S, Nakanishi N, Goto Y, Masuda Y, Miyatake K, Kangawa K 2000 Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. Circulation 101:498–503
40. Doi K, Itoh H, Ikeda T, Hosoda K, Ogawa Y, Igaki T, Yamashita J, Chun TH, Inoue M, Masatsugu K, Matsuda K, Ohmori K, Nakao K 1997 Adenovirus-mediated gene transfer of C-type natriuretic peptide causes G1 growth inhibition of cultured vascular smooth muscle cells. Biochem Biophys Res Commun 239:889–894
41. Komatsu Y, Itoh H, Suga S, Ogawa Y, Hama N, Kishimoto I, Nakagawa O, Igaki T, Doi K, Yoshimasa T, Nakao K 1996 Regulation of endothelial production of C-type natriuretic peptide in coculture with vascular smooth muscle cells. Role of the vascular natriuretic peptide system in vascular growth inhibition. Circ Res 78:606–614
42. Hippenstiel S, Witzenrath M, Schmeck B, Hocke A, Krisp M, Krull M, Seybold J, Seeger W, Rascher W, Schutte H, Suttorp N 2002 Adrenomedullin reduces endothelial hyperpermeability. Circ Res 91:618–625
43. Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M 2002 Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429–441
44. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Bruggen N, Chopp M 2000 VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J Clin Invest 106:829–838
45. Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, Uchiyama Y, Ogihara T, Kaneda Y, Morishita R 2004 Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. Circulation 109:424–431
46. Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA 2003 VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest 111:1843–1851
47. Sondell M, Lundborg G, Kanje M 1999 Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 19:5731–5740

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Regulatory Peptides 132 (2005) 17 – 22
REGULATORY PEPTIDES
www.elsevier.com/locate/regpep

Expression of the adrenomedullin gene in adipose tissue

Takuo Nambu, Hiroshi Arai *, Yasato Komatsu, Akihiro Yasoda, Kenji Moriyama, Naotetsu Kanamoto, Hiroshi Itoh, Kazuwa Nakao

Department of Medicine and Clinical Science Kyoto University Graduate School of Medicine 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Received 9 December 2004; received in revised form 16 February 2005; accepted 29 July 2005
Available online 8 September 2005

Abstract

Adrenomedullin (AM) is a potent vasodilating peptide originally isolated from human pheochromocytoma cells. This report concerns the expression and secretion of AM from adipose tissue. Northern blot analysis demonstrated marked expression of AM mRNA in mouse adipose tissue. Expression levels in adipose tissues were 2.5–3.2 times higher than in the kidney. AM mRNA level in mature adipocytes was 7.3 times higher than in the stroma–vascular fraction of adipose tissue. In mature adipocyte culture, time-dependent increase of AM peptide concentration in the culture medium was detected. AM expression was also detected in human subcutaneous adipose tissue. Adipose AM expression significantly increased in obesity mouse model, high-fat diet fed mice and ob/ob mice. These results suggest that adipose tissue, especially mature adipocytes, is major source of AM in the body, and that adipocyte-derived AM plays a pathophysiological role in obesity.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Adrenomedullin; Adiopocyte; Fat; Obesity; ob/ob mice

1. Introduction

It has recently been suggested that adipose tissue is an endocrine organ. A wide variety of factors secreted from adipocytes, including leptin, TNF alpha, adiponectin, resistin and free fatty acid, are playing crucial roles in energy expenditure and glucose metabolism [1]. Adipocytes produce a host of vasoactive substances including angiotensinII [2], endothelin-1 [3], nitric oxide [4], prostacyclin [5] and natriuretic peptide [6]. Recent studies have suggested the paracrine/autocrine involvement of these molecules in the regulation of adipocyte growth and differentiation.

Adrenomedullin (AM) is a potent vasodilating peptide that was originally isolated from human pheochromocytoma cells [7]. Structural analysis indicates that AM belongs to the calcitonin gene-related peptide (CGRP) superfamily. AM and CGRP share a common receptor known as the calcitonin-receptor-like-receptor (CRLR). The ligand specificity of CRLR is regulated by the receptor-activity-modifying proteins (RAMPs), which is a family of proteins with a single transmembrane domain [8]. CRLR associated with RAMP1 acts as a CGRP receptor, while it binds to AM when coexpressed with RAMP2/3. It has been reported that AM-producing cells are distributed widely throughout the body, including the adrenal glands, lungs, heart and kidneys [9]. In vitro studies have demonstrated that vascular smooth muscle cells and endothelial cells secrete AM, and have suggested that the major source of AM in the body is the vascular wall [10].

We have previously reported that RAMPs are highly expressed in rat adipose tissue [11], and have posited the existence of an AM system in adipose tissue. The study reported here found massive expression of the AM gene in mouse and human adipose tissue, and its upregulation in obesity mouse models.

2. Materials and methods

2.1. Ethics

This study conforms to the policy of the Ethics Committee on Human Research of the Kyoto University Graduate School of Medicine, and written informed consent was obtained from all subjects.

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; RAMPs, receptor-activity-modifying protein; sv-f, stroma–vascular fraction; BAT, intrascapular brown adipose tissue; VEC, vascular endothelial cells; VSMC, vascular smooth muscle cells.

* Corresponding author. Tel.: +81 75 751 3181; fax: +81 75 771 9452.
E-mail address: hiroarai@kuhp.kyoto-u.ac.jp (H. Arai).

0167-0115/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2005.07.006

2.2. Mouse adipose tissue

Male C57BL/6 and ob/ob mice aged 14 weeks provided by Shionogi Research Laboratories (Osaka, Japan) were used in this study. They were treated in accordance with our institutional guidelines for animal research, housed in an animal room maintained at 24 °C with a 12:12-h light-dark cycle, fed a standard laboratory diet and given water ad libitum. The retroperitoneal, subcutaneous, omental, epididymal white adipose tissue, intrascapular brown adipose tissue (BAT), kidney, lung and heart were removed and stored at −80 °C until total RNA preparation.

2.3. Mature adipocytes

Murine mature adipocytes were isolated from subcutaneous adipose tissue of C3H/He mice (female, 8 months old) with a modificied version of the method of Rodbell [12]. In brief, the adipose tissue was minced and digested in a 0.2% collagenase solution at 37 °C for 1 h with constant shaking. The digested fluid was filtered through 100 μm nylon mesh and separated by centrifugation performed three times at 180 g for 5 min. Mature adipocytes appeared and were collected as a floating layer, while the sediment consisted of stroma–vascular fraction (sv-f).

2.4. Human adipose tissue

Human abdominal subcutaneous adipose tissue was obtained during plastic surgery after written permission had been obtained. Human kidney and lung mRNA were commercially available (Clontech Laboratories, Inc., Palo Alto, CA, USA).

2.5. RNA extraction and Northern blot analysis

Total RNA was extracted with TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA). Northern blot analysis was performed as previously described using mouse and human AM cDNA as probes [11].

2.6. Measurements of AM released from isolated adipocytes

500 μl (2.5×10^5 cells) of isolated adipocytes from subcutaneous adipose tissue of ICR mouse (male, 12 weeks old) were incubated together with DMEM/H-12 (total volume: 500 μl) containing 10% fetal bovine serum in a CO_2 incubator for 24 h at 37 °C [13]. Aliquots of the incubation medium were removed at 6 and 24 h and stored at −20 °C for measurement of AM. The AM content in 100 μl of the incubation medium was determined by means of a radioimmunoassay (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA).

2.7. TaqMan real time PCR

The quantitative real time RT-PCR was employed to examine the murine AM gene expression. Briefly, AM cDNA was synthesized with Superscript II reverse transcriptase (Gibco-BRL, St. Louis, MO, USA) and used as a template. The primers and probes for TaqMan PCR analysis were designed with primer-express software (Applied Biosystems, Foster City, CA, USA) as follows:

AM forward, 5′-CTCGCTGATGAGACGACAGTTC-3′,
AM reverse, 5′-CTCTGGCGGTAGCGTTTGAC-3′,
detection probe: 5′-CAGCAATCAGAGCGAAGCCCA-CATT-3′.

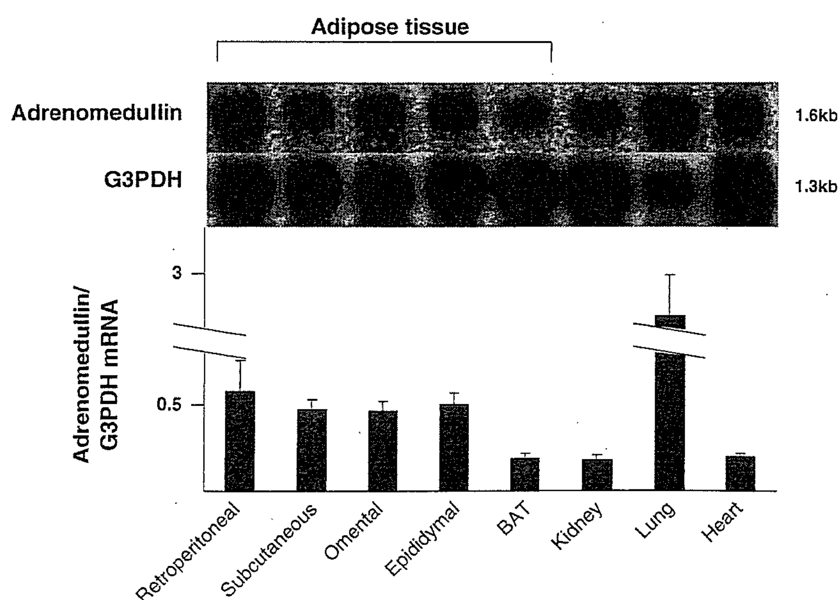

Fig. 1. Northern blot analysis of AM mRNA content of C57BL/6 mice. 10 μg of total RNA in each lane was electrophoresed and hybridized with mouse AM cDNA probes. The lower panel indicates hybridization with a G3PDH probe as an internal control. The ratio of AM to G3PDH mRNA is shown below. Bars represent mean ± S.E.M.

Rodent ribosomal 18S as an internal control was amplified using a commercially available kit (Applied Biosystems) at the same time. Thermal cycling was performed at 40 cycles of 95 °C for 15 s and of 60 °C for 1 min. Reactions were performed in triplicate with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Input RNA amounts were calculated with a multiplex comparative method for mRNAs of AM and 18S protein.

2.8. High-fat diet (HFD)

Male C57BL/6 mice aged 10 weeks were randomly divided into two groups, one fed a standard fat diet (11% fat by energy) and the other a high-fat diet (60% fat by energy, Research Diets, Inc., New Brunswick, NJ, USA). After 11 weeks, the retroperitoneal, subcutaneous, omental, epididymal white adipose tissue and kidney were removed and stored at −80 °C until total RNA preparation. Mouse plasma AM and leptin concentrations were determined with a radioimmunoassay (Phoenix Pharmaceuticals, Inc.) and an enzyme immunoassay (Immune Biological Laboratory, Gunma, Japan), respectively.

2.9. Statistical analysis

All data are expressed as the mean±S.E.M. Statistical analysis was performed with Student's t-test. Values of $P<0.05$ were considered to be statistically significant.

3. Results

3.1. AM gene expression in adipose tissue and mature adipocytes

The expression of AM gene was examined by means of Northern blotting (Fig. 1). A marked expression of AM mRNA was detected in all white adipose tissues from C57BL/6. The expression levels in the retroperitoneal, subcutaneous, omental, and epididymal adipose tissue were 3.2, 2.6, 2.5 and 2.7 times higher than the corresponding levels in the kidney. In addition to mature adipocytes, adipose tissue contains blood vessels, fibroblasts and preadipocytes, among which especially the vascular wall has been regarded as a site of AM production. We therefore separated the adipose tissue into a mature adipocytes fraction and sv-f, and examined AM expression in each fraction (Fig. 2). The AM mRNA level in the mature adipocyte

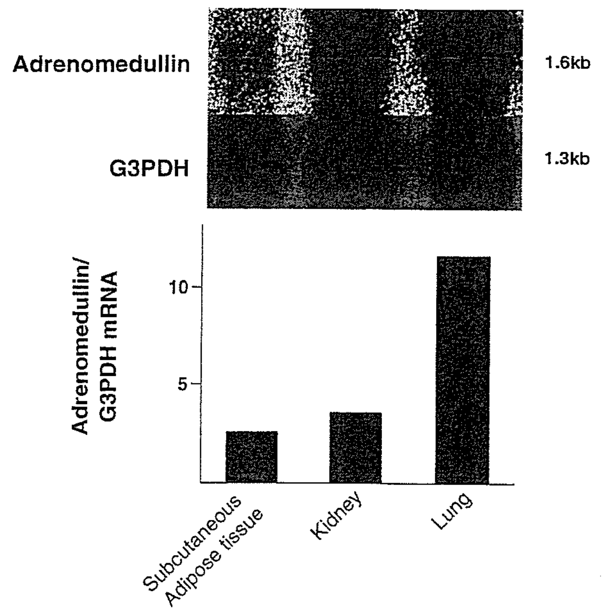

Fig. 3. Northern blot analysis of human AM mRNA content. 10 μg of total RNA in each lane was electrophoresed and hybridized with human AM cDNA probes. The lower panel indicates hybridization with a G3PDH probe as an internal control. The ratio of the AM to G3PDH mRNA is shown below.

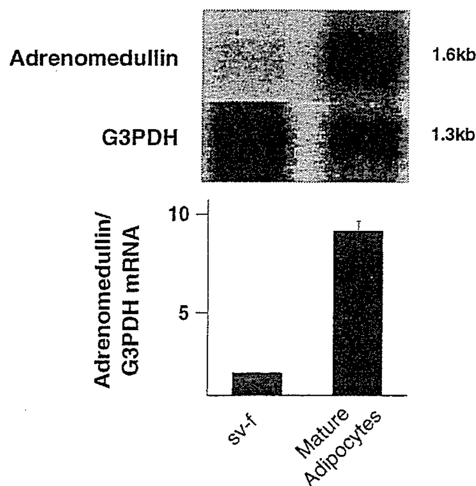

Fig. 2. Northern blot analysis of AM mRNA content in stroma–vascular fraction and isolated mature adipocytes of C3H/He mice. 10 μg of total RNA in each lane were electrophoresed and hybridized with mouse AM cDNA probes. The lower panel indicates hybridization with a G3PDH probe as an internal control. The ratio of AM to G3PDH mRNA is shown below. Bars represent the mean±S.E.M.

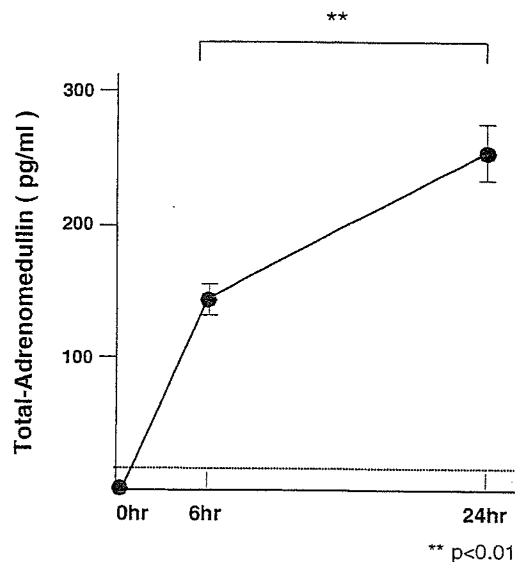

Fig. 4. Time course of AM levels in culture medium of mature adipocytes isolated from ICR mice (12 wk old, male, $n=5$). Mature adipocytes released AM, which significantly increased in a time-dependent manner. Bars represent the mean±S.E.M. Statistical analysis was performed with the t test. $^{**}P<0.01$.

Table 1
Body weight and plasma parameters in mice fed with high-fat diet or standard chow

	Standard chow	High-fat diet
Body weight(g)	31.3±1.2	48.6±2.1**
BS (mg/dl)	87.0±8.5	141.5±12.7**
FFA (mEq/ml)	0.51±0.08	0.94±0.18*
Leptin (ng/ml)	0.23±0.02	18.5±3.7***
Total AM (pg/ml)	56.3±17.5	84.9±6.2*

*$P<0.05$; **$P<0.01$, ***$P<0.001$ vs. standard chow group.

fraction was 7.3 times higher than that in the sv-f. In view of the tissue volume, this suggests that the major source of AM mRNA in the adipose tissue is mature adipocytes.

In the human subcutaneous adipose tissues obtained during plastic surgery, AM expression comparable to that in the kidney was detected (Fig. 3).

3.2. AM secretion from mature adipocytes

In addition to AM mRNA expression in the adipose tissue and mature adipocytes, we cultured mature adipocytes and measured AM peptide secretion in the medium. As shown in Fig. 4, AM peptide detected in the cultured medium increased in a time-dependent manner, indicating that AM is secreted from mature adipocytes.

3.3. Changes in AM gene expression in obesity

In order to assess the relationship between obesity and adipose AM production, we examined the AM mRNA expression after administration of the high-fat diet. As shown in Table 1, the HFD group weighed 1.5 times more than the standard chow group, and plasma leptin concentration was significantly higher as well as concentrations of plasma free fatty acid (FFA) and glucose. Plasma AM concentration in the HFD group was significantly higher than that in the standard chow group. After the high-fat diet, AM expression level was elevated in all adipose tissue compared with that in the control group, especially in retroperitoneal, subcutaneous and epididymal white adipose tissue (Fig. 5). In contrast, AM expression levels in the kidney of the HFD group and the control group were comparable.

In addition to HFD, we also examined AM mRNA expression in the obesity mouse model, ob/ob. Fig. 6 shows the AM mRNA level in the adipose tissues of ob/ob and the control, namely, C57BL/6 mice. In ob/ob mice, AM expression level was elevated in all adipose tissues compared with that in C57BL/6, especially in subcutaneous, omental, epididymal white adipose tissue and BAT.

4. Discussion

AM has been implicated in the regulation of circulation and the development of vasculature. Although AM was originally isolated from pheochromocytoma cells, the major site of production in the physiological state is believed to be vascular endothelial cells. Our findings demonstrate that adipose tissue strongly expresses the AM gene. In C57BL/6 mice, AM mRNA expression in all white adipose tissues examined was found to be higher than that detected in kidney. Adipose tissue is capillary-rich, containing blood vessels, fibroblasts and preadipocytes in addition to mature adipocytes. In order to exclude the possibility that AM mRNA in the adipose tissue is of vascular endothelial cell origin, we examined the AM expression in the mature adipocyte fraction and sv-f separately and found that the AM mRNA level in the mature adipocyte fraction was much higher than that in the sv-f, which consists of small vessels, preadipocytes and connective tissue. Moreover, we determined AM peptide secretion from isolated mature adipocytes in the medium. These findings indicate that the major source of AM in the adipose tissue consists of mature adipocytes. A few reports about adipose tissue and AM have

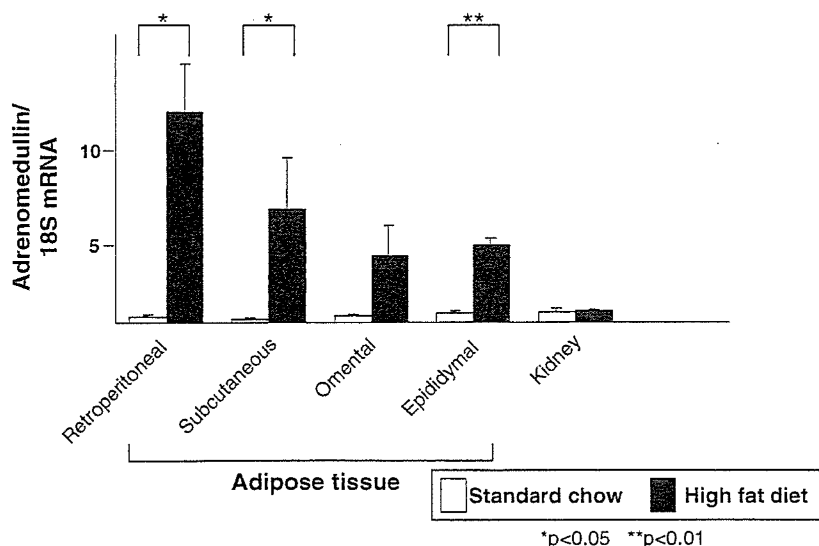

Fig. 5. AM mRNA levels from C57BL/6 mice (21 wk old, male, $n=5$) after 11 weeks of standard chow diet (open bars) or high-fat diet (closed bars). Bars represent the mean±S.E.M. Statistical analysis was performed with the t test. *$P<0.05$; **$P<0.01$.

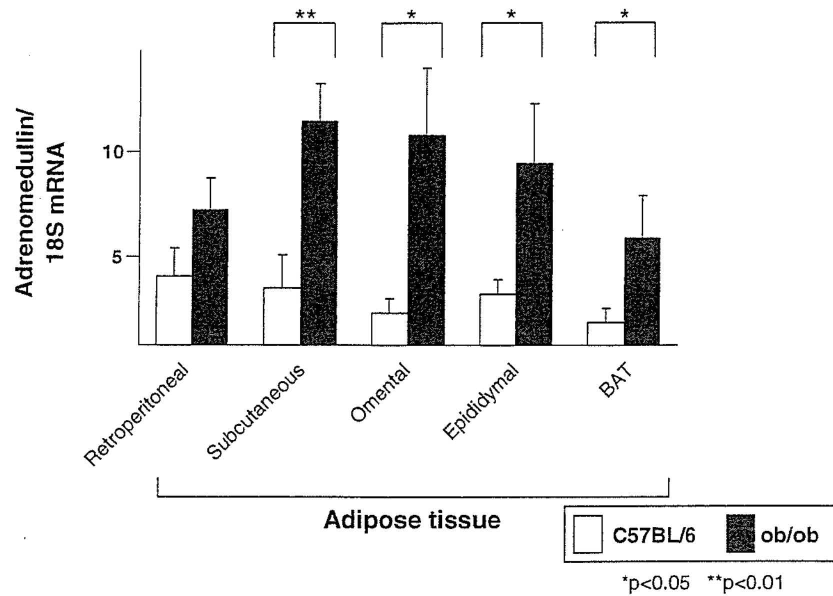

Fig. 6. AM mRNA levels in C57BL/6 (open bars) and ob/ob mice (closed bars) (14 wk old, male, $n=5$). Bars represent the mean±S.E.M. Statistical analysis was performed with the t test. $^{*}P<0.05$; $^{**}P<0.01$.

been published. Using NIH 3T3-L1 cells, Li et al. reported observing AM mRNA expression and secretion in mature adipocytes [14,15]. Recently Fukai et al. found AM mRNA expression in rat and human adipose tissue [16], which agrees with the results of our study.

Human subcutaneous adipose tissue obtained from healthy subjects during plastic surgery exhibited marked AM expression, too. In view of the tissue volume, these results suggest that adipose tissue is one of the major sites of AM production in the body.

The physiological significance of adipocyte-derived AM remains to be clarified. Since it has been reported that the plasma concentration of AM in the physiological state is not high enough to exert a vasodilatory effect [17], it stands to reason that AM secreted from adipocytes functions in an autocrine/paracrine manner. Adipose tissue is distributed throughout the body and works as supportive tissue. AM is a potent vasodilator and a growth factor for vascular endothelial cells (VEC) and vascular smooth muscle cells (VSMC) [18,19]. Many blood vessels are surrounded by fat and AM produced by the adipocytes and the vascular wall itself may make the local concentration of AM high enough to work as a vasodilator or a growth factor for VEC and VSMC. In reconstruction culture of the skin, the epidermal layer is known to grow better on the subcutaneous adipose layer [20]. It has also been reported that AM has a mitogenic effect on human keratinocytes [21]. Along with its enhancing effect on the proliferation and migration of VEC and VSMC, AM secreted from subcutaneous adipose tissue may be crucial for the maintenance and regeneration of the skin. Bone marrow is another organ which is rich in adipose tissue, while it has been reported that AM is expressed in cord blood hematopoietic cells and stimulates their clonal growth [22]. It thus seems reasonable to speculate that bone marrow fat may contribute to haematopoiesis.

It is extremely interesting that AM expression in adipose tissue and plasma AM concentration were significantly augmented with the increase in body weight after the high-fat diet. The same results were observed in ob/ob, leptin-deficient obesity model mice [23]. Furthermore, the massive increase in adipose tissue volume in obesity makes the total AM production in adipose tissue much greater. It has also been reported that the expression of the AM receptor components CRLR and RAMP2 was heightened in HFD rat adipose tissue [16]. Shimosawa et al. reported that heterozygous AM-deficient mice showed obesity, higher blood pressure and insulin resistance in their old age [24]. Taken together, these findings suggest that adipose tissue-derived AM protects against obesity.

The relationship between human plasma AM levels and obesity is not clear yet, although several studies have shown that plasma human AM levels are elevated in obesity [25,26].

In conclusion, our study presented here demonstrated that adipocytes produce AM in human and mouse and the AM production in adipose tissue is enhanced in obesity. Adipose AM may thus have a pathophysiological function in obesity.

Acknowledgements

This study was supported in part by the Smoking Research Foundation.

References

[1] Ahima RS, Flier JS. Adipose tissue as an endocrine organ. Trends Endocrinol Metab 2000;11:327–32.
[2] Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM. Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin type 1 receptors. Diabetes 2002;51:1699–707.

[3] Hauner H, Petruscheke T, Gries FA. Endothelin-1 inhibits the adipose differentiation of cultured human adipocyte precursor cells. Metabolism 1994;43:227–32.

[4] Nisoli E, Clementi E, Tonello C, Sciorati C, Brinscini L, Carruba MO. Effects of nitric oxide on proliferation and differentiation of rat brown adipocytes in primary cultures. Br J Pharmacol 1998;125:888–94.

[5] Aubert J, Saint-Marc P, Belmonte N, Dani C, Negrel R, Ailhaud G. Prostacyclin IP receptor up-regulates the early expression of C/EBPbeta and C/EBPdelta in preadipose cells. Mol Cell Endocrinol 2000;16:149–56.

[6] Sengenes C, Berlan M, De Glisezinski I, Lafontan M, Galitzky J. Natriuretic peptides: a new lipolytic pathway in human adipocytes. FASEB J 2000;14:1345–51.

[7] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.

[8] McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998;393:333–9.

[9] Washimine H, Kitamura K, Ichiki Y, Yamamoto Y, Kangawa K, Masuo H, Eto T. Immunoreactive proadrenomedullin N-terminal 20 peptide in human tissue, plasma and urine. Biochem Biophys Res Commun 1994;202:1081–7.

[10] Sugo S, Minamino N, Shoji H, Kangawa K, Kitamura K, Eto T, Matsuo H. Interleukin-1, tumor necrosis factor and lipopolysaccharide additively stimulate production of adrenomedullin in vascular smooth muscle cells. Biochem Biophys Res Commun 1995;207:25–32.

[11] Nagae T, Mukoyama M, Sugawara A, Mori K, Yahata K, Kasahara M, Suganami T, Makino H, Fujinaga Y, Yoshioka T, Tanaka I, Nakao K. Rat receptor-activity-modifying proteins (RAMPs) for adrenomedullin/CGRP receptor: cloning and upregulation in obstructive nephropathy. Biochem Biophys Res Commun 2000;270:89–93.

[12] Rodbell M. Metabolism of isolated fat cells. J Biol Chem 1964;239:375–80.

[13] Cassis LA, English VL, Bharadwaj K, Boustany CM. Differential effects of local versus systemic angiotensin II in the regulation of leptin release from adipocytes. Endocrinology 2004;145:169–74.

[14] Li Y, Totsune K, Takeda K, Furuyama K, Shibahara S, Takahashi K. Decreased expression of adrenomedullin during adipocyte-differentiation of 3T3-L1 cells. Hypertens Res 2003;26:S41–4.

[15] Li Y, Totsune K, Takeda K, Furuyama K, Shibahara S, Takahashi K. Differential expression of adrenomedullin and resistin in 3T3-L1 adipocytes treated with tumor necrosis factor-alpha. Eur J Endocrinol 2003;149:231–8.

[16] Fukai N, Yoshimoto T, Sugiyama T, Ozawa N, Sato R, Shichiri M, Hirata Y. Concominant expression of adrenomedullin and its receptor components in rat adipose tissues. Am J Physiol Endocrionol Metab 2005;288:E56–62.

[17] Shindo T, Kurihara H, Maemura K, Kurihara Y, Kuwaki T, Izumida T, Minamino N, Ju KH, Morita H, Oh-hashi Y, Kumada M, Kangawa K, Nagai R, Yazaki Y. Hypotension and resistance to lipopolysaccharide-induced shock in transgenic mice overexpressing adrenomedullin in their vasculature. Circulation 2000;101:2309–16.

[18] Shichiri M, Fukai N, Ozawa N, Iwasaki H, Hirata Y. Adrenomedullin is an autocrine/paracrine growth factor for rat vascular smooth muscle cells. Regul Pept 2003;112:167–73.

[19] Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi T, Nakao K. Adrenomedullin promotes proliferation and migration of cultured endothelial cells. Hypertens Res 2003;26:S93–8.

[20] Sugihara H, Toda S. Reconstruction culture of the skin. In: Doyle A, editor. Cell & tissue culture: laboratory procedures. Chichester: John Wiley & Sons Company Ltd.; 1995. p. 3A 2.1-2.12.

[21] Kapas S, Brown DW, Farthing PM, Hagi-Pavli E. Adrenomedullin has mitogenic effects on human oral keratinocytes: involvement of cyclic AMP. FEBS Lett 1997;418:287–90.

[22] Del Pup L, Belloni AS, Carraro G, De Angeli S, Parnigotto PP, Nussdorfer GG. Adrenomedullin is expressed in cord blood hematopoietic cells and stimulates their clonal growth. Int J Mol Med 2003;11:157–60.

[23] Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 1995;269:540–3.

[24] Shimosawa T, Ogihara T, Matsui H, Asano T, Ando K, Fujita T. Deficiency of adrenomedullin induces insulin resistance by increasing oxidative stress. Hypertension 2003;41:1080–5.

[25] Minami J, Nishikimi T, Ishimitsu T, Makino Y, Kawano Y, Takishita S, Kangawa K, Matsuoka H. Effect of a hypocaloric diet on adrenomedullin and natriuretic peptides in obese patients with essential hypertension. J Cardiovasc Pharmacol 2000;36:S83–6.

[26] Kato J, Kitamura K, Uemura T, Kuwasako K, Kita T, Kangawa K, Eto T. Plasma levels of adrenomedullin and atrial and brain natriuretic peptides in the general population: their relations to age and pulse pressure. Hypertens Res 2002;25:887–92.