cells were revealed to be hypotensive and resistant to vascular remodeling such as neointima formation caused by cuff injury, and atherogenesis associated with a high cholesterol diet (5). We have recently established that AM promotes endothelial regeneration in the wound healing assay using cultured endothelial cells and enhances neo-vascularization in vivo into subcutaneously implanted gel-plugs in mice (6-7). We and others have further demonstrated that the potentiating action of AM on vascular regeneration is mediated by activation of the phosphatidyl inositol-3 kinase (PI3K) - Akt pathway (8-11).

Recently, it has been known that AM is secreted from various organs including the heart, lung, kidney, adipose tissues and central nervous system (12). Moreover, AM expression has been demonstrated to be markedly enhanced by ischemia through the activation of hypoxia-responsive elements (HREs) in the AM gene via transcription factor hypoxia-inducible factor-1 (HIF-1). In the central nervous system, where AM is mainly expressed in neurons and the

endothelium (13), it is reported that transient ischemia boosted AM expression for more than 15 days (14). However, the role of augmented AM has remained unclear for inconsistent previous results: three studies reported neuro-protective effects of AM by demonstrating reduction of infarct size after transient ischemia (15-17), while one study detected exacerbation of infarction as a result of AM infusion (14).

In this context, our study presented here focused on the roles of augmented AM in ischemic brain and examined its therapeutic potential. We generated new lines of transgenic mice that overproduce AM (AM-Tg) in the liver which mimics chronic AM administration. After inducing 20-minute middle cerebral artery occlusion (20m-MCAO) to produce a non-fatal stroke model in the AM-Tg mice, we observed the long-term effects of AM on the ischemic brain up to postoperative day 56. We examined the mice for the recovery of blood flow in the ischemic region and impaired motor function after stroke, and

immunohistochemically examined the ischemic striatum to determine effects of AM on neuronal loss/apoptosis, gliosis, leukocyte infiltration, oxidative stress, vascular regeneration, and neurogenesis after 20m-MCAO. In addition, another stroke model, 2-hour middle cerebral artery occlusion (2h-MCAO), was performed to observe the effect of AM in acute phase of the fatal stroke. In vitro studies using neuronal progenitor cells or rat pheochromocytoma PC12 cells were performed to examine direct anti-apoptotic and neurogenic actions of AM on these neuronal cells. Finally, we investigated the effect of exogenous AM administration after 20m-MCAO to determine the appropriate amount and timing of AM treatment after cerebral ischemia.

Materials and Methods

Generation of transgenic mice which overproduce human AM but do not

overproduce mature PAMP.

The AM gene contains coding regions for not only AM but also proadrenomedullin N-terminal 20 peptide (PAMP), a different vasodilating peptide. Amidation at their carboxyl terminals after their synthesis is needed for both AM and PAMP to exert their biological activity. The bioactive amidated forms are known as mature AM and mature PAMP, respectively. To identify the specific effects of AM, we generated a transgene construct with a point mutation on the PAMP amidation signal in the full-length AM gene cDNA. Guanine was substituted for cytosine on the 3' end of the PAMP coding region so that glycine on the C' terminal of the PAMP product was replaced with alanine. In this way, amidation and maturation of PAMP by peptidylglycine α-hydroxylase and α-hydroxyglycine N-C lyase were inhibited (Figure 1A). The mutant AM gene cDNA was then inserted into a plasmid containing the human serum amyloid P component (SAP) promoter, which is widely used to target gene expression

specific to the liver. When the product is secreted from the liver, it mimics intravenous administration of the agent. The HindIII-XhoI fragment of the plasmid was microinjected into the pronucleus of fertilized C57BL/6J mice eggs.

The copy number of transgenes was quantified by means of genomic Southern blotting according to standard procedure. Plasma concentrations of human total AM and mature AM were measured with a commercially available immunoradiometric assay (Cosmic, Tokyo, Japan). Human mature PAMP concentration was measured with a recently developed enzyme immunoassay (18). To determine the brain concentration of AM, we used the RIA kits for measurement of human and mouse total AM (Phoenix, Belmont, CA), according to the manufacturer's instruction. Blood pressure (BP) was measured with tail cuff (Softron, Tokyo, Japan). Hindlimb ischemia was induced by ligating the right femoral artery and blood flow of the ischemic limb was estimated with a laser Doppler perfusion imager (LDPI; Moor Instruments Ltd., Devon, UK) to

confirm the angiogenic effect of AM-Tg mice. The perfusion ratio (%) was calculated as that of the ipsilatereal to the contralateral side. Animal care and experiments were in accordance with the guidelines for animal experiments of Kyoto University.

Induction of stroke by middle cerebral artery occlusion (MCAO)

We performed non-fatal 20m-MCAO and fatal 2h-MCAO by the standard trans-luminal method, which has been described in various previous reports (19). Briefly, a 8-0 nylon monofilament coated with silicone was inserted from the left common carotid artery via the internal carotid to the base of the left middle cerebral artery (MCA) of 12-week old mice anesthetized with 5% halothane and maintained on 1%. After 20 minutes or 2 hours of occlusion, the filament was withdrawn; and the arteries were re-perfused while the left common carotid artery was permanently ligated. Occlusion and reperfusion of

the MCA was confirmed by means of fiber-shaped laser Doppler perfusion imager (Omegawave, Tokyo, Japan). We observed the mice until postoperative day 56 to examine blood flow in the ischemic region with an LDPI and motor function with a rota-rod exercise test.

Immunohistochemical examination of the ischemic striatum

After the induction of 20m-MCAO, mice were sacrificed on postoperative days 0 to 56 and the harvested brains were subjected to immunohistochemical examination using a standard procedure described elsewhere (20). We used these primary antibodies: neuronal marker, NeuN (1:200, Chemicon); astrocyte marker, GFAP (1:400, Chemicon); apoptosis marker, ssDNA (1:50, DAKO); leukocyte marker, CD45 (1:100, Pharmingen); endothelial marker, PECAM-1 (CD31) (1:100, Pharmingen); and a marker for proliferating cells, BrdU (1:50, Molecular probe); to examine infarct area, gliosis, leukocyte infiltration,

apoptosis, vascular regeneration and neurogenesis. Briefly, free-floating 30-μm coronal sections at the level of the anterior commissure were stained and observed with a confocal microscope (LSM5 PASCAL, Carl Zeiss SMT AG, Oberkochen, Germany). The infarct area (mm^2/field) was defined and quantified as the region where loss of NeuN immunoreactivity was observed and gliosis (mm^2/field) as the area stained with glial fibrillary acidic protein (GFAP) in the ischemic striatum at x5 fields. CD45 or single strand DNA (ssDNA) positive cells (cells/mm^2) were quantified to serve as an index of leukocyte infiltration or of apoptosis, respectively, in the ischemic core at x20 magnification. Capillary density was quantified as the number of PECAM-1 positive cells (cells/mm^2). The vessel counts were performed in the region of ischemic core at 0.5 ~ 1.0 mm anterior from the bregma. We prepared two thin sections (6μm thickness) per a mouse for vessel counting and four representative fields from each section were evaluated for capillary density in the ischemic core. To examine neurogenesis,

mice were intraperitoneally injected with bromodeoxyuridine (BrdU 50 mg/kg; Sigma-Aldrich Co., St. Louis, MO) twice daily on postoperative days 4 to 6 and the number of BrdU - NeuN double positive cells (cells/mm^2), which are generally defined as regenerated neurons, were quantified to serve as an index of neurogenesis. We also examined the production of reactive oxygen species (ROS) in situ by using the oxidative fluorescent dye dihydroethidium (diHE; 2 x10^{-6} M; Sigma).

Quantification of CD34$^+$ mononuclear cells after 20m-MCAO

We counted peripheral CD34$^+$ mononuclear cells according to the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines (21). Briefly, peripheral blood was taken from the orbital vein and stained with CD34-PE and CD45-FITC monoclonal antibodies (BD Pharmingen, San Jose, CA) in a TruCOUNT tube (BD Pharmingen) according to the

manufacturer's instruction. After the reaction, $CD34^+$ - $CD45^{dim}$ cells were quantified as $CD34^+$ mononuclear cells by a FACS machine Aria (BD) by using the ISHAGE sequential gating strategy (21).

Analysis of infarct volume and brain edema after 2-hour MCAO

We performed 2h-MCAO to examine the effect of AM in the acute phase of fatal stroke. To estimate infarct or edema volume, mice were sacrificed 24 hours after the occlusion. The brain was removed and cut into 2mm-thick slices and immersed in saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 4C. Infarct or edema volume was calculated as the percentage volume of the contralateral hemisphere with a standard procedure as described elsewhere (22). We estimated Evans Blue leakage in the brain parenchyma as previously reported (23), to serve as an index of vascular permeability in situ. Briefly, 0.2 ml of 2.5 % Evans Blue solution was injected into mice via a tail vein

10 min before 2h-MCAO and mice were sacrificed at 24 hours after the ischemia. Brain tissues were weighed and homogenized in 50% trichloroacetic acid solution to extract the dye in the supernatant. The tissue content of Evans Blue was estimated from the absorbance of 620nM.

Estimation of apoptosis and differentiation of neuronal cells

The ratio of apoptotic cells was examined using normal human neuronal progenitor cells (NHNP; Cambrex Bioscience, Walkersville, MD). Cells were plated at a density of 5 x 10^4 cells/cm^2 on a laminin coated 24-well dish and incubated in serum-free NBM (Neuronal basal medium) for 48 hours. After the experimental period, the cell number was assessed by MMT assay (Nakalai Tesque), and the cells were stained with an anti-ssDNA antibody and nuclear staining propidium iodide (PI) to calculate the ratio of apoptotic cells to the total cells in each microscopic image.

Neuronal differentiation was examined as described previously (24), using rat pheochromocytoma PC12 cells (Riken Gene Bank, Tsukuba, Japan). Briefly, the length of the neuronal process (μm/cell) was calculated to serve as an index of neuronal differentiation after plating at a density of 10^4 cells/cm^2 on a collagen I-coated 24-well dish and incubated in 1% serum D-MEM (Dulbecco's modified eagle medium) for 7 days. The cells were treated with 10^{-5}mol/l AM or 100ng/ml nerve growth factor (NGF) as a positive control, and with the following inhibitors: the two AM antagonists, 10^{-5}mol/l AM(22-52) and 10^{-5}mol/l calcitonin gene-related peptide (8-37) (CGRP(8-37)) (Peptide Institute Inc., Osaka, Japan), the two PKA inhibitors, 10^{-5}mol/l adenosine 3P,5P-cyclic monophosphorothioate Rp-isomer (Rp-cAMP) and 10^{-6}mol/l myristoylated cell-permeable PKA inhibitor peptide sequence (14-22) (PKA Inh), and the two phosphatidylinositol 3-kinase (PI3K) inhibitors, 10^{-5}mol/l LY294002 and 10^{-7}mol/l wortmannin (Calbiochem, San Diego, CA). For EC co-culture

experiments, human umbilical vein endothelial cells (HUVEC; Cambrex) were plated into transwell membrane inserts at a density of 10^5 cells/cm^2.

Exogenous administration of AM and hydralazine

Recombinant human mature AM dissolved in 0.9% saline was exogenously administrated to C57BL/6J wild type mice by means of osmotic pumps (Alzet Model 2002; Alzet Osmotic Pumps Co., Cupertino, CA) at a rate of 50 ng/h, which is estimated to achieve a plasma concentration of 2 fmol/ml (25). To determine appropriate timing to start AM treatment after 20m-MCAO, we intra-peritoneally implanted the pump just after the operation (day 0), or at 24 (day 1) or 72 hours (day 3) later. We sacrificed the mice on day 7 for histological examination and the period of the exogenous AM-treatment was from day 0, 1, or 3 to day 7. In some experiments, low-dose (0.1mM) hydralazine was exogenously administrated in drinking water.

Statistics

All data were expressed as mean ± standard error (S.E.). Comparison of means between two groups was performed with Student's t test. When more than two groups were compared, ANOVA was used to evaluate significant differences among groups, and if significant differences were confirmed, each difference was further examined by means of multiple comparisons. We performed ANCOVA analysis when repeated-measurement had done, specifically, in the rota-rod test and laser Doppler flowmetry. Probability was considered to be statistically significant at $P<0.05$.

Results

Generation of transgenic mice that overproduce human AM but do not

overproduce mature PAMP

We generated seven lines of founder mice carrying the transgene and maintained three of them (lines #5, #6 and #15). Their plasma concentrations of human total AM were 585.5±117.7, 17.6±4.4 and 142.2±18.4 fmol/ml and the copy numbers of the transgene estimated by Southern blot densitometry analysis were 11, 8 and 30, respectively (Figure 1B). The physiological concentration of mouse total AM is reportedly 5~10 fmol/ml, so that the transgenic mice were expected to overproduce AM about 100, 3, and 30 times more than endogenous AM. The three lines were designated low (#6), medium (#15), and high (#5) concentration (conc.) line according to their plasma AM concentration. The high conc. line (#5) was used for further study unless otherwise indicated. The plasma concentration of human mature AM, the bioactive amidated form, increased to 2.6~24.9 fmol/ml in the AM-Tg mice (Table 1). On the other hand, plasma human mature PAMP did not change in

AM-Tg mice. The concentration (fmol/ml) was 2.21±0.58 in Wt versus 2.15±0.35 in AM-Tg (n=6), so that the point mutation on the amidation signal in the PAMP coding region was expected to successfully inhibit maturation of PAMP. There were no apparent differences in overall appearance, behavior, growth or fertility between Wt and AM-Tg mice. The systolic BP in 12-week old mice was significantly reduced in all three lines of AM-Tg compared to Wt. The BP (mmHg) was 122.7±1.6 in Wt versus 109.4±2.5 ~ 113.4±2.6 in AM-Tg, depending on the line ($P<0.05$; n=5; Table 1).

Therapeutic angiogenesis in hindlimb ischemia model was promoted in AM-Tg mice

The recovery of blood flow in the ischemic hindlimb of Wt and AM-Tg mice was compared, and was found to have significantly improved in AM-Tg mice after postoperative day 17. The hindlimb blood flow ratio on day 28 (ipsilateral/

contralateral, %) was 56.6±8.3 in Wt versus 73.8±5.3 in AM-Tg ($P<0.05$; n=6; Figure 1C-D). In this way, promotion of therapeutic angiogenesis by AM was confirmed in AM-Tg mice.

Brain remodeling in ischemic striatum after 20m-MCAO

We investigated the time course of neuronal loss, reactive gliosis, vascular regeneration and neuronal regeneration, the entire process can be defined as "Brain remodeling" after ischemia.

20m-MCAO caused selective loss of NeuN positive cells and marked reactive gliosis (Figure 2A) in the ipsilateral striatum within 24 hours after the operation; this condition was different from pan-necrosis caused by longer MCAO (e.g. 2h-MCAO). The infarct area, that is, the area of neuronal loss, expanded progressively up to day 7, and then showed gradual increase in size until day 56, while gliosis spread in parallel. The expansion of the infarct area

in the subacute to chronic phase after mild stroke was compatible with previously reported findings (26). Vascular regeneration in the striatum with enhanced capillary density was obvious after postoperative day 7, and subsequent neurogenesis became obvious after day 28.

The concentrations of the overproduced human AM (fmol/g tissue) in the ischemic brain of AM-Tg mice before and on postoperative day 1 and 28 after 20m-MCAO were 27.8±10.3, 87.4±4.0 and 30.3±16.8, respectively. Those of endogenous mouse AM (fmol/g tissue) were 3.7±2.1, 7.2±2.5 and 4.6±3.0.

Infarct area and gliosis were reduced in AM-Tg mice after 20m-MCAO along with suppression of leukocyte infiltration and ROS production

A significant decrease in infarct area and gliosis was observed in AM-Tg mice (Figure 2B-E) after postoperative day 7, but was not obvious on day 3. The infarct area (mm^2/field) on day 56 was 0.88±0.08 in Wt versus 0.64±0.08 in

AM-Tg ($P<0.05$; n=12; Figure 2D), and gliosis (mm^2/field) on the same day was 0.76±0.08 in Wt and 0.56±0.07 in AM-Tg ($P<0.05$; n=12; Figure 2E). Leukocyte infiltration quantified as the number of $CD45^+$ cells was significantly suppressed in AM-Tg mice especially from days 3 to 7. $CD45^+$ cells on day 3 ($/mm^2$) numbered 197.5±16.6 in Wt versus 140.7±14.6 in AM-Tg ($P<0.05$; n=12; Figure 3A-B, G). In situ ROS production detected by immunostaining for dihydroethidium (diHE), which stained the nucleus of $NeuN^+$ or $GFAP^+$ cells, was enhanced in Wt compared to that in AM-Tg mice (Figure 3C-D). Apoptotic cells quantified as the number of $ssDNA^+$ cells in the ischemic core were significantly reduced in the AM-Tg mice on days 3 to 7. $ssDNA^+$ cells ($/mm^2$) on day 3 numbered 214.8±19.6 in Wt versus 123.2±11.1 in AM-Tg ($P<0.01$; n=12; Figure 3E-F, H).

Vascular regeneration was augmented in AM-Tg mice after 20m-MCAO

associated with increased mobilization of CD34+ mononuclear cells

The blood flow in the ischemic brain estimated by LDPI was significantly higher in AM-Tg mice after postoperative day 7 and higher flow was maintained until day 56. The brain blood flow ratio (ipsilateral/ contralateral, %) on day 56 was 88.9±2.8 in Wt versus 97.6±3.0 in AM-Tg ($P<0.01$ by ANCOVA analysis; n=8; Figure 4C-D, H). We were also able to confirm that capillary density determined as the number of PECAM-1+ cells was augmented in AM-Tg mice. The density (/mm^2) on day 56 was 468.8±21.8 in Wt versus 536.6±13.6 in AM-Tg ($P<0.05$; n=8; Figure 4I). Thus, the physiological neo-vascularization in the ischemic core after stroke was augmented in AM-Tg mice. Peripheral CD34+ mononuclear cells were physiologically enhanced after 20m-MCAO and further increased in AM-Tg mice on days 3 to 7. The cells (/ml) on day 3 numbered 1774±272 in Wt versus 3199±562 in AM-Tg ($P<0.05$; n=6; Figure 5A-C).