Augmented neurogenesis and improved recovery of impaired neurological function were observed in AM-Tg mice after 20m-MCAO

BrdU injection on postoperative days 4~6 proved that most BrdU-positive cells were co-stained with GFAP (data not shown) and that there were far fewer BrdU-PECAM-1 or BrdU-NeuN double positive cells. We found that regenerated neurons defined as BrdU-NeuN double positive cells were frequently detected adjacent to the vasculature and the number of these cells on day 56 was correlated with capillary density ($P=0.003$; $n=12$; Figure 6A-B, Table 2). The cells increased from postoperative days 7 to 56 and their number was significantly higher in AM-Tg mice. The regenerated neurons (/mm^2) on day 56 numbered 20.4±3.9 in Wt versus 33.9±4.7 in AM-Tg ($P<0.05$; $n=12$; Figure 6C).

Recovery of impaired motor function after 20m-MCAO, quantified as the exercise time on an accelerating rota-rod from the start to collapse down, was significantly better in AM-Tg mice. The exercise time (second) on day 49 was

21.5±1.5 for Wt versus 27.1±2.0 for AM-Tg ($P<0.01$ by ANCOVA analysis; n=14; Figure 6D). To confirm whether vasculogenesis and neurogenesis are the contributing factor to the recovery from the ischemic damage, we analyzed the relation between capillary density, the number of regenerated neuron and the rota-rod result in AM-Tg mice after 20m-MCAO. As shown in Table 2, we found that the capillary density was significantly correlated with the rota-rod exercise time ($P=0.005$; n=24) and neurogenesis tended to be correlated with it ($P=0.08$; n=12).

Low-conc. AM-Tg mice also showed reduced infarct area and promoted vascular regeneration

We performed 20m-MCAO, using the low-conc. AM-Tg mice (plasma mature AM, 2.6±0.6 fmol/ml) as well as the high-conc. line (plasma mature AM, 24.9±4.2 fmol/ml) to determine appropriate concentration for AM treatment.

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

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

The survival rate of mice after the fatal stroke, 2h-MCAO, was 0% on day 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 hours after 2h-MCAO; although the infarct volume showed no significant difference between them.

Edema volume (% volume of contralateral hemisphere) was 13.5±1.2 in Wt versus 9.7±0.9 in AM-Tg ($P<0.05$; n=9, Figure 7C), while infarct volume (% volume of contralateral hemisphere) was 39.0±4.9 in Wt versus 44.5±7.3 in AM-Tg (not significant; n=9; Figure 7A-B). As shown in Figure 7D, we found that Evans Blue leakage into the ischemic core was significantly reduced in AM-Tg mice. The content of Evans Blue (ng/g tissue) in the ischemic brain at 24 hours after 2h-MCAO was 239.4±37.3 in Wt versus 133.9±9.4 in AM-Tg ($P<0.01$; n=4; Figure 7E).

AM exerted direct anti-apoptotic and neuro-differentiating effects on neuronal cells in vitro

After 48 hour incubation of normal human neuro-progenitor cells (NHNP) under serum-free apoptotic conditions, in which the number of the cells had decreased to half, the viable cell number was increased in the AM 10^{-8}mol/l

-treated group to 38.8±7.1% over the control ($P<0.01$; n=4; Figure 8C). The ratio of $ssDNA^+$ cells to total cells (%) was 9.8±1.9 in Wt versus 4.0±0.6 in the AM 10^{-8}mol/l-treated group ($P<0.05$; n=4; Figure 8A-B, D).

After 7-days 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; Figure 8E-I). Co-culture 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 day 7 to a comparable level to those in AM-Tg mice. Moreover, the treatment started at 24 hours after the operation (day 1) showed almost the same therapeutic effect. However, the implantation at 72 hours after the operation (day 3) failed to reveal any significant effect (Figure 9A-B). The rota-rod exercise time was significantly improved in the AM-treated group. The exercise time (second) on day 7 was 17.0±1.5 in vehicle group versus 18.1±2.0 in AM-treated group (n=6 for vehicle group and 12 for AM-treated group; $P<0.05$ by ANCOVA analysis).

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 Figure 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 non-fatal stroke. AM was also found to exert direct anti-apoptotic and neuro-differentiating effects on neuronal cells in vitro. Exogenous administration of AM in mice after 20m-MCAO also 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, while delayed neuronal loss, which may start several days

after transient ischemia, is considered to be apoptotic (27-28). Many studies have found that treatments which reduce inflammation or oxidative stress are beneficial for the prevention of apoptotic neuronal loss (29-30).

In this study, we demonstrated that AM exerts neuro-protective actions in the ischemic brain. A significant reduction in neuronal loss in AM-Tg mice after 20m-MCAO became obvious after postoperative day 7, but was not obvious before day 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 neuro-protective effects on the ischemic brain by reducing apoptotic neuronal loss through both its direct anti-apoptotic action on neurons and

indirect effect via anti-inflammation 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 day 7 after 20m-MCAO. Moreover, the infarct area kept expanding between days 7 to 28 in Wt mice, while 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 neuro-protection after 20m-MCAO, though we suppose that multiple actions of AM, as described above, could also contribute for neuro-protection.

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 vasculature, the so-called "Vascular niche" (35), where endothelial cells secrete neurogenic factors, including bFGF, VEGF and BDNF, 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. 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 day

7 with subsequent promotion of neurogenesis after day 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 neo-vascularization was preceded by augmented mobilization of $CD34^+$ mononuclear cells, which are known to differentiate into endothelial cells and contribute to vasculogenesis (37). Recently, intravenous infusion of $CD34^+$ cells has reported to promote not only neo-vascularization 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 following enhanced

neo-vascularization.

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 since 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 which is by two-order 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 SAP 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 intra-peritoneally 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 neuro-protection 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 hours after produced significant effects that were comparable to those seen in the two lines of AM-Tg mice. However, that from 72 hours postoperatively failed to reveal significant effects. These results showed that appropriate timing to start AM administration after stroke is less than 72 hours after the event.

We performed two different stroke models, non-fatal 20m-MCAO and fatal 2h-MCAO. In 2h-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 day 1 after 2h-MCAO. The result suggests that AM exerts more significant therapeutic effect on the brain tissue after non-fatal 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. bFGF and EGF) and angiogenic (e.g. VEGF and HGF) 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 neuro-protective 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 intravenous administration (39).

Thus, we are prompted to propose a new strategy to rescue ischemic brain by

using vascular hormone AM for the combined neuro-protective and vasculo-neuro-regenerative therapy to improve impaired neurological function.

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