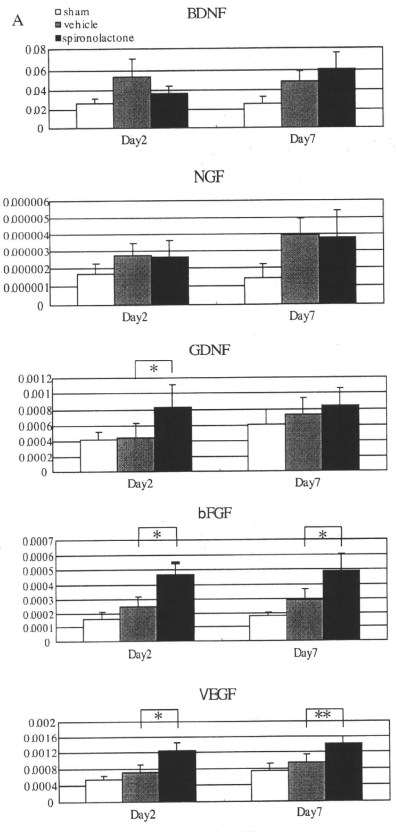

FIG. 5. Effects of spironolactone on the expression of neuroprotective and angiogenic factors in the ischemic brain on d 2 and 7 after 20-min MCAo. A, Quantitative real-time RT-PCR analysis of BDNF, NGF, GDNF, bFGF, and VEGF levels in the ipsilateral hemisphere in sham-operated (n = 8) and vehicle- (n = 12) and spironolactone-treated (n = 12) mice on d 2 and 7 after MCAo. *, $P < 0.05$; **, $P < 0.01$ spironolactone *vs.* vehicle. B–D, Representative photomicrographs showing immunostaining of bFGF (*red*), Neu-N (*blue*), and GFAP (*green*) on d 7 after MCAo in the nonischemic striatum (B) and the ischemic striatum of vehicle- (C) and spironolactone-treated (D) mice. E–G, Representative photomicrographs showing immunostaining of VEGF (*red*), Neu-N (*blue*), and GFAP (*green*) on d 7 after MCAo in the nonischemic striatum (E) and the ischemic striatum of the vehicle- (F) and spironolactone-treated (G) mice. H, Measurement of the area (square millimeters) of bFGF and VEGF positivity in the nonischemic (n = 3) and ischemic striatum of the vehicle- and spironolactone-treated mice (n = 8–11). **, $P < 0.01$. *Scale bar*, 100 μm (B–G); magnification, ×20.

$0.0039\ mm^2$ (n = 8) *vs.* $0.1335 \pm 0.0098\ mm^2$ (n = 10), $P < 0.01$; VEGF: $0.0516 \pm 0.0045\ mm^2$ (n = 10) *vs.* $0.1186 \pm 0.0067\ mm^2$ (n = 11), $P < 0.01$] (Fig. 6L).

Vascular density and blood flow in the infarct area after stroke

Because treatment with spironolactone appeared to increase the expression of angiogenic factors, we next examined the extent to which spironolactone could induce an increase in vascular density within the ischemic striatum. We found that by d 7 after MCAo, the PECAM-1-positive vascular density was clearly higher in the ischemic core of both vehicle- (Fig. 6B) and spironolactone-treated (Fig. 6C) mice than in the nonischemic striatum (Fig. 6A). At that point there was no significant difference in the vascular density (percent area) between the vehicle- and spironolactone-treated mice, however (vehicle: 7.4 ± 0.4%, n = 10; spironolactone: 8.3 ± 0.5%, n = 10) (Fig. 6F). By contrast, on d 14 after MCAo, the vascular density in the spironolactone-treated mice (n = 10) was significantly greater than in the vehicle-treated mice (n = 9) (11.2 ± 0.4 *vs.* 10.1 ± 0.4%, $P < 0.05$) (Fig. 6, D, E, and F). As shown in the representative photomicrographs (Fig. 6, G–I), the number of microspheres (*red*) in the ischemic core in vehicle- and spironolactone-treated mice was markedly higher than in the nonischemic striatum. Moreover, the relative blood flow in the spironolactone-treated mice (n = 11) was significantly higher than in the vehicle-treated mice (n = 11) (262.2 ± 36.8 *vs.* 215.1 ± 24.3%, $P < 0.05$) (Fig. 6J). Evans Blue leakage was clearly seen within the ischemic lesion in the striatum (n = 4) 24 h after MCAo but was not seen in any of the vehicle- (n = 7) or spironolactone-treated mice (n = 9) on d 14 after MCAo (data not shown). This suggests that the maturity of newly formed vessels in the ischemic striatum in both vehicle- and spironolactone-treated mice had been fully constructed at least on postoperative d 14 after cerebral ischemia, and spironolactone might have a potential to promote endogenous angiogenesis without attenuating the integrity of the vasculature.

Effect of MR blockade on neurogenesis

To examine the effect of MR antagonism on neurogenesis under ischemic conditions, we quantified the number of Dcx-positive neuroblasts migrating from the SVZ to the ischemic area on d 7 after MCAo. We detected no neuroblasts in the nonischemic striatum. On the other hand, we detected numerous migrating neuroblasts in the ischemic striatum, and there were significantly greater numbers in spironolactone-treated than vehicle-treated mice (237.9 ± 19.3 *vs.* 191.1 ± 8.4 counts/mm^2 (n = 6 in each group), $P < 0.05$) (Fig. 7, A–C).

Effect of MR blockade on infarct size after MCAo

Finally, we examined the effect of spironolactone treatment on infarct size on d 14 after MCAo. We found that, compared with vehicle, spironolactone reduced infarct area, especially at the level of bregma (section 3) and + 0.5 mm from bregma (section 4), which were seriously affected by ischemic damage in our stroke model (Fig. 8, A–C). As a

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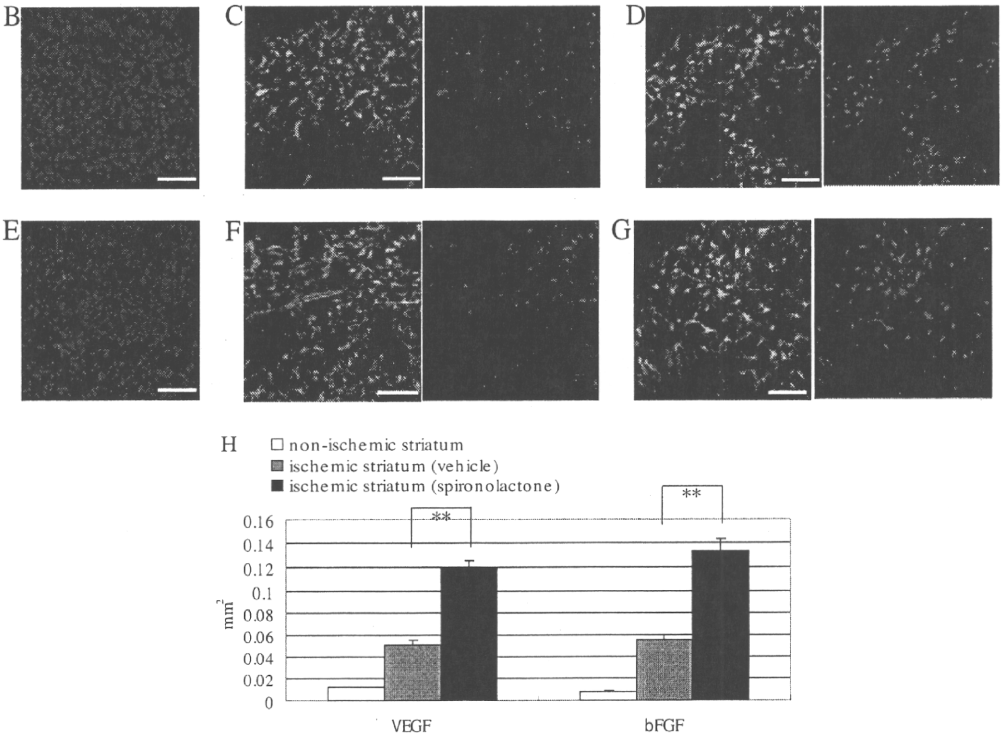

FIG. 5. *Cont.*

result, the infarct volume in spironolactone-treated mice was significantly (~10%) smaller than in vehicle-treated mice (spironolactone: 1.673 ± 0.032 mm^3, n = 8; vehicle: 1.87 ± 0.050 mm^3, n = 7; $P < 0.01$) (Fig. 8D).

Effect of MR blockade on recovery of motor function after MCAo

The exercise times of the sham-operated mice did not change during the 2-wk period of the experiment. By contrast, the exercise times of the vehicle- and spironolactone-treated mice were markedly reduced on d 2 after MCAo, after which time-dependent recovery of motor function was observed. Spironolactone-treated mice tended to have longer exercise times than vehicle-treated mice, but the difference was not significant (data not shown).

Effect of drug treatment on blood pressure and heart rate

The data summarized in Table 1 show that there were no differences between blood pressure and heart rate in vehicle- (n = 8) and spironolactone-treated (n = 8) mice and no change in blood pressure over the course of the 14-d follow-up after MCAo. Thus, the dose of spironolactone used had no effect on blood pressure. A significant increase of heart rate was observed in both groups on d 14. We assume that this is an effect of invasion itself.

Discussion

In the present study, we examined the time course of MR expression after transient cerebral ischemia, using a mouse nonfatal stroke model (20 min MCAo). In the brain, MR is generally expressed in the hippocampus and cerebral cortex but not in the striatum under normal conditions. In this study, however, we found that MR expression in the striatum was markedly increased under ischemic condition during the acute and, especially, subacute phases after MCAo. The majority of the cells expressing MR in the ischemic striatum were astrocytes, although a slight increase of the number of MR-positive neurons was detected during the acute phase. In addition, vascular endothelial cells in large vessels expressed MR, but only a small number of such vessels were detected in the nonischemic striatum, and that number was not increased under ischemic conditions. We therefore suggest that astrocytes are the key cell type involved in MR-mediated brain remodeling after cerebral ischemia.

Astrocytes, which are known to migrate to ischemic areas in the brain, are activated by chemokines and cytokines secreted from necrotic tissues and/or leukocytes infiltrating the infarct area. Once activated, astrocytes support tissue repair processes by removing debris (33) and secreting a number neurotrophic factors, including BDNF, GDNF, NGF, bFGF, ciliary neurotrophic factor and neutrotrophins 3, 4, and 5. On the other hand, they also produce various cytotoxic mediators and inflammatory cytokines, including nitric oxide (NO), TNF-α and IL-1, -6, and -8 (34). Consequently, whereas it is well recognized that astrocytes play an important role in brain remodeling after ischemia, it is less clear whether their activities are ultimately beneficial or harmful. Our present findings indicate that blockade of the up-regulated MRs on astrocytes during the acute and subacute phases after transient cerebral ischemia effectively reduces infarct size. We suggest that the neuroprotection provided

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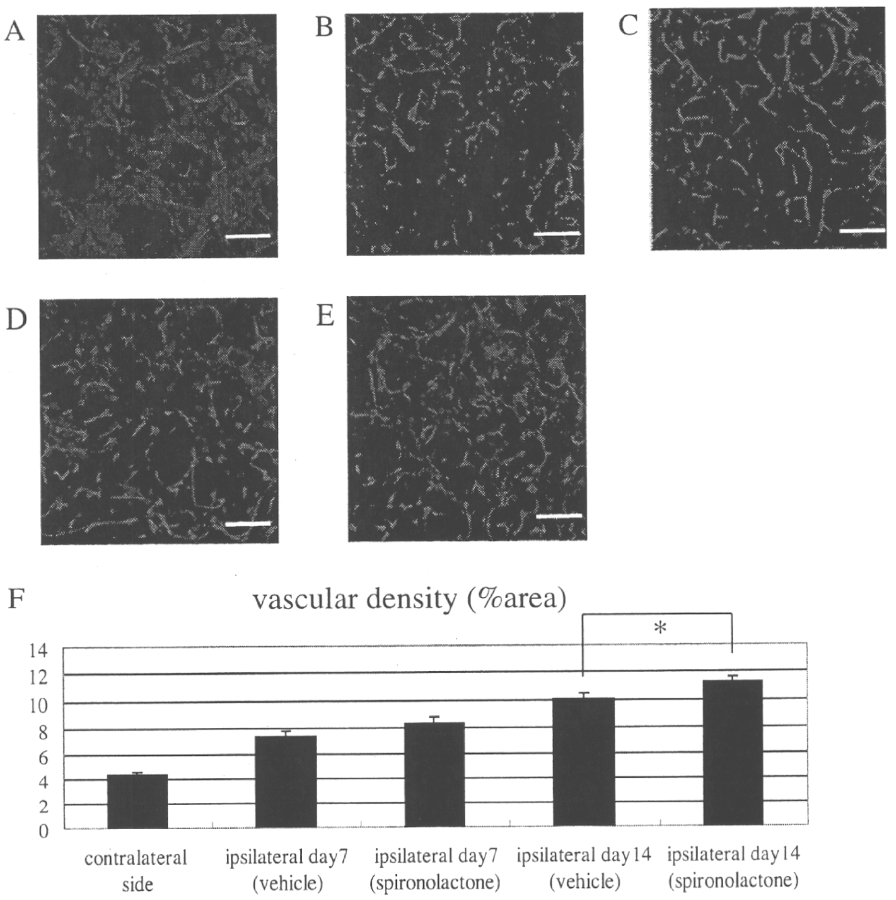

FIG. 6. Effects of spironolactone on vascular regeneration and blood flow in the ischemic striatum after 20-min MCAo. A–E, Histological examination of the vasculature in the ischemic core stained with mouse PECAM-1 (*red*) and Neu-N (*blue*). Shown are representative photomicrographs in the nonischemic striatum (A) and the ischemic striatum of vehicle- (B and D) and spironolactone-treated (C and E) mice on d 7 (B and C) and 14 (D and E) after MCAo. F, Quantitative analysis of the relative area of PECAM-1 positivity (percent area) in the nonischemic striatum (n = 5) and ischemic striatum of vehicle- and spironolactone-treated mice (n = 9–10) on d 7 and 14 after MCAo. G–I, Representative photomicrographs of sections of the nonischemic striatum of a sham-operated mouse (G) and the ischemic core of the striatum in vehicle- (H) and spironolactone-treated (I) mice on d 14 after MCAo. J, Quantitative analysis of the relative blood flow in the nonischemic striatum of sham-operated mice (n = 5) and ischemic core of the striatum in vehicle- (n = 11) and spironolactone-treated mice (n = 11) on d 14 after MCAo. The relative blood flow in the ischemic striatum in vehicle- and spironolactone-treated mice was expressed as the ratio of number of fluorescent microspheres in the ischemic core to that in the nonischemic striatum of sham-operated mice (set as 100%). *, $P < 0.05$ spironolactone *vs.* vehicle. *Scale bar*, 100 μm (A–E, G–I); magnification, ×20.

by the MR antagonist spironolactone was meditated via four mechanisms: 1) reduction of ROS production; 2) induction of bFGF and VEGF expression by astrocytes; 3) prevention of the apoptosis of neurons; and 4) enhancement of angiogenesis.

MR activation promotes oxidative stress by stimulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to increase ROS generation (35). In the heart, MR activation also stimulates activation of a number of downstream signaling pathways, leading to expression of inflammatory mediators (*e.g.* TNF-α, monocyte chemotactic protein-1, vascular cell adhesion molecule-1), fibrosis, and vascular endothelial and myocardial dysfunction. In addition, Edarabone, a potent scavenger of hydroxyl radicals, reportedly exerts an early neuroprotective effect and suppresses oxidative DNA damage in the ischemic brain (36). In the present study, ROS generation was markedly increased in the ischemic striatum after MCAo, and MR blockade by spironolactone effectively attenuated that generation. This suppression of oxidative stress led to a significant reduction in the incidence of apoptosis in the ischemic striatum of spironolactone-treated mice.

It is well known that aldosterone and cortisol (corticosterone in mice) bind to MRs with equal affinity and that MRs have a 10-fold higher affinity for corticosterone than glucocorticoid receptors (GRs) (37). It is also accepted that 11β-hydroxysteroid dehydrogenase type II (11β-HSD2) metabolizes cortisol to cortisone (11-dehydroxycorticosterone in mice), preventing it from binding to and activating MRs. In the brain, however, 11β-HSD2 activity is limited to the subcommissural organ, nucleus tractus solitarius, and amygdala (38). Because cells of the blood-brain barrier (BBB) have the ability to pump aldosterone back across the barrier, levels of aldosterone are normally low in brain tissue (39). Due to transient destruction of the BBB caused by the cerebral ischemia, however, aldosterone may enter the ischemic striatum and bind to MR. Spironolactone may thus provide neuroprotection in the damaged ischemic striatum, in part by suppressing MR activation by aldosterone until the BBB can be restored. On the other hand, adrenalectomy promotes neurogenesis in the hippocampus (40), which suggests glucocorticoid released from the adrenal glands readily enters the brain and exerts effects in the hippocampus via MRs and/or GRs. It therefore seems plausible to us that because 11β-HSD2 is not present in the striatum, and because MR expression is markedly up-regulated in the ischemic striatum, cortisol is able to exert effects in the ischemic striatum via formation of glucocorticoid-MR complexes. Furthermore, elevation of ROS levels reportedly leads to activation of the glucocorticoid-MR complex (41). Thus, by reducing ROS levels, spironolactone may also contribute to neuroprotection after cerebral ischemia by suppressing both oxidative DNA damage and activation of the cortisol-MR complex.

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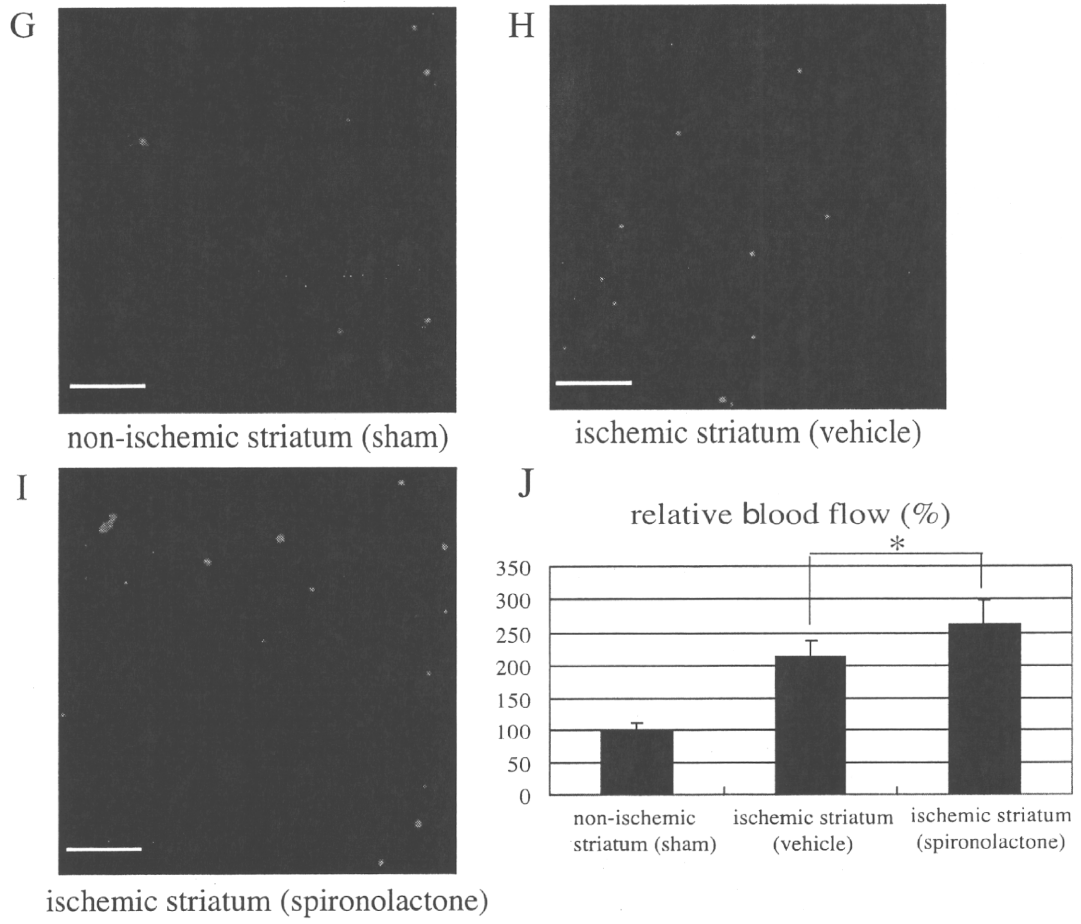

FIG. 6. *Cont.*

It is well known that cerebral ischemia up-regulates the expression of bFGF (42, 43). Recent studies suggest that bFGF supports the survival of brain neurons in culture and protects them from anoxia, hypoglycemia, and ROS (44–46). In addition, one report suggests bFGF is expressed by activated astrocytes in brain (47), whereas another suggests bFGF reduces DNA fragmentation and prevents down-regulation of the antiapoptotic protein Bcl-2 in the ischemic hemisphere after permanent MCA occlusion (48). In the present study, we detected numerous bFGF-positive astrocytes in the ischemic striatum on postoperative d 7 and found that bFGF antigenicity was up-regulated by spironolactone. This suggests spironolactone may act to protect damaged neurons from apoptosis, at least in part, by increasing of bFGF-expression in the ischemic core.

It also has been shown that that the angiogenic factor VEGF (49) is increased in the ischemic striatum (43). Consistent with those findings, we observed that after induction of MCAo in mice, expression of VEGF was clearly increased in the ischemic striatum, mainly in migrating astrocytes, and that spironolactone further enhanced VEGF expression in astrocytes on postoperative d 7. The up-regulated expression of both bFGF and VEGF would be expected to promote angiogenesis in the ischemic striatum, as was observed in spironolactone-treated mice on postoperative d 14. Increased vascularity is reportedly associated with improved neurological recovery in human stroke patients (50). Given that neovascularization provides trophic support to and removes toxic products from damaged cells, including neurons, we suggest that astrocytes also exert a neuroprotective effect in the ischemic brain by expressing VEGF and bFGF and that spironolactone enhances that effect, in part by promoting neovascularization via up-regulated expression of these angiogenic/neuroprotective factors. Moreover, the relative blood flow in the ischemic striatum was significantly increased in spironolactone-treated mice on d 14 after MCAo, which suggests spironolactone effectively increases the size of the vascular bed formed after cerebral ischemia.

Throughout the life of adult animals, neurogenesis occurs primarily in the SVZ of the lateral ventricle and in the dentate gyrus of the hippocampus (51, 52). In addition, one recent study demonstrated that after induction of transient ischemia, Dcx-positive neuroblasts migrate into damaged striatum and differentiate into mature neurons to replace the dead ones (53). However, it was further shown that more than 80% of these newly formed neurons ultimately die, most likely because of unfavorable environmental conditions, including a lack of trophic support and exposure to toxic products from damaged tissues. It was also shown that bFGF can increase the number of neuroblasts migrating from the SVZ

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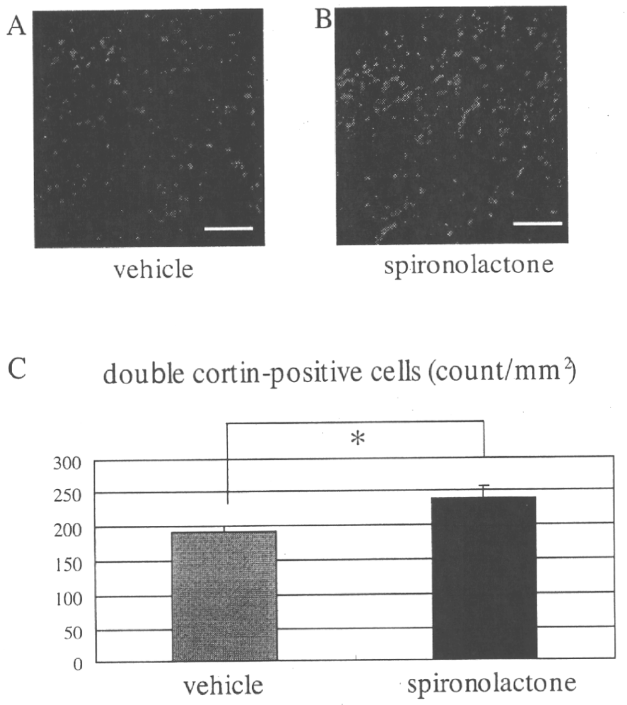

FIG. 7. Effects of spironolactone on migration of neuroblasts toward the ischemic striatum after 20-min MCAo. A and B, Immunostaining of Neu-N (*blue*) and Dcx (*red*) in the ischemic striatum of vehicle- (A) and spironolactone-treated (B) mice on d 7 after MCAo. C, Quantitative analysis of the numbers (counts per field) of Dcx-positive neuroblasts in the ischemic striatum of vehicle- and spironolactone-treated mice (n = 6/each group). *, $P < 0.05$. *Scale bar*, 100 μm (A and B); magnification, ×20.

(54). Perhaps the notable increase of the expression of bFGF and the promotion of neovascularization induced by MR suppression might contribute to protect Dcx-positive neuroblasts from ischemic damage until they are able to differentiate into new neurons.

Although the neuroprotective effects provided by spironolactone may contribute to a reduction in infarct volume after MCAo, significantly better recovery of motor function was not seen in spironolactone-treated mice after MCAo. We think that because the infarct area was confined to the striatum in our stroke model and the volume of the infarct induced by 20-min MCAo was not large, the significant reduction in infarct volume seen in spironolactone-treated mice was not sufficient to enable evaluation of neurological changes during the acute and subacute phases after MCAo.

It has been reported that aldosterone receptor blockade prevents up-regulation of vascular endothelin-1 and restores endothelial function after disruption by NO in the 11β-HSD2-deficient hypertensive rat (55). A more recent study using spontaneously hypertensive rats also suggests that treatment with eplerenone normalizes the aortic media to lumen ratio and acetylcholine-induced relaxation by enhancing expression of endothelial nitric oxide synthase and reduces oxidative stress. In addition, aldosterone reportedly contributes to alterations in vessel structure and function by reducing NO availability (56). Because in the present study MR expression in the ischemic striatum was already up-regulated during the acute phase after transient cerebral ischemia, we treated mice with spironolactone 48 h before induction of MCAo to fully examine its effects on brain remodeling during the that phase. However, large vessels, a small number of which are present in the striatum, express MR under normal conditions. Consequently, there is the possibility that spironolactone administered before MCAo might increase cerebral blood flow after transient cerebral ischemia, in part by enhancing production of endothelial nitric oxide synthase in blood vessels, thereby influencing brain remodeling.

It is noteworthy that despite the observed beneficial effects of spironolactone, some evidence suggests that MR activation is necessary for neuroprotection. For instance, MR blockade beginning 1 h before induction of transient global ischemia resulted in increased cell death (57). Moreover, overexpression of human MR in PC12 cells prevented staurosporine- and oxygen/glucose deprivation-induced cell death, and spironolactone attenuated that effect (58). Lai *et al.* (59) also demonstrated that, compared with wild-type mice, transgenic mice overexpressing MR specifically in their forebrain show significantly reduced neuronal death in the hippocampus, improved spatial memory retention, reduced anxiety, and altered behavioral responses to novelty after transient global cerebral ischemia. Our observation that some neurons in the ischemic striatum were expressing MR 1 d after MCAo suggests MR blockade may have some direct negative effect on ischemic neurons during the hyperacute phase. On postoperative d 2–28, however, the number of MR-expressing neurons declined, and the majority of the cells expressing MR in the ischemic striatum were astrocytes. In addition, our findings indicate that blockade of MR in astrocytes migrating to the ischemic core after MCAo appear to protect damaged neurons via indirect effects. Consequently, although our finding might seem to be inconsistent with that of Lai *et al.*, we think the significance of MR activation to brain remodeling differs in neurons and astrocytes.

There is an interesting report that suggests synaptic function and cellular integrity in the hippocampus can be preserved after unilateral cerebral hypoxia/ischemia (HI) by preventing an ischemia-induced rise in plasma corticosteroid levels (60). HI-induced impairment of synaptic transmission in the CA1 area of the hippocampus is exacerbated by concomitant corticosteroid treatment and alleviated by treatment with the steroid synthesis inhibitor metyrapone. Similarly, degenerative changes in the hippocampus seen after HI are exacerbated by corticosterone but reduced by metyrapone. Kloet and Derijk (61) suggested that although MRs maintain neuronal homeostasis and limit stress-induced disruption, GRs promote recovery after a challenge and storage of the experience, which aids in coping with future encounters. Imbalance in MR/GR-mediated actions compromises homeostatic processes in these neurons, which is thought to lead to maladaptive behavior and hypothalamic-pituitary-adrenal dysregulation that may, in turn, lead to aberrant metabolism, impaired immune function, and altered cardiovascular control. Our experiments are focused on the effects of MR suppression, mainly in the damaged ischemic striatum.

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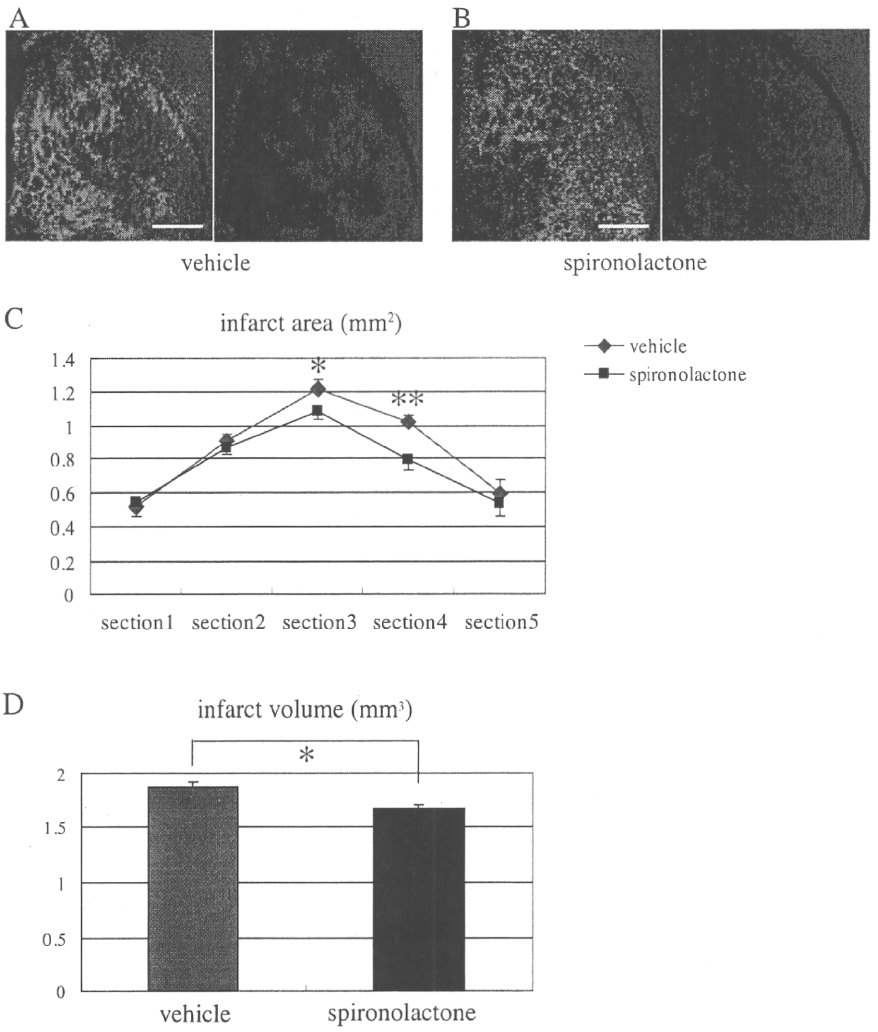

FIG. 8. Effects of spironolactone on infarct size after 20-min MCAo. A and B, Representative fluorescence photomicrographs showing the ischemic striatum of vehicle- (A) and spironolactone-treated (B) mice on d 14 after MCAo. The *black area*, in which Neu-N-positive neurons are not observed, is the infarcted area. C, Measurement of the infarct areas (square millimeters) in the ischemic striatum in five coronal sections (−1, −0.5, ± 0, +0.5, and +1 mm from bregma) in vehicle- (n = 7) and spironolactone-treated (n = 8) mice. *, $P < 0.05$; **, $P < 0.01$ spironolactone *vs.* vehicle in the corresponding section. D, Measurement of the infarct volume (cubic millimeters) in the two treatment groups. *, $P < 0.05$. *Scale bar*, 500 μm (A and B); magnification, ×5.

Because GR is more widely expressed in the brain, even under normal conditions, there is a possibility that administration of spironolactone might promote formation of a glucocorticoid-GR complex, which could lead to an imbalance in MR/GR-mediated actions in neurons within nonischemic lesions in areas such as hippocampus. In future experiments, it would be useful to clarify the effects of spironolactone in nonischemic lesions in areas other than the striatum in this animal stroke model.

In conclusion, our findings provide evidence that expression of MR is enhanced during the acute and subacute phases after transient cerebral ischemia, especially in the astrocytes that migrate into the ischemic core. Suppression of MR-mediated signaling by spironolactone induces several beneficial effects on brain remodeling, which appears to significantly reduce infarct size. Spironolactone thus appears to exert potentially therapeutic neuroprotective effects in the ischemic brain.

TABLE 1. Blood pressures (mm Hg) and heart rates (counts per minute) in vehicle- and spironolactone-treated mice before (d 0) and 14 d after MCAo

	Vehicle	Spironolactone
Blood pressure (d 0)	111.7 ± 3.6/75.1 ± 1.9	109.4 ± 4.1/74.1 ± 3.3
Blood pressure (d 14)	110.1 ± 2.3/67.9 ± 3.1	107.9 ± 3.3/68.3 ± 2.6
Heart rate (d 0)	563.9 ± 17.1	587.5 ± 15.2
Heart rate (d 14)	643.4 ± 11.7	658.3 ± 12.2

Values are means ± SE; n = 8 in each group.

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REVIEW

Translational research of novel hormones: lessons from animal models and rare human diseases for common human diseases

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Abstract Since the 1980s, a number of bioactive molecules, now known as cardiovascular hormones, have been isolated from the heart and blood vessels, particularly from the subset of vascular endothelial cells. The natriuretic peptide family is the prototype of the cardiovascular hormones. Over the following decade, a variety of hormones and cytokines, now known as adipokines or adipocytokines, have also been isolated from adipose tissue. Leptin is the only adipokine demonstrated to cause an obese phenotype in both animals and humans upon deletion. Thus, the past two decades have seen the identification of two important classes of bioactive molecules secreted by newly recognized endocrine cells, both of which differentiate from mesenchymal stem cells. To assess the physiological and clinical implications of these novel hormones, we have investigated their functions using animal models. We have also developed and analyzed mice overexpressing transgenic forms of these proteins and knockout mice deficient in these and related genes. Here, we demonstrate the current state of the translational research of these novel hormones, the natriuretic peptide family and leptin, and discuss how lessons learned from excellent animal models and rare human diseases can provide a better understanding of common human diseases.

Keywords Natriuretic peptide family (ANP, BNP, CNP) · Leptin · Translational research · Animal models · Genetically engineered mice

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Although a multitude of animal models have been developed to emulate various diseases, there are a few excellent animal models that mimic human disease remarkably well, such as spontaneously hypertensive rats (SHR) [1] and hereditary obese mice, ob/ob mice [2]. These models are very useful for translational research into the common human diseases, hypertension and obesity. Lessons from research on SHR, an excellent animal model for hypertension research, developed at Kyoto University led us to investigate the clinical importance of cardiovascular hormones and adipokines using appropriate animal models that mimic human diseases beyond species differences. In this review, we discuss the current state of translational research of the natriuretic peptide family and leptin and discuss the ways in which animal models and rare human diseases can educate about common human diseases.

Translational research of natriuretic peptide family

The natriuretic peptide family consists of three structurally related peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [3]. The biological actions of natriuretic peptides are mediated by activation of two subtypes of membranous guanylyl cyclase (GC), GC-A and GC-B, leading to

intracellular accumulation of cyclic guanine monophosphate (cGMP) [4]. The rank order of potency to induce cGMP production via GC-A is ANP ≥ BNP >> CNP, while that via GC-B is CNP > ANP ≥ BNP [5]. Thus, ANP and BNP serve as endogenous ligands for GC-A, while CNP is specific for GC-B. A third natriuretic peptide receptor with no intracellular GC domain, dubbed the clearance receptor (C-receptor), is thought to be engaged in the receptor-mediated degradation of natriuretic peptides [4]. The ANP, BNP/GC-A system plays a pivotal role in the regulation of cardiovascular homeostasis, as demonstrated by their augmentation in various pathophysiological states such as heart failure [6–10], myocardial infarction [11, 12], cardiac hypertrophy [13, 14], and hypertension [15–17]. ANP and BNP are cardiac hormones secreted primarily by the atrium and ventricle of the heart, respectively [10, 17], with strong diuretic, natriuretic, and vasodilatory activities [6, 7, 10]. ANP and BNP are used in the treatment of heart failure [18, 19] and serve as sensitive biochemical markers for heart failure and cardiac hypertrophy [8–10]. ANP infusion therapy has currently reached a greater than 30% share among drugs given for acute congestive heart failure in Japan.

CNP, the third member of natriuretic peptide family, was first purified from porcine brain [20]. While CNP is the primary natriuretic peptide in the human brain [21], it is also produced by vascular endothelial cells [22–24] and macrophages [25]. This hormone functions in the regulation of vascular endothelial function and arteriosclerosis via local effects, not by acting as a circulating hormone [26–28]. These observations indicate that CNP acts as an autocrine/paracrine regulator and as a neuropeptide [21].

The distribution of the natriuretic peptide system overlaps with the distribution of the renin–angiotensin system [21, 29–33], prompting us to examine the functional relationship of the natriuretic peptide system and the renin–angiotensin system. We demonstrated an antagonistic relationship between these two systems, both in their peripheral functions as well as their central actions [34–39]. Furthermore, the natriuretic peptide system has therapeutic implication in vascular regeneration in patients with arteriosclerosis obliterans [40].

Mice with genetic alterations in the ANP, BNP/GC-A system

Genetically engineered mice are useful tools to study the complex phenotypic effects of an altered gene in living animals. Overexpression or deficiency of each member of the natriuretic peptide family or its receptors has been generated through transgenic (Tg) or knockout (KO) technologies [41–45]. We generated Tg mice expressing BNP under the control of the serum amyloid P (SAP) component promoter, which targets hormone expression to the liver [43]. BNP-Tg mice exhibited a 100-fold increase in plasma BNP concentrations with concomitant elevations in plasma cGMP concentrations. These mice displayed significantly lower blood pressures and smaller hearts than non-Tg littermates. These results indicate that BNP functions in the long-term cardiovascular regulation and may be useful as a long-term therapeutic agent. In addition, the proteinuria and renal dysfunction observed in anti-GBM nephritis [46], the nephrosclerosis induced by subtotal nephrectomy [47], and the manifestations of diabetic nephropathy [48] were ameliorated in BNP-Tg mice compared to those in wild-type mice, indicating a possible application for the natriuretic peptide family in the treatment of renal disorders.

We also generated mice bearing a targeted disruption of the BNP gene [44]. At baseline, BNP-KO mice did not show any signs of systemic hypertension or ventricular hypertrophy; however, these animals developed multifocal fibrotic lesions within the cardiac ventricle even in the absence of additional stresses; these lesions increased in size and number in response to ventricular pressure overload, demonstrating that BNP is an antifibrotic factor acting within the ventricle of the heart as an autocrine/paracrine regulator for ventricular remodeling [44]. In addition to these cardiovascular manifestations, BNP-Tg mice exhibited marked skeletal overgrowth via endochondral bone formation [49]. Nevertheless, BNP-KO mice did not possess any skeletal abnormalities [44]. The skeletal overgrowth seen in BNP-Tg mice that express elevated plasma concentrations of BNP was similar to that seen in cartilage-specific CNP-Tg mice [49]. As the BNP/GC-A system does not have an abnormal skeletal phenotype [41, 42, 45], we postulated that the markedly increased circulating levels of BNP (100-fold greater than wild-type mice) may cross-react with GC-B to stimulate endochondral bone growth, even though the affinity of BNP for GC-B is lower than that for GC-A. This interpretation is supported by the finding that the skeletal overgrowth observed in BNP-Tg mice was not abrogated by a genetic deficiency of GC-A in BNP-Tg mice [50].

ANP transgenic mice expressing elevated levels of circulating ANP under the control of mouse transthyretin promoter [41] exhibited decreased arterial blood pressure without the induction of diuresis or natriuresis. ANP-KO mice and GC-A-KO mice displayed salt-sensitive and salt-resistant hypertension, respectively [42, 45]. Studies using GC-A-KO mice implicated the involvement of GC-A in antihypertrophic actions in the heart [51–53]. A more detailed analysis of GC-A was performed using mice bearing a conditional knockout of GC-A and indicated the importance of GC-A in vascular endothelial-cell-mediated blood pressure control [54–56].

As for the regulation of ANP and BNP gene expression, neuron-restrictive silencer elements (NRSEs) are located in the 5′-flanking region of the BNP gene and the 3′-untranslated region of the ANP gene [57]. The neuron-restrictive silencer factor (NRSF) can thus repress ANP promoter activity through binding to NRSE [58]. Studies examining dominant-negative NRSF Tg mice expressed under the control of the α-myosin heavy-chain promoter have demonstrated that NRSF plays an important role in the gene expression of both ANP and BNP and in the progression of cardiac dysfunction and lethal arrhythmia associated with heart failure [59].

Genetically engineered mice of the CNP/GC-B system

We generated mice with a targeted disruption of the CNP gene; the resultant CNP-KO mice exhibited markedly short stature due to impaired bone growth [60]. Mammalian bones are formed through two different mechanisms, endochondral ossification and membranous ossification. Most mammalian bones are formed through endochondral ossification, a process during which chondrocytes in the growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement [61]. The short-stature phenotype of CNP-KO mice resulted from impaired bone growth through endochondral ossification [60]. CNP-Tg mice with targeted overexpression of CNP at the growth plate cartilage exhibited prominent overgrowth of those bones formed through endochondral ossification [62]. GC-B-KO mice exhibit the same short-stature phenotype as observed in CNP-KO mice [63], demonstrating that the CNP/GC-B system is a physiologically important stimulator of endochondral bone growth. Dominant-negative GC-B transgenic rats displayed blood-pressure-independent cardiac hypertrophy, suggesting evidence linking GC-B signaling to the control of cardiac growth [64].

cGMP-dependent protein kinase (cGK) has been identified as a molecule activated downstream of the natriuretic peptide family and GC system [65]. Mice depleted with the gene of one subtype of cGK, cGKII (cGKII-KO mice), exhibit a short-stature phenotype secondary to impaired endochondral bone growth [66], similar to that observed in CNP-KO mice [60]. We demonstrated that cGKII affected endochondral bone growth by functioning downstream of the CNP/GC-B system by showing that the impaired endochondral bone growth observed in cGKII-KO mice could not be rescued by targeted overexpression of CNP in the growth plate cartilage [67].

Multiple spontaneous animal models with impairments in the CNP/GC-B system have been identified [68–71]. Two strains of dwarf mice, with an autosomal recessive mutant gene, named cn/cn [68] and short-limbed dwarfism (SLW) mice [69], possess spontaneous loss-of-function mutations in the *GC-B* gene. Spontaneous mutant mice with a loss-of-function mutation in the CNP gene, named long bone abnormality (Lbab) mice, exhibit short-stature owing to their impaired endochondral bone growth [70], and this phenotype could be abrogated by targeted overexpression of CNP in the growth plate cartilage [71].

Clinical application of CNP and its analogs for skeletal dysplasia

To explore the potential applications of CNP and its analogs for clinical use, we attempted to apply the strong effect of CNP and GC-B on endochondral bone growth to skeletal dysplasia, a group of genetic disorders characterized by severely impaired bone growth [72]. Achondroplasia (Ach), the most common form of skeletal dysplasia characterized by short-limbed dwarfism, is caused by constitutive activation of fibroblast growth factor (FGF) receptor 3 [73]. The current therapy for Ach is limited to distraction osteogenesis [74], an orthopedic procedure; no efficient medical therapies have been developed as yet. We demonstrated that targeted overexpression of a CNP transgene in the growth plate cartilage of a mouse model of achondroplasia (Ach mice) rescues their impaired bone growth and short-stature phenotypes [62] (Fig. 1). To elucidate the molecular

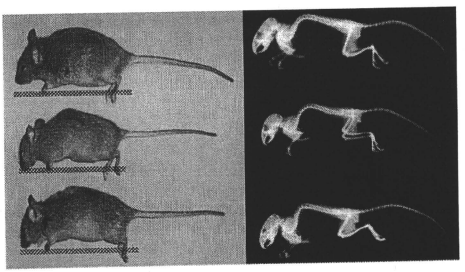

Fig. 1 Rescue of achondroplastic mice (Ach mouse) by targeted overexpression of CNP in growth plate cartilage. From *top* to *bottom* are shown the gross appearance (*left panel*) and skeletal phenotype (*right panel*, soft X-ray picture) of female wild-type mice (*Wt*), Ach mice (*Ach*), and Ach mice overexpressing CNP in the growth plate cartilage (*Ach/CNP-Tg*) at an age of 3 months

mechanism by which CNP ameliorates achondroplasia, we examined the effect of CNP on extracellular signal-regulated kinase (ERK) signaling. CNP inhibited FGF2-stimulated phosphorylation of ERK in a dose-dependent manner through cGMP activation via GC-B ligation, ultimately increasing matrix synthesis by chondrocytes [62].

We also demonstrated that systemic and continuous administration of synthetic CNP is safe and effective to reverse the impaired bone growth seen in Ach mice [75] (Fig. 2). The safety and efficacy of systemic CNP administration in preclinical studies with the observation that CNP has only a minimal effect of blood pressure in humans [76] suggest that systemic administration of CNP or CNP analogs provides a novel therapeutic strategy for the treatment of human skeletal dysplasia, including Ach.

One form of human skeletal dysplasia, acromesomelic dysplasia type Maroteaux, is caused by loss-of-function mutations in the GC-B gene [77]. This implicates the CNP/GC-B system as a physiologically important enhancer of endochondral bone growth in humans, suggesting a clinical application for CNP and CNP analogs to multiple types of human skeletal dysplasia [75].

In the near future, idiopathic short stature, a common disease of short-stature phenotype with an unknown etiology, and bone fracture, the healing of which is made through endochondral ossification, would be the next avenues to explore for a therapeutic effect of CNP treatment.

Translational research of leptin

Leptin, an adipocyte-derived hormone originally identified from hereditary obese mice (ob/ob mice) [78], plays crucial physiologic roles in the regulation of energy expenditure and food intake [79–83]. Mice [84] and rats [85, 86] bearing mutations in leptin receptors demonstrate identical phenotypes as ob/ob mice. The Koletsky rat, an obese substrain of SHR serving as a model of metabolic syndrome exhibiting both hypertension and morbid obesity, was discovered to carry an additional nonsense mutation of the leptin receptor [86].

In obese animals and subjects, plasma leptin concentrations are increased in proportion to the degree of adiposity [87–89], indicating that leptin is a satiety signal communicating the size of adipose stores to the brain [90–92] and that leptin resistance is related to obesity [87, 93–95]. Leptin deficiency in human subjects is associated with morbid obesity with insulin resistance, indicating the physiological role of leptin in both animal models and humans [96, 97]. Leptin is implicated in a number of manifestations seen in obese animal models [91, 98–101], especially obesity-related hypertension [99], abnormal reproduction [98], bone changes [100], and Cushing syndrome [102]. Leptin is also produced by human placenta [103] and choriodecidual tumors [104].

Generation of Tg mice overexpressing leptin

To explore the clinical implications of leptin *in vivo*, we generated leptin-Tg mice displaying elevated plasma leptin concentrations comparable to those seen in obese subjects [105]. A fusion gene comprised of the human SAP promoter upstream of the mouse leptin cDNA coding sequences was designed to target hormone expression to the liver [43, 106]. Overexpression of leptin in the liver resulted in the complete disappearance of both white and brown adipose tissues in mice [105]. Such a phenotype did not occur when transgene expression was targeted to adipose tissue, the endogenous site of leptin production, using adipocyte-specific promoters [107]. The hyperlepti-

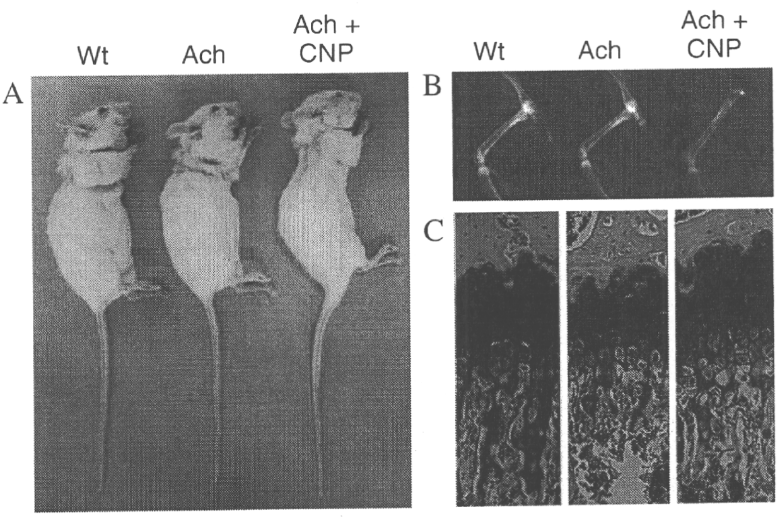

Fig. 2 Rescue of Ach mice by administration of synthetic CNP. Three-week-old female wild-type (*Wt*) or Ach mice were continuously administered CNP intravenously. The gross appearances (**a**), soft X-ray pictures of femurs (**b**), and histological pictures of tibial growth plates stained with safranin-O and hematoxylin and eosin (**c**) are shown for wild-type mice treated with vehicle (*left*), Ach mice treated with vehicle (*middle*), and Ach mice treated with 1 μg/kg per minute CNP (*right*) after a 4-week administration period. *Scale bar* in **c**, 50 μm

nemia seen in these transgenic "skinny" mice provides a unique experimental system in which the long-term effects of leptin are investigated in vivo [98–101, 105, 108, 109]. Skinny mice exhibit augmented glucose metabolism and increased insulin sensitivity of both skeletal muscle and liver [105], supporting the concept that leptin acts as an antidiabetic hormone in vivo [110–112]. These studies suggest the potential usefulness for leptin treatment of diabetes and obesity.

Crossbreeding of transgenic skinny mice with A-ZIP/F-1 mice, a mouse model of severe lipoatrophic diabetes

Generalized lipodystrophy, caused by a systemic deficiency of adipose tissue, is characterized by severe insulin resistance and hypertriglyceridemia [113]. A form of diabetes, called lipoatrophic diabetes, eventually develops, although the precise mechanism by which this paucity of fat results in diabetes has remained to be elucidated. Plasma leptin concentrations are markedly reduced or absent in patients with lipoatrophic diabetes and in rodent models of this disease [114–117]. Given leptin's antidiabetic action, leptin deficiency may play a role in the pathogenesis of lipoatrophic diabetes; thus, leptin may be a drug for lipoatrophic diabetes.

A mouse model of severe lipoatrophic diabetes (A-ZIP/F-1) was generated by expressing in adipose tissue a protein that inactivates basic-zipper transcription factors [116]. To assess the pathophysiological role and therapeutic potential of leptin in lipoatrophic diabetes, we crossed transgenic skinny (LepTg/+) and A-ZIP/F-1 (A-ZIPTg/+) mice to produce double transgenic mice (LepTg/+:A-ZIPTg/+) virtually lacking adipose tissue and expressing approximately tenfold higher levels of leptin than normal controls [118]. LepTg/+:A-ZIPTg/+ mice were hypophagic in comparison to A-ZIPTg/+ mice and exhibited decreased hepatic steatosis. Glucose and insulin tolerance tests displayed increased insulin sensitivity and normal glucose tolerance in LepTg/+:A-ZIPTg/+ mice, which was comparable to LepTg/+ mice. Pair-feeding experiments demonstrated that the effects of leptin were not solely due to decreased food intake. Leptin also helped to prevent diabetic nephropathy in generalized lipoatrophic diabetes mice [101]. These results demonstrate that leptin can improve insulin resistance and diabetic manifestations in a mouse model of severe systemic lipodystrophy, indicating that leptin is therapeutically useful in the treatment of lipoatrophic diabetes [118].

Leptin replacement therapy in Japanese patients with generalized lipodystrophy

We previously reported a novel homozygous mutation of *MC4R* in a Japanese woman with severe obesity (body mass index (BMI) 62 kg/m^2) [119]. *MC4R* mutations have been identified at a relatively high frequency (3–4%) in morbidly obese patients in Europe; all of the mutations reported to date occur in an autosomal-dominant fashion, with the exception of a single unique pedigree in the UK. [120, 121]. Although both parents were heterozygous for the mutation, neither exhibited such a severe obese phenotype (BMI 27 and 26 kg/m^2, respectively, which are preobese according to WHO criteria). As genetic backgrounds and lifestyles vary significantly between European and Asian countries, it is necessary to examine the effect of lifestyle on the phenotypes resulting from genetic mutations and on treatment efficacy in each country.

Four-month leptin replacement therapy has been reported to improve glucose and lipid metabolism in lipodystrophy patients in the USA [122]. To elucidate the efficacy, safety, and mechanisms underlying leptin replacement therapy in Asian patients with generalized lipodystrophy, we treated seven Japanese patients, two acquired and five congenital types, with physiological replacement dose of leptin [123, 124]. Leptin replacement therapy dramatically improved fasting glucose (mean±SE, 172±20 to120±12 mg/dl, $P<0.05$) and triglyceride (mean ± SE, 700±272 to 260±98 mg/dl, $P<0.05$) levels within 1 week. Leptin replacement reduced insulin resistance, as demonstrated by the euglycemic clamp method. Improvement of

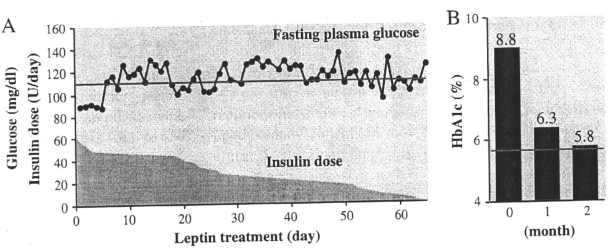

Fig. 3 **a** Daily insulin doses and fasting plasma glucose levels and **b** HbA1c levels during the first 2 months of leptin therapy in a 19-year-old male patient with congenital generalized lipodystrophy (Seipin gene mutant)

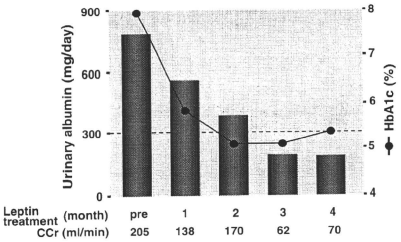

Fig. 4 Time course of daily urinary albumin secretion, creatinine clearance, and HbA1c levels during leptin treatment of a 16-year-old female patient with acquired generalized lipodystrophy

fatty liver was also confirmed by changes in computed tomography (CT) attenuation, and liver volume was calculated by CT imaging. By 4 months, six of seven patients were able to discontinue all antidiabetic drugs, including insulin (Fig. 3). The decreased fasting plasma glucose levels, triglyceride levels, and liver volumes in all seven patients were well maintained throughout the therapy period with no adverse effects. The longest period of leptin replacement therapy has now extended beyond 7 years.

Leptin treatment was also effective at combating diabetic complications. The macroalbuminuria seen in two patients regressed to microalbuminuria, while microalbuminuria in two additional patients normalized. The creatinine clearance of patients with glomerular hyperfiltration decreased with improved glucose tolerance (Fig. 4), which was consistent with previous findings in the lipoatrophic diabetes model mice [101].

We also examined the effect of leptin therapy on a 16-year-old girl with severe hypertriglyceridemia who suffered from repeated episodes of acute pancreatitis (Fig. 5). After the initiation of leptin therapy, her triglyceride levels normalized; she did not have any additional episodes of acute pancreatitis (Fig. 5). These results clearly demonstrate the safety and efficacy of the long-term leptin replacement therapy in patients with generalized lipodystrophy. While these results are impressive, it is important to remember that the efficacy of leptin replacement therapy in patients from Japan, a country in which the prevalence of obesity is relatively low, is excellent.

Leptin therapy for more prevalent forms of diabetes

To assess the therapeutic potential for leptin treatment in insulin-deficient diabetes, we generated diabetic animals by treating wild-type and LepTg/+ mice with a relatively low dose of streptozotocin (STZ 180 g/g body weight) [125]. Plasma insulin concentrations were reduced (<0.10 ng/ml), resulting in severe hyperglycemia in both wild-type and LepTg/+ mice 2 weeks after STZ treatment. LepTg/+ mice were more sensitive to exogenously administered insulin than wild-type mice; STZ-treated LepTg/+ mice became normoglycemic at doses of insulin that did not improve the hyperglycemia in STZ-treated wild-type mice. To clarify if combination therapy with leptin and insulin is beneficial for insulin-deficient diabetes, we also examined the effect of chronic coadministration of leptin and insulin in STZ-treated wild-type mice. We demonstrated that subthreshold doses of insulin, which do not affect glucose homeostasis, are effective at improving diabetes in STZ-treated wild-type mice in combination with leptin. These results indicate that leptin therapy may be used as an adjunct for insulin therapy in insulin-deficient diabetes.

We also investigated the therapeutic usefulness of leptin in a mouse model of type 2 diabetes mellitus with increased adiposity [126], generated using a combination of a low-dose STZ (120-g/g body weight) and a high-fat diet (HFD, 45% of energy as fat; STZ/HFD). In STZ/HFD mice, continuous infusion of leptin (20-ng/g body weight per hour) reduced food intake and body weight gain and improved glucose and lipid metabolism with enhanced insulin sensitivity. Leptin therapy also decreased the triglyceride content of both the liver and skeletal muscle.

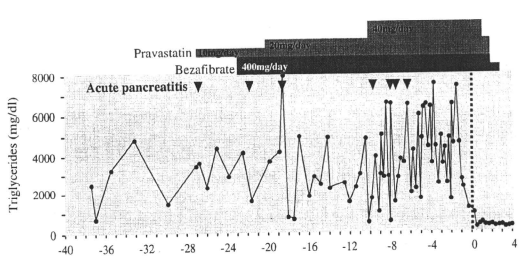

Fig. 5 Fasting serum triglyceride levels, doses of lipid-lowering drugs, and episodes of acute pancreatitis (red inverted triangle) before and after leptin therapy in a 16-year-old girl with acquired generalized lipodystrophy

These results indicate a beneficial effect of leptin therapy for type 2 diabetes mellitus with increased adiposity, which corresponds to a BMI in the range of 25–30 kg/m^2 [126].

Our previous and ongoing studies utilizing transgenic skinny mice and other animal models have demonstrated the pleiotropic actions of leptin in the regulation of energy homeostasis and food intake [98–101, 105, 108, 109] and its clinical usefulness as a therapy for multiple conditions, particularly diabetes mellitus [108, 118, 124, 125]. Tg skinny mouse may be a useful model to study the long-term effects of leptin therapy in vivo and to evaluate the clinical implications of leptin therapy.

Conclusions

Currently, the primary targets of our ongoing translational research of CNP and leptin are achondroplasia and lipoatrophic diabetes, respectively. Demonstration of the efficacy of CNP therapy for achondroplasia and leptin replacement therapy for lipoatrophic diabetes has relied heavily on basic and preclinical studies using excellent animal models. Although lipoatrophic diabetes is a rare disease in humans, the safety and efficacy of leptin replacement therapy for patients with lipoatrophic diabetes have been well established. Achondroplasia, while also a rare disease in humans, may be effectively managed with CNP therapy.

It has been possible to establish the safety and efficacy of these hormones in rare human diseases through studies that began with excellent animal models. These studies provided us with novel treatments for common human diseases, which were explored as adjacent to or in extension of these rare human diseases, as seen in the study of hypertension. Research on the SHR animal model and study of a relatively rare cause of hypertension, renovascular hypertension, led to more detailed studies on the blockade of renin–angiotensin system, bringing research forward to the current widespread field of cardiovascular disorders in translational research. These lessons teach us the importance of the breakthroughs using animal models and rare human diseases.

Conflict of interest statement The authors declare that they have no conflict of interests.

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Clinical characteristics and efficacy of pioglitazone in a Japanese diabetic patient with an unusual type of familial partial lipodystrophy

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Abstract

This report describes a 46-year-old Japanese diabetic woman with an unusual type of familial partial lipodystrophy. She has marked loss of subcutaneous fat in her lower limbs and buttocks, with sparing of the face, neck, upper limbs, and trunk. This distribution of fat atrophy appears to be rare in comparison with previous reports. Sequencing of candidate genes *LMNA*, *PPARG*, *AKT2*, caveolin-1, as well as the *PPARG4* promoter gene, which are known to be associated with familial partial lipodystrophy, revealed no genetic abnormalities, suggesting that this case may involve a novel gene. Pioglitazone was markedly effective in glycemic control in this case. Her diabetes remained uncontrolled despite a total daily dose of insulin of 30 U and combined treatment with 10 mg of glibenclamide and 0.6 mg of voglibose. We therefore attempted combined treatment with 30 mg of pioglitazone and 30 U/d insulin injection. The hemoglobin A_{1c} level was reduced from 11.2% to 6.1% after 6 months of treatment and has since remained stable. Her body weight increased from 62.0 to 71.0 kg after 12 months of treatment, suggesting that weight gain may result from synergism between thiazolidinediones and insulin-promoting adipogenesis. Pioglitazone increased the fat mass in the upper limbs and trunk, while inducing less increase in the lower limbs, where fat atrophy exists in this patient. Pioglitazone may thus have improved the glycemic control in this case through adipocyte differentiation from progenitor cells mainly in the upper limbs and trunk.
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1. Introduction

Understanding of the pathophysiology in lipodystrophy has recently improved [1-4]. Lipodystrophy is a rare disorder characterized by partial or generalized loss of adipose tissue deposits. It is commonly associated with dyslipidemia, hepatic steatosis, and insulin-resistant diabetes. Familial partial lipodystrophy (FPLD) is named after Dunnigan et al [5], who provided a detailed description of the syndrome. In some cases, the lipodystrophy is confined to the limbs, with sparing of the face and trunk, whereas the trunk is also affected with sparing of the face and vulva in other cases. Many cases of FPLD of European origin have been reported to be of the Dunningan et al type, whereas Asian cases of FPLD have only rarely been reported [2,3]. It is thus unclear whether differences in phenotype or genotype of FPLD exist between cases of European origin and of Asian origin. We present here an instructive case of a Japanese diabetic patient with an unusual type of FPLD, with the results of mutational analysis for the *LMNA*, *PPARG*, *AKT2*, and caveolin-1 gene and *PPARG4* promoter gene. We also describe the effectiveness of pioglitazone on glycemic control and the changes of fat and lean mass as measured by dual-energy x-ray absorptiometry (DEXA) scan during pioglitazone treatment.

2. Subject and methods

2.1. Blood samples

Blood was collected after a 12-hour overnight fast for analysis of glucose, insulin, leptin, and adiponectin.

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