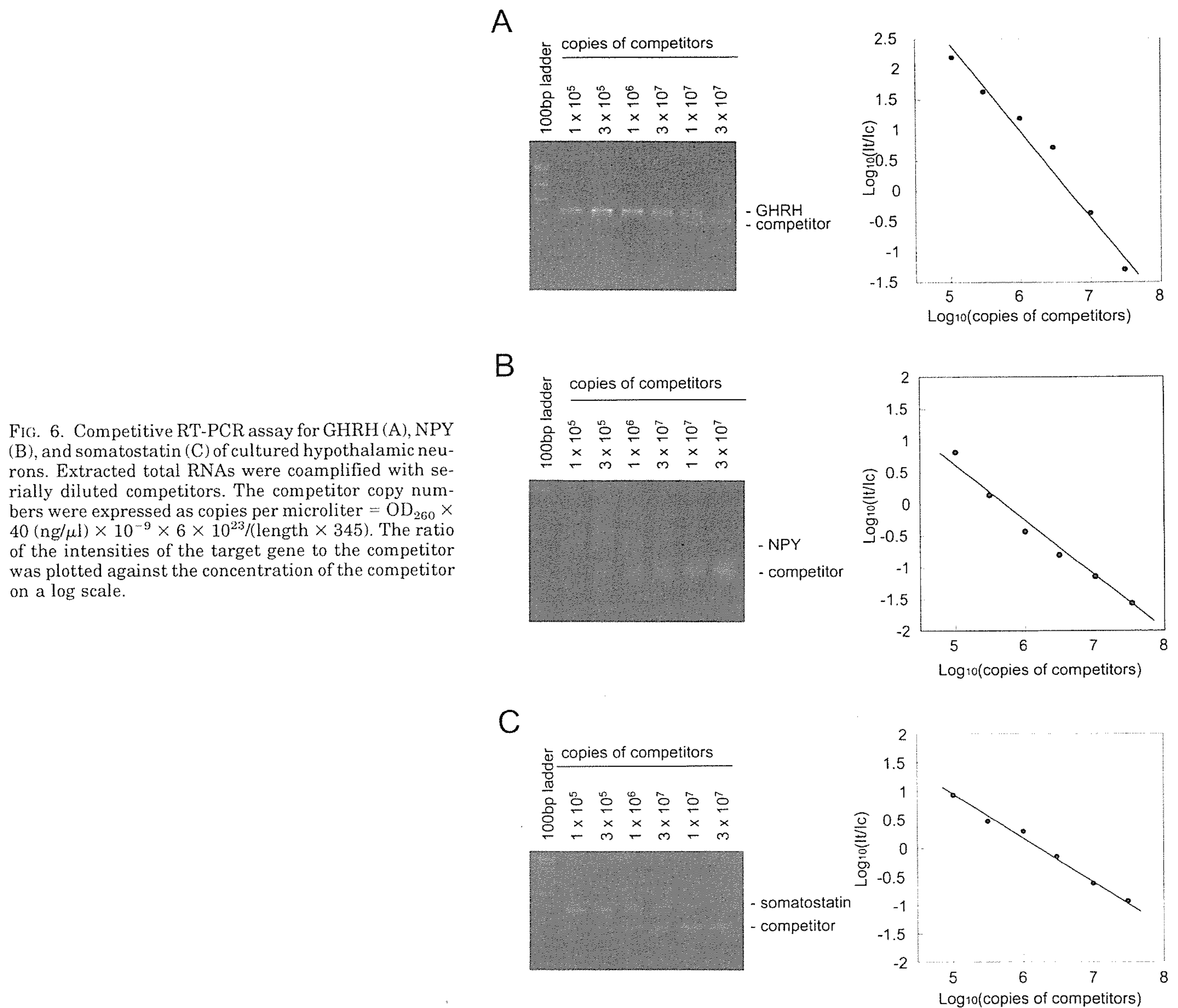

FIG. 6. Competitive RT-PCR assay for GHRH (A), NPY (B), and somatostatin (C) of cultured hypothalamic neurons. Extracted total RNAs were coamplified with serially diluted competitors. The competitor copy numbers were expressed as copies per microliter = $OD_{260} \times 40\ (ng/\mu l) \times 10^{-9} \times 6 \times 10^{23}/(\text{length} \times 345)$. The ratio of the intensities of the target gene to the competitor was plotted against the concentration of the competitor on a log scale.

were distributed in the portion medial to that of the GHRH neurons in the Arc (Fig. 1, E and F). The mean number of NPY neurons did not differ between Tg and WT rats (Tg rats, 468 ± 49 *vs.* WT rats, 478 ± 49) (Fig. 1G).

Coexpression of GHS-R with GHRH or NPY in the Arc

GHS-R-positive neurons (*green*), GHRH neurons (*red*), and GHS-R-positive GHRH neurons (*yellow*) in the Arc of WT rats (A–C) and Tg rats (D–F) are shown in Fig. 2. The numbers of GHRH neurons and GHS-R-positive GHRH neurons in Tg rats were significantly reduced than those in WT rats, respectively (GHRH neurons, 41 ± 5, GHS-R-positive GHRH neurons, 20 ± 3 in Tg rats; GHRH neurons, 82 ± 5, GHS-R-positive GHRH neurons, 54 ± 7 in WT rats, $P < 0.05$). GHS-R-positive neurons (*green*), NPY neurons (*red*), and GHS-R-positive NPY neurons (*yellow*) in the Arc of WT rats (A–C) and Tg rats (D–F) are shown in Fig. 3. There was no difference in the number of NPY neurons and GHS-R-positive NPY neurons between Tg rats (NPY neurons, 322 ± 27, GHS-R-positive NPY neurons, 315 ± 28) and WT rats (NPY neurons, 311 ± 18, GHS-R-positive NPY neurons, 309 ± 18). The number of GHRH neurons in Fig. 2 or NPY neurons in Fig. 3 was lower than that in Fig. 1. These differences are explained by the methodological difference between glucose oxidase diaminobenzidine nickel intensify method used in Fig. 1 and double-labeled immunofluorescence used in Figs. 2 and 3, the former is more sensitive than the latter.

Fos expression in response to KP-102 in the Arc

Fos-positive neurons in the Arc of WT and Tg rats in response to vehicle are shown in Fig. 4, A and C, respectively. In response to ICV administration of KP-102, Fos-positive

neurons distributed widely in the Arc of WT and Tg rats are shown in Fig. 4, B and D, respectively. The mean number of Fos-positive neurons after ICV administration of KP-102 was significantly reduced to 46% of that seen in WT rats (Fig. 4E).

The distributions of Fos-positive GHRH neurons of the Arc in response to saline or KP-102 in the WT and Tg rats are shown in Fig. 5. The distribution of Fos-positive GHRH neurons in response to saline in WT rats is shown in Fig. 5A and that of Fos-positive GHRH neurons in response to KP-102 in WT rats is in Fig. 5B. The distribution of Fos-positive GHRH neurons in response to saline in Tg rats is shown in Fig. 5C, whereas that of Fos-positive GHRH neurons in response to KP-102 in Tg rats is in Fig. 5D. The mean number of Fos-positive GHRH neurons in response to KP-102 was significantly less in Tg rats than in WT rats (Fig. 5E).

GHRH mRNA expression in primary cultured hypothalamic neurons

The results of a typical RT-PCR analysis of GHRH, NPY, and somatostatin mRNA levels in cultured hypothalamic neurons are shown in Fig. 6. Treatment of cultured neurons with KP-102 at concentrations ranging from 2–200 nM for 2 h did not significantly affect the level of GHRH mRNA expression (Fig. 7A). KP-102 at 20 nM did not significantly affect the GHRH mRNA expression level during 1, 2, 8, and 24 h, although with 2-h incubation, there was a trend to increased GHRH mRNA expression (Fig. 7B). Treatment of cultured neurons with KP-102 for 2 h did not significantly increase the level of NPY or somatostatin mRNA expression at a concentration of 0.2, 2.0, or 20 nM (Fig. 7, C and D). However,

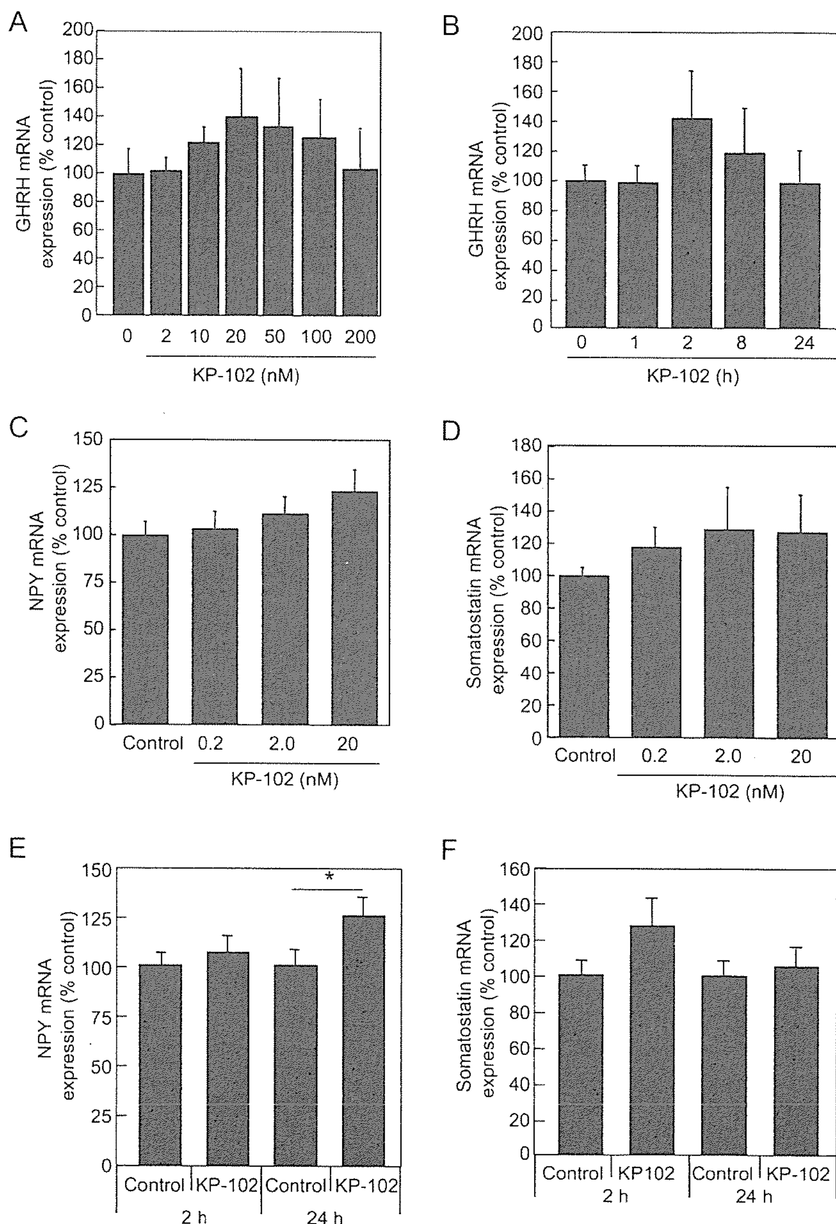

FIG. 7. Effects of KP-102 on GHRH, NPY, and somatostatin mRNA expression in cultured rat hypothalamic neurons. A, Dose-response effect of KP-102 on GHRH mRNA expression. B, Effect of duration of treatment with KP-102 on GHRH mRNA expression. C, Effect of KP-102 at concentrations of 0.2, 2.0, and 20 nM on NPY mRNA expression for 2 h. D, Effect of KP-102 on somatostatin mRNA expression at concentrations of 0.2, 2.0, and 20 nM for 2 h. E, Effect of KP-102 at a concentration of 20 nM on NPY mRNA expression for 2 or 24 h. F, Effect of KP-102 at a concentration of 20 nM on somatostatin mRNA expression for 2 or 24 h. The numbers of wells in each experimental group (A–F) were 12, 12, 9, 9, 8, and 8, respectively. *, $P < 0.05$.

treatment of cultured neurons with KP-102 at a concentration of 20 nM for 24 h but not 2 h significantly increased the NPY mRNA expression level (Fig. 7E). The level of somatostatin mRNA expression was not affected by KP-102 given at a concentration of 20 nM for either 2 or 24 h (Fig. 7F).

NPY at a concentration of 1 nM but not 0.1 nM significantly decreased the GHRH mRNA expression level (Fig. 8A). The inhibitory effect of 1 nM NPY on GHRH mRNA expression was completely blocked by anti-NPY IgG (3.6 μg/ml) (Fig. 8B). Anti-NPY IgG itself did not affect the level of GHRH mRNA expression. KP-102 significantly increased the level of GHRH mRNA expression approximately 2-fold in the presence of anti-NPY IgG during 2-h incubation, but it did not induce significant change in the expression level when given with normal rabbit serum IgG or without IgG (Fig. 8C).

Somatostatin significantly decreased the GHRH mRNA expression level at concentrations of 10 and 100 nM during the 2-h incubation period (Fig. 8D). The 10 nM somatostatin-induced suppression of GHRH mRNA expression level was completely reversed in the presence of antisomatostatin IgG (3.6 μg/ml) but not in the presence of normal rabbit serum IgG (Fig. 8E). The antisomatostatin IgG did not affect the level of GHRH mRNA expression in the presence of KP-102 (Fig. 8F). Treatment of neurons with somatostatin at a concentration of 10 nM for 2 h induced no significant change in NPY mRNA expression (Fig. 9A). The somatostatin-induced suppression of GHRH mRNA expression was partially reversed by anti-NPY IgG (Fig. 9B). GH at concentrations ranging from 10–500 ng/ml for 2 h significantly increased somatostatin mRNA expression level and suppressed GHRH mRNA expression level without influence on NPY mRNA expression (Fig. 10).

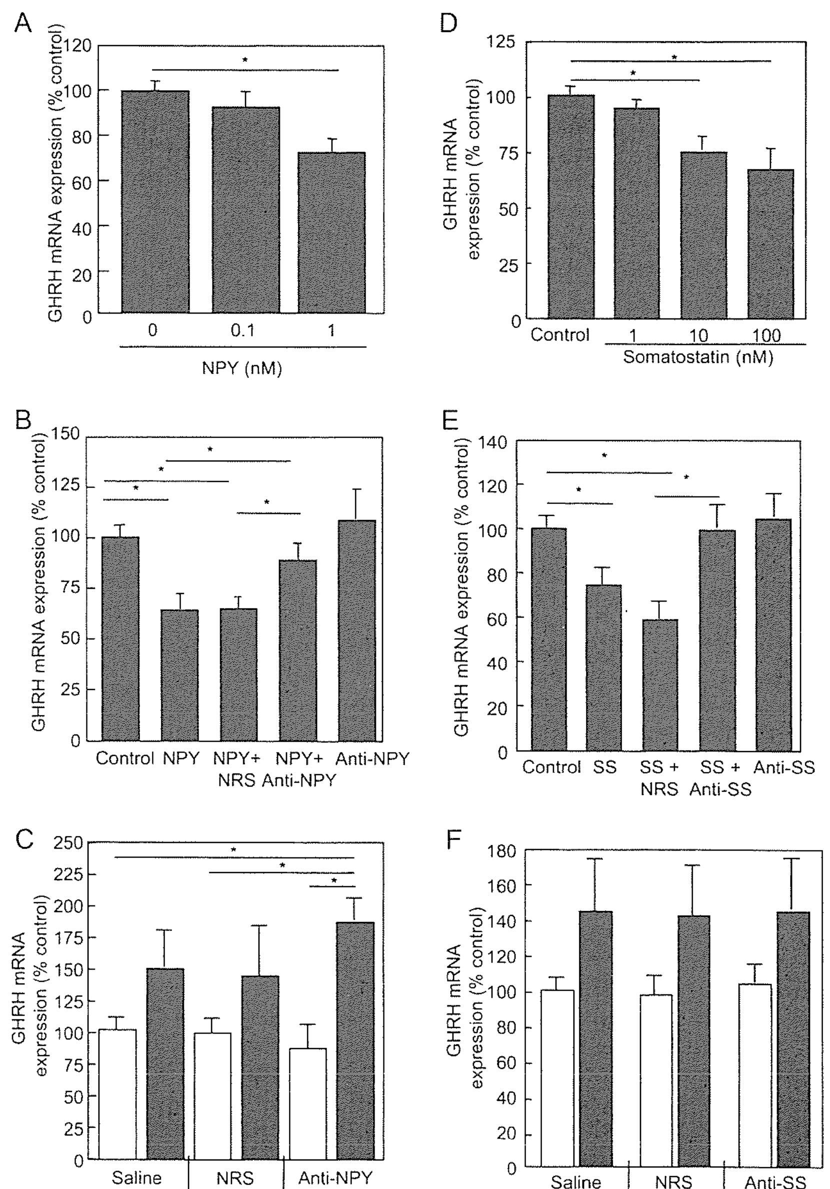

FIG. 8. Effects of NPY and somatostatin on GHRH mRNA expression and the KP-102-induced changes in GHRH mRNA expression level. A, Inhibitory effect of NPY on GHRH mRNA expression. B, Effect of anti-NPY IgG on NPY-induced inhibition of GHRH mRNA expression. C, Effect of anti-NPY IgG on KP-102-induced changes in GHRH mRNA expression. Cells were treated with vehicle (*white bar*) or 20 nM KP-102 (*black bar*) for 2 h in the presence of NRS or anti-NPY IgG. D, Inhibitory effect of somatostatin on GHRH mRNA expression. E, Effect of antisomatostatin IgG on somatostatin-induced inhibition of GHRH mRNA expression. F, Effect of antisomatostatin IgG on KP-102-induced changes of GHRH mRNA expression. Cells were treated with vehicle (*white bar*) or 20 nM KP-102 (*black bar*) for 2 h in the presence of NRS or antisomatostatin IgG. The number of wells in each experimental group (A–F) were 8, 9, 12, 8, 8, and 8, respectively. NRS, Normal rabbit serum IgG (3.6 μg/ml); Anti-NPY, anti-NPY IgG (3.6 μg/ml); Anti-SS, antisomatostatin IgG (3.6 μg/ml). *, $P < 0.05$.

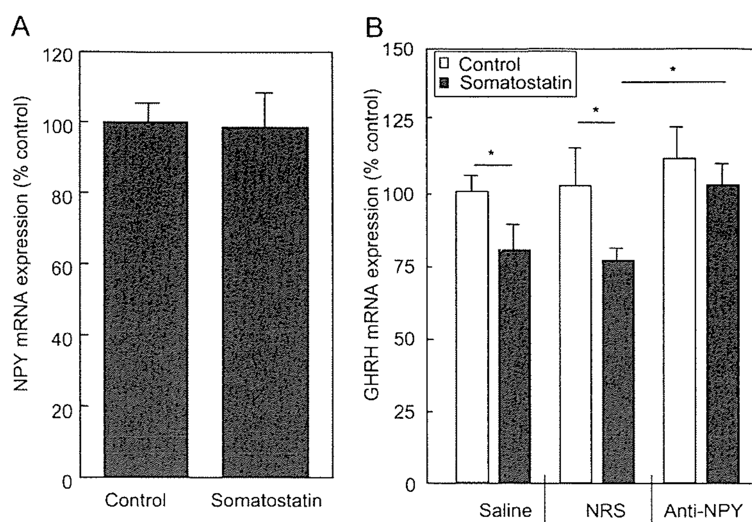

FIG. 9. Effect of somatostatin on NPY mRNA expression and effect of anti-NPY IgG on somatostatin-induced inhibition of GHRH mRNA. A, Effect of 10 nM somatostatin for 2 h on NPY mRNA expression. B, Effect of anti-NPY IgG on somatostatin-induced inhibition of GHRH mRNA expression. NRS, Normal rabbit serum IgG (3.6 μg/ml); Anti-NPY, anti-NPY IgG (3.6 μg/ml). The numbers of wells in each experimental group of A and B were nine. *, $P < 0.05$.

Concentrations of GHRH in cultured neurons

Although the basal secretion of GHRH from cultured hypothalamic neurons was undetectable level by RIA, the cellular concentrations of GHRH after 4-h treatment with KP-102 at concentrations of 2.0 and 20 nM were significantly increased (control, 567.5 ± 11.7 pg/well, 2.0 nM KP-102, 602.0 ± 13.6 pg/well, $P < 0.001$, *vs.* control, 20 nM KP-102, 651.0 ± 10.9 pg/well, $P < 0.001$ *vs.* control n = 6). The cellular concentrations of GHRH after 4-h treatment with somatostatin at a concentration of 1.0 and 10 nM did not show any significant changes.

Discussion

We have previously created Tg rats expressing an antisense GHS-R mRNA under the control of the promoter for TH and have reported that the concentrations of GHS-R protein in the Arc determined by Western blot analysis are lower in Tg rats than in WT rats (7). In the present study, we found that the numbers of GHS-R-positive neurons, GHRH neurons, and GHS-R-positive GHRH neurons were significantly lower in Tg rats compared with WT rats, whereas the number of NPY neurons or GHS-R-positive NPY neurons did not differ between the two groups. The expression level of NPY mRNA in the Arc of the Tg rats is thought not to be affected by the induction of GHS-R antisense due to the absence of TH in most of the NPY neurons located in the Arc (18, 19). The present study shows that, in response to the ICV injection of KP-102, one of the GHSs, Fos-positive neurons, and Fos-positive GHRH neurons in the Arc were reduced in Tg rats, reflecting the reduced expression of GHS-R in neurons including GHRH neurons in the Arc of Tg rats. These results suggest that the ghrelin/GHS-R system plays a role in up-regulating GHRH mRNA expression. This hypothesis is supported by a previous study, which found that Tg mice that constitutively overexpress GHS-R in the GHRH neurons show an increase in hypothalamic GHRH expression (12). However, unexpectedly, the ICV administration of ghrelin has been reported to induce no changes in the levels of GHRH mRNA expression levels, although it increases the levels of NPY and agouti-related peptide mRNA expression in the Arc (20, 21). Therefore, it seems that some mechanism masks the stimulatory effect of exogenous ghrelin on GHRH mRNA expression level in *in vivo* experiments.

Because GH, NPY, and somatostatin are involved in the feedback mechanism of the regulation of GH secretion, these hormones are the likely candidates that mask the stimulatory effect of ghrelin on GHRH mRNA expression. ICV administration of ghrelin increases GH secretion in nonanesthetized and anesthetized rats (22, 23). It is reported that iv administration of GH induces the expression of *c-fos* mRNA in NPY neurons of the Arc (24), where the GH receptor is expressed (25, 26). However, the present study showed that GH did not affect NPY mRNA expression. The difference in the effects of GH on NPY neurons may be explained by the difference of GH signal transport between *in vivo* study and *in vitro* study. Although these experiments do not show how GH modifies the release of NPY, the results of our study do not suggest a direct action of GH on NPY neurons.

When it is administered ICV, NPY inhibits GH secretion in rats (27, 28) at least in part through somatostatin release because NPY stimulates somatostatin release from the hypothalamus *in vitro* (29, 30). NPY neurons in the Arc project to the periventricular nucleus (PeV) (31), and synaptic connections between NPY axons and somatostatin neurons have been demonstrated in the PeV (32). These findings suggest that GH secreted in response to ghrelin may act on somatostatin neurons because GH receptor mRNA is present in somatostatin neurons in the PeV (33), and the iv administration of GH induces *c-fos* mRNA expression in somatostatin neurons in the PeV and Arc (24). The results of the present study actually showed that GH directly stimulated somatostatin mRNA expression and inhibited GHRH mRNA expression, suggesting the inhibitory role of GH in the GHRH expression.

Furthermore, NPY and somatostatin neurons activated by ghrelin directly may inhibit GHRH expression because somatostatin and NPY inhibit GHRH release from the hypothalamus *in vitro* (14, 30, 34). Histological connections between these neurons support this possibility; GHRH neurons are present in the ventrolateral part of the Arc where NPY

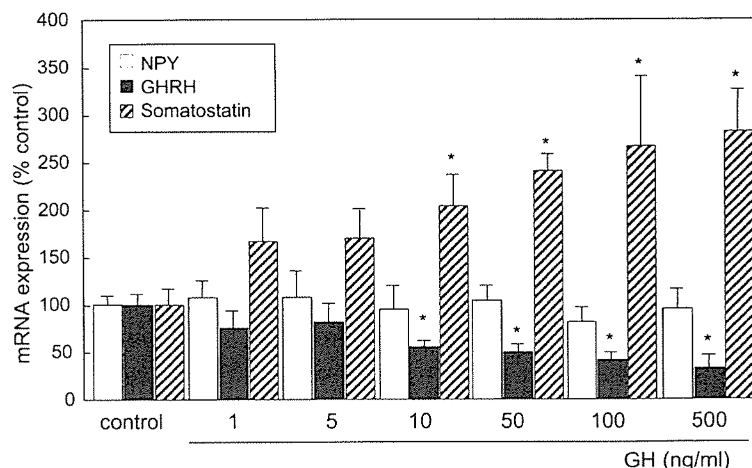

FIG. 10. Effects of GH on GHRH, NPY, and somatostatin mRNA expression in cultured rat hypothalamic neurons. The number of wells in each treatment group was six. *, $P < 0.05$.

fibers are concentrated (8, 35). GHRH neurons are innervated by somatostatin fibers, and somatostatin receptors are present on GHRH neurons (36–39). However, deleting the influence of GH, the present study showed that KP-102 significantly stimulated NPY mRNA expression and did not affect somatostatin mRNA expression, although both NPY and somatostatin inhibited GHRH mRNA expression. Therefore, NPY but not somatostatin activated by ghrelin seems to inhibit the activity of GHRH neurons. Furthermore, the inhibitory effect of somatostatin on GHRH mRNA expression was partially blocked by anti-NPY IgG, although somatostatin did not significantly affect NPY mRNA expression in the present study. Therefore, NPY released in response to somatostatin may also be involved in the inhibitory mechanism of GHRH mRNA expression by somatostatin in addition to direct action of somatostatin on GHRH neurons.

In contrast to our results, several reports showed that ICV administration of somatostatin stimulates GH release (40–42) and that ICV administration of antisense oligonucleotides against somatostatin 1 receptor suppresses GH tone in rats (43). Furthermore, somatostatin stimulates *in vitro* GHRH release in rat hypothalamic perfusion system (44). These reports suggest that somatostatin has a dual effect on GH secretion, although we found only inhibitory action of somatostatin on GHRH mRNA expression. The mechanism by which somatostatin stimulates GH secretion still remains unclear. Further studies are needed to clarify the complex mechanism.

We have found that KP-102 significantly increased the GHRH mRNA expression level only in the presence of anti-NPY rabbit IgG. Although the concentrations of NPY in the culture media were not measured in the present study, ghrelin has already been shown to stimulate *in vitro* NPY release from the rat hypothalamus (45). Therefore, these results suggest that KP-102 not only up-regulates GHRH mRNA expression but also stimulates NPY release in the Arc and that the NPY that is released by KP-102 attenuates the stimulatory effect of KP-102 on GHRH mRNA expression. The results of the present study are in agreement with a report that fasting-induced inhibition of GHRH mRNA expression in WT mice is abolished in *npy* null mice (46).

The present study showed that somatostatin inhibited the GHRH mRNA expression level. The results of the present study also showed that the effect of KP-102 on the GHRH mRNA expression level was not influenced by antisomatostatin IgG and that KP-102 did not significantly affect somatostatin mRNA expression, suggesting that somatostatin does not play a significant role downstream of the action of KP-102 on the expression of GHRH mRNA expression. These results are in agreement with other studies showing that ghrelin and GHSs have no effect on *in vitro* release of somatostatin from rat hypothalamus (30, 45).

In summary, the present study has shown that the number of GHS-R-positive GHRH neurons is reduced in Tg rats whose GHS-R expression is attenuated. It has also been shown that KP-102, one of the GHSs, stimulates the NPY mRNA expression level of cultured rat hypothalamic neurons and that NPY reduces the GHRH mRNA expression level. The present study also demonstrated that KP-102 stimulates the level of GHRH mRNA expression when NPY action is deleted. Furthermore, GH and somatostatin inhibit GHRH mRNA expression. These results indicate that GHS-R is involved in the up-regulation of GHRH and NPY expression in the Arc and that NPY as well as GH and somatostatin down-regulate GHRH mRNA expression. It is also suggested that the reduction of GHRH neurons in the Arc of Tg rats is induced by the decrease in GHS-R expression.

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All authors have nothing to declare.

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Inhibitory Effects of Amlodipine and Fluvastatin on the Deposition of Advanced Glycation End Products in Aortic Wall of Cholesterol and Fructose-Fed Rabbits

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Recent studies suggest that advanced glycation end products (AGEs) can promote the development of atherosclerotic lesions in a similar manner to oxidatively modified low density lipoproteins. As oxidative stress accelerates the formation of AGEs, antioxidant drugs may exert atheroprotective effects *via* suppression of AGE formation. Although amlodipine, a calcium channel blocker, and fluvastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, show antioxidant and atheroprotective effects, the relation of AGEs to their effects is unknown. We immunohistochemically investigated the inhibitory effects of chronic treatment with amlodipine (5 mg/kg per day) or fluvastatin at a dose insufficient to reduce plasma cholesterol levels (2 mg/kg per day) on the accumulation of AGEs in atherosclerotic aortas of rabbits fed 1% cholesterol diet and 10% fructose containing water. After eight weeks of treatment, AGEs, namely argpyrimidine, carboxymethyllysine and pyrraline, markedly accumulated with intimal thickening in cholesterol and fructose-fed control rabbits, while the drugs inhibited those changes other than the pyrraline deposition without plasma lipid-lowering effects. Enhanced lipid peroxidation was observed in plasma from cholesterol and fructose-fed rabbits only, and lipid peroxidation was not suppressed by the drugs. These results suggest that the atheroprotective effects of the drugs are at least partly due to the suppression of AGE accumulation although the exact mechanism of AGE suppression is ambiguous.

Key word amlodipine; atherosclerosis; advanced glycation end product; fluvastatin; hypercholesterolemia; rabbit

It has been well established that oxidative modification of low density lipoproteins (LDL) plays an important role in atherosclerosis.[1,2] The uptake of oxidatively modified LDL by macrophages and smooth muscle cells results in the formation of foam cells which accumulate lipid droplets with consequent cell-mediated responses leading to vascular dysfunction, matrix expansion and atherosclerosis. Although the structure of oxidatively modified LDL has not been well characterized, malondialdehyde (MDA)-lysine,[3,4] 4-hydroxy-2-nonenal (HNE)-protein[4] and oxidized phosphatidylcholine[5] have been recognized as the oxidatively modified moieties. Recently, non-enzymatic glycation (Maillard reaction) and the resultant advanced glycation end products (AGEs) such as carboxymethyllysine (CML),[5] pyrraline,[3] and 3-deoxyglucosone (3-DG)-hydroimidazolone,[6] have been reported to contribute to the progression of atherosclerosis in humans according to the similar mechanism as in oxidatively modified LDL.[7,8] It has also been reported that oxidative stress accelerates glycation processes to form AGEs,[9] and conversely glycation reactions generate reactive oxygen species.[10] Furthermore, it has been found that AGEs accelerate oxidative stress *via* recognition and internalization by their receptors on vascular cells.[11] Thus glycation and oxidative stress are closely linked, and AGEs probably promote the development of atherosclerosis in combination with oxidatively modified LDL.[3]

Use of antioxidants is considered to have beneficial effects in the prevention of atherosclerosis because oxidative stress is said to play an integral role in the development of atherosclerotic lesions as described above.[12] Recently, there has been a growing amount of evidence that calcium channel blockers, widely used in the treatment of hypertension, myocardial ischemia and arrhythmia, have atheroprotective effects due to their antioxidant activities.[13] Amlodipine, a long acting and charged dihydropyridine-type calcium channel blocker, exerts antioxidant actions based on not only its electron-donating activity but also the intimate physicochemical interaction with cell membrane.[14] It has shown significant antioxidant activities on isolated membrane vesicles while no effect was observed with other representative calcium channel blockers such as felodipine, verapamil and diltiazem.[14] The inhibitory effects of amlodipine on copper-induced oxidation of LDL *in vitro* have been also reported.[13,15] Amlodipine has lowered the level of MDA, an index of lipid peroxidation, in plasma and aorta with reduction in the accumulation of aortic cholesterol in cholesterol-fed rabbits.[16,17]

On the other hand, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors are the most potent antihypercholesterolemic agents, and effective in the prevention of atherosclerosis and its accompanying cardiovascular events.[18,19] Fluvastatin, the first entirely synthetic HMG-CoA reductase inhibitor, has been reported to suppress atherosclerosis due to its antioxidant activities related to its unique chemical structure[20] and the cholesterol-lowering effects. This drug showed strong free radical scavenging activity, whereas other HMG-CoA reductase inhibitors such as pravastatin, simvastatin, and lovastatin yielded only very weak effects.[21] Fluvastatin but neither pravastatin nor simvastatin showed inhibitory effects on copper-induced oxidation of LDL *in vitro*.[21,22] In cholesterol-fed rabbits, fluvastatin at a dose insufficient to reduce plasma cholesterol levels significantly decreased susceptibility of LDL to *ex vivo* copper-induced oxidation, reduced lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS) in serum, plaque area, formation of superoxide anion and accumulation of macrophages in aortic wall, and reversed the suppression

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of acetylcholine-induced relaxation of aortic wall.[21–23] These inhibitory effects of fluvastatin were not observed in cholesterol-fed rabbits treated with pravastatin.[22,23]

These studies seem to suggest that amlodipine and fluvastatin help reduce the risk of atherosclerosis *via* their antioxidant activities. However, no work has been done on their inhibitory effects on the formation and deposition of AGEs related to the development of atherosclerotic lesions. Thus, in the present study, we have investigated the inhibitory effects of these drugs on the deposition of AGEs and progression of atherosclerosis assessed by intimal thickening in aortas of hypercholesterolemic rabbits.

MATERIALS AND METHODS

Chemicals Amlodipine besilate and fluvastatin sodium were supplied by Sumitomo Pharmaceuticals (Osaka, Japan) and Novartis Pharma (Tokyo, Japan), respectively. Phosphate buffered saline powder and Tris–HCl buffer powder were purchased from Wako Pure Chemical Industries (Osaka, Japan), and dissolved with distilled water to prepare phosphate buffered saline solution (PBS, 10 mM, pH 7.2) and Tris buffer solution (50 mM, pH 7.6), respectively. All other chemicals were purchased from Wako and Kanto Chemical (Tokyo, Japan).

Antibodies Vectastain Elite ABC Mouse IgG kit and monoclonal anti-apolipoprotein-B (apo-B) antibody T3 (0.5 mg/ml, clone No. 4C11) were purchased from Funakoshi (Tokyo, Japan). Monoclonal anti-CML antibody (0.25 mg/ml, clone No. CMS-10), anti-pyrraline antibody (0.25 mg/ml, clone No. H12) and anti-rabbit macrophage antibody (0.25 mg/ml, clone No. RbM2) were obtained from Trans Genic (Kumamoto, Japan). Monoclonal anti-argpyrimidine antibody (0.1 mg/ml, clone No. 5B3CMAH13) was obtained from NOF CORPORATION (Tokyo, Japan).

Animal Studies and Sample Collection Male New Zealand White rabbits weighing 2—2.5 kg (starting age 10 weeks) were divided into four groups: Group NN=standard chow ($n=4$); Group CF=standard chow containing 1% cholesterol and 10% fructose in drinking water ($n=6$); Group AM=the high cholesterol and fructose diet plus amlodipine, 5 mg/kg body weight per day ($n=6$); Group FL=the high cholesterol and fructose diet plus fluvastatin, 2 mg/kg body weight per day ($n=6$). These diets were prepared by mixing amlodipine and fluvastatin directly into each respective high cholesterol diet. Each treatment was continued for eight weeks. About 100 g of food were given to the rabbits every morning. All rabbits were allowed free access to drinking water.

Blood (5 ml) was drawn from an ear vein after 48 h fasting at eight weeks of dietary. A vacuum collection tube containing 7.5 mg of ethylenediaminetetraacetic acid disodium (Venoject II VP-NA050, Terumo, Tokyo, Japan) for anticoagulation and prevention of autooxidation of lipoproteins was used. Plasma was separated from the blood by centrifugation, and stored in plastic tube until analysis at −80 °C. Total cholesterol and HDL cholesterol in plasma were measured by enzymatic methods using Hitachi (Tokyo, Japan) 7170 automatic analyzer. Lipid peroxides in plasma were assessed by TBARS which was measured by a kit (Wako) and represented in nmol MDA. After blood collection, the rabbits were anesthetized by administration of pentobarbital sodium (25 mg/kg) into the marginal ear vein, dissected, and killed by exsanguination from vena cava. The vascular segments (*ca.* 5 mm length) between the heart and the bifurcation of the carotid arteries were excised, cleared of adhering fat and connective tissue, rinsed with saline, and embedded in Tissue-Tek O.C.T. compound (SAKURA, Tokyo, Japan). The embedded samples were immediately frozen on dry-ice, and stored at −80 °C until histological examination.

Histochemical Methods Thin sections of 5 μm thickness were made of the embedded samples by a cryostat, mounted onto APS-coated slides (Matsunami, Osaka, Japan), fixed with acetone, and rinsed with phosphate buffered saline. The sections were stained with hematoxylin and eosin (HE) following a standard procedure. Intimal thickening of each aorta was estimated as an index of the extent of atherosclerosis by measuring the cross-sectional area of intima and media of five serial sections using an AX80 microscope coupled with a DP50 digital camera system (Olympus Optical, Tokyo, Japan).

Immunostaining of AGEs was performed using the Vectastain Elite ABC Mouse IgG kit based on an avidin/biotin/peroxidase system. The sections were incubated with 10% normal horse serum in PBS at room temperature for 30 min to block nonspecific binding of the second antibody and then reacted with the primary antibodies against different AGE structures, apo-B or macrophages at 4 °C over night. After they were washed in PBS, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol at room temperature for 40 min. They were washed in PBS and exposed to the biotinylated secondary antibody at room temperature for 30 min. After washing in PBS, the specimens were incubated with an avidin–biotin–peroxidase complex at room temperature for 30 min. Deposition was visualized by treating the sections with a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB, 30 mg), sodium azide (65 mg) and 30% hydrogen peroxide (18 μl) in Tris buffer (100 ml). After washing with PBS, the sections were stained with methyl green. Control tests for the specificity of immunostaining included the substitution of PBS for the primary antibodies. No significant staining was observed in the control tests.

Statistical Analysis Data were expressed as means± standard deviation (S.D.). Statistical analysis was done using *t*-test.

RESULTS

Plasma Lipids and TBARS Levels In this study, fluvastatin was administered at a dose insufficient to reduce plasma cholesterol levels to exclude the effects to inhibit atherogenesis through the lipid-lowering property.[21,23] There was no significant difference in body weight among the different groups of rabbits at the end of experiment. Plasma lipids and TBARS levels were summarized in Table 1. Plasma total cholesterol levels were significantly greater in rabbits fed cholesterol and fructose, whether or not supplementation with amlodipine or fluvastatin was provided. There were no significant differences in plasma HDL cholesterol levels among the different groups of rabbits. The plasma TBARS level of the Group NN rabbits was 1.0±0.1 nmol/ml, while

Table 1. Effects of Amlodipine and Fluvastatin on Plasma Lipids and TBARS in High Cholesterol and Fructose-Fed Rabbits[a]

	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	TBARS (nmol/ml)
Group NN	34±12	18.3±11.4	1.0±0.1
Group CF	943±252*	19.2±13.9	2.3±0.7*
Group AM	1312±744*	39.4±40.2	1.9±0.5*
Group FL	1380±412*	19.3±12.7	2.3±0.6*

a) Data of Group NN and other groups are represented as mean±S.D. for four and six rabbits, respectively, except for the HDL cholesterol value ($n=5$) of Group AM. Group NN=standard chow only; Group CF=high cholesterol and fructose; Group AM=high cholesterol and fructose plus amlodipine; Group FL=high cholesterol and fructose plus fluvastatin; TBARS=thiobarbituric acid reactive substances. * $p<0.01$ *versus* Group NN.

that of the Group CF rabbits nearly doubled. The decrease in plasma TBARS was not observed by supplementation with amlodipine or fluvastatin.

Morphological Examination of Atherosclerotic Aorta The representative appearance of HE stained sections of atherosclerotic aortas from the four experimental groups is shown in Fig. 1. Group NN rabbits fed standard chow for eight weeks exhibited no intimal thickening in aortas. A remarkable intimal thickening of aortas was induced by cholesterol and fructose feeding, and it tended to be reduced by supplementation with amlodipine or fluvastatin, the latter being more effective than the former (Fig. 2).

Immunohistochemical Analysis of Atherosclerotic Aorta The presence of different AGEs including argpyrimidine, CML and pyrraline was immunohistochemically examined in the tissue specimens of the aortas from the four experimental groups. Figures 3, 4 and 5 show the representative appearance of argpyrimidine, CML, and pyrraline immunoreactivity, respectively. Immunostaining of argpyrimidine was observed in both of the intima and media of Group CF rabbits (Fig. 3B). Immunostaining of CML and pyrraline was mainly observed in the intima and the inner media of Group CF rabbits, respectively (Figs. 4B, 5B). Amlodipine and fluvastatin treatment effectively reduced the appearance of argpyrimidine and CML (Figs. 3C, D, 4C, D), and the effects by both drugs were comparable. The effects by these drugs on the deposition of pyrraline were, on the whole, ambiguous although the speckled stain indicated by arrows was suppressed, as shown in Figs. 5C, D. Immunoreactivity of macrophages and apo-B was found in the intima of all rabbits fed cholesterol and fructose, and that of apo-B was also in the media of all groups of rabbits, and the density of immunostaining was similar irrespective of supplementation with amlodipine or fluvastatin (Figs. 6, 7).

DISCUSSION

Deposition of AGEs in Atherosclerotic Lesions It has been well recognized that AGEs have close connection with the pathophysiological processes of various chronic diseases.[3,5,6,8,9] The *in vivo* accumulation of AGEs has been studied in various tissues such as artery, kidney and skin in relation to atherosclerosis, diabetic nephropathy and aging, respectively. Imanaga *et al.*[5] have demonstrated that CML accumulates in human atheroscrelotic lesions. Sakata *et al.*[3] have shown that CML and MDA-protein mainly colocalize within macrophage/foam cells in human atherosclerotic lesions while pyrraline and apo-B localize in the extracellular matrix. In these studies the AGE structures have been suggested to form on LDL molecules.

Although hypercholesterolemic rabbits have been generally used for the study of atherosclerosis, there had been no report of AGE accumulation in their atherosclerotic aortas before our previous report[24] except for 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole whose presence *in vivo* is still controversial.[25] In the present study, we examined old well-known AGEs, *i.e.*, CML and pyrraline, and a relatively new AGE structure, argpyrimidine in cholesterol and fructose-fed rabbits. Fructose was given in addition to cholesterol in order to accelerate the formation of AGEs related to atherosclerotic lesions[24] because fructose promotes *in vitro* AGE formation about 8—10 times faster than glucose.[26,27] Consequently, CML and pyrraline were found to accumulate in the atherosclerotic aortas of the control cholesterol and fructose-fed New Zealand White rabbits as in humans and cholesterol and fructose-fed Japanese White rabbits.[24] In addition, argpyrimidine remarkably accumulated with intimal thickening. No data has been reported to date on the accumulation of argpyrimidine in atherosclerotic aortas although the AGE structure has been found in intima and media of small artery wall of kidney from diabetic patients.[28] The simultaneous deposition of argpyrimidine and CML, macrophage, and apo-B in the intima is apparently consistent with the proposed mechanism[7,8] where modified LDL is incorporated into macrophages, which accumulate in the proliferated intima. However, further examination is required to clarify the exact mechanism of AGE deposition in aortic wall because the localization of pyrraline in the intima was ambiguous, and the immunostaining of the three AGEs in the media was considerably different as shown in Figs. 3B, 4B, 5B. The observed accumulation of argpyrimidine, CML and pyrraline accompanied by intimal thickening suggests that the AGEs are important factors in the development of atherosclerosis of cholesterol and fructose-fed rabbits, and various AGEs may contribute differently to the development of atherosclerotic lesions.

Recently, carbonyl stress compounds originating from sugars, lipids and amino acids, whose accumulation is mainly accelerated by oxidative stress, have been suggested to play a major role in the formation of chemically modified proteins such as AGEs and advanced lipoxidation end products rather than the conventional glycation pathways accompanying formation of Amadori compounds, and exhibit direct actions related to the pathogenesis of various chronic diseases including atherosclerosis.[9] The carbonyl stress compounds comprise of glyoxal, methylglyoxal, 3-DG, MDA, HNE *etc.*... In particular, the former three compounds, namely α-ketoaldehydes, have lately attracted special interest as the main carbonyl stress compounds because they have higher chemical reactivity to form various AGEs such as argpyrimidine, CML and pyrraline and direct actions to cardiovascular systems.[6,9,29] The latter two compounds are of importance in the sense that they are well-known as lipid peroxidation products and the precursors of MDA-lysine and HNE-protein recognized as the oxidatively modified LDL.[3,4,8,9] Argpyrimidine and pyrraline are exclusively formed from methylglyoxal and 3-DG, respectively, while CML is formed by the condensa-

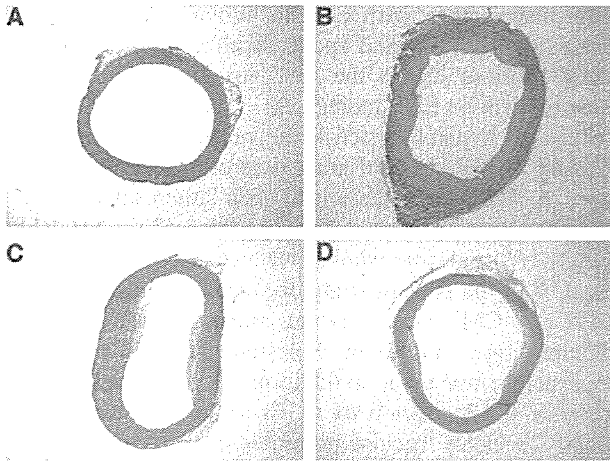

Fig. 1. Comparative HE Staining of Atherosclerotic Aorta Wall

A, Group NN=standard chow only; B, Group CF=high cholesterol and fructose; C, Group AM=high cholesterol and fructose plus amlodipine; D, Group FL=high cholesterol and fructose plus fluvastatin. Original magnification is ×100 for all photographs.

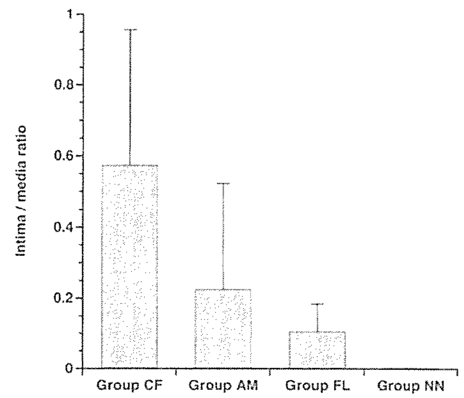

Fig. 2. Inhibitory Effects of Amlodipine or Fluvastatin Treatment on Intimal Thickening in Thoracic Aorta of Rabbits Fed High Cholesterol and Fructose for 8 Weeks

Data are represented as mean±S.D. Group CF=high cholesterol and fructose ($n=5$); Group AM=high cholesterol and fructose plus amlodipine ($n=6$); Group FL=high cholesterol and fructose plus fluvastatin ($n=6$); Group NN=standard chow only ($n=4$). Intima/media ratio is the ratio of intimal area to medial area.

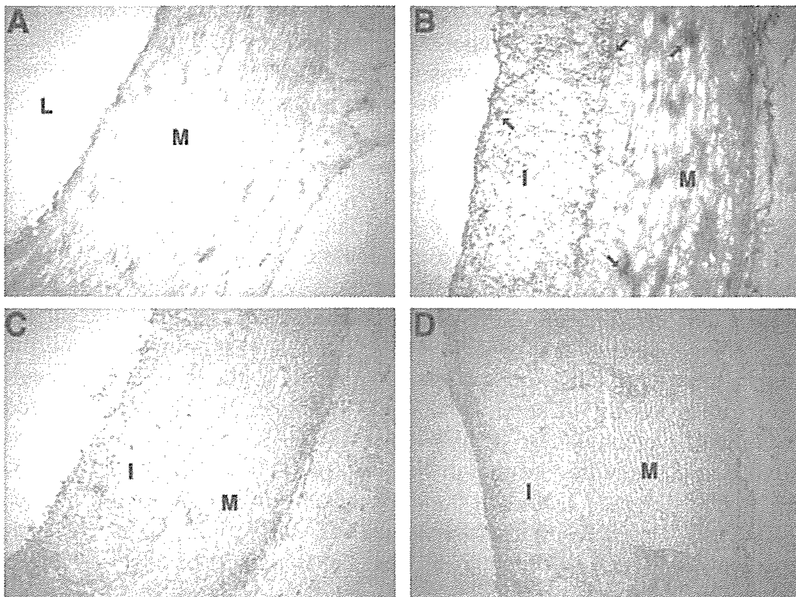

Fig. 3. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for Argpyrimidine

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was positive in the intima and media (B, arrows). Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

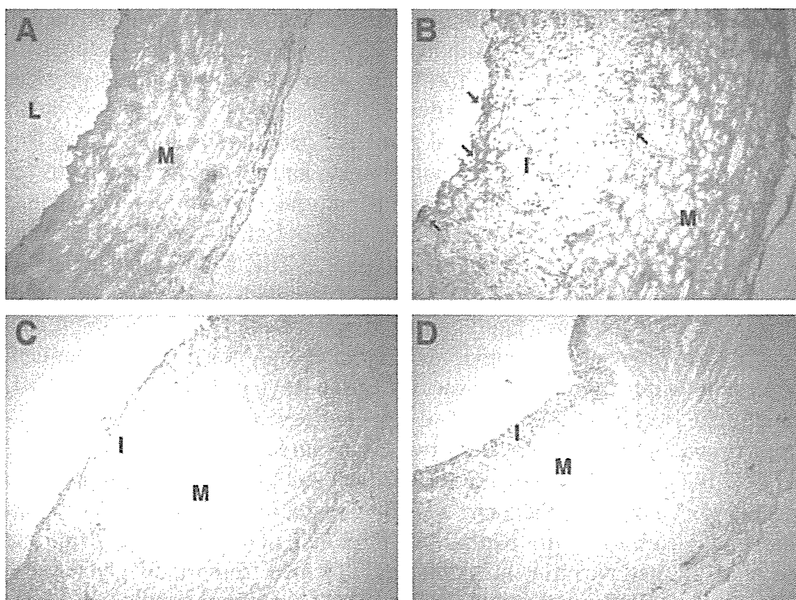

Fig. 4. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for CML

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was positive in the intima (B, arrows). Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

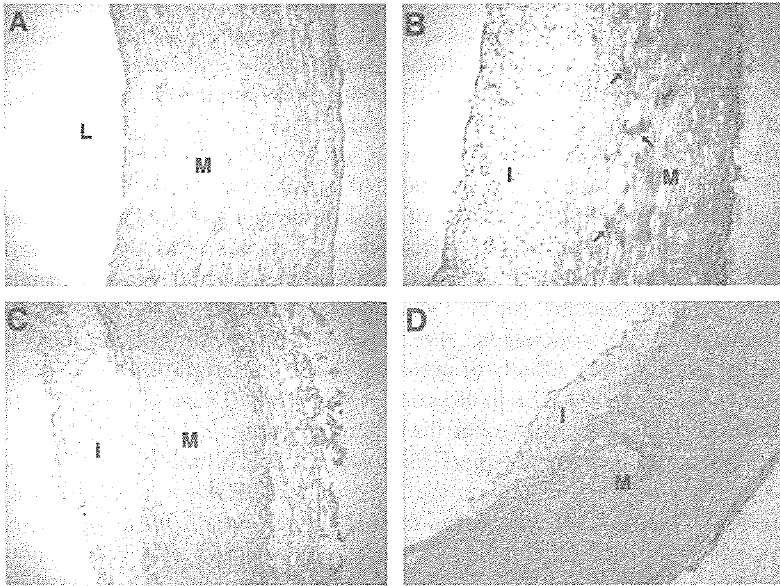

Fig. 5. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for Pyrraline

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was positive in the inner media (B, arrows). However, that in the intima was slight. Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

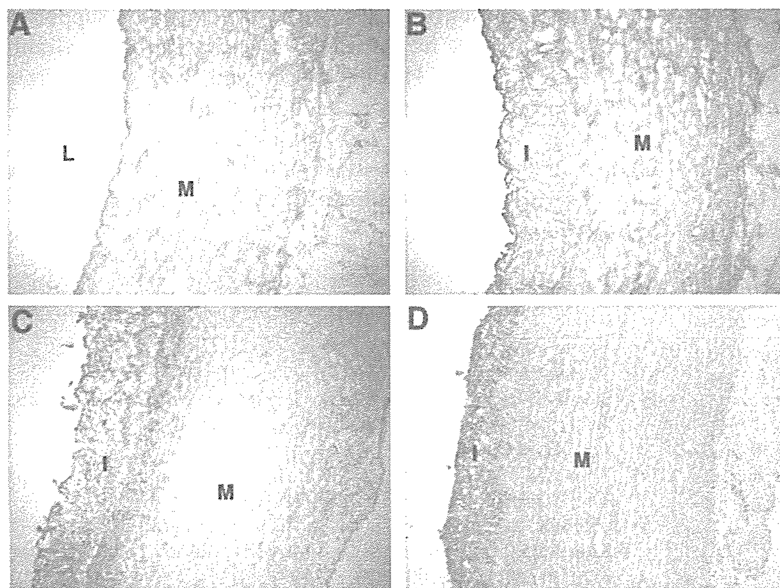

Fig. 6. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for Rabbit Macrophages

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was observed in the intima (B, C, D). Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

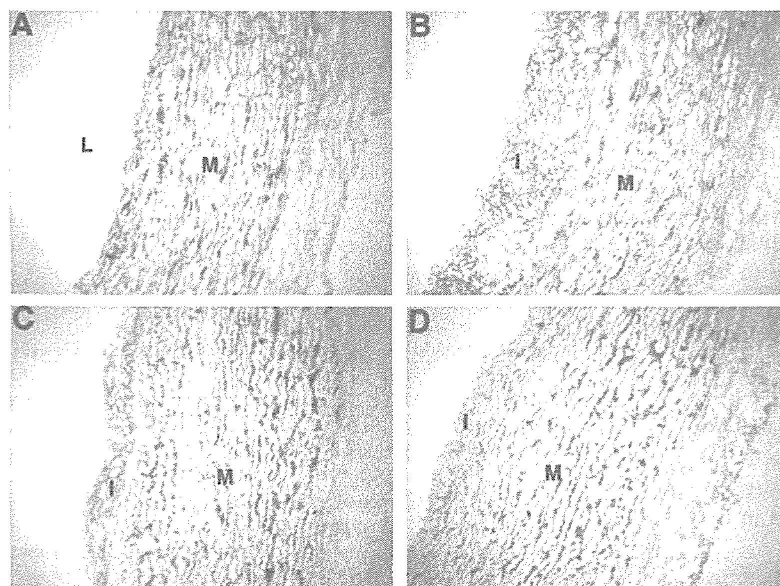

Fig. 7. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for apo-B

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was observed in the intima and media. Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

tion of glyoxal with proteins as well as the oxidative degradation of Amadori compounds.[8,9] Thus, these AGEs may be useful as the integrative biomarkers for carbonyl stress. The observed accumulation of these AGEs in the control cholesterol and fructose-fed rabbits probably reflects the increase in the concentrations of the corresponding α-ketoaldehydes.

The increase of plasma TBARS indicated the acceleration of oxidative stress in the Group CF rabbits compared with the Group NN rabbits. Similar acceleration of oxidative stress has been observed previously in cholesterol-fed rabbits.[16,17,23,24] This enhanced oxidative stress has been reported to be explained by the increase of NADPH oxidase which forms superoxide anion in artery.[30] Oxidative stress not only accelerates formation of some AGEs including CML from Amadori products but also promotes formation of glyoxal and suppresses detoxification of α-ketoaldehydes by glyoxalase,[29] leading to the acceleration of carbonyl stress. In addition, fructose fed to rabbits contributes to the accumulation of the above AGEs because it is converted to Amadori compounds and carbonyl stress compounds including methylglyoxal and 3-DG.[6,9,25] The accelerated oxidative and carbonyl stress are thus considered to be responsible for the marked deposition of argpyrimidine, CML and pyrraline.[9] Needless to say, cholesterol feeding increases the level of plasma LDL so that formation of AGE-modified LDL accelerates.[8]

Inhibitory Effects of Amlodipine and Fluvastatin on the Deposition of AGEs in Atherosclerotic Lesions The deposition of argpyrimidine and CML in the aortic walls of the cholesterol and fructose-fed control rabbits was remarkably suppressed by amlodipine and fluvastatin, independent of their lipid-lowering effects. These results are the first presentation of pharmacological effects to inhibit the deposition of AGEs in atherosclerotic aortas. In the literature,[17,21,23] atherosclerotic lesions are suppressed at the dose used in the present experiments. Treatment by the drugs showed, in fact, a tendency to reduce intimal thickening although the reduction was statistically insignificant. Thus, the observed inhibitory effects on the deposition of AGEs are likely associated with their atheroprotective effects.

The suppressive effects for AGEs were thought to be due to the reduced formation of AGEs by the antioxidant activities of the drugs. However, no significant decrease in plasma TBARS, an index of lipid peroxidation in the circulation, was observed. Turgan *et al.*[17] have reported that amlodipine (5 mg/kg/d) reduces plasma MDA in New Zealand White rabbits fed 1% cholesterol for eight weeks. Bandoh *et al.*[23] have reported that fluvastatin (2 mg/kg/d) reduces serum TBARS in Japanese White rabbits fed 0.5% cholesterol for 17 weeks. The contradiction concerning lipid peroxidation between this paper and the literature is probably due to the differences in the various experimental conditions including the additional feeding of fructose in the present experiments and the index of lipid peroxidation. As mentioned above, oxidative stress increases the levels of argpyrimidine and CML. It should be noted that CML has been proposed as a marker of oxidative stress.[31—33] Thus, the suppressive effects for AGE deposition by amlodipine and fluvastatin may suggest that the drugs exerted their local antioxidant activities in the aortic walls.

Amlodipine and fluvastatin have been reported to show indirect antioxidant activities other than the direct quenching of reactive oxygen species. Amlodipine induced antioxidant nitric oxide production accompanied by increased inducible nitric oxide synthase mRNA expression and protein accumulation in rat vascular smooth muscle cells.[34] Fluvastatin upregulated endothelial nitric oxide synthase mRNA expression and reduced production of superoxide anion,[35] and suppressed the increase of lipid peroxides and NADPH oxidase component mRNA expression,[30] in aortas of 0.5% cholesterol-fed rabbits. These biological activities may be partly responsible for the local antioxidant effects of the drugs.

In conclusion, the present results demonstrate the inhibitory effects of amlodipine and fluvastatin on the deposition of AGEs in atherosclerotic aortas. These effects seem to be associated with the atheroprotective effects although the exact mechanism of AGE suppression is ambiguous. The inhibitory effects on the deposition of AGEs in artery wall of hypercholesterolemic animal models might be a significant indication to evaluate the atheroprotective effects of candidate drugs.

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REVIEWS

Impact of Lipoprotein Glomerulopathy on the Relationship Between Lipids and Renal Diseases

Takao Saito, MD, PhD, Akira Matsunaga, MD, PhD, and Shinichi Oikawa, MD, PhD

● Lipoprotein glomerulopathy (LPG) is a unique entity of renal lipidosis characterized by peculiar histopathologic characteristics of lipoprotein thrombi and an abnormal plasma lipoprotein profile resembling type III hyperlipoproteinemia, with a marked increase in serum apolipoprotein E (apoE) concentrations. At present, 65 cases have been reported worldwide, although most patients are found in Japan and east Asian countries. Recently, we identified 4 types of novel apoE mutations associated with LPG. In particular, a mutation designated apoE Sendai, in which arginine 145 is substituted with proline, occurs in the majority of Japanese patients. The virus-mediated transduction of apoE Sendai resulting in the development of LPG in apoE-deficient mice confirms the etiologic role of apoE mutation in LPG. Conversely, experimental graft-versus-host disease induced in Fc receptor γ-chain–deficient mice showed LPG-like lesions in glomeruli without apoE mutations. Considered together, we believe that intrinsic factors in the kidney also contribute to the induction of LPG. Today, apoE and related lipid abnormalities are reported to have an important role in the development of various renal diseases, eg, diabetic nephropathy and immunoglobulin A nephropathy. In this article, we review clinical and histopathologic features of LPG, describe the etiologic role of apoE variants and intrinsic renal factors, and discuss the impact of LPG on mechanisms of other renal diseases. *Am J Kidney Dis* 47:199-211.
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INDEX WORDS: Lipoprotein glomerulopathy; type III hyperlipoproteinemia; apolipoprotein E; Fc receptor.

LIPID ABNORMALITIES exist in many renal disorders. In particular, it is well known that nephrotic syndrome is associated with hypercholesterolemia. Conversely, renal lesions sometimes are present in patients with primary lipidoses involving an inborn error, most of which are caused by abnormalities of specific enzymes, transfer proteins, and lipoprotein receptors.[1] However, there is no information on the association between apolipoprotein abnormality and renal lesions, although some groups reported that glomerulosclerosis with foam cells is seen occasionally in type III hyperlipoproteinemia (HLP).[2-5]

In 1989, we reported a case of excessive storage of lipoproteins in the intraglomerular capillaries, so-called lipoprotein thrombi and dyslipidemia, fulfilling diagnostic criteria for type III HLP.[6] Since then, other patients with similar features were reported by other Japanese groups.[7-9] We then collected these cases and examined the common characteristics. Cases were categorized into a single disease entity,[10] and we proposed that it be called lipoprotein glomerulopathy (LPG).[6,10] In 1995, this disease entity was included in the second edition of *Renal Diseases: Classification and Atlas of Glomerular Diseases*, in collaboration with the World Health

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Organization.[11] Subsequently, we found that LPG was associated with a novel apolipoprotein E (apoE) variant.[12-15] These findings provide evidence that LPG is, at least in part, an inherited disease caused by special mutations in the apoE gene.[16] In this regard, recent reports suggested that apoE mutation may be involved in some renal diseases, eg, diabetic nephropathy and immunoglobulin A (IgA) nephropathy. Accordingly, studies of apoE mutation in patients with LPG will serve as a timely impetus to readdress the large question of the role of lipids in renal diseases.[16]

The purpose of this article is to present the unique clinical and histological features of LPG and discuss the pathogenetic role of apoE and other elements in the glomerulus and their interaction, which may be responsible for the development of renal diseases.

LIPOPROTEIN GLOMERULOPATHY

Geographic Distribution of Reported Cases

We previously reviewed 32 cases of LPG and described their clinical features.[17-19] However, in the last few years, 33 other cases have been reported. Thus, a total of 65 cases have been reported, although most are from the eastern area of Japan. Reported cases from China and Taiwan increased recently.[20-25] One case from the United States is a patient of Chinese ancestry.[26] Several cases were reported in Europe, but 3 of these cases are not certified to be LPG because they lacked specific findings in histological characteristics and lipoprotein profile.[27,28] To date, only 2 French cases are patients of Caucasian origin.[29,30]

Clinical Features

Clinical features of patients with LPG were noted in our previous articles.[17-19] However, these features should be modified slightly after the addition of newly reported cases. The summary is listed in Table 1. Fourteen cases in a recent study from China,[24,25] the United Kingdom,[27] and Macedonia[28] were excluded from Table 1 because they lacked detailed data. Age at onset varies widely, from 4 to 69 years, and male-female ratio is approximately 3:2. All patients initially were found to have mild to severe proteinuria, and most had nephrotic syndrome during the clinical course. Hematuria is usually negative microscopically. In most cases, systemic manifestations, eg, hypertension, atherosclerosis, hepatic dysfunction, and skin eruption, are mild, even when recognized. Accordingly, characteristic symptoms appear to be limited compared with those of other glomerular diseases. In several cases, LPG was associated with

Table 1. Summary of Clinicopathologic Features of LPG in 51 Reported Patients

	No. of Cases Analyzed	Mean ± SEM (range) or No. of Patients
Country†	51	Japan, 32; China, 12; Taiwan, 4; France, 2; US, 1
Age (y)	51	32.0 ± 2.2 (4-69)
Sex (male:female)	51	30:21
Urinary protein (g/d)	45	4.8 ± 0.6 (0.3-18)
Creatinine clearance (mL/min/1.73 m^2)	30	88.9 ± 4.4 (35-143)
Plasma triglycerides (mg/dL)	47	313 ± 28 (74-1,019)
Plasma total cholesterol (mg/dL)	47	272 ± 16 (107-720)
Plasma apoE (mg/dL)	40	17.1 ± 1.9 (3.9-71.0)
ApoE phenotype	32	E2/3, 21; E2/4, 5; E1/3, 2; E2/2, 1; E3/3, 1; others, 2
ApoE gene mutation	29	E Sendai, 18; E Kyoto, 4; E Tokyo (Maebashi), 5; E1 (del 156-173), 1; E5 (Gln3Lys), 1
Outcome	32	Renal failure, 16 (4 patients, transplantation); nephrotic syndrome, 9; asymptomatic or unchanged, 7

NOTE. To convert plasma triglycerides in mg/dL to mmol/L, multiply by 0.0113; total cholesterol in mg/dL to mmol/L, multiply by 0.2586; creatinine clearance in mL/min to mL/s, multiply by 0.01667.

†Fourteen other cases were reported from China,[24,25] the United Kingdom,[27] and Macedonia,[28] but were excluded because they lacked detailed data.

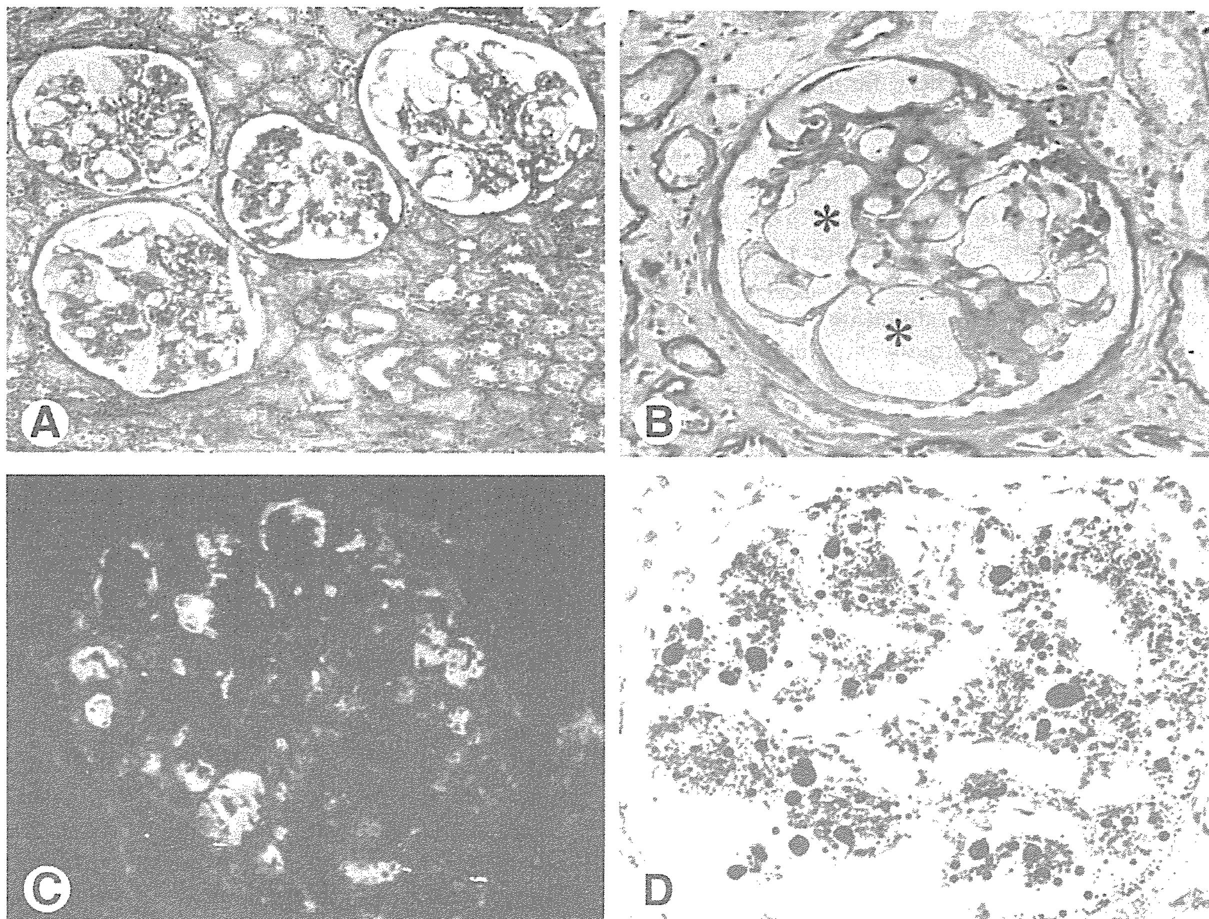

Fig 1. Glomerular findings in LPG. (A, B) Light microscopy: capillary lumina are markedly dilated and contain (B) pale-stained thrombus-like substances (*). (Periodic acid–Schiff stain; original magnification: [A] ×100, [B] ×200.) (C) Immunofluorescence study: apoE is present mainly in the capillary lumina. (Frozen section; original magnification ×200.) (D) Oil red-O stain: numerous red droplets are seen in the capillary lumina. (Frozen section; original magnification ×300.)

other glomerulopathies, such as IgA nephropathy, membranous nephropathy, and lupus nephritis.[17-19] Furthermore, patients with psoriasis vulgaris recently were reported.[22] Because familial cases also were reported,[6,8,9] it is possible that LPG is a genetically based condition.

Lipoprotein Profile

Most patients show hyperlipidemia with a predominance of triglycerides. In detailed assays for plasma lipoproteins isolated by means of ultracentrifugation, high cholesterol levels in very-low-density (VLDL) and intermediate-density lipoprotein (IDL) fractions were observed.[31] These findings satisfy criteria for type III HLP as defined by Hazzard et al[32] and Fredrickson et al.[33] However, hyperlipidemia often is milder than that in familial type III HLP and is not even recognized in some cases.[15,34] Clinical symptoms characteristic of lipidosis, eg, corneal arcus, xanthoma, and Achilles tendon thickening, rarely are observed. A high serum apoE level is a characteristic finding in patients with LPG, and the level is approximately 2-fold the upper normal range.[17-19] The apoE phenotype, determined by means of isoelectric focusing polyacrylamide gel electrophoresis (IEF), shows heterozygosity for E2 (E2/3 or E2/4) in most cases of LPG,[18] which is different from that of familial type III HLP (homozygous for E2). However, in some cases, the protein band by IEF is recognized at the position of E1[13,15] or between E2 and E3.[30,35] Conversely, the apoE genotype routinely determined by means of re-

striction fragment length polymorphism analysis with restriction enzyme *Hha*I shows E3/3 or E3/4 and is inconsistent with the apoE phenotype.[12] This problem is resolved by means of DNA sequence analysis, as mentioned later.

Histopathologic Findings

Histopathologic examination provides the most important information for the diagnosis of LPG (Fig 1).[17-19] Light microscopic examination of renal biopsy material shows marked dilatation of the capillary lumen in glomeruli by a pale-stained substance. Foam cells characterizing lipidosis rarely are seen in either glomeruli or interstitium. Electron microscopy shows that thrombus-like substances in glomerular capillaries are composed of granules and vacuoles of various sizes, forming concentric lamellate, like a fingerprint.[17,18,36] We indicated that granules become smaller under the influence of hypertriglyceridemia and particularly elevated plasma VLDL and high-density lipoprotein–triglyceride levels.[36] In snap-frozen renal sections, immunohistochemical studies showed deposition of apoB and apoE, and Sudan or oil red O staining shows lipid droplets in the thrombus-like substances. Using an immunoelectron microscopic technique, Zhang et al[26] showed that lipids are sur-

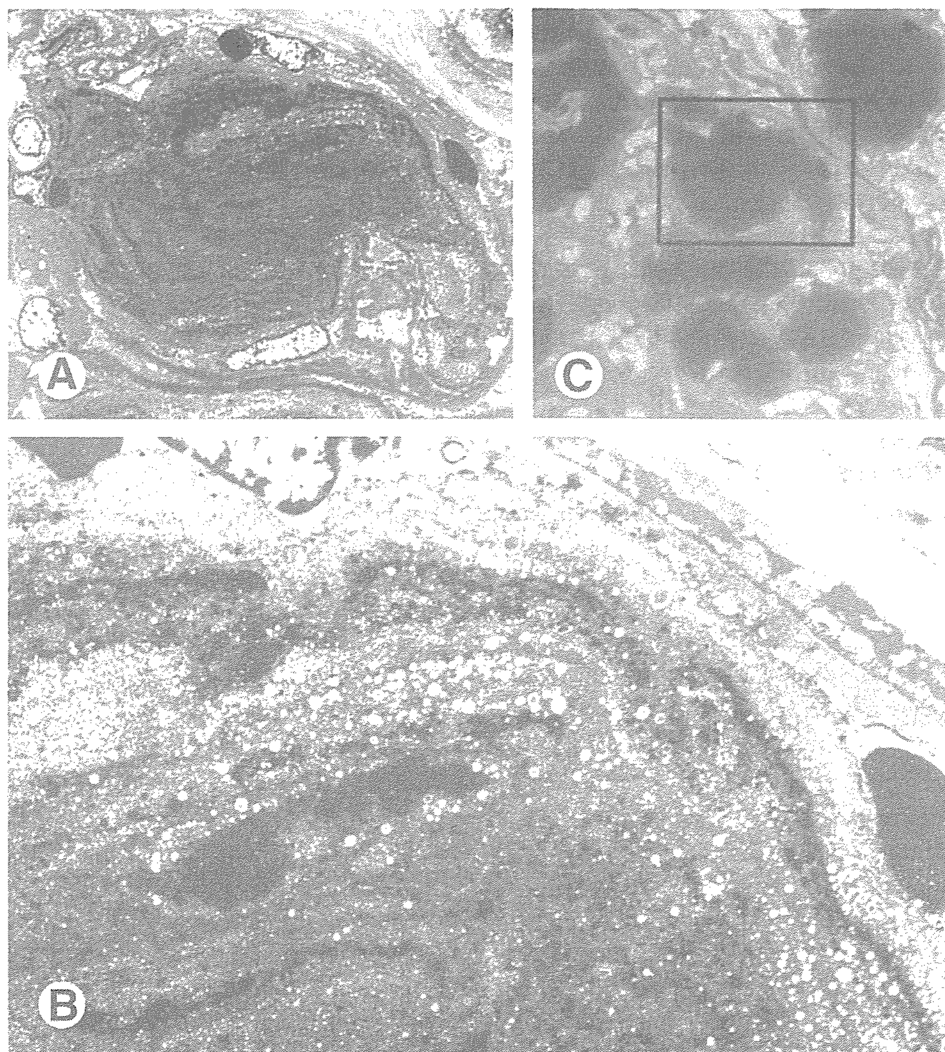

Fig 2. Serial observations using an Epon-embedded renal tissue block of lipoprotein thrombi in LPG. A fingerprint-like substance with fine granules in (A, B) electron micrographs is defined as (C) Sudan-positive in the boxed area of the serial section. (Electron micrographs; original magnification: [A] ×1,600, [B] ×6,500; [C] Sudan-IV stain; original magnification ×400.)

rounded by apoE and the thrombus-like substances consist of lipoproteins. Recently, we also investigated serial sections of Epon-embedded tissues (TAAB laboratory, Aldermaston, UK) from patients with LPG and found that the fine granules contain lipids (Fig 2).[37]

Criteria for Diagnosis of LPG

Based on the information obtained from reported cases, the following is a summary of criteria for LPG by clinical, pathological, and laboratory surveys: (1) mild to severe proteinuria; (2) dilatation of glomerular capillary lumina with pale-stained substances on light microscopy; (3) stone or sand-like granules occupying the capillary lumina on electron microscopy, the so-called lipoprotein thrombi; and (4) type III HLP with high apoE concentration, usually associated with a heterozygous apoE phenotype, E2/3 or E2/4, by means of IEF, but sometimes with an uncommon type, eg, E1/3 or others.

However, to establish the diagnosis of LPG, DNA sequence analysis should be conducted. Novel apoE variants were detected in all patients with LPG for whom DNA sequence was determined (Table 1).

Response to Therapy and Prognosis

Regular treatments for patients with nephrotic syndrome, eg, steroids, immunosuppressants, and anticoagulants, were reported to be ineffective.[17,18] Initially, reported effects of lipid-lowering agents were controversial because such agents did not seem to decrease urinary protein excretion, even in patients who showed remission of dyslipidemia.[17] In only 1 case, probucol was effective against proteinuria.[38] Recently, however, intensive therapy using lipid-lowering agents, including fibrates, was reported to result in clinical remission with histological resolution.[39,40] In these studies, complete disappearance of lipoprotein thrombi was shown in serial renal biopsies, in addition to decreases in serum cholesterol, triglyceride, and apoE levels.[39,40]

Half the patients developed renal failure at 1 to 27 years after onset (Table 1). Renal transplantation was performed in 4 patients. Kidneys were donated by the patients' relatives in 2 Japanese cases[32,41] and from cadavers in French and American-Chinese cases.[30,42] Unfortunately, LPG was reported to recur in all transplanted kidneys.

ABNORMALITY OF THE ApoE GENE IN LPG

Routine restriction fragment length polymorphism for apoE is used to discriminate wild-type apoE3/3 from apoE2 (Arg158Cys) or apoE4 (Cys112Arg), but is not available to detect other specific mutations.[43] The apoE isoforms (apoE2, apoE3, and apoE4) obtained by means of IEF are divided by their electrophoretic charge.[44] Therefore, the discrepancy between phenotype and genotype of apoE in LPG predicted a novel apoE variant in patients with LPG.[12] Moreover, reports of familial cases and recurrence in transplanted kidneys also suggest a genetic factor in LPG. In 1997, three patients in 2 families were subjected to analysis of apoE gene mutation.[12] In all, DNA sequence analysis showed a nucleotide substitution of G to C at codon 145 of the apoE gene. This missense mutation denoted amino acid substitution of the proline for arginine residue at position 145 of apoE. We recognized that apoE(Arg145Pro) was a novel apoE variant and termed it apoE Sendai, representing the name of the city where the patients lived (Fig 3).[12] Subsequently, we found 3 other apoE variants in patients with LPG (Fig 3), although the majority had apoE Sendai. ApoE Kyoto(Cys25Arg) is another missense mutation,[14] and apoE Tokyo (Leu141 to Lys143del) is an in-frame deletion.[15] In 1 patient, an 18–amino acid deletion in apoE (Gln156 to Gly173del) was detected.[13] Ogawa et al[45] reported apoE Maebashi (Arg142 to Leu144del) as a novel variant, but this is recognized to be the same as apoE Tokyo because leucine is placed at both positions 141 and 144. Miyata et al[41] also reported apoE5 (Gln3Lys) in a patient after kidney transplantation.

The discovery of novel mutants in apoE suggested that lipoproteins with these variants have a causative role in LPG. In 2000, we showed that LPG was induced by virus-mediated transduction of apoE Sendai in apoE-knockout mice, and apoE Sendai has a reduced affinity for the low-density lipoprotein (LDL) receptor in in vitro studies.[46] A proportion of mice in that study showed marked dilatation of glomerular capillaries by lipoprotein thrombi, which closely resembled histological characteristics in human

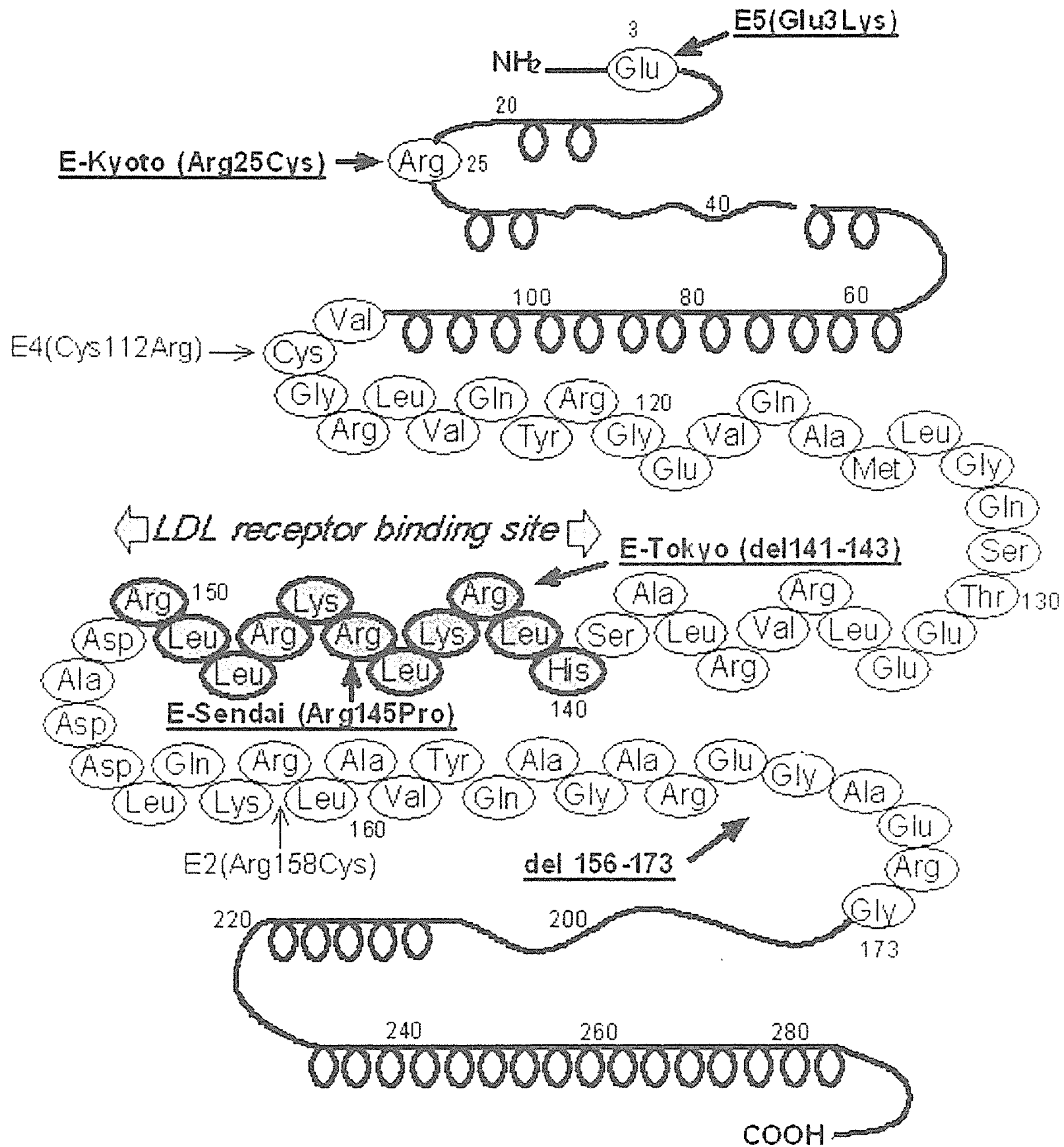

Fig 3. Structure and mutations of apoE. The underlined mutations were detected in patients with LPG. Abbreviation: del, deletion of amino acid residues. Adapted from Saito et al.[19]

LPG. These findings confirmed that apoE variants contribute to the pathogenesis of LPG.[47]

As mentioned, polymorphism of apoE variants is identified in LPG. Detection of apoE Sendai was restricted to eastern Japan. Conversely, apoE Kyoto recently was found in an American-Chinese patient who underwent renal transplantation in New York,[42] as well as in patients born in western Japan.[14,35] ApoE Tokyo-Maebashi also was reported in both Japan[15,45] and China.[21] However, at present, the significance of distribution has not yet been elucidated.

Apart from various apoE mutants, there are no differences in histopathologic findings in the glomerulus. However, some atypical features may depend on the peculiarity of mutants. Severe aneurysm of the aorta and lipoprotein particles in artery walls were reported in a patient with apoE (Gln156 to Gly173del).[13] Furthermore, normal

apoE levels in sera were reported in a few patients with apoE Kyoto[35] and apoE Tokyo.[15]

PATHOGENETIC ROLE OF ApoE VARIANTS IN LPG

ApoE composed of 299 amino acids with a relative molecular mass of 34kDa is 1 of the essential apolipoproteins and a major constituent of various plasma lipoproteins.[48] In particular, binding activities of apoE to LDL receptor, LDL receptor–related protein, and VLDL receptor determine plasma lipoprotein levels.[49,50] As recognized in apoE2/2 homozygosity, reduction of binding activity frequently induces type III HLP.[51-53] Occasionally, renal diseases are involved in this relationship.[54,55] Moreover, as reviewed by Liberopoulos et al,[56] recent studies focused on apoE2 and other variants to examine their role in the pathogenesis and progression of renal diseases. In several cases of familial type III HLP, a specific glomerulopathy with sclerosis and marked accumulation of foam cells was associated with severe hyperlipidemia.[2-5] Similarly, most apoE variants in patients with LPG have mutation around the receptor-binding domain and show type III HLP in the lipoprotein profile.[18] However, features of LPG are different from those of familial type III HLP. In principle, lipoprotein thrombi, but not foam cells, are detected in glomeruli in patients with LPG. Plasma lipid and lipoprotein levels in patients with LPG are slightly lower or within normal ranges compared with those in patients with familial type III HLP. Thus, apoE variants in patients with LPG may be directly responsible for glomerular lesions. It is presumed that the substitution of proline (Arg145Pro) in apoE Sendai produces severe structural changes in the middle of the α helix in apoE and may alter the 3-dimensional conformation of the protein.[12] Although such transformation reduces the receptor-binding activity, as reported by our group[46] and Hoffmann et al,[57] it also is predicted that abnormal lipoproteins containing ApoE Sendai are concentrated and aggregated in the glomerular flow[12] and deposited in capillary walls or around the mesangium. We[17,36] and another group[26] observed under electron microscopy the accumulation of osmiophilic substances, structurally similar to lipoprotein thrombi, in the subendothelial space and paramesangial area in the immature stage of LPG. Conversely, in some apoE variants of LPG, eg, apoE Kyoto[14] and apoE5(Gln3Lys),[41] positions of mutation are far from the receptor-binding site (Fig 3). This means that these variants are not involved in the mechanisms of familial type III HLP inducing marked hyperlipidemia and systemic lipidosis, but are directly injurious to the glomerulus. ApoE Kyoto showed increased binding capacity to endothelial cells.[35] Despite the different positions of mutations, the crucial transformation in the apoE molecule may form similar aggregated deposits of lipoproteins that have high affinity or low clearance in glomeruli.

Miyata et al[41] identified various kinds of lipid peroxidation protein adducts in glomeruli of a patient with LPG who underwent transplantation. We also showed an increase in plasma electronegative LDL in active LPG cases by using capillary isotachophoresis (Fig 4),[58] which is a new and useful technique for lipoprotein analysis.[59,60] As reviewed by Sanchez-Quesada et al,[61] recent studies provided new evidence on the putative atherogenic role of plasma electronegative LDL subtraction, which is understood to reflect small dense LDL, glycated LDL, and diasylated LDL, as well as oxidized LDL. Conversely, impaired catabolism of the VLDL-IDL-LDL cascade in patients with hypertriglyceridemia promotes the increase in apoE, apoC-III, and electronegative LDL.[62] These conditions may be applicable to LPG and III HLP, in which hypertriglyceridemia is caused by negatively charged apoE variants. Taken together, the modified LDL related to apoE abnormality may have an important role in renal lesions in patients with LPG.

It was reported that apoE concentration in plasma is an important determinant independently of apoE polymorphism, but that significantly influences lipid metabolism in patients with renal failure[63] and coronary artery disease[64] in combination with apoE polymorphism. Accordingly, apoE concentration seems an important factor in the development of LPG. Conversely, apoE is synthesized in human kidney[65] and regulates mesangial cell proliferation and matrix overproduction in experimental models.[66] Therefore, apoE seems to act as an autocrine regulator of mesangial and glomerular function,[56,66] and intrarenal dysfunction induced by apoE abnormality may contribute to the induc-