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Biphasic effects of angiotensin II and receptor antagonism on aggregability and protein kinase C phosphorylation in human platelets

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Summary

In order to define the role of angiotensin II (AngII) receptor subtypes, AT1 and AT2, in platelet activation, we examined the effects of AngII and receptor antagonists on both aggregability and phosphorylation status of protein kinase C (PKC) isoforms in human platelets obtained from 56 healthy volunteers. AngII promoted both spontaneous and agonist (collagen and ADP) stimulated platelet aggregation at concentrations of 10 nM or less, but the promotion effects were lost at 100 nM. Antagonism of AT1 receptor inhibited the promotion effects of AngII at 10 nM or less. On the other hand, antagonism of AT2 receptor enhanced platelet aggregability modestly with AngII at 10 nM or less, and markedly with 100 nM AngII. Furthermore, with 10 nM AngII, phospho-PKCα/βII expression in platelets was increased after collagen stimulation and was inhibited by antagonism of AT1 receptor. With 100 nM AngII, expression levels of phospho-PKCα/βII remained low even after collagen stimulation but were markedly enhanced by antagonism of AT2 receptor. These findings suggest that at 10 nM or below, AngII promotes aggregability and PKC phosphorylation in human platelets through the AT1 receptor, which can be inhibited by AT1 receptor antagonists, but at higher concentrations, the promotion effects were lost through the opposing action of the AT2 receptor. The present study may provide an additional mechanism for AT1 receptor antagonism, which would provide clinical benefit to patients with stroke or cardiovascular disease accompanied by hypertension.

Keywords

Angiotensin II, AT1 receptor, platelet aggregation, protein kinase C, sartan

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Introduction

The functions of two major angiotensin II (AngII) receptor subtypes, AT1 and AT2, have been studied using specific receptor antagonists (1–4). Mounting evidence indicates that the AT1 receptor is responsible for the majority of the classical and known biological effects of AngII, including hypertension, vasoconstriction and proliferation of vascular cells, and that it functions as a significant contributor in the pathophysiology of ischemic stroke (1–5). AT1 receptor stimulation results in cerebral vasoconstriction, thus contributing to the shift in cerebrovascular autoregulation toward elevated blood pressure (1–4). Antihypertensive therapy, which blocks the AngII system, is beneficial not only by lowering blood pressure but also by reducing brain vulnerability to ischemia in hypertension (1–4, 6). AngII synthesis or AT1 receptor blockade improves neurological outcome and reduces infarct volume in experimental cerebral ischemia (1–4, 6). These beneficial effects of AT1 receptor blockade do not depend exclusively on normalizing cerebral blood flow (7, 8). We recently demonstrated that AT1 receptor blockade protects neuronal cells against AngII-promoted hypoxic neuronal damage through the inhibition of protein kinase C (PKC) δ (8).

Platelet activation also plays a key role in the pathogenesis of thromboembolic diseases, such as ischemic stroke. As AngII receptors are present on the platelet surface (9, 10), it is important to define whether AngII activates platelets via the AT1 receptor and whether blockade of platelet AT1 receptor serves as an additional mechanism for clinical benefit. However, previous studies have reported conflicting results (9, 10) and the issues remain to be fully elucidated. Ding et al. (9) demonstrated that AngII at physiological concentrations facilitates agonist-induced platelet aggregation and can contribute to thrombotic events. In contrast, it has also been reported that exogenous AngII does not affect *in*

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vitro platelet activation (10), and that some AT1 receptor antagonists function as competitive antagonists to the thromboxane A2 (TxA_2)/prostaglandin H2 (PGH_2) receptor, in addition to blocking the AT1 receptor (10).

In platelets, PKC inhibitors inhibit platelet aggregation and granule release, and phorbol ester stimulates both functions, thus suggesting that PKC is an important signaling molecule in platelet activation (11, 12). Although there is conflicting evidence concerning the sensitivity of agonist-induced platelet aggregation to pharmacological inhibition of PKC (13–15), a variety of mechanisms in the activation and localization of different PKC isoforms is likely to be involved; among these is phosphorylation of serine, threonine, and as recently reported, tyrosine residues (16).

In an effort to clearly define the involvement of AngII receptors in platelet activation, we examined the effects of AngII and receptor antagonists on both aggregability and phosphorylation status of PKC isoforms in human platelets. In the present study, we found that AngII can promote both aggregability and PKC phosphorylation in platelets via the AT1 receptor at concentrations of 10 nM or less, but at higher concentrations, the promotion effects were lost, probably due to the opposing action of the AT2 receptor.

Subjects and methods

Subjects

Subjects were 56 healthy volunteers, non-smokers, between the ages of 26 and 74 (mean age, 47.1±16.2 years; 31 males and 25 females). None of the subjects had a history of stroke, cardiac disease including atrial fibrillation, occlusive peripheral vascular disease, inflammatory disease, diabetes mellitus, or hypertension. Informed consent was obtained from all subjects prior to blood sampling.

Platelet aggregation studies

Venous blood was collected in one-tenth volume of 3.8% (0.13 M) trisodium citrate, and was then centrifuged at 150×g for 10 min to obtain platelet-rich plasma (PRP). PRP obtained from individual subjects was subdivided into four or eight 300 µl-tubes for use with the 4-channel AG10 aggregometer (Kowa, Tokyo) (18, 19). Platelet-poor plasma was used as a reference (17–19). Within 30 min after venipuncture, PRP aggregation was examined simultaneously by conventional evaluation methods for maximum percent decrease in optical density (OD) and partly by assessing the light scattering (LS) intensity using the AG10 (18, 19). Changes in OD and LS intensity were recorded at 10 s intervals for 5 min. The value of OD% was determined at the 5 min time point. Quantitative estimation of LS intensities of small, medium, and large aggregates was performed by determining the areas under the curve representing the sum of the 30 measurements of LS intensity. This method has been described in detail elsewhere (18, 19).

Platelet aggregation studies using a set of four consecutive agent concentrations (i.e., 0, 1, 10, 100 nM of AngII) were performed with PRP from the same subject. When a combination of agents; i.e., AT1 or AT2 antagonist plus AngII was used, the antagonist was preincubated for 2 min with PRP in the aggregometer with continuous stirring, after which AngII was added, immediately followed by agonistic stimulation with collagen or ADP.

We used valsartan (10, 20) and candesartan (8) as specific antagonists for the AT1 receptor, and PD123319 (8) for the AT2 receptor. The following 3 experiments were designated: spontaneous platelet aggregation with four concentrations (0, 1, 10 or 100 nM) of AngII in the presence of 10 µM valsartan, 100 nM PD123319, 10 µM valsartan+100 nM PD123319, or without AngII receptor antagonist; platelet aggregation after stimulation with agonists (collagen at 1 µg/ml or ADP at 1 µM) with AngII (0, 1, 10 or 100 nM) in the presence of valsartan, PD123319, valsartan+PD123319, or without AngII receptor antagonist; platelet aggregation after stimulation with 1 µg/ml collagen with both 10 nM AngII and four concentrations of AT1 receptor antagonist [candesartan (0, 10 nM, 100 nM, 1 µM) or valsartan (0, 1 µM, 10 µM, 100 µM)].

Statistical analysis for changes in platelet aggregability

Results are expressed as mean value ± SD (n=10 to 14 in each condition). Changes as a function of AngII concentration and differences between agent conditions at the same AngII concentration were evaluated by One-way ANOVA and Post hoc tests. The significance level was p=0.05.

Western blotting

PRP samples incubated in the aggregometer with continuous stirring were collected at 0, 1, 3, 5 min after addition of 10 nM AngII alone or both 10 µM valsartan and 10 nM AngII; and 100 nM AngII alone or both 100 nM PD123319 and 100 nM AngII. Isolated PRP was quickly cooled on ice, centrifuged at 3000×g at 4°C for 5 min, and the pellet was used for Western blotting.

Cell lysates were prepared in lysis buffer (50 mM HEPES, 1 mM EDTA, 150 mM NaCl, pH 7.5) containing 1% NP40, 1 mM sodium orthovanabate (SV), 1 mM dithiothreitol (DTT), 10 mM NaF and Protein Inhibitor Cocktail (Calbiochem). Proteins were subjected to electrophoresis in SDS-8% polyacrylamide gel, and were then transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell). Membranes were blocked with 5% dried milk/Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TTBS), and were incubated with primary antibodies for 1 h at room temperature. After washing with TTBS, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (Amersham) or anti-rabbit IgG (Amersham) antibodies diluted 1:3000, and were again washed with TTBS. Primary antibodies used were anti-PKCα (ABGENT), anti-PKCβ (ABGENT), anti-phospho-PKCα/βII (phosphorylated at Thr638/641, Cell Signaling Technology), anti-PKCδ (Santa Cruz), anti-PKCθ (Santa Cruz), anti-phospho-PKCδ/θ (phosphorylated at Ser643/676, Cell Signaling Technology), anti-AT1 (Santa Cruz), anti-AT2 (Santa Cruz), and anti-actin (PROGEN). Detection was performed using an enhanced chemiluminescence (ECL) system (NEN). The chemiluminescence signals from the CCD camera were integrated for 60 s into the computer memory directly from the membranes using the Chemi Doc system (BIO RAD). The density of each pixel and the number of pixels were measured and calculated. Density equal to or lower than the background was eliminated.

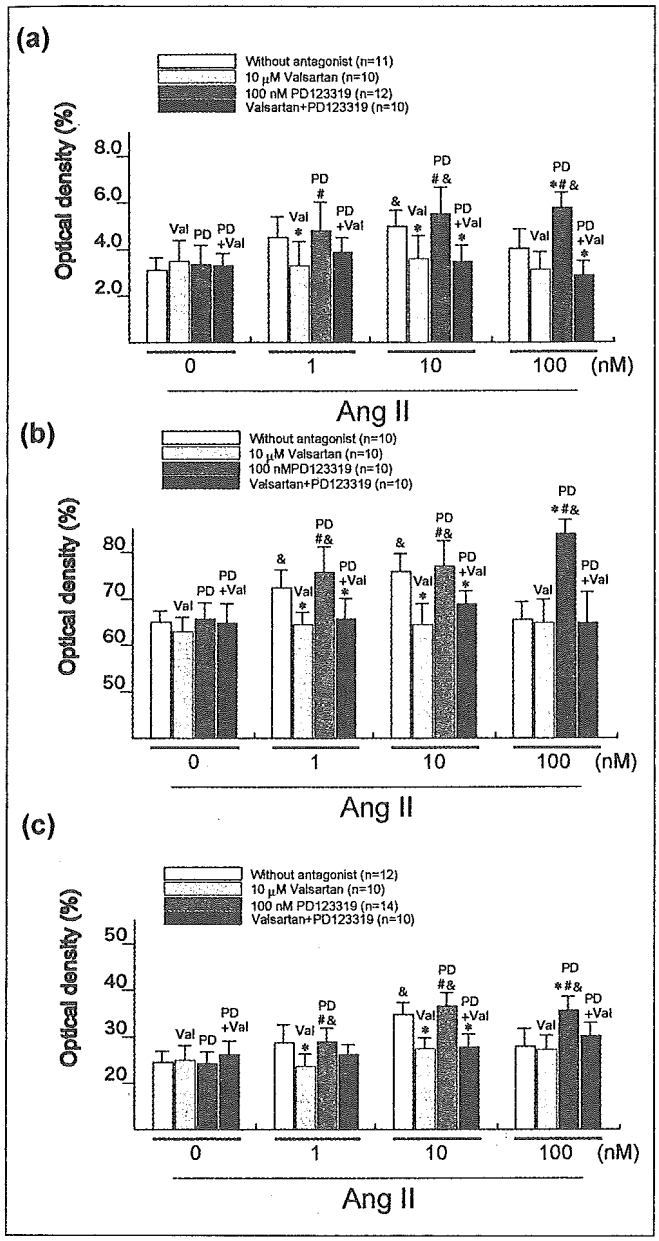

Figure 1: Effects of AngII and receptor antagonism on platelet aggregability. Spontaneous platelet aggregation [optical density (OD)] (a), Collagen (1 μg/ml)- induced platelet aggregation (OD) (b), and ADP (1 μM)-induced platelet aggregation (OD) (c) at four AngII concentrations (0, 1, 10 or 100 nM) without AngII receptor antagonist or in the presence of 10 μM valsartan (AT1 receptor antagonist), 100 nM PD123319 (AT2 receptor antagonist) and 10 μM valsartan+100 nM PD123319. Results are expressed as mean value±SD (n=10 to 14 in each condition). Changes as a function of AngII concentration and differences between agent conditions at the same AngII concentration were evaluated by One-way ANOVA and Post hoc tests. &p<0.05 when compared to 'without (0 nM) Ang II'. *p<0.05 when compared to 'without antagonist' at the same AngII concentration. #p<0.05 when compared to '10 μM valsartan' at the same AngII concentration.

Statistical analysis for semi-quantitative determinations for Western blots

Results are expressed as mean value ± SEM of 3 independent experiments. Changes as a function of time and differences in means between agent conditions at the same time point were evaluated by One-way ANOVA and Post hoc tests. The significance level was p=0.05.

Results

Effects of AngII and receptor antagonism on platelet aggregability

Although human platelets have been clearly shown to possess AT1 receptors (10), the presence of AT2 receptors in platelets has yet to be established (10). As shown in Fig. 3-a, anti-AT2 antibody (Santa Cruz) clearly detected a major band at approximately 50 kDa corresponding to the AT2 receptor in Western blotting for human platelets. To examine the effects of AngII and receptor antagonism on platelet aggregability, we performed the experiments for spontaneous and agonist-stimulated (collagen at 1 μg/ml or ADP at 1 μM) platelet aggregation by OD method with four concentrations (0, 1, 10 or 100 nM) of AngII in the presence of 10 μM valsartan (AT1 receptor antagonist), 100 nM PD123319 (AT2 receptor antagonist), 10 μM valsartan+100 nM PD123319, or without AngII receptor antagonist.

Spontaneous platelet aggregation without agonistic stimulation was faint (3.1±0.6%), but increased with 1 nM AngII and further increased at 10 nM, the effects were lost with AngII at 100 nM (Fig. 1-a). The stimulation effects of AngII on platelets at 1 and 10 nM were inhibited by valsartan (Fig. 1-a). Incubation with PD123319 enhanced spontaneous platelet aggregation modestly with AngII at 1 and 10 nM, and markedly at 100 nM (Fig. 1-a).

Incubation with AngII promoted both collagen (1 μg/ml)-induced and ADP (1 μM)-induced platelet aggregation at 1 nM, and this promotion was more pronounced at 10 nM, but the promotion effects were lost at 100 nM (Fig.1-b, c). The promotion effects of AngII at 1 and 10 nM were inhibited by valsartan (Fig.1-b, c). Incubation with PD123319 enhanced agonist stimulated platelet aggregation modestly with AngII at 1 and 10 nM, and markedly at 100 nM (Fig.1-b, c).

Effects of AngII on large aggregate formation

Incubation with AngII promoted both collagen (1 μg/ml)-induced and ADP (1 μM)-induced large aggregate formation by LS method at 1 nM, and this promotion was more pronounced at 10 nM, but the promotion effects were lost at 100 nM (Table). Generally, smaller aggregates are formed in the first phase of aggregation, and larger aggregates are formed in the second phase (18, 19). The main effect of AngII at 10 nM or below therefore appeared to be on the secondary phase, which was consistent with previous reports (9).

Effects of AT1 receptor antagonists on platelet according to plasma concentration

We examined the ability of AT1 receptor antagonists to inhibit AngII-promoted platelet activation at the plasma concentration

within therapeutic levels. We used candesartan (10 nM, 100 nM, 1 μM) and valsartan (1, 10, 100 μM) as specific antagonists for the AT1 receptor. The plasma concentration of candesartan can peak at 100 nM after oral administration of 4 mg (clinically available at oral doses up to 12 mg per day) (data on file, Takeda Pharmaceuticals Corp.). Valsartan plasma concentration can peak at 10 μM after oral administration of 80 mg (clinically available at oral doses up to 160 mg per day) (data on file, Novartis Pharmaceuticals Corp.).

In the presence of AngII at 10 nM, the concentration exhibiting marked stimulation effects on platelets (Fig. 1), collagen (1 μg/ml)-induced platelet aggregation was inhibited by candesartan at 1 μM (Fig. 2-a), a concentration exceeding therapeutic levels. However, valsartan inhibited aggregation at 10 μM (Fig. 2-b), which is within therapeutic levels.

Effects of AngII and AT1 or AT2 receptor antagonism on phosphorylation of PKC isoforms during platelet aggregation

PKC isoforms are subdivided into three groups based on their lipid and cofactor requirements (21): the diacylglycerol- and calcium-sensitive conventional isoforms (α, βI, βII, and γ), the diacylglycerol-sensitive and calcium-insensitive novel isoforms (δ, η, θ, and ε), and the diacylglycerol- and calcium-insensitive atypical isoforms (ξ, ι, μ, and λ). Human platelets are known to express PKC isoforms α, β, δ, η, θ, ε, and ξ (22). In the present study, we examined the effects of AngII and receptor antagonists on phosphorylation of serine or threonine residues in conventional isoforms (α, βII) and novel isoforms (δ, θ) of PKC.We first analyzed platelets in the absence of agonist at 0, 1, 3, 5 min after addition of 10 nM AngII alone or 10 μM valsartan together with10 nM AngII; 100 nM AngII alone or 100 nM PD123319 together with 100 nM AngII (Fig. 3-a, b). Representative immunoblots for phospho-PKCα/βII (phosphorylated at Thr638/641), actin (internal control), phospho-PKCδ/θ (phosphorylated at Ser643/676), and AT1 and AT2 receptors are shown in Fig. 3-a. Semi-quantitative determinations for each band of phospho-PKCα/βII, relative to actin, are shown as a function of time in Fig. 3-b.

After incubation with 10 nM AngII alone, the ratio of phospho-PKCα/βII to actin was modestly increased, 1.3-fold at 3 min after incubation, whereas pre-incubation with 10 μM of valsartan and 10 nM AngII decreased the ratio, 0.5–0.7-fold at 1–5 min, relative to that before incubation (Fig. 3-a, b). In contrast, while 100 nM AngII alone decreased the expression levels of phospho-PKCα/βII to approximately 0.5-fold at 1–5 min after incubation, pre-incubation with 100 nM PD123319 and 100 nM AngII increased the levels to 1.5–1.6-fold when compared with those before incubation (Fig. 3-a, b). Expression of phospho-PKCδ/θ was faint and difficult to quantify, but appeared to change in parallel with that of phospho-PKCα/βII (Fig. 3-a). Throughout the course of examination, before and up until 5 min after incubation with AngII, the expression levels of both AT1 and AT2 receptors were unchanged, and did not differ among the experimental conditions (Fig. 3-a). Expression levels of PKCα, PKCβ, PKCδ and PKCθ proteins did not change throughout the course of examination and not differ among the experimental conditions (not shown).

Table 1: Platelet aggregation measured by Light scattering (LS) method under conditions without or with Ang II (1, 10, 100 nM). LS total intensity is expressed as area under the curve [(volt × count/10sec) representing 30 determinations (5 min)] for small, medium, and large aggregates.

	Without Ang II	Ang II 1 nM	Ang II 10 nM	Ang II 100 nM
Collagen (1 μg/ml) (n=11)				
LS (×10⁵)				
Small	7.0 ± 4.2	9.1 ± 5.0	7.0 ± 3.4	7.1 ± 4.3
Medium	5.6 ± 3.1	6.4 ± 3.4	6.7 ± 3.7	5.4 ± 2.4
Large	10.4 ± 9.0	13.9 ± 12.8	15.09 ± 10.4**	11.9 ± 8.6#
ADP (1μM) (n=11)				
LS (×10⁵)				
Small	26.9 ± 16.5	33.1 ± 15.7	28.2 ± 20.3	26.0 ± 19.4
Medium	6.4 ± 6.9	11.5 ± 11.8*	8.0 ± 2.6	9.0 ± 2.9
Large	3.0 ± 4.5	4.5 ± 6.2*	8.0 ± 6.3*	5.5 ± 7.2

*p < 0.05, **p<0.01 compared with 'without Ang II' (Wilcoxon signed rank test). #p<0.05, ##p<0.01 compared with 'Ang II 10 nM' (Wilcoxon signed rank test).

We then analyzed platelets after stimulation with 1 μg/ml collagen (Fig. 4-a, b). Representative immunoblots for phospho-PKCα/βII, actin and phospho-PKCδ/θ are shown in Fig. 4-a. Semi-quantitative determinations for phospho-PKCα/βII, relative to actin, are shown as a function of time in Fig. 4-b.

With 10 nM AngII alone, the ratio of phospho-PKCα/βII to actin was increased approximately 1.8-fold at 3 min after collagen stimulation when compared with that before stimulation,

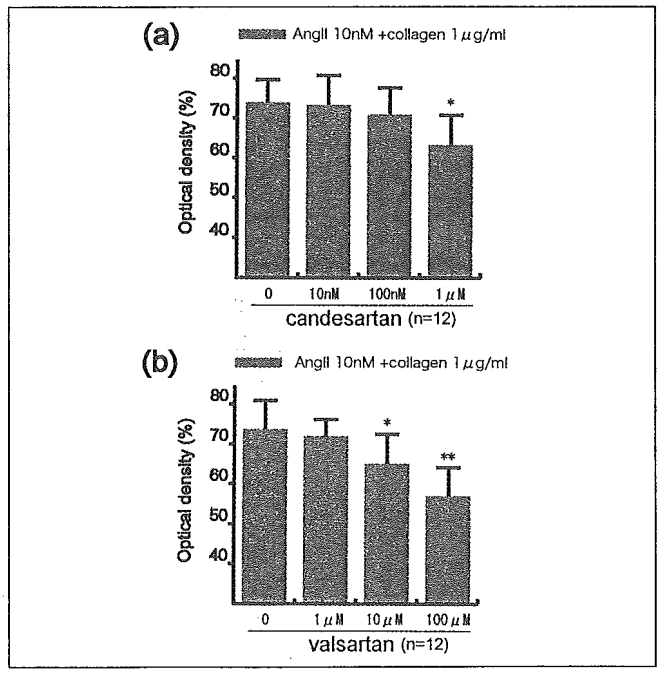

Figure 2: Effects of AT1 receptor antagonists on platelet aggregability according to plasma concentration. Effects of candesartan (10 nM, 100 nM, 1 μM) **(a)** and valsartan (1, 10, 100 μM) **(b)** on collagen (1 μg/ml)-induced platelet aggregation (OD) with 10 nM AngII. Results are expressed as mean value±SD (n=12 in each condition). Changes in aggregability as a function of AngII receptor antagonist concentration were evaluated by One-way ANOVA and Post hoc tests. *p < 0.05, **p<0.01 when compared to 'without (0 nM) antagonist'.

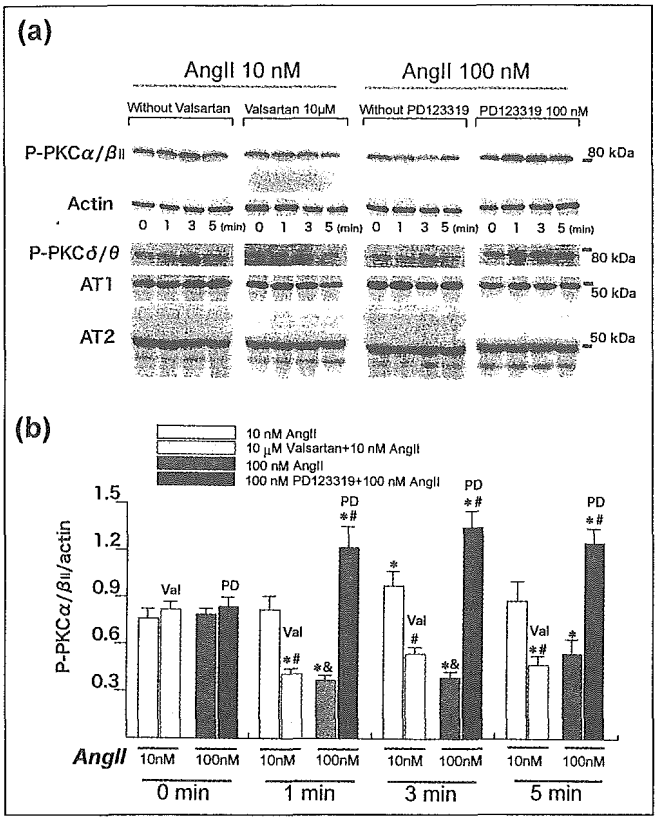

Figure 3: Western blotting for phospho-PKCα/βII, actin, phospho-PKCδ/θ, AT1 receptor, and AT2 receptor in platelets under conditions without agonist at 0, 1, 3, 5 min after addition of 10 nM AngII alone, or 10 μM valsartan together with 10 nM AngII; 100 nM AngII alone, or 100 nM PD123319 together with 100 nM AngII. (a) Representative immunoblots. (b) Semi-quantitative determinations for each band of phospho-PKCα/βII, relative to actin (internal control), are shown as a function of time. Each value represents mean±SEM of 3 independent experiments. Changes as a function of time and differences in means between agent conditions at the same time point were evaluated by One-way ANOVA and Post hoc tests. *p<0.05 when compared to 'before AngII addition'. #p<0.05 when compared 'without AngII receptor antagonist' at the same time point. &p<0.05 when compared to 10 nM AngII alone at the same time point.

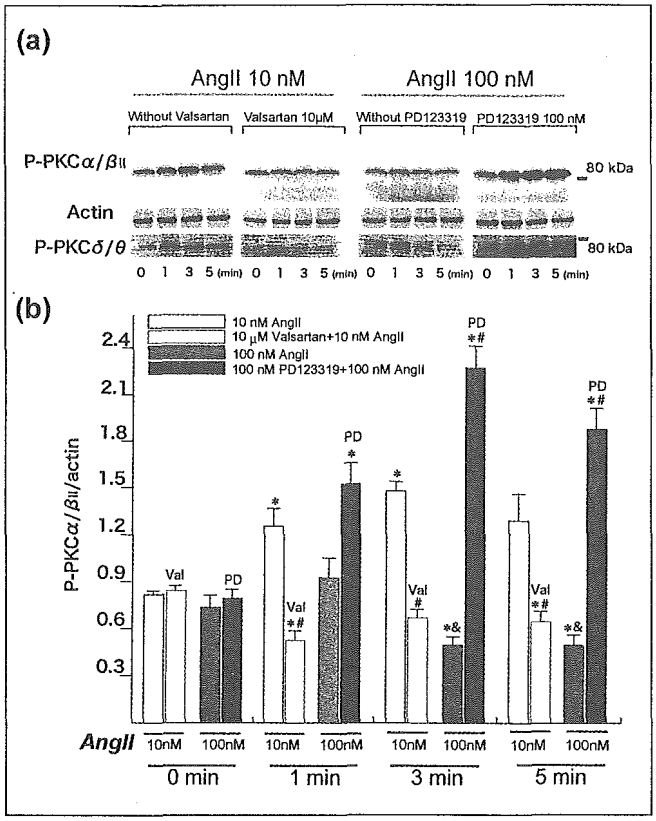

Figure 4: Western blotting for phospho-PKCα/βII, actin, and phospho-PKCδ/θ in platelets at 0, 1, 3, 5 min after stimulation with collagen (1 μg/ml) in the presence of 10 nM AngII alone, or 10 μM valsartan together with 10 nM AngII; 100 nM AngII alone, or 100 nM PD123319 together with 100 nM AngII. (a) Representative immunoblots. (b) Semi-quantitative determinations for each band of phospho-PKCα/βII, relative to actin (internal control) as a function of time. Val, valsartan. PD, PD123319. Each value represents mean ± SEM of 3 independent experiments. Changes as a function of time and differences in means between agent conditions at the same time point were evaluated by One-way ANOVA and Post hoc tests. *p<0.05 when compared to 'before collagen addition'. #p<0.05 when compared to 'without AngII receptor antagonist' at the same time point. &p<0.05 when compared to incubation with 10 nM AngII alone at the same time point.

whereas pre-incubation with 10 μM valsartan markedly inhibited the ratio to 0.5-fold lower than that with AngII (10 nM) alone (Fig. 4-a, b). With 100 nM AngII alone, the expression levels of phospho-PKCα/βII decreased by approximately 0.7-fold at 3–5 min after stimulation, whereas pre-incubation with 100 nM PD123319 markedly enhanced the levels by 3.0–4.0-fold when compared with AngII (100 nM) alone (Fig. 4-a, b). AngII-promoted increases (10 nM AngII alone or PD123319 and 100 nM AngII) in the expression of phospho-PKCα/βII after collagen stimulation was more marked when compared to those without agonistic stimulation (Fig. 3, 4). Expression of phospho-PKCδ/θ was faint in some experimental conditions, and was difficult to quantify, but appeared to change in parallel with that of phospho-PKCα/βII (Fig. 4-a). Expression levels of PKCα, PKCβ, PKCδ and PKCθ proteins did not change throughout the course of examination and did not differ among the experimental conditions (not shown).

Discussion

AngII has been reported to increase intracellular free calcium in platelets and promote agonist-induced platelet aggregation (9). It is postulated that activation of AngII receptors on platelets potentially contributes to thrombotic events (9, 10, 20). However, it has also been reported that exogenous 100 nM AngII failed to modify agonist-induced platelet activation, although the platelets apparently expressed AT1 receptors (10). The ability of some AT1 receptor antagonists to inhibit TxA_2-dependent platelet activation by interacting with TxA_2/PGH_2 receptors has been demonstrated under conditions without exogenous AngII

(10). The biphasic effects of AngII depend on its concentration, as noted by Ding et al. (9): low concentrations of AngII (<1 nM) enhanced adrenaline-induced platelet aggregation, but higher concentrations had an inhibitory effect. However, no studies have examined the involvement of AT1 and AT2 receptors in that phenomenon (9).

In the present study, AngII apparently promoted both spontaneous and agonist (collagen and ADP) stimulated platelet aggregation at concentrations of 10 nM or less, which is near physiological concentrations (<1 nM) (23), but the promotion effects were lost at 100 nM. Antagonism of the AT1 receptor inhibited the promotion effects of AngII at 10 nM or less. On the other hand, antagonism of the AT2 receptor enhanced platelet aggregability modestly with AngII at 10 nM or less, and markedly with 100 nM AngII. These findings suggest that AngII promotes platelet aggregability at lower concentrations predominantly via the AT1 receptor and the promotion effects are inhibited by the predominant action of AT2 receptor at higher AngII concentrations. Thus the biphasic effects of AngII on platelets are probably related with opposite actions of the AT1 and AT2 receptor in activating and inactivating platelet aggregability with or without agonistic stimulation and predominant actions of the AT1 or AT2 receptor at lower or higher AngII concentrations.

Several reports indicated that AT1 and AT2 receptors have opposite actions on signal pathways of serine/threonine kinases including PKC (24-27). The AT2 receptor reportedly inhibits AT1-stimulated inositol triphosphate generation and stimulates serine/threonine phosphatase PP2A (27, 28). Direct interaction by binding of the AT2 to the AT1 receptor is also one of the mechanisms by which the AT2 exerts antagonistic effects on the AT1-induced signal pathways (28). In the present study, at a lower concentration, AngII enhanced the phosphorylation of PKCα/βII in platelets and AT1 antagonism blocked this action, whereas at a higher concentration, AngII downregulated phosphorylation of PKCα/βII, which was markedly enhanced by AT2 antagonism. Considering that PKC is an important signaling molecule in platelet activation (11-13, 15), the opposite actions of the AT1 and AT2 receptor in activating and inactivating platelets may be related to their opposite actions on PKC in platelets.

However, in the present study, the mechanisms of predominant actions of the AT1 or AT2 receptor at lower or higher AngII concentrations remain unclear. One possible explanation is that once AT1 receptor is saturated, AT2 receptor may function as the principal receptor for AngII in platelets. The dissociation constant (Kd) values for AngII on the AT1 receptor and AT2 receptor are reportedly 1.6 ± 0.12 nM and 0.78 ± 0.04 nM, respectively, as for COS1 cells expressed with rat AT1 and AT2 receptors (29), suggesting that AngII binding affinity to the AT2 receptor appears not to be smaller than that to the AT1 receptor. However, as the binding ability of AngII may differ according to cell types and the expression levels of receptor subtypes, it is necessary to test this possibility.

Valsartan inhibited the AngII-promoted aggregation at 10 μM, which is within therapeutic levels, while candesartan inhibited the promotion effects at 1 μM, a concentration exceeding therapeutic levels. Therefore, the efficacy or usefulness of AT1 receptor antagonists in inhibiting AngII-promoted platelet activation appears to differ. On the other hand, under conditions without exogenous AngII, valsartan was reported to exhibit potency in diminishing platelet-fibrinogen binding and expression of P-selectin and vitronectin receptors on the platelet surface (20). However, AT1 receptor-independent platelet inhibition was reported to require valsartan at concentration of 100 μM or above, a concentration exceeding therapeutic levels (20). The ability of some AT1 receptor antagonists to inhibit TxA_2/PGH_2 receptors independently of Ang II involvement has also been reported to require valsartan at 50 μM or more (10). Therefore, the anti-platelet action of AT1 receptor antagonists is worth considering as an inhibitory action for AngII involvement, although some AT1 receptor antagonists have alternative targets in platelets (10, 20).

Although the ability of the AT1 receptor to enhance phosphorylation of PKCα/βII in platelets was observed in the presence and absence of collagen stimulation, the phosphorylation levels were more marked after collagen stimulation. Thus, PKCα/βII appears to function downstream of both AT1 receptor and collagen stimulation.

The expression levels of phospho-PKCδ/θ were modest when compared with those of phospho-PKCα/βII under the present experimental conditions. These findings do not necessarily suggest the insignificance of PKCδ/θ as a signal transducer downstream to the AT1 receptor. Phosphorylation of PKCδ at Ser643, a major phosphorylation site for enzymatic activity, is induced by AngII in a candesartan-inhibited manner in the rat pheochromocytoma cell line PC12 (8). A variety of mechanisms associated with different PKC isoforms seems to be regulated in platelet activation (22). The conventional and novel PKC isoforms play different roles in platelet degranulation (22, 30). While conventional PKCα/β isoforms play an important role in the dense granule secretion mediated by glycoprotein VI, which also acts as a receptor for collagen (22), the novel PKCδ/θ isoforms do not play such a role (22). Therefore, differences in AngII-induced or collagen-induced phosphorylation levels between PKCα/βII and δ/θ observed in the present study may be specific for platelets or platelets under the present experimental conditions. However, in the present study, our focus was to examine the effects of AngII and receptor antagonism on the phosphorylation status of PKC isoforms, and thus the correlations between PKC isoforms and platelet activation could not be clarified.

In conclusion, we demonstrated that AngII promotes aggregability and phosphorylation of PKCα/βII in human platelets through the AT1 receptor at concentrations of 10 nM or less, and this action can be inhibited by AT1 receptor antagonists. However, the ability is lost at higher concentrations probably through the opposing action of the AT2 receptor. Recent studies on antihypertensive treatment by AT1 antagonists indicate a conceptual shift from lowering blood pressure to specific tissue-protective effects (31). Considering the presence of AngII receptors on platelets, the present study may demonstrate an additional mechanism of AT1 receptor antagonism for the clinical benefit of patients with stroke or cardiovascular disease accompanied by hypertension. However, thrombosis is a multicellular event in which other blood cells and endothelium are involved in the regulation of platelet reactivity. Whether AT1 receptor antagonists reduce vascular thrombotic events via additional pathways of platelet inhibition requires further clinical research.

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Increase of total homocysteine concentration in cerebrospinal fluid in patients with Alzheimer's disease and Parkinson's disease

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Abstract

We determined the concentrations of free homocysteine (HC) and total HC in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) or Parkinson's disease (PD) in order to elucidate whether HC is related to the pathogenesis of these neurodegenerative diseases. The concentration of free HC did not differ significantly from that of the normal controls, while the concentration of total HC was significantly higher in the AD and PD patients (+31% in AD,+31% in PD; $p<0.05$). These findings suggest that an increase of total HC concentration in the brain is commonly seen in patients with AD and PD, and this may be related to the pathogenesis of these two diseases.

Keywords: Alzheimer's disease; Parkinson's disease; Free homocysteine; Total homocysteine; Cerebrospinal fluid

Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are neurodegenerative diseases that frequently develop with aging, and the incidence of these diseases is increasing in the elderly population. Furthermore, environmental factors, daily activity and life style, as well as hereditary predisposition are also related to the pathogenesis of AD and PD, and there has been much interest in identifying the causes of these diseases.

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Among the essential amino acids, homocysteine (HC) is generated from the metabolism of methionine (Met), the only sulfur-containing amino acid. HC contains an SH-radical, and thus, oxidative stress occurs from the process of excessive oxidation of HC, which may lead to cell damage (Starkebaum and John, 1986; Nishinaga et al., 1993). Most HC in plasma is the of oxidized type, and 70–80% of HS is bound to albumin. The remaining 20–30% exists as mixed disulfide with HC, thereby creating an HC dimer, or with cysteine. Free HC, that is, the reduced type, comprises only about 1% of total HC (Fig. 1). A high level of free HC has been shown to be an independent risk factor for coronary artery and cerebrovascular arteriosclerotic disease (Welch and Loscalzo, 1998).

Recent studies have identified a point mutation from C to T in the 677th nucleoside (C677T) in the methylenetetrahydrofolate reductase (MTHFR) gene (Frosst et al., 1995). Enzyme activity has also been shown to change due to MTHFR gene polymorphism, and the plasma total HC concentration is high in individuals with the polymorphic homo (Val/Val) type. Plasma total HC concentration increases with aging, and thus the relationship between the pathogenesis of AD and HC is under investigation (Selhub et al., 1993). Studies have also suggested that plasma total HC is significantly higher in AD than in normal controls, and a significant correlation has been found between plasma total HC concentration and dementia scale (Lehmann et al., 1999).

On the other hand, plasma HC concentration is higher in patients with PD (Kuhn et al., 1998) and the polymorphic homo (Val/Val) type of the MTHFR gene has a significantly higher frequency in cases of PD. Yasui et al. reported that plasma HC concentration is higher in patients with PD and the polymorphic homo (Val/Val) type of the MTHFR has a significantly higher frequency in cases of PD (Yasui et al., 2000). Using mouse models of AD and PD, Mattson recently reported that HC promotes neurodegeneration in the substantia nigra and hippocampus (Kruman et al., 2002; Duan et al., 2002). In the present study, the concentrations of free HC and total HC in the CSF of untreated patients with AD or PD were measured, and attempts made to determine whether HC is related to the mechanism of development of AD and PD and to the progression of disease stage.

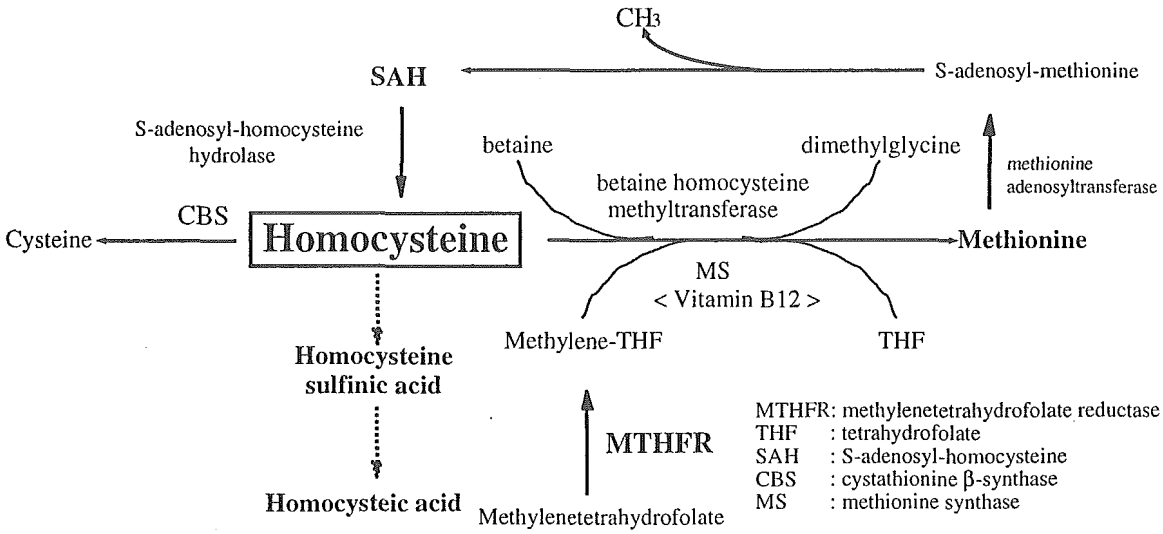

Fig. 1. Synthesis and metabolic pathway of homocysteine.

Patients and methods

Patients

The subjects were 17 untreated patients with AD (age; 67.4 ± 5.0 years (mean ± S.D.), 7 men and 10 women, duration of illness 3.1 ± 2.2 years, Mini-Mental State Examination (MMSE) score 16.5 ± 3.6), 16 untreated patients with PD (65.2 ± 10.3 years, 7 men and 9 women, duration of illness 2.2 ± 1.7 years, MMSE score 27.0 ± 3.2, Hoehn and Yahr's stage 2.7 ± 0.8, relatively high), the reason that the mean duration of illness 2.2 ± 1.7 (two cases over 5 years) is that Iwate is a rural area, and thus the number of untreated PD patients is fewer in urban areas and thus mean is higher and 16 age-matched neurologically normal controls (65.7 ± 9.2 years, 9 men and 7 women) (Table 1). AD and PD were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (4th) (DSM-IV) (Frances et al., 1994) the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and Alzheimer's Disease and Related Disorders Association (ADRDA) (Mckhann et al., 1984) and Koller's criteria (Koller and Montgomery, 1997). Cell counts and protein concentration in the CSF were within the normal range in both AD and PD patients (1.5 ± 0.9 mm^3 and 31.2 ± 6.7 mg/dl, respectively, in AD and 1.3 ± 0.8 mm^3 and 30.1 ± 5.9 mg/dl, respectively, in PD) and in the controls (1.8 ± 1.2 mm^3 and 30.4 ± 6.7 mg/dl, respectively). All patients provided informed consent, and the study protocol was approved by the Committee for Ethics in biomedical research of The Iwate Medical University (Morioka, Japan).

CSF Analysis

CSF was obtained between 9:00 and 10:00 AM after overnight bed-rest and before breakfast. The CSF samples were rapidly frozen and stored at −80 °C until assayed. A tube filled with 100 μl distilled water was used to determine free HC concentration, and a tube filled with 75 μl distilled water and 25 μl tris-2-carboxy-ethylphosphine (TCEP) was used to determine total HC concentration. To each of these tubes, 300 μl CSF was added. After mixing for 60 s and shaking for 10 min, 500 μl of 0.3 N $HClO_4$ was added. After shaking for 1 min, centrifugation at 25 °C was performed at 10,000 g for 10 min. From the mixed solution thus obtained, 100 μl of supernatant was taken, and 20 μl of the supernatant was injected into a high performance liquid chromatography (HPLC) Coulochem electrochemical detector system to determine the concentration of HC. As the mobile phase, 0.15 M phosphate buffered saline was used (Table 2).

Table 1
Subjects

	n	Age (years)	Duration of disease (years)	H and Y stage	MMSE score
AD	17	67.4 ± 5.0	3.1 ± 2.2	–	16.5 ± 3.6
PD	16	64.6 ± 10.3	2.2 ± 1.7	2.7 ± 0.8	27.0 ± 3.2
Control	16	65.7 ± 9.2	–	–	–

MMSE: Mini-Mental State Examination.
H and Y stage: Hoehn and Yahr's stage.
mean ± S.D.

Table 2
Chromatographic conditions for homocysteine

- Pump: Shimadzu LC-10 AD
- Flow: 1.0 mL/min
- Column: 80 × 4.6 mm MCM C 18 reversed phase column (MC Medical)
- Mobile phase: 20 mM NaH_2PO_4, 8% CH_3OH, pH 2.7 with H_3PO_4
- Temp: 26 °C
- Injection volume: 80 μL
- Detector: Coulochem II Model 5300 (ESA)
- Potentials: Guard cell 750 mV, E1 550 mV, E2 700 mV

Statistical Analysis

Statistical analysis was performed using nonparametric Mann–Whitney *U* test or Spearman's rank test correlation coefficient (r_s) with Stat View 5.0 (SAS Institute Inc.). The significance level was set at $p<0.05$.

Results

The concentration of free HC (nM) did not differ significantly between controls (10.9 ± 3.4) and AD patients (10.6 ± 6.0) or PD patients (10.6 ± 4.5) (Fig. 2a). The concentration of total HC (nM) was 84.9 ± 24.5 in controls, 110.6 ± 31.6 in AD patients and 111.4 ± 28.4 in PD patients. AD and PD patients showed a significant increase in total HC compared with controls (+31% for AD,+31% for PD, $p<0.05$) (Fig. 2b).

There was no significant correlation between the duration of illness and the concentration of total HC in AD and PD. In patients with AD, there was no significant correlation between MMSE score and the concentration of total HC. In patients with PD, there was no significant correlation between Hoehn and Yahr's stage and the concentration of total HC.

Discussion

The involvement of HC in AD has not been evaluated in the autopsied brain, and most in vivo studies have been performed on plasma total HC. The plasma total HC concentration was significantly higher in AD patients than in normal elderly subjects, while the concentrations of plasma folate and vitamin B_{12} were significantly lower. Many researchers have suggested that this decrease in vitamin levels might be related to the concentration of total HC (Joosten et al., 1997; Clarke et al., 1998). Furthermore, significant correlation between the concentration of total HC and dementia scale has been demonstrated (Clarke et al., 1998). Selley recently examined the CSF of 8 patients with AD, and found that total HC in the CSF was high in the AD patients and that positive correlation existed between total HC concentration and the concentration of 4-hydroxy-2-nonenal, which is a biochemical marker of neurotoxic products of lipid peroxidation (Selley et al., 2002). The results of that study suggested that HC may induce neuronal damage via oxidative stress. HC has also been reported to enhance susceptibility of the hippocampus to

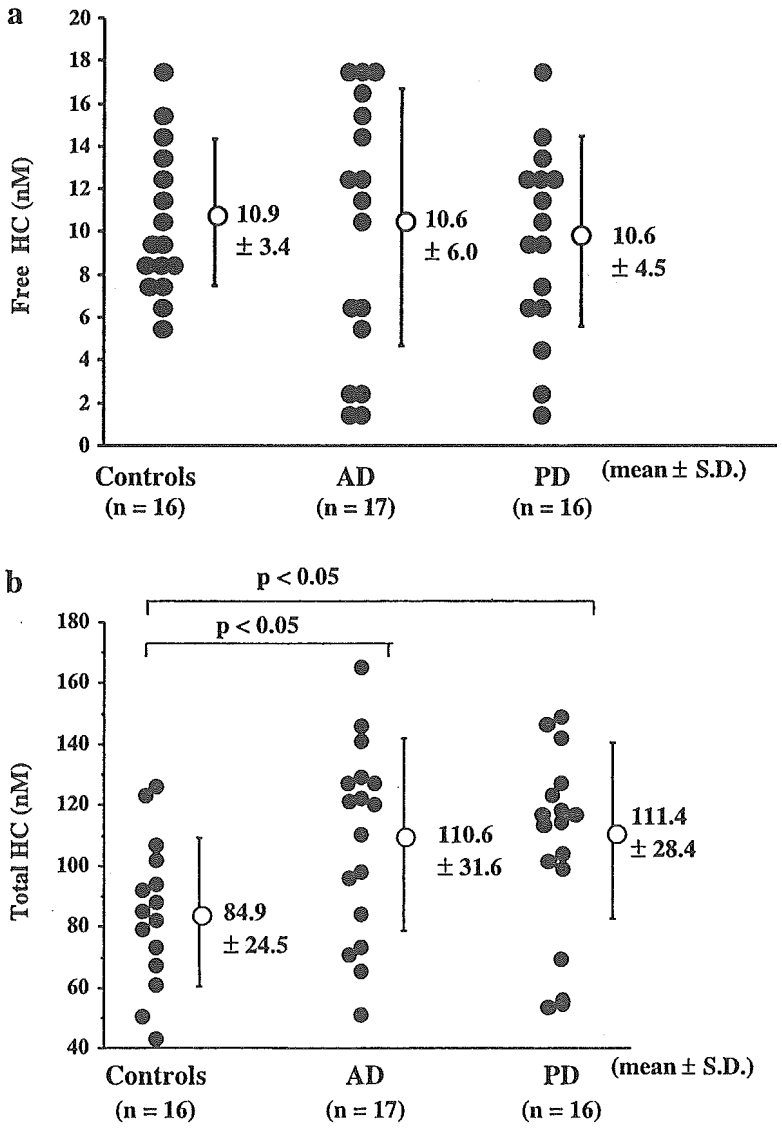

Fig. 2. (a) Concentration of free homocysteine in cerebrospinal fluid of patients with AD and PD. (b) Concentration of total homocysteine in cerebrospinal fluid of patients with AD and PD.

neuronal damage and HC may promote peroxidation of amyloid β protein, i.e., the major protein component in senile plaques, or enhance neurotoxicity (White et al., 2001).

On the other hand, in PD, Allain et. al reported no change in plasma concentrations of cysteine and sulfate but found that total HC concentration was significantly higher than normal. They suggested that HC may be related to peroxidation of iron ions (Allain et al., 1995). Kuhn et al. reported high plasma total HC concentrations in patients with PD receiving treatment with L-DOPA/DCI, and the concentration correlated with severity of the disease (Kuhn et al., 1998). Müller reported that total HC concentration was higher in patients treated with L-DOPA/DCI, while there was no change in the untreated group, compared to the controls (Müller et al., 1999), suggesting that the increase in plasma

total HC concentration may have been caused by the L-DOPA/DCI treatment. Recently, the above mentioned Yasui found that the polymorphic homo type of the MTHFR gene was present at a significantly higher frequency (as much as 17% higher) in PD patients than in controls, and that HC concentration showed a negative correlation with plasma folic acid level, which is related to the metabolism of MTHFR, in homo type patients (Yasui et al., 2000). Moreover, HC is toxic to cultured nerve cells, and at low concentration, selectively damages dopaminergic neurons. At high concentration, HC is toxic to all cultured nerve cells in addition to dopaminergic neurons (Yasui et al., 2000).

These combined findings suggest that a mild increase in HC concentration in the brain may selectively promote degeneration of dopaminergic neurons. In the study of the involvement of HC in neurodegenerative diseases, it is important to analyze the concentrations of both free HC and total HC in the brain. As HC is water-soluble, its migration from the blood into the brain is physiochemically blocked by the brain. Thus, free HC and total HC in the brain presumably originate there. In fact, total HC concentration in the CSF in normal subjects is reported to be about 5% of that in plasma (Hyland and Bottigliere, 1992), which is very low. On the other hand, in the above-mentioned study by Selley, total HC concentrations in plasma and CSF were higher in AD patients than in controls, and there was positive correlation between the two. However, the increase in HC concentration was greater in CSF than in plasma (+32% in plasma; +48% in CSF), while the concentration of folate (coenzyme required to metabolize HC) was significantly lower (−41%) in plasma, but merely showed a tendency to decrease (−28%) in CSF. The researchers noted that the increased HC concentration in the CSF may have derived from the brain (Selley et al., 2002). These results indicate that the concentrations of free and total HC in the CSF determined in the present study were primarily derived from the brain. In the present study, total HC concentrations in the CSF in AD and PD patients were significantly increased, and by almost the same degree. However, no significant change was found in free HC concentration, which is considered to have biochemically high activity such as promotion of vascular injury (arteriosclerosis, arterial occlusive disease). Total HC is metabolized to various products, including homocysteic acid (Fig. 1) (Parnetti et al., 1997), which has a structure similar to that of glutamate, which itself is an excitatory neurotransmitter. Studies have shown that homocysteine metabolites such as homocysteic acid (Fig. 1) activate the excitatory *N*-methyl-D-aspartate (NMDA) receptor and induce apoptosis (Ratan et al., 1994a). The cytotoxicity of glutamate causes cell damage from excitotoxicity via the receptor, and from competitive inhibition of cysteine transport by an increase in glutamate concentration. The latter causes a deficiency in intracellular glutathione, and consequently, neurons succumb to apoptosis due to oxidative stress (Ratan et al., 1994a). Rantan examined the apoptosis-inducing action of the sulfur-containing excitatory amino acids using cultured rat neurons, and demonstrated that homocysteic acid induces apoptosis at a lower concentration than does glutamate (Ratan et al., 1994b). The results of the present study suggest that homocysteic acid, which is a metabolite of total HC, may be increased in the brain of AD and PD patients, as no significant change was observed in free HC concentration, which is thought to have high activity. Most of the total HC is of the oxidized type, and this suggests the possibility that the superoxide radical (O_2^-) or hydroxyl radical (OH^-) may have been generated in the peroxidation process associated with the increase in HC, thereby inducing nerve cell damage. In the present study, the total HC concentration in the CSF was significantly higher in untreated AD and untreated PD subjects, while no significant correlation between disease duration and severity was observed. Generally, homocysteine is a risk factor for cerebrovascular disease and AD may be linked to its presence. In AD, one sees a decrease in density of capillary, focal stenosis in vessels, endothelial dysfunction, decrease in mitochondoria in endothelium, increase in pinocytic vesicle, increase in collagen basal membrane,

irregular arrangement of smooth muscle cells and accumulation of amyloid (Buee et al., 1994). The above mentioned researchers have all reported such vascular disorders in their AD patients. On the otherhand, in PD, an increased number of endothelial cell nuclei in the substantia nigra pars compacta has been reported by Faucheux (Faucheux et al., 1999). These changes in vascularisation density could contribute to nigral iron accumulation, which is known as "nigral endothelial dysfunction" in Parkinson's disease and may be related to homocysteine (Müller et al., 1999). Based on the results of the present study, both hypotheses have merit, namely that an increase in total HC in the brain is a finding common to AD and PD, and that total homocysteine is a contributor to degeneration but also could also reflect an epiphenomenon.

Conclusions

Total HC and HC metabolites (such as homocysteic acid) may be suggested to be related to the pathogenesis of AD and PD.

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Perivascular infiltrate of memory lymphocytes and mature dendritic cells in MG thymomas

Abstract—In thymomas associated with myasthenia gravis (MG), the authors found that perivascular infiltrates of memory lymphocytes and mature dendritic cells (DCs) were more frequent in patients with early improvement after thymectomy than in patients without response to thymectomy. Although these findings may be limited to particular types of thymoma, thymectomy may interrupt the recruitment of mature DCs in thymus and export of activated T cells to extra-thymic tissues, thereby improving the disease.

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Myasthenia gravis (MG) is an autoimmune disease generally mediated by antibodies against the acetylcholine receptor (AChR) of skeletal muscle.[1] Production of these antibodies in B cells is T cell-dependent.[2] MG-associated thymomas are enriched with autoreactive T cells with specificity for AChR subunits,[3] and these cells appear to be generated by nontolerogenic thymopoiesis occurring in abnormal thymic microenvironments with neoplastic epithelial cells.[3] However, the presence of potentially AChR-reactive T cells alone would not be sufficient to cause MG. Sustained activation of autoreactive lymphocytes resulting from self antigen presentation by dendritic cells (DCs)[4,5] is crucial for the development of autoimmune disease.[6] Efficient triggering of MG pathogenesis would require DCs to present an AChR-like antigen to AChR-reactive T cells in an MHC-restricted manner.[6]

It is postulated that MG-associated thymomas export naive and potentially autoreactive T cells to extratumoral sites and that T-cell activation outside the thymoma may initiate the autoimmune process.[3] However, the pathogenesis in the thymus would differ among patients with and without early improvement following thymectomy. Although there is no convincing evidence that thymectomy is of benefit in MG,[7,8] the activation of AChR-specific T cells possibly occurs in the thymus in patients with MG with early improvement following thymectomy.[6] In thymus with lymphofollicular hyperplasia (TLFH) from patients with early onset MG (EOMG) who exhibit marked improvement after thymectomy, CD44- and secondary lymphoid-tissue chemokine-associated mechanisms appear to promote migration of both memory lymphocytes and mature DCs from the blood to the thymus.[6] Mature DCs from the vascular system may activate potentially autoreactive T cells in MG thymus, and thymectomy may interrupt the recruitment of mature DCs to the thymus and the export of activated autoreactive T cells to extrathymic organs, thereby improving the disease.[6] We report herein that CD44-associated perivascular infiltrates of memory lymphocytes and mature DCs were marked in thymomas from patients with MG with early improvement after thymectomy.

Methods. Subjects were nine patients with thymoma-associated MG with generalized symptoms (table). They were selected out of patients with thymoma-associated MG in our institute according to definite clinical course of early remission or the absence of response after thymectomy. The clinical symptoms of three patients (Patients 1 to 3 in the table) had improved after thymectomy, enabling complete stable remission (CSR) according to the Myasthenia Gravis Foundation of America (MGFA) Postintervention Status classification[9] (asymptomatic for more than 1 year with no medication) within 1 year after thymectomy. Six patients (Patients 4 to 9 in the table) were administered both pyridostigmine bromide (120 mg 180 mg) and oral prednisolone for more than 2 years after thymectomy to achieve a MGFA Postintervention Status of Minimal Manifestations (MM)-3.[9] Three of these six patients also received the immunosuppressant FK506. Dose of prednisolone and FK506, and total observation period after thymectomy for individual patients, are described in the table. None of the patients had received any immune treatment prior to thymectomy. After receiving informed consent, thymoma tissue was obtained from the patients. At least five solid blocks were taken from each thymoma tissue to analyze thymoma histology. Specimens were immediately frozen, without fixation, in liquid isopentane cooled in liquid nitrogen and stored at −80 °C until analysis. Thymic histology was classified according to the criteria for histologic diagnosis of thymic epithelial tumors defined by the World Health Organization (WHO) (see table).[3]

The monoclonal antibodies used were CD83-RPE and CD44-FITC (dilution 1:20, Immunotech), CD11c-FITC (dilution 1:20, eBioscience), and CD45R0-RPE (dilution 1:20, Ancell Corporation). Cryostat-cut 10-μm-thick sections were quickly semifixed in 95% ethanol, dried, and immersed in tris-HCl-buffered saline (pH 7.5). After blocking, the specimens were incubated for 1 hour at 37 °C with the primary antibodies and then washed three times in tris-HCl-buffered saline for 10 minutes. Double staining for laminin and CD44 was performed by incubating for 1 hour with both anti-rabbit IgG-RPE (dilution 1:200, ICN Biomedicals, Inc.) and CD44-FITC after reaction with rabbit anti-laminin (dilution 1:500, LSL Co. Ltd.) and subsequent wash. Fifty to 20 sections were prepared from each specimen for each staining. Immunofluorescence images were obtained using a confocal fluorescence microscope (LSM510 Ver3.2, Carl Zeiss Co. Ltd.). Details of the semiquantitative evaluation of immunoreactivity against CD44 using an image processor and computerized measuring system have been reported elsewhere.[6,10]

Results. CD44-highly positive ($CD44^{high}$) cells are a phenotype associated with memory lymphocytes and mature DCs in adults,[6,10] and CD44 serves as a homing receptor

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Table *Patient background and frequency of perivascular infiltrates of memory lymphocytes and mature dendritic cells (DCs)*

Pt./age, y/sex	Duration of symptoms before thymectomy, mo	MGFA clinical classification	Anti-AChR antibody titer, nmol/L	Type of thymoma, WHO classification[3]	MGFA Postintervention Status[9]	Dose of immunosuppressive medication, mg/day	Total observation period after thymectomy, y	Frequency of perivascular infiltrates/cm^2
1/65/F	6	II	46.9	B2	CSR	—	9.4	30
2/60/F	9	III	46.0	B2	CSR	—	4.2	24
3/57/F	7	II	63.9	B2	CSR	—	2.4	15
4/67/F	12	II	23.7	B2	MM-3	PSL 20	10.2	4
5/66/M	11	III	20.9	B3	MM-3	PSL 15 +FK506 3	7.8	3
6/64/F	9	II	17.1	AB	MM-3	PSL 20	13.6	0
7/61/F	6	III	3.1	B2	MM-3	PSL 20	4.4	0
8/60/F	9	II	78.9	B2	MM-3	PSL 15 +FK506 3	3.0	5
9/46/F	28	III	17.0	AB	MM-3	PSL 20 +FK506 3	2.8	0

CSR = complete stable remission; MGFA = Myasthenia Gravis Foundation of America; MM = minimal manifestations; PSL = prednisolone; WHO = World Health Organization.

during extravasation of these cells into lymphoid tissues.[6,10] In TLFH from EOMG, CD44-associated mechanisms are likely to promote blood-thymus circulation of both memory lymphocytes and mature DCs.[6,10] We studied the distribution of CD44^{high} cells and the co-expression of other markers (CD45R0 for memory cells, CD83 for mature DCs[4], and CD11c for myeloid DCs[5]) in the MG thymomas. We also studied the local relationship between CD44^{high} cells and laminin, a marker for basement membrane.

In thymomas from Patients 1 to 3 (all were type B2 thymoma; see the table) who achieved early CSR[9] after thymectomy, infiltrate of CD44^{high} cells was frequently observed around blood vessels with a diameter 30 to 100 μm. These cells appeared to have migrated from the vascular system into the thymoma tissue (figure, A to G). These

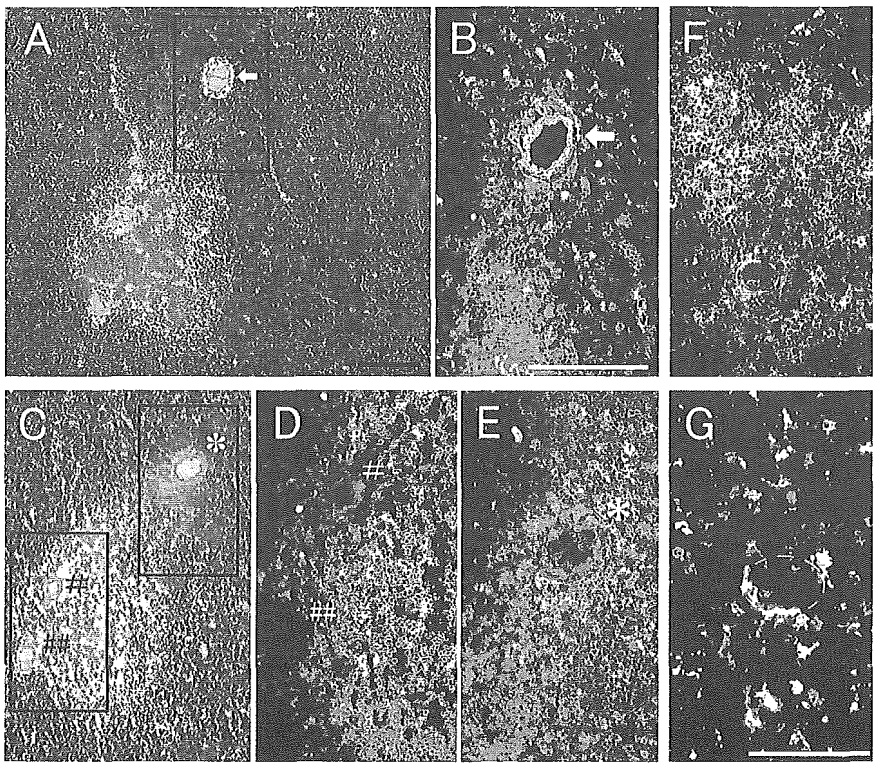

*Figure. Histologic findings of thymomas from patients who achieved early CSR after thymectomy (A, B, C, D, E, Patient 1; F, Patient 2; G, Patient 3). A and B are identical sections, as are C, D, and E. Following immunohistochemical observations (B or D and E), the section was stained with hematoxylin-eosin (HE) (A or C). A and C are identical portions of serial sections. (A, B) Infiltrate of CD44^{high} cells around a blood vessel (arrow indicates identical vessel). A: HE stain, original magnification ×40, bar = 100 μm; B: CD44 (green) and laminin (red), original magnification ×100, bar = 100 μm. (C, D, E) Some CD44^{high} cells strongly co-express CD45R0 (#, ##, and * indicate identical vessels). C: HE stain, original magnification ×40, D and E: CD44 (green) and CD45R0 (red), original magnification ×100. (F) Cells positive for CD83 are included in the population of CD44^{high} cells around a blood vessel (broken circle) and strongly co-express CD44. CD44 (green) and CD83 (red), original magnification ×100. (G) CD83$^+$CD11c$^+$ cells around a blood vessel (broken circle). CD 11c (green) and CD83 (red), original magnification ×200, bar = 50 μm.*

vessels often exhibited enlargement of the perivascular space (see the figure, A and C) and appeared to be small arteries or tumor vessels rather than functional venules involved in immature lymphocyte migration in the thymus. The basement membrane surrounding the vessels was fenestrated or permeated by $CD44^{high}$ cells (see the figure, B), and there was no separation between the areas of infiltrate and the parenchyma (see the figure, B). Some $CD44^{high}$ cells strongly co-expressed CD45R0 (see the figure, D and E). $CD83^{+}CD11c^{+}$ cells, mature DCs probably originating from the vascular system, were frequently encountered around the blood vessels and displayed the dendritic morphology characteristic of mature DCs (see the figure, G). Cells expressing CD83 were included in the population of $CD44^{high}$ cells, and strongly co-expressed CD44 (see the figure, F). However, such perivascular infiltrates of $CD44^{high}$ cells were very rarely observed in thymomas from Patients 4 to 9, who did not exhibit early response to thymectomy (type AB, two cases; type B2, three cases; type B3, one case, see the table). The frequency of perivascular infiltrates incorporating memory lymphocytes and mature DCs was significantly higher in the patients who achieved early CSR[9] (15 to 30 infiltrates/cm^2) than in the patients who were still MM-3[9] (0 to 5 infiltrates/cm^2) ($p < 0.05$, Mann–Whitney U test, see the table).

Discussion. The pathogenesis of thymoma-associated MG may differ from that of TLFH-associated MG.[3] Potentially autoreactive but naive T cells with specificity for AChR subunits are generated and exported to extratumoral sites, and it is postulated that T-cell activation initiating the autoimmune process occurs outside the thymus.[3] Hence, excision of thymomas rarely succeeds in immediate improvement of MG symptoms.[3] Thymoma-associated MG is generally considered to be treatment resistant. However, interactions between an AChR-like antigen constitutively expressed in the thymus and DCs may be very important in pathogenesis in certain patients with MG.[6] In the present study, CD44-associated perivascular infiltrates of memory lymphocytes and mature DCs were frequent in thymomas from patients with MG with early CSR after thymectomy but not in thymomas from patients without early response to thymectomy. Although type B2 thymoma is known to exhibit small perivascular spaces filled with immature lymphocytes,[3] the vessels that we reported in the present study were larger in diameter (>30 μm) and appeared to be small arteries or tumor vessels rather than functional venules involved in immature lymphocyte migration in the thymus. Although the present findings may be limited to particular types of MG thymomas that demonstrate hypervascularity, mature DCs from the vascular system may present an AChR-like antigen expressed in the thymus, thereby activating potentially autoreactive T cells against AChR. Accordingly, thymectomy may interrupt the recruitment of mature DCs in the thymus and the export of activated autoreactive T cells to extrathymic organs, thereby improving the disease. However, the present study is preliminary because of the small number of patients; additional patients should be studied.

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症　例

ラクナ梗塞後の高用量アスピリン投与により脳出血が疑われた症例

寺山靖夫

41歳女性。右穿通枝領域の脳梗塞発症後からアスピリン500 mgの内服を開始。アスピリン開始1年3ヵ月後に梗塞に一致した部位に脳出血を発症した。ラクナ梗塞に代表される穿通枝領域の小梗塞は穿通枝のlipohyalinosis, microatheroma（微小粥腫）およびプラークによる主幹動脈の穿通枝入口部の閉塞などで生ずる。エビデンスレベルの高い二次予防薬はアスピリンであるが，本症例のような出血性合併症の危険性を常に念頭におく必要がある。また，microatheromaに関連する陳旧性小出血巣を脳梗塞と誤診することもあり，アスピリンの投与は慎重でなければならない。

症　　例

患　者：U.P，41歳，女性。

主　訴：左上下肢のしびれと脱力。

現病歴：ドイツ人の主婦。1998年5月7日，昼食の準備中に左上下肢のしびれと脱力を自覚。近医に入院，右中大脳動脈領域の脳梗塞の診断のもとに治療を受けた。約2週間で退院。その後はアスピリン500 mgの投与を受け，9ヵ月間のリハビリテーションを行い，左上下肢にごくわずかのしびれ感を残すまでに回復した。1999年8月20日，夫の転勤で家族とともに来日。翌21日子供3人をつれてプールに出かけた後，疲労感強く夕方自宅で臥床していた。午後7時頃から左上下肢のしびれと脱力が増強してきたため，救急車にて22日午前1時56分来院し入院となる。

既往歴：25歳頃から高血圧を指摘され降圧薬（β-ブロッカー：アテノロール25 mg）を服用している。

家族歴：特記事項なし。

初診時の一般身体所見：身長168 cm，体重58 kg。血圧152/94 mmHg，脈拍54整，呼吸数20/分，体温36.4℃。貧血・黄疸（−），頸部リンパ節触知せず。甲状腺腫なし。頸部bruit（−）。肺野清，心音純。肝脾触知せず。腹部平坦，圧痛なし。グル音正常。下腿浮腫なし。下腿静脈瘤なし。

初診時の神経学的所見：意識清明，高次脳機能障害なし。脳神経系：左鼻唇溝やや浅薄である以外異常なし。運動系：左上下肢Barre兆候(+)。左上下肢（遠位筋）の筋力低下あり（MMT 4）。感覚系：左上下肢に知覚障害を訴える。協調運動：筋力低下のためと思われる変換運動障害を左上肢に認めるが測定障害なし。深部腱反射：左上腕二頭筋・三頭筋および膝蓋腱反射の軽度亢進を認める。病的反射なし。

検査所見：末梢血所見（WBC 7020/μL，RBC 480万/μL，Hb 13.6 g/dL，Ht 43%，Plt 24.5万/μL），凝固系（異常なし），血液生化学所見（TP 7.4 g/dL，AST 12 IU/L，ALT 20 IU/L，LDH 120 IU/L，γ-GTP 32 IU/L，T. Chol 248 mg/dL，TG 158 mg/dL，LDL 156 mg/dL，HDL 44 mg/dL，血糖101 mg/dL，HbA_{1c} 4.8%，BUN 12.3 mg/dL，Cr 0.7 mg/dL，Na 138 mmol/L，K 4.5 mmol/L，Cl 106 mmol/L，CRP<0.3，HBS-Ab（−），HCV（−），STS（−））。頭部MRI（入院時）（図1），頭部CT（入院時）（図2）。

頸動脈エコー：異常なし。経胸壁心エコー：異常

てらやま やすお：岩手医科大学神経内科