knockout. The results from both experiments strongly supported the idea that MMP-2 plays a critical role in BBB disruption and WM lesions.

Materials and Methods

Chronic Cerebral Hypoperfusion in Rats and Treatment With an Matrix Metalloproteinase Inhibitor

Chronic cerebral hypoperfusion with bilateral common carotid artery occlusion (BCAO) was induced in male Wistar rats (weight 150 to 200 g; Shimizu Experimental Supply; Kyoto, Japan) by double ligation of the common carotid arteries as previously described.[2] After the operation, the rats were kept in animal quarters with food and water ad libitum.

AG3340 (Agouron Pharmaceuticals) was dissolved at 75 mg/mL in 50% DMSO in propylenglycol. The rats were treated twice a day with an intraperitoneal injection of AG3340 (100 mg/kg) or vehicle (DMSO/propylenglycol) from just before the operation until 14 days after the operation. Similar doses and treatment paradigms have been shown to be effective in inhibiting MMP activity in gliomas in model animals.[9] Because our previous study demonstrated that the number of microglia peaked on 3 days and WM lesion started to become evident on 14 days after BCAO,[2] the animals were subjected to the analyses described subsequently.

Mice

The generation of C57BL/6J mice carrying the MMP-2-null allele has been described elsewhere.[10] In this mutant allele, a region containing the promoter and the first exon of the MMP-2 gene is replaced by the pgk-neo cassette. MMP-2^{+} parents were mated to obtain both wild-type and MMP-$2^{-/-}$ (MMP-2-null) littermates. Genotyping was performed by polymerase chain reaction using the following primers: wild-type forward, CAACGATGGAGGCACGAGTG; wild-type reverse, GCCGGGGAACTTGATGATGG; mutant forward, CTTGGGTGGAGAGGCTATTC; and mutant reverse, AGGTGAGATGACAGGAGATC.

Chronic Cerebral Hypoperfusion in Mice and Cerebral Blood Flow Measurement

Adult male mice (weight 20 to 25 g) were subjected to bilateral common carotid arteries stenosis (BCAS) by applying the microcoils with an inner diameter of 0.18 mm to both common carotid arteries as previously described.[3] The cerebral blood flow (CBF) was recorded by laser Doppler flowmetry by placing a straight probe (OmegaFLO-N1; Neuroscience Inc) on 1 mm posterior and 2 mm lateral from bregma perpendicular to the skull bone through the guide cannula. The baseline CBF recordings were obtained just before and at 2 hours and 3, 7, 14, and 30 days after the surgery. The CBF values were expressed as a percentage of the baseline value.

Histochemical Evaluation of White Matter Lesions and Glial Activation

Under deep anesthesia, the animals were perfused with 10 mmol/L phosphate-buffered saline (300 mL for rats, 100 mL for mice) and then with a fixative consisting of 4% paraformaldehyde, 0.2% picric acid, and 0.1 mol/L phosphate buffer at pH 7.4 (300 mL for rats, 100 mL for mice). The brains were removed and postfixed for 24 hours in 4% paraformaldehyde in 0.1 mol/L phosphate buffer and then stored in 15% sucrose in 0.1 mol/L phosphate buffer. The fixed brains were embedded in paraffin and sliced into 2-μm-thick coronal sections. Klüver-Barrera staining and Bielschowsky staining were used to visualize the myelin sheaths and axons, respectively. As previously described,[2] the severity of the WM lesions was semiquantitatively graded as normal (grade 0), disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and disappearance of myelinated fibers (grade 3) by an investigator blind to the experimental condition. For immunohistochemistry, serial sections (20-μm-thick) were cut in a cryostat and incubated overnight with a primary antibody at 4°C followed by incubation with the appropriate biotinylated secondary antibody (1 hour, room temperature), treatment with an avidin–biotin complex (diluted 1:200; Vector Laboratories), and visualization with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in 50 mmol/L Tris-HCl (pH 7.6). The primary antibodies used were as follows: monoclonal anti-rat glial fibrillary acidic protein (GFAP) (diluted 1:5000; Sigma-Aldrich; Mo, USA), polyclonal rabbit anti-mouse GFAP (diluted to 1:5000; Dako Cytomation, Denmark), polyclonal rabbit anti-MMP-2 (diluted to 1: 1,000, Chemicon International, Inc), monoclonal rat anti-mouse MHC class II antigen antibodies (diluted to 1:5000; Dako Cytomation), and rabbit anti Iba-1 antibody (1 μg/mL; Wako Pure Chemical Industries, Ltd; Osaka, Japan). Some sections were incubated with a biotinylated goat anti-rat IgM (μ), biotinylated goat anti-mouse IgM (μ) (diluted 1:1000; Kirkegaad & Perry Laboratories; Md, USA), or biotinylated Ricinus communis agglutinin-1 (diluted 1:1000; Vector Laboratories; Calif, USA) and were incubated directly with the avidin–biotin complex. To confirm the cellular source of IgM, sections were labeled by biotinylated anti-mouse IgM and rabbit anti-mouse GFAP followed by fluorescein isothiocyanate-labeled avidin (diluted 1:100; Dako Cytomation) and rhodamine-labeled goat anti- rabbit IgG (2.5 μL/mL; Dako). In the sections immunostained for Ricinus communis agglutinin-1, MHC class II antigen, Iba-1, GFAP, and IgM, we counted the number of immunopositive cells in at least 6 representative fields (per 0.25 mm^2) in the corpus callosum, the caudoputamen, and the optic tract for the quantitative analysis.

Zymography and Matrix Metalloproteinases-2 Activity Assay

Minced forebrain tissues were incubated with gentle rotation at 4°C for 20 hour in an extraction buffer consisting of 0.5% Triton-X 100, 0.5 U/mL aprotinin, and 0.01% sodium azide in 0.01 mol/L phosphate-buffered saline. The samples were then centrifuged at 14 000 rpm for 15 minutes at 4°C and the supernatants were collected. The protein content was adjusted to 10 mg/mL. The gelatinolytic activity of these samples was detected by SDS-PAGE zymography as described elsewhere,[8] although MMP-2 activity in the gray matter may interfere a sensitive detection of the activity in the WM. Equal amounts of tissue extract (50 μg) were then subjected to electrophoresis. To restore the activity of the protein, sample gels were agitated in 0.01 mol/L Tris-HCl (pH 8.0) containing 2.5% Triton X-100 (30 minutes×2). After washed in 0.05 mol/L Tris-HCl (pH 8.0) for 30 minutes, the gels were incubated overnight twice at 37°C in 0.05 mol/L Tris-HCl (pH 8.0) containing 0.5 mmol/L $CaCl_2$ and 1.0 mol/L $ZnCl_2$. After incubation, the gels were stained with Coomassie blue R-250. The amount of activated and latent forms of MMP-2 in the whole forebrain extracts were also assessed using the Matrix Metalloproteinase-2 Biotrak Activity Assay System (Amersham Biosciences), which is based on a 2-site enzyme-linked immunosorbent assay "sandwich" format and recognizes both the proform and active form of MMP-2.

Evans Blue Extravasation

The mice were killed at 3 hours and 1, 3, 5, 7, and 14 days after BCAS. One hour before each time point, 1 mL of 4% Evans blue (EB; Nakalai Chemicals Ltd) in normal saline was injected intraperitoneally. The animals were anesthetized and then perfused transcardially with 200 mL of 10 mmol/L phosphate-buffered saline. The brains were snap-frozen, sectioned into 20-μm-thick slices, and examined by fluorescence microscopy. For quantitative measures, the images were analyzed within 4 structurally similar areas (2 paramedian portions of the corpus callosum on each hemisphere) in each mouse and digitally level-adjusted by Adobe Photoshop (Adobe Systems) so that intravascular EB would be reported as white (pixel value 255) on a black background (pixel value 0). Using the public domain NIH Image 1.61 program (National Institutes of Health), the images were then binarized with intensity threshold set at pixel value 50 so that the white pixels represent intravascular and extravasated EB. The number of white pixels was divided by the total pixel

number in the selected area to estimate percent area containing intravascular and extravasated EB as an approximate index of BBB breakdown. Image analysis was focused on the paramedian portion of the corpus callosum facing the dorsal part of the lateral ventricle, because WM lesions were most intense in this region.[3]

Statistical Analysis

All data are presented as means±SE. A one-factor ANOVA followed by Fisher protected least significant difference procedure was used to compare the differences between groups. P values <0.05 were considered to be statistically significant.

Results

The amount of total MMP-2 in the forebrain extracts was comparable between the vehicle-treated and AG3340-injected rats after BCAO as assessed using the Biotrak Activity Assay System. The percentage of activated MMP-2 was only 7% on day 3 after the sham operation but was elevated to approximately 80% on day 3 after the BCAO (supplemental Figure I, available online at http://stroke.ahajournals.org). We also confirmed almost complete suppression of MMP-2 activation with AG3340 administration.

The operation was successful in rats (n=40) except 3, which developed convulsions and was killed within 7 days, and in mice (n=62) except 4, which developed cerebral infarction. These animals with unsuccessful operations were excluded from the statistical analysis. In the vehicle-treated animals, severe WM lesions, as shown by an increased number of disarranged nerve fibers and vacuolation, were found on day 14 after the BCAO in the optic nerve, medial part of the corpus callosum (Figure 1B and 1E), the internal capsule, and the fiber bundles of the caudoputamen. In such WM regions, the number of Ricinus communis agglutinin-1-positive microglia and GFAP-positive astroglia increased (2- to 3-fold) on day 3 after the BCAO (Figure 1H and 1K). Both WM lesions and gliosis were less severe in the AG3340-treated animals (Figure 1C, 1F, 1I, 1L, 1P through 1R, and Table 1).

The BBB integrity in rats subjected to BCAO was also assessed by the immunostaining for IgM. IgM-immunoreactive glial cells represent those cells that have taken up the serum proteins, which leaked into the brain parenchyma, and their number serves as an indicator of BBB dysfunction.[8] Some IgM-immunoreactive glial cells were found in the vicinity of the microvessels in the corpus callosum in the vehicle-treated animals on day 3 after the BCAO (Figure 1R), suggesting BBB dysfunction in this region. In contrast, much fewer IgM-immunoreactive glia were found in the same area of the AG3340-treated animals (Figure 1O and 1R).

These results strengthen the notion that MMPs play a role in BBB impairment and WM lesions. To further elucidate the roles of MMPs in the WM damage after chronic cerebral hypoperfusion, we applied BCAS (the established technique for mice hypoperfusion)[3] for mice lacking functional MMP-2 gene (MMP-2-null mice), which showed no obvious developmental abnormalities[10] or brain anomalies[11] and examined its effects using histochemical methods. The reduction of CBF after BCAS was comparable between wild-type and MMP-2-null mice. The CBF reductions (wild-type versus MMP-2-null; mean±SE %, n=3 each) were 42.5±4.3% versus 39.1±3.2% (2 hours after BCAS), 38.1±4.3 versus 39.4±4.0 (3 days), 35.2±4.6 versus 33.6±6.2 (7 days), 20.8±1.4 versus 26.9±3.1 (14 days), and 11.2±3.0 versus 24.0±4.0 (30 days). In wild-type mice, MMP-2-immunoreactive glial cells increased after BCAS compared with sham-operated mice (Figure 2A and 2B). MMP-9-immunoreactive cells were not induced after BCAS in both wild-type (Figure 2C and 2D) and MMP-2-null mice (Figure 2E). Consistently, zymography using forebrain homogenates revealed only a faint band of MMP-9 in the samples after BCAS for 3 days in both wild-type and MMP-2-null mice (n=4), whereas a robust band was found in the sample from a mouse with an incidental cerebral infarction after BCAS (Figure 2G). A band of MMP-2 was detected in the samples in wild-type mice but not in MMP-2-null mice after BCAS (n=4). However, zymography using such homogenates failed to show the upregulation of MMP-2 after 3 days of BCAS; regional upregulation of MMP-2 in the WM seemed obscured.

Klüver-Barrera staining revealed that WM lesions were predominant in the corpus callosum, caudoputamen, and internal capsule but not in optic tract on day 30 after BCAS in the wild-type mice. The medial part of the corpus callosum adjacent to the lateral ventricles was most severely affected (Figure 3E). In MMP-2-null mice, such WM lesions were far less severe (Figure 3I; Table 2). The mouse model showed little damage to the visual pathway and no difference was found between the wild-type mice and MMP-2-null mice after the operation. This may be attributable to the fact that BCAS in mice induces a milder decrease in the CBF than in the rat model and maintains a residual blood flow within the common carotid arteries and its branch, the ophthalmic artery.

In the wild-type mice on day 14 after BCAS, numerous activated microglia, as visualized by immunostaining with anti-MHC class II antibodies, were found in some WM regions (Figure 3F). In addition, the number of GFAP-immunoreactive astroglia increased in these mice (Figure 3G). In the MMP-2-null mice, the number of microglia and astroglia was much fewer in the WM as compared with the wild-type animals (Figure 3J, 3K, 3P, 3Q). Thus, both WM lesions and glial activation after chronic hypoperfusion were dramatically reduced in the MMP-2-null mice. There was no difference of the number of microglia, astroglia, and IgM-positive cells in optic tract (Figure 3P).

The BBB integrity in mice subjected to BCAS was assessed by the immunostaining for IgM and EB extravasation assay. After BCAS, the number of IgM-positive cells increased in the WM of the wild-type mice (Figure 3H) as compared with the sham-operated wild-type animals (Figure 3D). Intriguingly, the IgM-immunoreactive cells significantly decreased in the WM of MMP-2-null mice after BCAS (Figure 3L and 3R). IgM-immunoreactive cells were identified as astroglia based on their colabeling with GFAP in the perivascular areas (Figure 3L through 3O). Three days after BCAS, EB apparently leaked into the perivascular area in the corpus callosum (Figure 4B) and the cerebral cortex (data not shown). This extravasation was most notable in the paramedian portion of the corpus callosum. At all time points after BCAS, no extravasation of EB could be detected in the MMP-2-null mice (Figure 4C). The estimated percent area

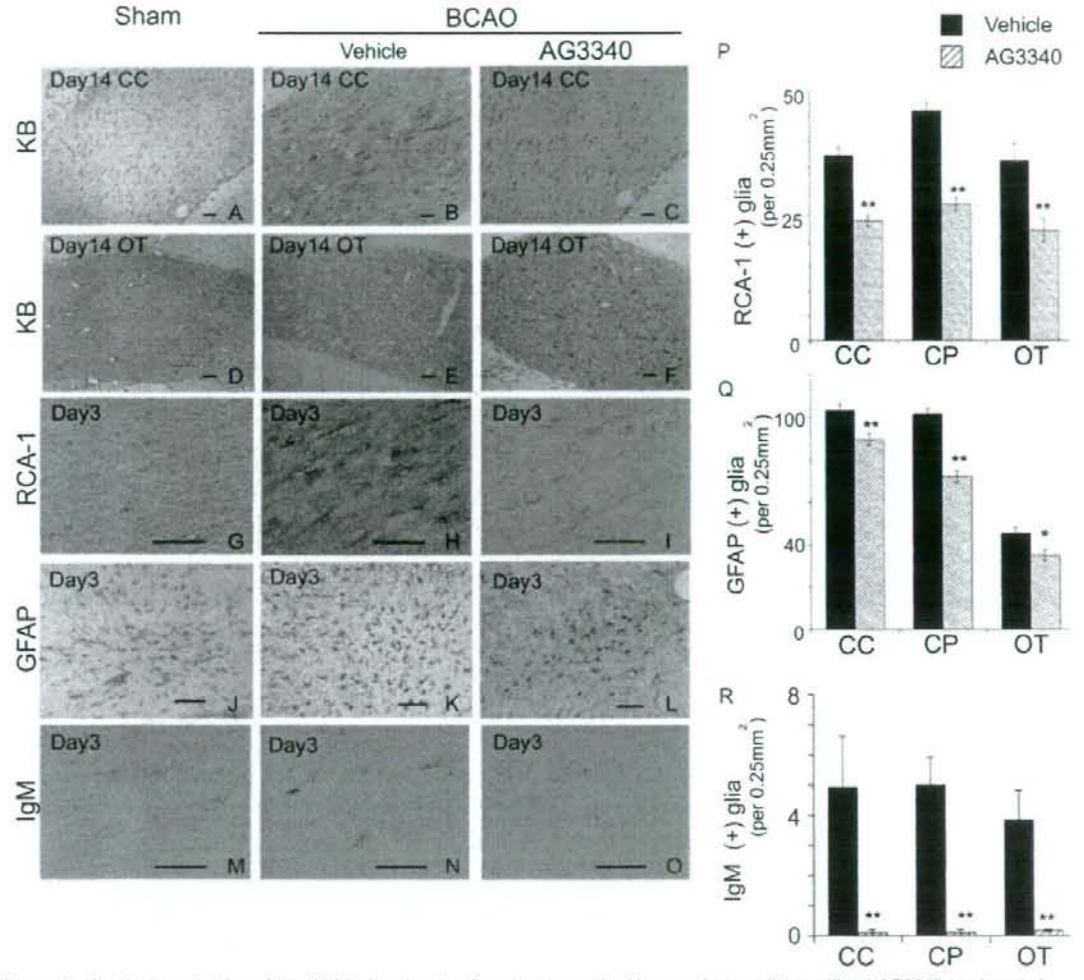

Figure 1. Histologic evaluation of the WM lesions in rats after chronic cerebral hypoperfusion with or without AG3340-treatment. A through O, Klüver-Barrera staining on day 14 (A through F; myelin sheath) or immunostaining on day 3 for Ricinus communis agglutinin-1 (G through I; microglia), GFAP (J through L; astroglia), or IgM (M through O) of the corpus callosum (A through C, G through O) and optic tract (D through F) of rats that had undergone sham operation (A, D, G, J, M) or BCAO operation, which had been treated either with vehicle (B, E, H, K, N) or AG3340 (C, F, I, L, O). Scale bar, 50 μm. (P through R) A histogram representing the density of cells immunoreactive for Ricinus communis agglutinin-1 (P), GFAP (Q), or IgM (R) in sections from the corpus callosum (CC), caudoputamen (CP), and optic tract (OT) in rats 3 days after a BCAO (n=4 each: $^{*}P<0.05$, $^{**}P<0.01$).

stained with EB was approximately 8% in wild-type mice after BCAS, which significantly reduced to 2% in MMP-2-null mice after BCAS (Figure 4D). Taken together, these results indicated that loss of MMP-2 alleviated BBB damage after BCAS and suggested a causative role for MMP-2 in the WM lesions after hypoperfusion.

TABLE 1. Histologic Grading of the WM Lesions in Untreated and AG3340-Treated Rats on Day 14 After BCAO

	Corpus Callosum	Caudoputamen	Optic Tract
Vehicle, N=5	1.3±045	1.4±0.54	2.6±0.55
AG3340, N=4	0.5±0.4*	0.63±0.25*	1.13±0.63*

*$P<0.05$.

Discussion

The synthetic MMP inhibitor AG3340 is known to inhibit several MMP family members, including MMP-2 (Ki=0.05 nmol/L), MMP-9 (0.26 nmol/L), MMP-13 (0.03 nmol/L), and MT1-MMP (0.33 nmol/L).[12] As a lipophilic, low-molecular-weight (Mr 423.5) compound, AG3340 can readily cross the BBB.[12] Using this compound, we have demonstrated that AG3340 shows protective effects against the WM lesions after chronic cerebral hypoperfusion in rats. This is consistent with our previous data using the same model, which showed a correlation of WM lesions with MMP-2 upregulation.[8] Then, AG3340 may have reduced the severity of WM lesions by inhibiting MMP-2 activation. In support of this notion,

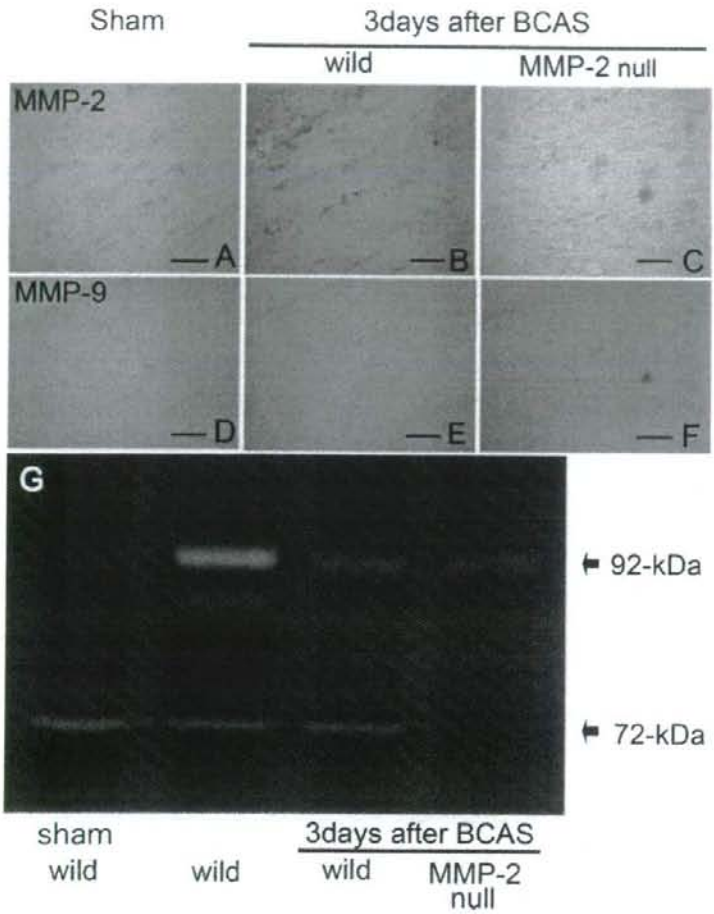

Figure 2. A through F, Immunohistochemical analysis for MMP-2 (A through C) and MMP-9 (D through F) in the corpus callosum of wild-type mice (A, B, D, E) or MMP-2-null mice (C, F) on day 3 after sham operation or BCAS (B, D). G, Zymography assay of the samples from a mouse with incidental cerebral infarction (CI), a wild-type mouse and an MMP-2-null mouse 3 days after BCAS. Note the absence of compensatory upregulation of MMP-9 in MMP-2-null mice.

genetic deletion of MMP-2 attenuated the WM lesions after chronic cerebral hypoperfusion in mice. These data jointly suggest that MMP-2 upregulation plays a major role in the WM lesions.

Previous studies have established the importance of the upregulation and activation of MMPs in acute brain ischemia.[13–15] Among the members of the MMP family (n≥20), MMP-9 is of particular interest in the context of acute brain ischemia, because the selective upregulation of MMP-9 has been observed in the brains of patients with stroke.[15] More importantly, the neuronal damage after cerebral ischemia was attenuated in the MMP-9-null mice compared with the wild-type mice.[16] Furthermore, Heo et al demonstrated association of MMP-9 upregulation with hemorrhagic transformation in the nonhuman primates.[17] Thus, MMP-9 upregulation may contribute to the BBB damage and infarct size, especially in the acute setting. Although previous study demonstrated the upregulation of MMP-9 in MMP-2-null mice,[18] no upregulation of MMP-9 was observed in our model, which suggested a negligible role of MMP-9 in chronic cerebral hypoperfusion.

What then would be the role of MMPs in cerebral ischemia? Hamann et al reported disappearance of the basal lamina around the microvessels during cerebral ischemia and reperfusion.[7] Fukuda et al demonstrated that the ischemic primate brain contained elevated levels of activity enough to digest basal lamina components such as type IV collagen.[19] In fact, Heo et al indicated that MMP-2 upregulated significantly by 1 hour after MCAO[18] and was persistently elevated thereafter in primates, and Chan et al demonstrated the upregulation of activation system for latent MMP-2 after focal cerebral ischemia.[20] These findings support the hypothesis that excessive degradation of the vascular basal lamina is a mechanism by which MMP triggers BBB dysfunction, edema, hemorrhage. The most marked extravasation of Evans blue in the paramedian portion of the corpus callosum facing the lateral ventricle was consistent with a previous report on a rat model of chronic cerebral hypoperfusion[21] and further indicated a vulnerability of the BBB in this area. In the case of chronic hypoperfusion, a previous study suggested the association of MMP-2 but not MMP-9 upregulation with BBB disruption. Consistently, Rosenberg et al showed that the activated

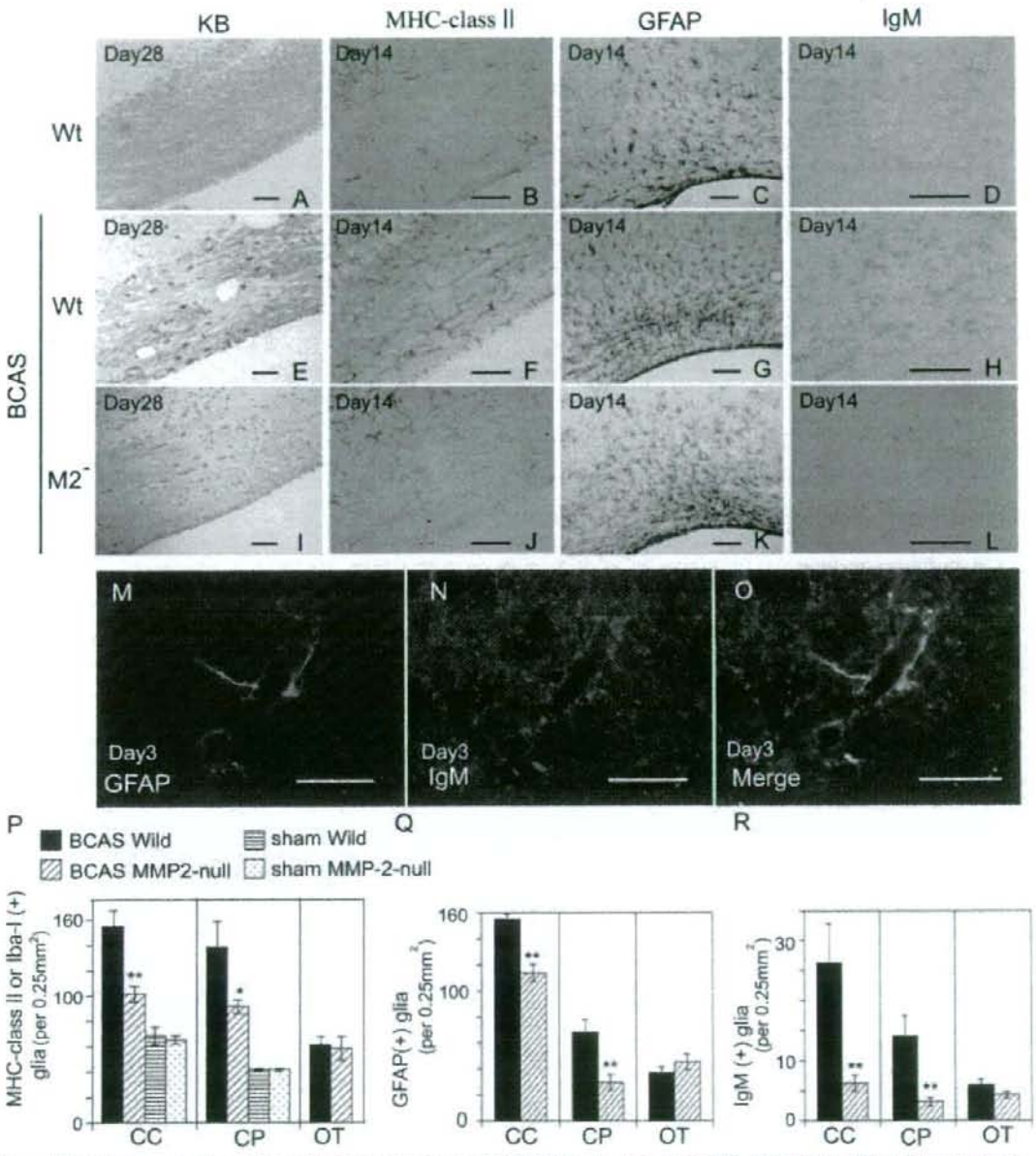

Figure 3. Histologic evaluation of the WM lesions in wild-type and MMP-2-null mice after BCAS. A through L, Klüver-Barrera staining 28 days after BCAS (A, E, I) and immunostaining 14 days after BCAS for MHC class II (B, F, J), GFAP (C, G, K), or IgM (D, H, L) of corpus callosum sections from wild-type (Wt) mice (A through H) or MMP-2-null (M2⁻) mice (I through L) that had undergone either a sham operation (A through D) or BCAS (E through L). Note that MMP-2 gene knockout recover the decrease of Klüver-Barrera staining in the WM after BCAS (compare E with I) and glial activation (compare F with J for microglia and G with K for astroglia). Scale bar, 50 μm. M through O, Double staining with GFAP and IgM of the WM lesions in wild-type mice after BCAS. IgM was observed on endfeet of GFAP-positive glia (O). Scale bar, 10 μm. P through R, A histogram representing the density of cells immunoreactive for MHC-class II or Iba-I (P), GFAP (Q), or IgM (R) in sections from the corpus callosum (CC), caudoputamen (CP), and optic tract (OT) of mice that had undergone BCAS (n=6 each: $^{*}P<0.05$, $^{**}P<0.01$). For the microglial count, anti-MHC-class II antibodies were used for mice with BCAS operation, whereas anti-Iba-I antibodies were used for mice with sham operation (P). Note that glial activation was not observed in the optic tract, being consistent with the absence of rarefaction of this structure.

TABLE 2. Histologic Grading of the WM Lesions in Wild-Type and MMP-2-Null Mice on Day 30 After BCAS

	Corpus Callosum	Caudoputamen	Anterior Commissure
Wild-type, N=6	1.5±0.8	1.3±0.58	0.5±0.5
MMP-2-null, N=6	0.5±0.8*	0.58±0.37*	0±0

*$P<0.05$.

astroglia and microglia/macrophages around the arterioles expressed MMP-2 and MMP-3, but not MMP-9, in the brains of patients with vascular dementia.[6]

Caplan[22] proposed that the major pathologic features of WM lesions such as demyelination and gliosis may result from a BBB dysfunction, which allows the leakage of proteins and fluid through the compromised barrier of the penetrating arteries. This hypothetical pathway is consistent with our present findings. Given the overlapping substrate specificity between MMP-2 and MMP-9, in the case of chronic cerebral hypoperfusion, MMP-2 may contribute to the BBB disruption through the excessive digestion of the vascular basal lamina and activation of glia. In addition, MMP-2 may be directly involved in demyelination associated with WM lesions, because MMP-2 can digest myelin more efficiently than MMP-9.[24]

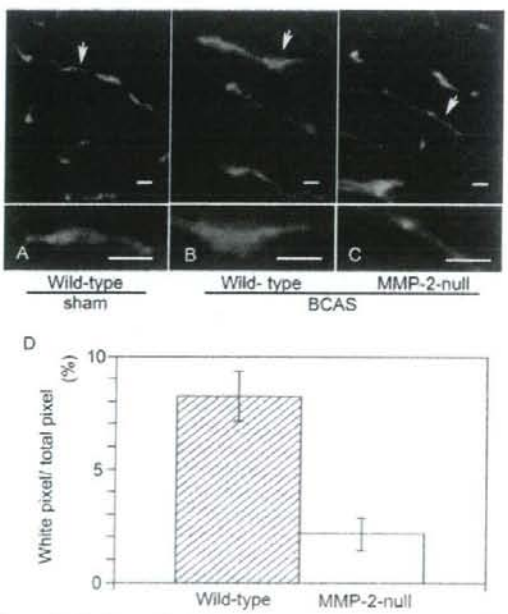

Figure 4. Evaluation of BBB dysfunction in the corpus callosum of mice. The Evans blue extravasation assay was performed on day 3 after a sham operation in wild-type mice (A) or on day 3 after BCAS in wild-type (B) and MMP-2-null mice (C). A magnified view of the area indicated by an arrow in the upper panel is shown in the lower panel (A through C). The experiments were repeated in triplicate with similar findings (n=4 each). Scale bar, 100 μm. A histogram representing the degree of Evans blue extravasation as an approximate index of BBB breakdown (see "Methods") (D).

In conclusion, the present study has provided direct evidence that MMP-2 is involved in the pathogenesis of WM lesions in the mouse model. Although the species difference between rodents and humans should be taken into consideration, our data also suggest the potential value of MMP inhibitors in preventing subcortical ischemic vascular dementia resulting from BBB dysfunction and chronic cerebral ischemia in humans. Activation of MMP-2 is reported to participate in matrix injury during focal cerebral ischemia. An elucidation of the exact roles of MMP-2 in BBB disruption may also provide information useful in developing strategies for controlling neuroinflammation in general.

Acknowledgments

The authors are indebted to Miss Nakabayashi for her excellent technical assistance.

Disclosures

None.

References

1. Meguro K, Hatazawa J, Yamaguchi T, Itoh M, Matsuzawa T, Ono S, Miyazawa H, Hishinuma T, Yanai K, Sekita Y. Cerebral circulation and oxygen metabolism associated with subcortical perivascular hyperintensity as shown by magnetic resonance imaging. *Ann Neurol*. 1990;28:378–383.
2. Wakita H, Tomimoto H, Akiguchi I, Kimura J. Glial activation and white matter changes in the rat brain induced by chronic cerebral hypoperfusion: an immunohistochemical study. *Acta Neuropathol*. 1994;87:484–492.
3. Shibata M, Ohtani R, Ihara M, Tomimoto H. White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. *Stroke*. 2004;35:2598–2603.
4. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol*. 2001;17:463–516.
5. Clark AW, Krekoski CA, Bou SS, Chapman KR, Edwards DR. Increased gelatinase A (MMP-2) and gelatinase B (MMP-9) activities in human brain after focal ischemia. *Neurosci Lett*. 1997;238:53–56.
6. Rosenberg GA, Sullivan N, Esiri MM. White matter damage is associated with matrix metalloproteinases in vascular dementia. *Stroke*. 2001;32:1162–1168.
7. Hamann GF, Okada Y, Fitridge R, del Zoppo GJ. Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion. *Stroke*. 1995;26:2120–2126.
8. Ihara M, Tomimoto H, Kinoshita M, Oh J, Noda M, Wakita H, Akiguchi I, Shibasaki H. Chronic cerebral hypoperfusion induces MMP-2 but not MMP-9 expression in the microglia and vascular endothelium of the white matter. *J Cereb Blood Flow Metab*. 2001;21:828–834.
9. Price A, Shi Q, Morris D, Wilcox ME, Brasher PM, Reweastle NB, Shalinsky D, Zou H, Appelt K, Johnston RN, Yong VW, Edwards D, Forsyth P. Marked inhibition of tumor growth in a malignant glioma tumor model by a novel synthetic matrix metalloproteinase inhibitor AG3340. *Clin Cancer Res*. 1999;4:845–854.
10. Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T, Itohara S. Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. *J Biol Chem*. 1997;272:22389–22392.
11. Asahi M, Sumii T, Fini ME, Itohara S, Lo EH. Matrix metalloproteinase 2 gene knockout has no effect on acute brain injury after focal ischemia. *Neuroreport*. 2001;12:3003–3007.
12. Shalinsky DR, Brekken J, Zou H, McDermott CD, Forsyth P, Edwards D, Margosiak S, Bender S, Truitt G, Wood A, Varki NM, Appelt K, Broad. antitumor and antiangiogenic activities of AG3340, a potent and selective MMP inhibitor undergoing advanced oncology clinical trials. *Ann N Y Acad Sci*. 1999;878:236–270.
13. Rosenberg GA, Navratil M, Barone F, Feuerstein G. Proteolytic cascade enzymes increase in focal cerebral ischemia in rat. *J Cereb Blood Flow Metab*. 1996;16:360–366.

14. Horstmann S, Kalb P, Koziol J, Gardner H, Wagner S. Profiles of matrix metalloproteinases, their inhibitors, and laminin in stroke patients: influence of different therapies. *Stroke*. 2003;34:2165–2170.
15. Maier CM, Hsieh L, Yu F, Bracci P, Chan PH. Matrix metalloproteinase-9 and myeloperoxidase expression: quantitative analysis by antigen immunohistochemistry in a model of transient focal cerebral ischemia. *Stroke*. 2004; 5:1169–1174.
16. Lee SR, Tsuji K, Lee SR, Lo EH. Role of matrix metalloproteinase in delayed neuronal damage after transient global ischemia. *J Neurosci*. 2004;24:671–678.
17. Heo JH, Lucero J, Abumiya T, Koziol JA, Copeland BR, del Zoppo GJ. Matrix metalloproteinases increase very early during experimental focal cerebral ischemia. *J Cereb Blood Flow Metab*. 1999;19:624–633.
18. Esparza J, Kruse M, Lee J, Michaud M, Madri JA. MMP-2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity. *FASEB J*. 2004;18:1682–1691.
19. Fukuda S, Fini CA, Mabuchi T, Koziol JA, Eggleston LL Jr, del Zoppo GJ. Focal cerebral ischemia induces active proteases that degrade microvascular matrix. *Stroke*. 2004;35:998–1004.
20. Chang DI, Hosomi N, Lucero J, Heo JH, Abumiya T, Mazar AP. Activation system for latent matrix metalloproteinase-2 are upregulated immediately after focal cerebral ischemia. *J Cereb Blood Flow Metab*. 2003;23:1408–1419.
21. Ueno M, Tomimoto H, Akiguchi I, Wakita H, Sakamoto H. Blood–brain barrier disruption in white matter lesions in a rat model of chronic cerebral hypoperfusion. *J Cereb Blood Flow Metab*. 2002; 22:97–104.
22. Caplan LR. Dilatative arteriopathy (dolichoectasia): what is known and not known. *Ann Neurol*. 2005;57:472–479.
23. Chandler S, Coates R, Gearing A, Lury J, Wells G, Bone E. Matrix metalloproteinases degrade myelin basic protein. *Neurosci Lett*. 1995; 201:223–226.

Circulating CD34-Positive Cell Number Is Associated With Brain Natriuretic Peptide Level in Type 2 Diabetic Patients

SADANORI OKADA, MD[1]
HISASHI MAKINO, MD, PHD[1]
AYAKO NAGUMO, MD[1]
TAKAKO SUGISAWA, MD, PHD[1]
MUNEYA FUJIMOTO, MD, PHD[1]
ICHIRO KISHIMOTO, MD, PHD[1]
YOSHIHIRO MIYAMOTO, MD, PHD[1]
AKIE KIKUCHI-TAURA[2]
TOSHIHIRO SOMA, MD[2]
AKIHIKO TAGUCHI, MD, PHD[3]
YASUNAO YOSHIMASA, MD, PHD[1]

Patients with type 2 diabetes often suffer from asymptomatic left ventricular (LV) injury, including increased LV mass, without apparent myocardial ischemia. The mechanisms underlying diabetic LV injury remain unclear; however, it has been suggested that endothelial dysfunction plays a role. Accumulating evidence indicates that bone marrow–derived endothelial progenitor cells (EPCs) contribute to neovascularization of ischemic tissue and endothelialization of denuded endothelium. Recent studies have shown that circulating bone marrow–derived immature cells, including $CD34^+$ cells, contribute to the maintenance of the vasculature, both as a pool of EPCs and as the source of growth/angiogenesis factors (1). We hypothesized that circulating $CD34^+$ cells might be associated with LV dysfunction in patients with type 2 diabetes. Therefore, we studied the correlation between circulating $CD34^+$ cell levels and plasma brain natriuretic peptide (BNP) levels, an LV dysfunction marker, in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

The institutional review board of the National Cardiovascular Center approved this study, and all subjects provided informed consent. We examined 26 patients with type 2 diabetes (12 men and 14 women, duration of diabetes 16.1 ± 10.7 years) who were over 60 years of age (70.5 ± 6.4 years). Statin was given to nine subjects. ACE inhibitor or angiotensin receptor blocker was given to nine subjects, and thiazolidinedione was given to two subjects. Subjects were excluded from the study if they had known cardiovascular disease or chronic renal failure (defined as serum creatinine ≥180 μmol/l). No study subject showed hypokinesis by echocardiography or electrocardiogram change, indicating myocardial ischemia. Systolic (SBP) and diastolic (DBP) blood pressure and anthropometric parameters were determined. Blood samples were taken after 12-h fasting to measure circulating $CD34^+$ cells, plasma BNP, fasting plasma glucose (FPG), and A1C. Circulating $CD34^+$ cells were quantified by flow cytometry according to the manufacturer's protocol (ProCOUNT; Becton Dickinson Biosciences) as previously reported (2). BNP was quantified by enzyme immunoassay (Tohso, Tokyo, Japan). We further examined LV fractional shortening (LVFS), LV mass index (LVMI) (3), and peak flow velocity of the early filling wave (E), the late filling wave (A), and the E/A-wave ratio (E/A) by echocardiography. All echocardiograms were performed by several expert physicians who were blinded to $CD34^+$ cell level.

All statistical analyses were performed using JMP version 5.1.1 software (SAS Institute). Data are expressed as means ± SD. Comparisons of number of $CD34^+$ cells by sex were made using the two-tailed unpaired *t* test. Correlations between number of $CD34^+$ cells and clinical parameters were assessed by univariate liner regression analysis and multiple regression analysis. LVMI and plasma BNP concentrations were analyzed after logarithmic transformation.

RESULTS

FPG levels, A1C levels, and BMIs in the study subjects were measured to be 9.5 ± 2.6 mmol/l, 9.2 ± 1.8%, and 26.4 ± 4.3 kg/m^2, respectively. A total of 88% of the patients had hypertension (SBP 142 ± 18 mmHg, DBP 75.7 ± 13.5 mmHg). Plasma BNP levels were measured to be 95 ± 319 pg/ml. Although it has been reported that the level of BNP ≥100 pg/ml has a sensitivity of 90% of diagnosing congestive heart failure (CHF) in patients with CHF symptoms (4), none of the subjects in this study, including subjects with ≥100 pg/ml of BNP, showed symptoms of CHF. The level of circulating $CD34^+$ cells was measured to be 0.76 ± 0.39 cells/μl, and there was no significant difference between sexes. The range of LVMI was 73.3–340.2, and 11 subjects applied to the definition of LV hypertrophy (LVMI ≤131 in men and ≤100 in women) (3).

Plasma BNP levels had a significant inverse correlation with the number of circulating $CD34^+$ cells (Fig. 1*A*), whereas FPG, A1C, BMI, SBP, DBP, and age showed no significant correlations. There was a significant correlation between the number of circulating $CD34^+$ cells and LVMI by echocardiography (Fig. 1*B*). LVFS and E/A were not associated with circulating $CD34^+$ cell numbers (LVFS $r = -0.07$, $P = 0.72$; E/A $r = -0.11$, $P = 0.59$). There was also a significant correlation between BNP levels and LVMI ($r = 0.59$, $P = 0.001$).

In multiple regression analysis, the

From the [1]Department of Atherosclerosis, National Cardiovascular Center, Suita, Japan; the [2]Department of Hematology, Osaka Minami National Medical Center, Osaka, Japan; and the [3]Department of Cerebrovascular Disease, National Cardiovascular Center, Suita, Japan.

Address correspondence and reprint requests to Hisashi Makino, MD, Atherosclerosis, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail: makinoh@hsp.ncvc.go.jp.

Received for publication 14 June 2007 and accepted in revised form 13 October 2007.

Published ahead of print at http://care.diabetesjournals.org on 24 October 2007. DOI: 10.2337/dc07-1125.

Abbreviations: BNP, brain natriuretic peptide; CHF, congestive heart failure; DBP, diastolic blood pressure; EPC, endothelial progenitor cell; FPG, fasting plasma glucose; LV, left ventricular; LVFS, LV fractional shortening; LVMI, LV mass index; SBP, systolic blood pressure.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

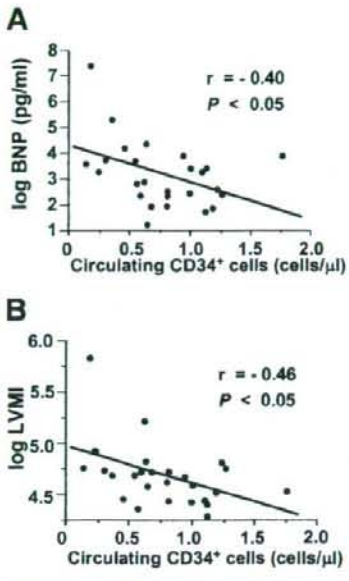

Figure 1—*Correlation between CD34$^+$ cell numbers and plasma BNP levels (A) and correlation between CD34$^+$ cell numbers and LVMI (B) in type 2 diabetic patients* (n = 26).

level of CD34$^+$ cells was an independent correlate of both BNP ($\beta = -1.64$, $P = 0.017$) and LVMI ($\beta = -0.337$, $P = 0.031$) in the model including age, A1C, SBP, BMI, and medication (ACE inhibitor/angiotensin receptor blocker, statin, and thiazolidinedione).

CONCLUSIONS — In this study, circulating CD34$^+$ cell number was found to significantly correlate with plasma BNP level, a marker of LV dysfunction. To the best of our knowledge, this is the first report that circulating bone marrow–derived cells are associated with diabetic LV abnormality. Circulating CD34$^+$ cell numbers also significantly correlated with LVMI, whereas they did not correlate with LVFS (an LV systolic function marker) or E/A (an LV diastolic function marker). LV hypertrophy is a well-known predictor of cardiovascular events independent of coronary artery disease. The Framingham Heart Study identified an association between diabetes and increased LV wall thickness and mass (5). Although the precise mechanisms underlying the association between diabetes and LV hypertrophy remain unknown, our results suggest that reduced circulating CD34$^+$ cell numbers may be involved in the progression of LV hypertrophy in diabetic patients. However, further investigations are necessary to demonstrate this hypothesis.

We measured the level of CD34$^+$ cells in this study but not the levels of circulating CD34$^+$/kinase insert domain receptor (KDR)$^+$ cells that are regarded as EPCs. Circulating CD34$^+$ cell levels are associated with ischemic stroke (6), and administration of CD34$^+$ cells ameliorates cerebral ischemia in mice (7). This indicates that CD34$^+$ cells may be involved in cardiovascular disease. Indeed, another recent report indicated that levels of circulating CD34$^+$ cells are more strongly correlated with cardiovascular risk than levels of EPCs (8). Therefore, our results suggest that measurement of CD34$^+$ cells may provide an indicator for diabetic LV hypertrophy.

Our study had several limitations. First, the study was performed only by cross-sectional analysis; therefore, a prospective study is needed to clarify whether circulating CD34$^+$ cell numbers predict LV injury in diabetic patients. Second, although systemic blood pressure did not significantly associate with CD34$^+$ cell numbers, further investigation of normotensive diabetic patients is needed to exclude the possible effects of hypertension on circulating CD34$^+$ cell numbers, as most of the subjects in this study were hypertensive. Despite this caveat, these results may be of practical use in elderly patients with type 2 diabetes, as hypertension is a very common comorbid condition in this population.

In conclusion, reduced circulating CD34$^+$ cell numbers are significantly associated with plasma BNP concentration and LVMI in elderly patients with type 2 diabetes. These results suggest that decreased circulating CD34$^+$ cells may be involved in LV hypertrophy and that measurement of circulating CD34$^+$ cell numbers may be useful for the identification of diabetic patients at high risk of LV injury.

References

1. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, Gewirtz AM, Emerson SG, Ratajczak MZ: Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. *Blood* 97:3075–3085, 2001
2. Kikuchi-Taura A, Soma T, Matsuyama T, Stern DM, Taguchi A: A new protocol for quantifying CD34(+) cells in peripheral blood of patients with cardiovascular disease. *Tex Heart Inst J* 33:427–429, 2006
3. Devereux RB, Reichek N: Echocardiographic determination of left ventricular mass in man: anatomic validation of the method. *Circulation* 55:613–618, 1977
4. McCullough PA, Nowak RM, McCord J, Hollander JE, Herrmann HC, Steg PG, Duc P, Westheim A, Omland T, Knudsen CW, Storrow AB, Abraham WT, Lamba S, Wu AHB, Perez A, Clopton P, Krishnaswamy P, Kazanegra R, Maisel AS, BNP multinational study investigators: B-type natriuretic peptide and clinical judgment in emergency diagnosis of heart failure: analysis from Breathing Not Properly (BNP) Multinational Study. *Circulation* 106: 416–422, 2002
5. Galderisi M, Anderson KM, Wilson PW, Levy D: Echocardiographic evidence for existence of a distinct diabetic cardiomyopathy (the Framingham Heart Study). *Am J Cardiol* 68:85–89, 1991
6. Taguchi A, Matsuyama T, Moriwaki H, Hayashi T, Hayashida K, Nagatsuka K, Todo K, Mori K, Stern DM, Soma T, Naritomi H: Circulating CD34-positive cells provide an index of cerebrovascular function. *Circulation* 109:2972–2975, 2004
7. Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, Tsukamoto Y, Iso H, Fujimori Y, Stern DM, Naritomi H, Matsuyama T: Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J Clin Invest* 114:330–338, 2004
8. Fadini GP, de Kreutzenberg SV, Coracina A, Baesso I, Agostini C, Tiengo A, Avogaro A: Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. *Eur Heart J* 27:2247–2255, 2006

Original Article

Impact of Metabolic Syndrome Components on the Incidence of Cardiovascular Disease in a General Urban Japanese Population: The Suita Study

Yoshihiro KOKUBO[1], Tomonori OKAMURA[1], Yasunao YOSHIMASA[2], Yoshihiro MIYAMOTO[2], Katsuyuki KAWANISHI[3], Yasushi KOTANI[3], Akira OKAYAMA[1),4)], and Hitonobu TOMOIKE[1]

Abdominal obesity is a prerequisite for some definitions of metabolic syndrome (MetS). We investigated the impact of MetS defined by two different criteria, which either did or did not require abdominal obesity as a prerequisite, on cardiovascular disease (CVD) incidence in an urban Japanese cohort study. We studied 5,332 Japanese (aged 30–79 years, without CVD at baseline), who completed a baseline survey (September 1989 to March 1994) and were followed up through December 2005. MetS was defined by the NCEP-ATPIII (modified by Asian obesity criteria) and the Japanese criteria. After 61,846 person-years of follow-up, we documented 317 CVD incidences. The MetS frequencies of the Japanese and of the modified NCEP-ATPIII criteria were 17.7% and 25.1% for men and 5.0% and 14.3% for women, respectively. The multivariate hazard ratios (HRs; 95% confidence intervals [CI]) of CVD incidence for MetS by the modified NCEP-ATPIII criteria were 1.75 (1.27–2.41) in men and 1.90 (1.31–2.77) in women, and those for MetS by the Japanese criteria were 1.34 (0.96–1.87) in men and 2.20 (1.31–3.68) in women. The multivariate HRs of CVD incidence for MetS for the Japanese and for the modified NCEP-ATPIII criteria were 2.92 (1.54–5.55) and 1.94 (0.98–3.82) in men under 60 years old, respectively. The CVD incidence risks increased according to the number of MetS components. The risks were similar among participants with the same number of MetS components, regardless of abdominal obesity. In conclusion, the number of MetS components (modified NCEP-ATPIII criteria) may be more strongly associated with CVD incidence than the abdominal obesity essential criteria (the Japanese criteria) in a general urban Japanese population. (*Hypertens Res* 2008; 31: 2027–2035)

Key Words: **metabolic syndrome, cardiovascular risk factor, cohort study, general population**

Introduction

Metabolic syndrome (MetS) is a clustering of impaired glucose metabolism, abdominal fat accumulation, dyslipidemia, and elevated blood pressure (*1*). Previous papers have shown an association between MetS and cardiovascular disease (CVD) (*2*), but most studies conducted thus far have been based on Western populations. There have been several well-designed prospective studies of Asian populations, and those

From the [1]Department of Preventive Cardiology and [2]Department of Atherosclerosis and Diabetes, National Cardiovascular Center, Suita, Japan; [3]The Suita Medical Foundation, Suita, Japan; and [4]Japan Anti-Tuberculosis Association, Tokyo, Japan.

This study was supported by grants-in-aid from the Japanese Ministry of Health, Labour and Welfare (H14-Kouka-027, H19-SeiShu-017, and H18-SeiShu-012) and by a Research Grant for Cardiovascular Disease from the Ministry of Health, Labour and Welfare (18S-2, 19K-8), Japan.

Address for Reprints: Yoshihiro Kokubo, M.D., Ph.D., Department of Preventive Cardiology, National Cardiovascular Center, 5–7–1, Fujishiro-dai, Suita 565–8565, Japan. E-mail: ykokubo@hsp.ncvc.go.jp

Received May 30, 2008; Accepted in revised form September 7, 2008.

studies had various limitations, including the use of body mass index (BMI) (*3*, *4*), non-fasting triglyceride and glucose levels (*3*, *4*), mortality (*4*, *5*), or small sample size (*4–7*). In order to properly define MetS, it is essential to use data on waist circumference and on the levels of both fasting glucose and fasting triglycerides.

MetS has been defined in several ways by several groups, including the World Health Organization (*8*), the European Group for the Study of Insulin Resistance (*9*), the American Association of Clinical Endocrinologists, and the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATPIII) (*10*). However, these definitions are aimed mainly at Western countries. The International Diabetes Foundation (IDF) (*11*) and the American Heart Association (*12*) have recently introduced alternative definitions that can be applied worldwide (*10*). Stroke incidence is relatively higher in Japan than in Western countries (*13*). It is uncertain whether these criteria can be applied well to Japanese populations. A MetS definition needs to be tailored to the epidemiological background of the area in question.

The Japanese Committee on the Criteria for MetS has recently proposed a definition of Japanese MetS (*14*, *15*). Under both the IDF and the Japanese definitions, the presence of abdominal obesity is necessary for a diagnosis of MetS. However, no prospective study has examined the association between MetS based on the Japanese criteria and CVD, particularly in urban areas, where most Japanese live. Therefore, we undertook this study to examine the impact of MetS under the Japanese and modified NCEP-ATPIII criteria on CVD incidence in a general urban Japanese population.

Methods

Study Population

The Suita study (*16*, *17*), an epidemiological survey of cerebrovascular disease and CVD, was based on a random sampling of 12,200 residents of Suita, a city of approximately 350,000 people in northern Osaka, Japan. As a baseline, in 1989, participants between the ages of 30 and 79 were arbitrarily selected from the municipality population registry and stratified into groups by sex and age in 10-year increments. Of these, 6,406 men and women participated in regular health checkups between September 1989 and March 1994. Since then, these participants have participated in regular health checkups at the National Cardiovascular Center every 2 years and answered health questionnaires every year.

Some cohort members in the study population were excluded from these analyses because they met one or more of the following criteria: past or present CVD illness at baseline (n=208), failure to fast for at least 10 h before venipuncture or missing data (n=170), or failure to follow up after their baseline examination (n=696). After these exclusions, 5,332 individuals remained for analysis.

Baseline Survey

We performed routine blood tests that measured fasting serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose levels. Physicians or nurses administered questionnaires covering the subjects' personal habits and any present illnesses. The subjects were classified as current smokers if they smoked at least one cigarette per day, as non-smokers if they had never smoked, and as past smokers if they had stopped smoking. Blood pressure was measured three times in a sitting position after at least 5 min of rest. Systolic and diastolic blood pressures (SBP and DBP) were taken to be the average of the second and third measurements that were recorded at least 1 min apart by well-trained doctors. Waist circumference was measured in a standing position at the umbilical level to the nearest 1 cm by well-trained technicians. Informed consent was obtained from all participants. This study was approved by the Institutional Review Board of the National Cardiovascular Center.

Definitions of Metabolic Syndrome

MetS was defined using two criteria. First, in accordance with NCEP-ATPIII (*18*) criteria, it was defined as the presence of three or more of the following five components: 1) abdominal obesity modified by the International Obesity Task Force central obesity criteria for Asia (waist circumference ≥90 cm in men and ≥80 cm in women) (*19*), 2) elevated blood pressure (SBP/DBP ≥130/85 mmHg and/or current use of antihypertensive medication), 3) hypertriglyceridemia (serum triglyceride levels ≥1.7 mmol/L [150 mg/dL] and/or current use of cholesterol-lowering medication), 4) low HDL cholesterol (serum HDL levels of ≤1.0 mmol/L [40 mg/dL] in men and of ≤1.3 mmol/L [50 mg/dL] in women), and 5) elevated blood glucose levels (fasting blood glucose ≥6.1 mmol/L [110 mg/dL] and/or current use of insulin or oral medication for diabetes).

Second, we used the definition of MetS recommended by the Japanese Committee on the Criteria for MetS (*14*, *15*). MetS was defined by abdominal obesity (waist circumference ≥85 cm in men and ≥90 cm in women) (*20*) and least two of the following three components: 1) elevated blood pressure (SBP/DBP ≥130/85 mmHg), 2) hyperlipidemia (serum triglyceride levels ≥1.7 mmol/L [150 mg/dL] and/or HDL levels <1.0 mmol/L [40 mg/dL]), and 3) elevated blood glucose levels ≥6.1 mmol/L (110 mg/dL). Subjects taking medication for hypertension, hyperlipidemia, or diabetes were included as having that component.

Endpoint Determination

The endpoint of the follow-up period for each participant was whichever one of the following occurred first: 1) the date of the first myocardial infarction (MI) or stroke event, 2) the date of death, 3) the date the participant moved out of Suita,

Table 1. Baseline Distributions of Cardiovascular Disease Risk Factors According to Metabolic Syndrome under the NCEP-ATPIII Modified by Asian Obesity Definitions

	Men (n=2,492)			Women (n=2,840)		
	MetS(−) (n=2,043)	MetS(+) (n=449)	p*	MetS(−) (n=2,253)	MetS(+) (n=587)	p*
Age at baseline, years	55.4±13.3	58.1±11.5	<0.001	52.2±12.6	61.3±9.8	<0.001
Systolic blood pressure, mmHg	126±20	140±19	<0.001	120±20	141±20	<0.001
Diastolic blood pressure, mmHg	78±12	85±11	<0.001	73±11	83±12	<0.001
Total cholesterol, mg/dL	200±34	210±35	<0.001	210±38	227±38	<0.001
HDL cholesterol, mg/dL	51±13	40±10	<0.001	60±12	45±10	<0.001
Triglyceride, mg/dL†	121±73	241±156	<0.001	90±44	178±113	<0.001
Waist circumference, cm	81.0±7.3	89.7±7.0	<0.001	74.7±8.9	87.4±8.5	<0.001
Elevated blood pressure, %	41.8	85.8	<0.001	30.4	82.1	<0.001
Hypertriglyceridemia, %	21.6	82.9	<0.001	7.2	63.7	<0.001
Lower-HDL cholesterol, %	15.5	64.8	<0.001	18.7	80.1	<0.001
Hyperglycemia, %	8.9	43.9	<0.001	3.6	29.6	<0.001
Current smoker, %	50.5	47.6	0.278	11.9	11.8	0.958
Current drinker, %	75.5	72.6	0.207	34.6	25.4	<0.001

Elevated blood pressure: antihypertensive drug use or >130/85 mmHg; hypertriglyceridemia: antilipidemic drug use or triglyceride >150 mg/dL; lower-HDL cholesterol: HDL cholesterol <40 mg/dL. MetS, metabolic syndrome; HDL, high-density lipoprotein. *ANOVA or χ^2 tests were performed. †Log-transformed triglyceride was performed to statistical analysis.

Table 2. Age-Adjusted Hazard Ratios (Confidence Intervals) for Incidence of Cardiovascular Disease According to Abdominal Obesity at Baseline Examination

	Men				Women			
	Case, n	Person-year	HR (95% CI)	p	Case, n	Person-year	HR (95% CI)	p
Japanese criteria								
<85 cm (men)/<90 cm (women)	111	17,112	1		96	29,960	1	
≥85 cm (men)/≥90 cm (women)	77	11,247	0.97 (0.72–1.30)	0.844	33	3,890	1.64 (1.09–2.46)	0.019
Asian criteria								
<90 cm (men)/<80 cm (women)	145	23,136	1		53	21,139	1	
≥90 cm (men)/≥80 cm (women)	43	5,223	1.18 (0.84–1.67)	0.327	76	12,711	1.44 (1.00–2.07)	0.048
NCEP-ATPIII criteria								
<102 cm (men)/<88 cm (women)	182	27,976	1		91	28,730	1	
≥102 cm (men)/≥88 cm (women)	6	384	2.00 (0.88–4.54)	0.095	38	5,121	1.47 (1.00–2.17)	0.048

HR, hazard ratio; CI, confidence interval.

or 4) December 31, 2005 (censored). As a first-step survey to detect MI and stroke incidence, each participant's health status was checked during a clinical visit at the National Cardiovascular Center every 2 years. Furthermore, every year a health questionnaire was given to each participant *via* mail or telephone.

Confirmation of Strokes and Myocardial Infarctions

In total, five hospitals in this area were capable of performing computed tomographic scans and/or magnetic resonance imaging, and all were major hospitals that admitted acute stroke and MI patients. Medical records were reviewed by registered hospital physicians or research physicians who were blinded to the baseline information. Strokes and MI events were registered if they occurred after the date on which the baseline health examination was held and before January 1, 2006. Strokes were defined according to the National Survey of Stroke criteria (*21*). These criteria require the rapid onset of a constellation of neurological deficits lasting at least 24 h or until death. For each stroke subtype (cerebral infarction [thrombotic or embolic infarction], intracerebral hemorrhage, and subarachnoid hemorrhage), a definite diagnosis was established based on examination of computed tomographic scans, magnetic resonance images, or autopsy. Defi-

Table 3. Age-Adjusted Hazard Ratios (95% Confidence Intervals) for Incidence of Cardiovascular Disease, Myocardial Infarction, and All Strokes According to Metabolic Syndrome under the Japanese and NCEP-ATPIII Definitions

	Men			Women		
	MetS(−)	MetS(+)	*p* value	MetS(−)	MetS(+)	*p* value
Cardiovascular disease						
MetS Japanese definition						
Cases, *n*	140	48		110	19	
Person-year	23,542	4,817		32,325	1,526	
Age-adjusted	1	1.31 (0.94–1.82)	0.109	1	2.16 (1.31–3.54)	0.002
Multivariate-adjusted	1	1.34 (0.96–1.87)	0.080	1	2.20 (1.31–3.68)	0.003
<60 years old						
Cases, *n*	27	15		25	4	
Person-year	14,752	2,366		22,085	529	
Age-adjusted	1	2.76 (1.46–5.23)	0.002	1	5.39 (1.82–15.98)	0.002
Multivariate-adjusted	1	2.92 (1.54–5.55)	0.001	1	6.25 (2.08–18.79)	0.001
≥60 years old						
Cases, *n*	113	33		85	15	
Person-year	8,790	2,451		10,240	997	
Age-adjusted	1	1.04 (0.70–1.53)	0.841	1	1.83 (1.05–3.18)	0.033
Multivariate-adjusted	1	1.06 (0.71–1.57)	0.764	1	1.80 (1.01–3.20)	0.046
MetS NCEP-ATPIII (Asian) definition						
Cases, *n*	133	55		73	56	
Person-year	23,373	4,986		27,405	6,446	
Age-adjusted	1	1.70 (1.23–2.34)	0.001	1	1.93 (1.35–2.77)	<0.001
Multivariate-adjusted	1	1.75 (1.27–2.41)	<0.001	1	1.90 (1.31–2.77)	<0.001
<60 years old						
Cases, *n*	30	12		19	10	
Person-year	14,509	2,606		19,872	2,742	
Age-adjusted	1	1.79 (0.91–3.52)	0.089	1	2.72 (1.23–5.99)	0.013
Multivariate-adjusted	1	1.94 (0.98–3.82)	0.055	1	2.96 (1.34–6.57)	0.007
≥60 years old						
Cases, *n*	103	43		54	46	
Person-year	8,864	2,381		7,533	3,704	
Age-adjusted	1	1.67 (1.16–2.40)	0.005	1	1.78 (1.19–2.66)	0.005
Multivariate-adjusted	1	1.73 (1.20–2.48)	0.003	1	1.70 (1.12–2.59)	0.012
Myocardial infarction						
MetS Japanese definition						
Cases, *n*	56	22		32	7	
Person-year	22,962	4,663		31,697	1,457	
Age-adjusted	1	1.48 (0.90–2.44)	0.117	1	2.36 (1.02–5.46)	0.043
Multivariate-adjusted	1	1.51 (0.91–2.48)	0.105	1	2.70 (1.15–6.35)	0.023
MetS NCEP-ATPIII (Asian) definition						
Cases, *n*	52	26		18	21	
Person-year	22,833	4,795		26,944	6,211	
Age-adjusted	1	2.09 (1.30–3.37)	0.002	1	2.68 (1.41–5.10)	0.003
Multivariate-adjusted	1	2.12 (1.31–3.43)	0.002	1	2.77 (1.44–5.32)	0.002
All strokes						
MetS Japanese definition						
Cases, *n*	84	26		78	12	
Person-year	23,177	4,659		32,078	1,487	
Age-adjusted	1	1.21 (0.78–1.89)	0.381	1	2.09 (1.12–3.88)	0.019
Multivariate-adjusted	1	1.27 (0.81–1.97)	0.292	1	2.05 (1.07–3.92)	0.031
MetS NCEP-ATPIII (Asian) definition						
Cases, *n*	81	29		55	35	
Person-year	23,010	4,826		27,266	6,299	
Age-adjusted	1	1.52 (0.99–2.34)	0.053	1	1.70 (1.09–2.64)	0.018
Multivariate-adjusted	1	1.58 (1.02–2.43)	0.037	1	1.62 (1.02–2.58)	0.041

Multivariate adjusted for age, smoking and drinking status. MetS, metabolic syndrome.

nite and probable MI was defined according to the criteria set out by the MONICA (Monitoring Trends and Determinants of Cardiovascular Disease) project (*22*), which requires evidence from ECGs, cardiac enzymes, and/or autopsy. Sudden deaths of unknown origin were deaths that occurred within 24 h from onset and were included in MI. However, there was little difference in hazard ratios between the groups with and without sudden death from CVD, because sudden death constituted a small sample size ($n=6$).

To complete surveillance for fatal stroke and MI, we also systematically searched for death certificates, the purpose of which were permitted to use by the Ministry of Health, Labour and Welfare. We checked for possible stroke and MI using data from 1) the health examination and questionnaire for the stroke and MI registry, without informed consent for the medical records survey and 2) death certificates without registration of CVD incidence, which were defined as probable stroke or MI. CVD was defined as stroke and MI in this study. Informed consent to review in-hospital medical records was obtained from 86.2% of participants who were suspected of having any signs or information suggesting the incidence of stroke or MI. For 13.8% of subjects from whom informed consent was not obtained, final diagnoses of CVD were confirmed by physicians or epidemiologists who had been involved in the diagnostic process throughout the study, in order to avoid the misclassification of diagnoses.

Statistical Analysis

Analyses of variance and χ^2 tests were used to compare mean values and frequencies by sex, respectively, according to MetS based on the modified NCEP-ATPIII criteria. For each subject, the person-years of follow-up were calculated from September 1, 1989, to whichever came first: the first endpoint, MI or stroke event, death, emigration, or December 31, 2005. A Cox proportional hazards regression model was used to detect associations between abdominal obesity for Japanese (≥85 cm in men or ≥90 cm in women), Asian (≥90 cm in men or ≥80 cm in women), and American criteria (≥102 cm in men or ≥88 cm in women) and CVD during the follow-up period. The Cox proportional hazard regressions were fitted to the grouping (positive or negative MetS) after adjusting for age and the other potential confounding factors: baseline age, smoking status (never, ex-smoker, or current smoker), and drinking status (never, ex-drinker, or current drinker). Trend tests were conducted by assigning the number of MetS components to test the significance of these variables. All statistical analyses were conducted using the SAS statistical package (release version 8.2; SAS Institute Inc., Cary, USA).

Results

During the follow-up period (averaging 12.5 years), 200 strokes were documented (160 definite strokes and 40 probable strokes). These strokes comprised 130 cerebral infarctions, 31 intracerebral hemorrhages, 22 subarachnoid hemorrhages, and 17 unclassified strokes. In addition, 117 MIs were documented (61 definite MIs and 56 probable MIs or sudden cardiac deaths).

Table 1 shows the distribution of CVD risk factors at the baseline according to MetS as defined by the modified NCEP-ATPIII criteria. Compared with the non-MetS groups, men and women with MetS were more likely to be older and to have higher frequencies of each MetS component.

Table 2 presents the age-adjusted HRs (95% confidence intervals [CI]) for the incidence of CVD according to waist circumference by the NCEP-ATPIII, Japanese, and Asian obesity criteria. Regardless of the criteria set, abdominal obesity was associated with CVD only in women.

Table 3 shows the association of MetS by the Japanese and the modified NCEP-ATPIII criteria with CVD incidence according to age category and sex. Using the Japanese criteria, MetS was associated only in women with the incidence of CVD, MI, and all strokes (HR [95% CI]: 2.20 [1.31–3.68], 2.70 [1.15–6.35], and 2.05 [1.07–3.92], respectively), whereas in men overall MetS was not associated with the incidence of CVD or its subtypes. However, among men under 60 years old, MetS based on the Japanese criteria was associated with CVD incidence (HR=2.92, 95% CI: 1.54–5.55). Using the modified NCEP-ATPIII definition, MetS was associated with each CVD subtype in both men and women. Multivariate adjusted HRs of CVD incidence for MetS based on the NCEP-ATPIII criteria were 1.94 (0.98–3.82) and 1.73 (1.20–2.48) in men less than or equal to and over 60 years old, respectively.

Figure 1 shows that the frequency of MetS increased with age for men and women based on the NCEP-ATPIII (A) and Japanese (B) criteria, respectively. The frequency based on the NCEP-ATPIII modified by the Asian obesity criteria (25.1% for men and 14.3% for women) was higher than that based on the Japanese criteria (17.7% for men and 5.0% for women), especially in women.

The risk of CVD incidence increased according to the number of components combined in men and women with and without abdominal obesity (Fig. 2). In addition, compared with the non-abdominal obesity and non-component groups, the risks of CVD incidence were similar among participants who had the same numbers of components, regardless of the presence or absence of abdominal obesity in men and women combined.

Figure 3 shows the multivariate HRs for MetS based on the Japanese and NCEP-ATPIII definitions modified by the obesity criteria for waist circumference. When the Japanese definition was adopted and the risk of MetS was monitored through sequential waist circumference changes, the cut-off points for waist circumference, which conferred a risk of CVD in men and women, were 84 cm and 92 cm, respectively. When the definition of MetS-indicative waist circumference was higher than those values, the risk was not statistically significant. When the NCEP-ATPIII definition

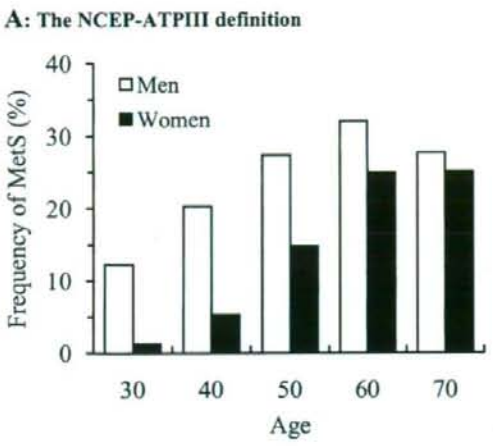

Fig. 1. *Frequencies of MetS components (A: the NCEP-ATPIII definition; and B: the Japanese definition, modified by the Asian waist circumference criteria) by sex. White and solid bars indicate men and women, respectively.*

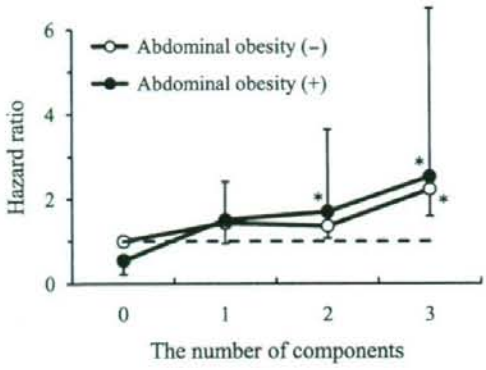

Fig. 2. *Multivariate HRs for the risks of CVD incidence according to the number of components based on the NCEP-ATPIII definition with and without abdominal obesity. White and solid circles indicate non-abdominal and abdominal obesity according to the Asian obesity criteria. *$p<0.05$ compared to the reference of non-abdominal obesity and no-components. Bars show 95% CI for the HRs.*

was used, the value of waist circumference did not modify the risk of CVD, implying that the clustering of risk factors may be more important than waist circumference itself for determining CVD risk.

Discussion

In the current cohort study of a general urban Japanese population, the association between MetS and CVD was significant when the NCEP-ATPIII (modified by the Asian criteria) definition was applied. MetS based on the Japanese criteria was associated with CVD incidence in women, whereas in men the association was found only in those under 60 years old. In addition, the risk of CVD incidence was similar among participants who had the same numbers of components regardless of whether they were abdominally obese. To the best of our knowledge, this is the first study of an urban Japanese cohort.

Compared to the previous studies, this study has several methodological strengths. First, previous Japanese cohort studies associating MetS with CVD were based predominantly on BMI (*3*, *4*), non-fasting blood collection (*3*, *4*), and mortality as the endpoint (*4*, *5*). Our baseline subjects were observed in the fasting state, and we used waist circumference and a wide age range. Second, we evaluated a large prospective cohort of people randomly selected from a general Japanese population. A prospective study has little recall bias as well as results from a general population cohort that is more representative than occupational, hospital-based, or volunteer cohorts. Third, our sample size was relatively large for a cohort study and we could therefore perform sub-analysis by age and CVD subtypes. Fourth, our cohort population was selected at random from an urban population, in contrast to most of the other MetS cohort populations, which were selected from rural populations. Our study is the first of its kind in an urban area. Finally, our study examined the risk of CVD incidence, which is a more direct measure of CVD risk than the rate of CVD mortality, because the time to death from CVD is influenced by treatment.

Abdominal obesity induces inflammation in adiposities (*23*), endothelial dysfunction (*24*, *25*), and oxidative stress (*26*), thereby contributing to CVD development (*27*, *28*).

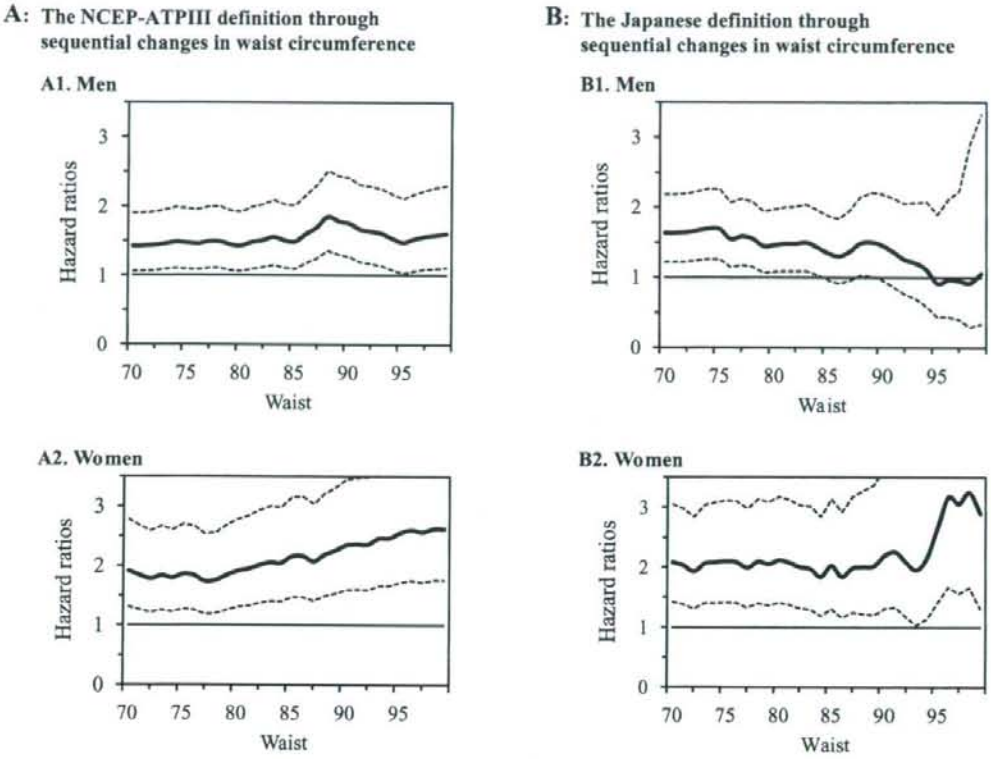

Fig. 3. *Multivariate HRs for MetS based on the NCEP-ATPIII (A) and Japanese (B) definitions through sequential changes in waist circumference by sex. Solid and dotted lines indicate HRs and 95% CI, respectively.*

Accumulating evidence suggests that MetS increases the risk of CVD (*29*). However, there has been a lack of convincing evidence (*29*) that MetS is associated with CVD in Japan. Iso *et al.* reported that MetS was associated with a risk for ischemic CVD in Japan (*3*), although they used BMI as well as non-fasting blood glucose and triglyceride levels to define MetS. Ninomiya *et al.* reported that MetS was a significant risk factor for CVD in a rural Japanese population (*6*). However, that study examined a rural population half the size of that in our study. Takeuchi *et al.* reported that MetS was a risk factor for cardiac disease in a rural cohort (*7*), but their data were based on a small sample that comprised only men. Kadota *et al.* reported that MetS, defined by BMI and non-fasting blood samples, was associated with CVD mortality (*4*).

We have shown that the components of MetS synergistically increase CVD risk. Abdominal obesity did not affect the association between the number of MetS components and the risk of CVD incidence. The risk of CVD was also not related to waist circumference when the NCEP-ATPIII definition was applied (data not shown), suggesting that the combination of risk factors *per se* is more important than abdominal obesity for conferring risk.

The definition of MetS may be reconsidered on the basis of age and sex. According to our results, lifestyle modifications may not be needed for older men who are free of cardiovascular risk factors even if they have abdominal obesity. Therefore, to prevent CVD, it is not adequate for only subjects with MetS to change their lifestyles; subjects with one or two MetS components, even without abdominal obesity, should modify their lifestyles.

When the waist-circumference threshholds were sequentially changed in the Japanese criteria for MetS, our data showed that the clustering of metabolic risk factors was statistically significant for CVD at waist circumferences less than 85 cm for men and 93 cm for women. When the definition of MetS-indicative waist circumferences was higher than those values, the risk clustering was not statistically significant for

CVD in men, and the 95% CI was much wider but still significant in women. Subjects with high risks and non-abdominal obesity with risk clustering aside from abdominal obesity will drop out when the waist-circumference definitions are raised.

Our study has several limitations. First, the annual emigration rate (1.5%) is relatively higher than that in rural areas. Second, about 10% of the subjects who underwent a baseline examination did not respond to our questionnaires afterward. We found no clinical background difference between participants and non-participants, because the main denial reason for participation in this study was not health problems. The frequencies of MetS according to NCEP-ATPIII modified by Asian criteria were 19% and 21% for participants and non-participants, respectively (χ^2 test $p=0.09$). In this study, the main reasons for emigration included job transfer, but not health problems.

In conclusion, the current prospective study for a general urban population showed that MetS, as defined by the Japanese criteria, was associated with CVD in women and middle-aged men; a stronger association was found when the NCEP-ATPIII definition modified by the Asian obesity criteria was applied. The number of MetS components may be more strongly associated with CVD incidence than the essential waist-circumference criteria.

Acknowledgements

We would like to express our deepest gratitude to all members of the Suita City Health Center and the Suita Medical Association. We also thank all researchers and the staff members of the Division of Preventive Cardiology for performing medical and follow-up examinations. We also thank Satsuki-Junyukai, the volunteer administrator for the Suita study.

References

1. Eckel RH, Grundy SM, Zimmet PZ: The metabolic syndrome. *Lancet* 2005; **365**: 1415–1428.
2. Galassi A, Reynolds K, He J: Metabolic syndrome and risk of cardiovascular disease: a meta-analysis. *Am J Med* 2006; **119**: 812–819.
3. Iso H, Sato S, Kitamura A, *et al*: Metabolic syndrome and the risk of ischemic heart disease and stroke among Japanese men and women. *Stroke* 2007; **38**: 1744–1751.
4. Kadota A, Hozawa A, Okamura T, *et al*: Relationship between metabolic risk factor clustering and cardiovascular mortality stratified by high blood glucose and obesity: NIPPON DATA90, 1990–2000. *Diabetes Care* 2007; **30**: 1533–1538.
5. Niwa Y, Ishikawa S, Gotoh T, Kayaba K, Nakamura Y, Kajii E: Metabolic syndrome mortality in a population-based cohort study: Jichi Medical School (JMS) Cohort Study. *J Epidemiol* 2007; **17**: 203–209.
6. Ninomiya T, Kubo M, Doi Y, *et al*: Impact of metabolic syndrome on the development of cardiovascular disease in a general Japanese population: the Hisayama study. *Stroke* 2007; **38**: 2063–2069.
7. Takeuchi H, Saitoh S, Takagi S, *et al*: Metabolic syndrome and cardiac disease in Japanese men: applicability of the concept of metabolic syndrome defined by the National Cholesterol Education Program–Adult Treatment Panel III to Japanese men—the Tanno and Sobetsu Study. *Hypertens Res* 2005; **28**: 203–208.
8. Hunt KJ, Resendez RG, Williams K, Haffner SM, Stern MP: National Cholesterol Education Program *versus* World Health Organization metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study. *Circulation* 2004; **110**: 1251–1257.
9. Kahn R, Buse J, Ferrannini E, Stern M: The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2005; **28**: 2289–2304.
10. Arenillas JF, Moro MA, Davalos A: The metabolic syndrome and stroke: potential treatment approaches. *Stroke* 2007; **38**: 2196–2203.
11. Alberti KG, Zimmet P, Shaw J: Metabolic syndrome—a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med* 2006; **23**: 469–480.
12. Grundy SM, Cleeman JI, Daniels SR, *et al*: Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005; **112**: 2735–2752.
13. Tanaka H, Yokoyama T, Yoshiike N, Kokubo Y: Cerebrovascular disease, in Detels R, McEwen J, Beaglehole R, Tanaka H (eds): Oxford Textbook of Public Health: The Scope of Public Health. Oxford, Oxford University Press, 2002, pp 1193–1254.
14. Matsuzawa Y: Metabolic syndrome—definition and diagnostic criteria in Japan. *J Atheroscler Thromb* 2005; **12**: 301.
15. Definition and the diagnostic standard for metabolic syndrome—Committee to Evaluate Diagnostic Standards for Metabolic Syndrome. *Nippon Naika Gakkai Zasshi* 2005; **94**: 794–809 (in Japanese).
16. Kokubo Y, Inamoto N, Tomoike H, *et al*: Association of genetic polymorphisms of sodium-calcium exchanger 1 gene, NCX1, with hypertension in a Japanese general population. *Hypertens Res* 2004; **27**: 697–702.
17. Kokubo Y, Tomoike H, Tanaka C, *et al*: Association of sixty-one non-synonymous polymorphisms in forty-one hypertension candidate genes with blood pressure variation and hypertension. *Hypertens Res* 2006; **29**: 611–619.
18. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001; **285**: 2486–2497.
19. James PT, Leach R, Kalamara E, Shayeghi M: The worldwide obesity epidemic. *Obes Res* 2001; **9** (Suppl 4): 228S–233S.
20. New criteria for 'obesity disease' in Japan. *Circ J* 2002; **66**: 987–992.
21. Walker AE, Robins M, Weinfeld FD: The National Survey of Stroke. Clinical findings. *Stroke* 1981; **12**: I13–I44.

22. Tunstall-Pedoe H, Kuulasmaa K, Amouyel P, Arveiler D, Rajakangas AM, Pajak A: Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation* 1994; **90**: 583–612.
23. Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW: C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 1999; **19**: 972–978.
24. Meigs JB, O'Donnell C J, Tofler GH, *et al*: Hemostatic markers of endothelial dysfunction and risk of incident type 2 diabetes: the Framingham Offspring Study. *Diabetes* 2006; **55**: 530–537.
25. Lteif AA, Han K, Mather KJ: Obesity, insulin resistance, and the metabolic syndrome: determinants of endothelial dysfunction in whites and blacks. *Circulation* 2005; **112**: 32–38.
26. Ceriello A, Motz E: Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler Thromb Vasc Biol* 2004; **24**: 816–823.
27. Despres JP, Lemieux I: Abdominal obesity and metabolic syndrome. *Nature* 2006; **444**: 881–887.
28. Van Gaal LF, Mertens IL, De Block CE: Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; **444**: 875–880.
29. Balkau B, Deanfield JE, Despres JP, *et al*: International Day for the Evaluation of Abdominal Obesity (IDEA): a study of waist circumference, cardiovascular disease, and diabetes mellitus in 168,000 primary care patients in 63 countries. *Circulation* 2007; **116**: 1942–1951.

DIABETES RESEARCH AND CLINICAL PRACTICE 81 (2008) 327–330

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/diabres

Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients

Hisashi Makino[a,*], Sadanori Okada[a], Ayako Nagumo[a], Takako Sugisawa[a], Yoshihiro Miyamoto[a], Ichiro Kishimoto[a], Taura K. Akie[b], Toshihiro Soma[b], Akihiko Taguchi[c], Yasunao Yoshimasa[a]

[a] National Cardiovascular Center, Department of Atherosclerosis and Diabetes, Suita, Osaka 565-8565, Japan
[b] National Hospital Organization Osaka Minami Medical Center, Department of Hematology, Osaka, Japan
[c] National Cardiovascular Center, Department of Cerebrovascular Disease, Suita, Osaka, Japan

ARTICLE INFO

Article history:
Received 30 October 2007
Received in revised form
31 March 2008
Accepted 12 May 2008
Published on line 17 July 2008

Keywords:
Endothelial progenitor cell
PPARγ agonist
Atherosclerosis
Chronic inflammation
Adiponectin

ABSTRACT

Circulating bone marrow derived immature cells, including CD34-positive ($CD34^+$) cells, contribute to maintenance of the vasculature, not only as a pool of endothelial progenitor cells (EPCs), but also as a source of growth/angiogenesis factor. We hypothesized that the thiazolidineone compound pioglitazone could stimulate the circulating $CD34^+$ cells in diabetic patients. Thirty-four patients with type 2 diabetes received 15–30 mg pioglitazone for 24 weeks. The number of circulating $CD34^+$ cells significantly increased at 12 and continued this effect for 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 cells/μl at 0, 12 and 24 weeks, respectively). The change of $CD34^+$ cell levels ($\Delta CD34^+$ cells) between 0 and 12 weeks was significantly correlated with the change of high sensitive C reactive protein levels (Δhs-CRP) and change in adiponectin levels (Δadiponectin) ($r = -0.412$, $r = 0.359$, respectively). Our study demonstrated that pioglitazone treatment increased circulating $CD34^+$ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

1. Introduction

Endothelial dysfunction plays a pivotal role in the progression of the atherosclerosis. Circulating EPCs contribute to the maintenance of vascular homeostasis and repair. They also play an important role in the maintenance of vascular endothelial function [1,2]. In diabetic patients, both a decrease in number and function of circulating EPCs are reported, suggesting that circulating EPCs participate in diabetic vascular complications [3].

Recent studies have identified circulating bone marrow derived immature cells, including $CD34^+$ cells, contribute to maintenance of the vasculature, not only as a pool of EPCs, but also as a source of growth/angiogenesis factor [4]. In fact, one recent report indicates that circulating $CD34^+$ cells are more strongly correlated with cardiovascular risk than circulating $CD34^+$/kinase insert domain receptor $(KDR)^+$ cells generally regarded as EPCs [5]. We have also reported that circulating $CD34^+$ cell levels are associated with cerebral infarction [6]. These findings indicate that persistent stimulation of $CD34^+$ cells may be a useful method to repair endothelial injury and microcirculation, and to suppress the progression of atherosclerotic disease at least theoretically. Recent experimental and clinical studies demonstrate that thiazolidinediones, peroxisome-proliferator-activated receptor γ (PPARγ) agonists, has the effects on the prevention of atherosclerosis including the maintenance of vascular endothelial function [7–9]. Therefore, we hypothesized that the thiazolidineone

* *Corresponding author*. Tel.: +81 6 6833 5012; fax: +81 6 6872 7486.
E-mail address: makinoh@hsp.ncvc.go.jp (H. Makino).
0168-8227/$ – see front matter
doi:10.1016/j.diabres.2008.05.012

compound pioglitazone could stimulate the circulating CD34⁺ cells in diabetic patients.

2. Methods

2.1. Study subjects

All subjects gave a written informed consent. The study was approved by the local ethics committee. Thirty-four patients with type 2 diabetes (age 60 ± 10, M/F; 18/16, HbA1c 9.3 ± 1.4%) received 15 or 30 mg pioglitazone for 24 weeks (15 mg; 31 patients, 30 mg; 3 patients). Other medications for diabetes, hypertension and hyperlipidemia were unchanged throughout the study. Insulin was given to 9 patients. Sulfonylurea was given to 15 patients. Biguanide was given to 21 patients. Alpha glucosidase inhibitor was given to 10 patients. Angiotensin converting enzyme inhibitor and/or angiotensin receptor blocker was given to 21 patients. Statin was given to 18 patients. Sixteen patients afflicted with cardiovascular diseases (CVD). Eighteen patients afflicted with nephropathy, 14 patients afflicted with retinopathy, and 15 patients afflicted with neuropathy.

2.2. Measurement of CD34⁺ cells

Three milliliters of heparinized peripheral blood were obtained after 12-h fasting and measured CD34⁺ cells. The precise number of circulating CD34⁺ cells was quantified as we described previously [10]. We evaluated circulating CD34⁺ cells with Stem-Kit™ (BeckmanCoulter, Marseille, France) according to manufacturers' protocols. These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines [11], and are frequently used for quantification of CD34⁺ cells mobilized into peripheral blood. To increase the reproducibility of CD34⁺ cell counts, the protocol of Stem-Kit was modified as follows: the blood sample volume, antibodies and lysing solution were doubled. After adding 30 μl of internal control (Stem count: BeckmanCoulter), samples were centrifuged for 5 min at 450 × *g* and 3860 μl of supernatant was removed carefully with a pipet. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (BeckmanCoulter) for 6 min each.

2.3. Other laboratory analysis

Blood samples were taken after 12-h fasting to measure adiponectin and, high sensitive C-reactive protein (hs-CRP) concentrations. Serum adiponectin and concentration was measured by enzyme-linked immunosorbent assay (SRL, Tokyo, Japan). Serum hs-CRP concentration was measured by latex nephelometry method (SRL, Tokyo, Japan). We also measured HbA1c, total cholesterol, HDL cholesterol and triglyceride levels.

2.4. Statistical analysis

Data was expressed using the mean ± S.D. The Student's *t*-test was used to compare parameter changes over time. The strength of correlation between variables was performed using Spearmann's correlation coefficient.

3. Results

3.1. Effects of pioglitazone on glucose and lipid metabolism

Treatment of pioglitazone significantly decreased HBA1c levels (9.3 ± 1.4, 7.4 ± 1.2 and 7.5 ± 1.7% at 0, 12 and 24 weeks, respectively). Systemic blood pressure levels did not change throughout the study period. BMI did not change throughout the study period (26.8 ± 3.2, 27.5 ± 3.0 and 27.9 ± 3.3 at 0, 12 and 24 weeks, respectively). Total cholesterol and triglyceride levels did not change throughout the study, whereas HDL cholesterol levels significantly increased at 12 and 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 mmol/l at 0, 12 and 24 weeks, respectively).

3.2. Effects of pioglitazone on adiponectin and inflammatory marker

The inflammatory marker, hs-CRP significantly decreased at 12 and 24 weeks (1518 ± 2350, 840 ± 975, and 838 ± 904 ng/ml at 0, 12, and 24 weeks, respectively). Serum adiponectin levels significantly increased at 12 and 24 weeks (5.0 ± 2.2, 13.5 ± 6.7 and 13.8 ± 8.4 μg/ml at 0, 12 and 24 weeks, respectively). The change in adiponectin levels between 0 and 12 weeks (Δadiponectin) of 30 mg pioglitazone was significantly larger than 15 mg of pioglitazone (15 mg; 7.9 ± 4.7 vs. 30 mg; 19.6 ± 2.5, $p < 0.05$), whereas there was no significant difference in the change in hs-CRP levels (Δhs-CRP) between 15 mg and 30 mg of pioglitazone (15 mg; 267 ± 322 vs. 30 mg; 480 ± 1883).

3.3. Effects of pioglitazone on circulating CD34⁺ cell level

The number of circulating CD34⁺ cells significantly increased at 12 and 24 weeks (0.90 ± 0.48, 1.10 ± 0.50, and 1.10 ± 0.57 cells/μl at 0, 12, and 24 weeks, respectively (Fig. 1). This effect was found in both patients with CVD and without CVD (patients with CVD; 0.81 ± 0.51, 1.05 ± 0.46 and 1.04 ± 0.50 cells/μl at 0, 12 and 24 weeks, respectively, $n = 16$, patients without CVD; 0.98 ± 0.41,

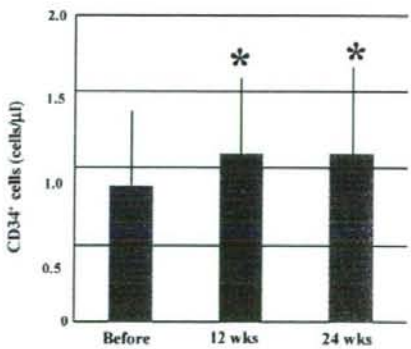

Fig. 1 – CD34+ cell level at 0, 12 and 24 weeks, *$p < 0.05$ vs. 0 week.