Differences in Cellular Responses to Mitogens in Arterial Smooth Muscle Cells Derived From Patients With Moyamoya Disease

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Background and Purpose—Moyamoya disease is a progressive cerebrovascular occlusive disease affecting primarily children. The etiology remains unknown. We examined the chemotactic and proliferative activities of inflammatory cell products from arterial smooth muscle cells (SMCs) derived from moyamoya patients and compared them with those from control subjects.

Methods—We used 12 SMC strains from moyamoya patients and eight from control subjects. SMC migration was examined in a micro chemotaxis chamber. DNA synthesis was measured by an immunoperoxidase technique.

Results—Platelet-derived growth factor (PDGF)-BB markedly stimulated cell migration and DNA synthesis in control SMCs. PDGF-AA stimulated only DNA synthesis in control SMCs. In moyamoya SMCs, PDGF-AA and PDGF-BB stimulated cell migration but not DNA synthesis. Basic fibroblast growth factor had little migratory activity but stimulated DNA synthesis in moyamoya SMCs and control SMCs. Conversely, hepatocyte growth factor stimulated cell migration but not DNA synthesis in moyamoya SMCs and control SMCs. In contrast, interleukin-1β (IL-1β) significantly stimulated the migration and DNA synthesis of control SMCs, while it inhibited moyamoya SMC migration. The levels of IL-1β–induced nitric oxide production did not differ between moyamoya SMCs and control SMCs, suggesting that IL-1β inhibits the migration of moyamoya SMCs through a nitric oxide–independent pathway.

Conclusions—The differences in responses to PDGF and IL-1 in moyamoya SMCs are involved in the mechanism by which intimal thickening develops in moyamoya disease. (Stroke. 1998;29:1188-1193.)

Key Words: cytokines ■ growth factors ■ moyamoya disease ■ muscle, smooth

Moyamoya disease is an unusual form of chronic cerebrovascular occlusive disease characterized by progressive stenosis or occlusion at the distal ends of the bilateral internal carotid arteries. The onset of the disease is seen primarily during the first decade of life, and the variety of neurological symptoms depends on the specific occluded arteries. The etiology of the disease is undefined. The findings that the incidence of the disease is highest in, but not confined to, Japanese and that the condition is frequently familial suggest the involvement of a genetic factor in its pathogenesis. Histopathological investigations have demonstrated that the main vascular lesions are stenosis or occlusion by fibrocellular thickening of the intima. Previous reports suggest the involvement of systemic arteries as well as intracranial arteries in moyamoya disease.

In atherosclerosis with intimal thickening, attention has been directed to the pathobiology of the arterial wall and the key role of SMC proliferation in lesion formation. SMCs in the media have low mitogenic activity. The proliferation and migration of SMCs in the intima may be induced by endothelial injury that alters the endothelial barrier to the passage of blood constituents, facilitating various growth factors and cytokines to act in lesion formation. With this in mind, we have postulated that alterations in cellular responses to growth factors and cytokines in vascular cells are responsible for the development of intimal thickening in moyamoya disease. We previously reported the decrease in growth response to PDGF-BB in cultured moyamoya SMCs, a fact explained by the reduced number of the PDGF receptors on moyamoya SMCs. Other groups suggest the involvement of angiogenic growth factors, especially b-FGF, in the development of intimal thickening of moyamoya disease. The elevation of b-FGF concentrations in the cerebrospinal fluid of moyamoya patients may be due to pathogenic factors affecting the basal vessels of the brain, although it may be caused by the hypoxic stress in the ischemic brain of moyamoya patients. These previous studies concerning moyamoya disease have focused on limited kinds of growth factors that act on SMCs.

PDGF has been isolated first from human platelets, and two polypeptide chains termed PDGF-A and PDGF-B are...
Selected Abbreviations and Acronyms

- b-FGF = basic fibroblast growth factor
- BrdU = 5-bromo-2'-deoxyuridine
- EGF = epidermal growth factor
- FBS = fetal bovine serum
- HCSMC = arterial SMCs derived from control subjects
- HGF = hepatocyte growth factor
- HMSMC = arterial SMCs derived from patients with moyamoya disease
- IFN-γ = interferon gamma
- IL = interleukin
- L-NAME = N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester
- MEM = minimum essential medium
- NO = nitric oxide
- PDGF = platelet-derived growth factor
- SMC = smooth muscle cell
- TGF = transforming growth factor
- TNF-α = tumor necrosis factor-α

encoded by different genes and dimerize to form either homodimeric (PDGF-AA or PDGF-BB) or heterodimeric (PDGF-AB) forms of the PDGF molecule. The mitogenic activity of PDGF-BB and -AB has been shown to be greater than that of PDGF-AA in SMCs derived from human and experimental animals. However, cultured SMCs derived from experimentally induced atherosclerotic lesions have been reported to express the gene for A chain of PDGF and to secrete PDGF-AA into the conditioned medium. The b-FGF is known as an angiogenic growth factor and also targets a wide variety of cell types in vitro, inducing the proliferation of SMCs and fibroblasts in addition to endothelial cells. The effects of PDGF-AA and b-FGF on mitogenesis of cultured moyamoya SMC have never been investigated. Furthermore, recent evidence suggests that chronic inflammatory responses may be involved in the pathogenesis of moyamoya disease. The inflammatory responses involve the activation of various cytokines such as IL-1, IL-6, TNF-α, and IFN-γ, all of which have recently been implicated as important factors in atherosclerosis and tissue remodeling after arterial wall injury. We have continued to establish the SMC strains derived from the superficial temporal arteries of patients with moyamoya disease. Recent evidence suggests that the migration of SMCs plays an important role in the development of intimal thickening and is controlled by different mechanisms from the cell proliferation. In the present study we examined the effects of various growth factors and cytokines on the proliferation and migration of cultured SMCs in a larger number of moyamoya SMC strains and compared them with those of SMCs from age-matched control subjects.

Materials and Methods

Materials

Recombinant human PDGF-AA, b-FGF, EGF, and IL-6 were purchased from Boehringer Mannheim. Recombinant PDGF-BB was obtained from Amersham International plc. Recombinant HGF and IFN-γ were purchased from Becton Dickinson Labware. Recombinant IL-1β was obtained from King Brewing Co. Recombinant TNF-α was purchased from Genzyme Co. L-NAME was purchased from Sigma Chemical Co. Eagle’s MEM was obtained from Gibco. FBS was obtained from Biosett (6201B304).

Cell Culture

Articular SMC strains derived from Japanese patients with moyamoya disease (HMSMC) and control subjects (HCSMC) were established as described previously. Arterial specimens were obtained from branches of scrap arteries (superficial temporal arteries) requiring division during indirect bypass or other cranial operations. Informed consent was obtained from the patients or their relatives, and the study was approved by the Ethical Committee of the Tokyo Metropolitan Institute of Gerontology.

We used 12 SMC strains from moyamoya patients and eight from control subjects. The cells were cultured in 60-mm Falcon dishes (3002) in 5 mL of MEM supplemented with 15% FBS at 37°C under humidified 5% CO\textsubscript{2}/95% air. The medium was renewed every 3 or 4 days. Confluent cultures were treated with 0.25% trypsin/0.02% EDTA in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free phosphate-buffered saline for 10 minutes at 37°C and subcultured at a 1:2 split ratio. The number of cells was counted with a hemocytometer after trypan treatment. For the present study, we used cells within 50% of the final population doubling levels that showed no signs of senescence in vitro. The cells were carefully examined for mycoplasma contamination by the method described previously.

Migration Assay

SMC migration was monitored in a Micro Chemotaxis Assembly (Neuro Probe) with the use of polyn vinylpyrrolidone-free polycarbonate membranes with 8-μm pores. SMCs grown to confluence were suspended in MEM containing 2% FBS, and 220 μL of cell suspension (1×10\textsuperscript{5} cells per milliliter) was placed in the upper compartment of the chamber. The lower compartment contained 30 μL of MEM supplemented with 2% FBS, growth factors (0 to 10 ng/mL) except for HGF (0 to 50 ng/mL), and cytokines (0 to 500 U/mL). These concentrations of these mitogens had optimal effects in both moyamoya and control SMCs. Incubation was performed in a CO\textsubscript{2} incubator for 18 hours at 37°C. Nonmigrated cells on the upper surface of the membranes were scraped off gently, and the membranes were fixed in methanol for 30 minutes at room temperature and stained with Diff-Quick solution (International Reagents Co). SMCs that migrated to the lower surface of the membranes were quantified by visual determination in five or more randomly selected fields per membrane at ×400 magnification. The area was measured with an image analyzer (SPICCA-II, Olympus). The assays were performed in a blinded fashion.

Incorporation of BrdU into Cellular DNA

The cell proliferation was assessed by estimating BrdU incorporation into cellular DNA. Estimation of DNA synthesis, as measured by BrdU incorporation into cellular DNA, is essentially an accurate measure of cell proliferation and correlates well with the findings of cell proliferation in HMSMC and HCSMC. SMCs grown to confluence were arrested in MEM containing 0.5% FBS for 24 hours. The cells were incubated in MEM containing 0.5% FBS, test mitogens, and a labeling reagent (BrdU) for 48 hours. BrdU incorporation into cellular DNA was measured by an immunoperoxidase technique (cell proliferation kit; Amersham) as previously described. The percentage of labeled nuclei was determined by counting more than 200 cells in each experiment. The test mitogens were PDGF-AA (0 to 10 ng/mL), PDGF-BB (0 to 10 ng/mL), b-FGF (0 to 10 ng/mL), EGF (0 to 10 ng/mL), HGF (0 to 50 ng/mL), TGF-β1 (0 to 10 ng/mL), and IL-1β (0 to 500 U/mL). The experiments were performed in a blinded fashion.

Determination of NO Production

SMCs grown to confluence were washed with MEM containing 0.5% FBS. The medium was replaced with fresh MEM containing 0.5% FBS, 500 U/mL IL-1β, and 1 mmol/L L-NAME, and the cells were incubated for 24 hours at 37°C. The medium was collected and filtered through a 0.22-μm filter. NO secreted into the culture was detected using a chemiluminescent detection kit (Amersham).
medium was measured with a nitrate/nitrite assay kit (Cayman Chemical Co). The detection limit for nitrite is approximately 2 μmol/L. The assays were performed in a blinded fashion.

**Statistical Analysis**

Data are expressed as mean±SD. Differences in data between groups were assessed by unpaired t test. A value of P<0.05 is considered statistically significant.

**Results**

**Clinical Characteristics**

Patients with moyamoya disease comprised 6 females and 6 males. The age of the moyamoya patients was 9.6±3.8 (mean±SD) years, and the age at disease onset was 6.1±3.4 years. The initial symptoms were transient ischemic attacks in 9, cerebral infarction in 2, and intracerebral hemorrhage in 1 patient. No associated diseases were found in any of the 12 patients with moyamoya disease. Control subjects comprised 5 females and 3 males. The age of the control subjects was 8.8±7.2 years, which was not statistically different from that of the moyamoya patients. The primary diseases in the control subjects were head injury in 4, cranial bone disease in 3, and intracerebral hemorrhage from the rupture of a small angioma in 1 patient.

**Migration of Moyamoya SMCs**

We examined the migration of HMSMC and HCSMC. The number of cells migrating in MEM with 2% FBS (no test mitogens) did not differ significantly between moyamoya (76.5±31.0) and control (68.5±17.6) SMC strains (Figure 1). Cell migration was markedly stimulated by PDGF-BB (5 to 10 ng/mL) in both HMSMC and HCSMC strains in a dose-dependent manner (Figures 1 and 2). HGF (20 to 50 ng/mL) also had a stimulatory effect on the migration of both HCSMC and HMSMC strains. PDGF-AA (5 to 10 ng/mL) stimulated cell migration in HMSMC strains in a dose-dependent manner but not in HCSMC strains. IL-1β (200 to 500 U/mL) also had a stimulatory effect on the migration of both HCSMC and HMSMC strains. PDGF-AA (5 to 10 ng/mL) stimulated cell migration in HMSMC strains in a dose-dependent manner but not in HCSMC strains. IL-1β (200 to 500 U/mL) also had a stimulatory effect on the migration of both HCSMC and HMSMC strains. IL-1β (200 to 500 U/mL) also had a stimulatory effect on the migration of both HCSMC and HMSMC strains. IL-6 also significantly inhibited the migration of moyamoya SMCs, while it neither stimulated nor inhibited the migration of control SMCs. b-FGF, EGF, TGF-β1, TNF-α, and IFN-γ...
had little stimulatory effect on cell migration in either HMSMC or HCSMC strains (Figure 2). A differing dose response to the mitogens was hardly observed between HMSMC and HCSMC strains.

**BrdU Incorporation into Intracellular DNA**

We examined the effects of growth factors and IL-1β on DNA synthesis. The basal (no test mitogens) labeling indices of HCSMC strains were 20.1%, and those of HMSMC strains were 15.5%, which were not statistically different. PDGF-AA (5 to 10 ng/mL), PDGF-BB (5 to 10 ng/mL), and IL-1β (200 to 500 U/mL) produced a significant stimulation of BrdU incorporation into intracellular DNA in HCSMC strains in a dose-dependent manner but not in HMSMC strains (Figure 3). b-FGF (5 to 10 ng/mL) significantly stimulated the initiation of DNA synthesis in both HCSMC and HMSMC strains in a dose-dependent manner, whereas HGF (0 to 50 ng/mL) was unable to stimulate DNA synthesis in either strain (Figure 3). EGF and TGF-β1 treatment (0 to 10 ng/mL) resulted in little BrdU incorporation into cellular DNA in HCSMC and HMSMC strains (data not shown).

**NO Release into Culture Medium**

Previous studies indicate that IL-1 stimulates the release of large amounts of NO from vascular SMCs in vitro and that NO released from vascular SMCs induces SMC death and inhibits the angiotensin II–induced migration of aortic SMCs. We then examined the production of NO by arterial SMCs from moyamoya patients and control subjects. As shown in Figure 4, IL-1β induced NO production in HMSMC strains, but the amount did not differ significantly from that induced in HCSMC strains. The simultaneous addition of L-NAME (1 mmol/L) with the cytokine suppressed NO production in both HCSMC and HMSMC strains.

**Discussion**

The migration of medial SMCs and their proliferation in the intimal layer contribute to the intimal thickening of injured and atherosclerotic vessels. It has been proposed that these events are regulated by growth factors and cytokines. PDGF has been implicated as a major potent factor that stimulates the migration and replication of SMCs during the development of intimal thickening in atherosclerosis or after arterial wall injury. We previously reported a decrease in the growth response to PDGF-BB in moyamoya SMCs, a fact explained by the reduced number of PDGF receptors on moyamoya SMCs. The differences in cellular responses to mitogens may be due to an underlying disease process, but not induced by culturing, because we culture both moyamoya and control SMC strains under the same conditions and use cells within 50% of the final population doubling levels (phase II) that are characterized by rapid cell multiplication. The results obtained in the present study confirm the previous findings in a larger number of moyamoya cell strains. Furthermore, we found a decrease in DNA synthesis in response to PDGF-AA and IL-1β compared with control SMCs. Only b-FGF pro-
motes DNA synthesis in moyamoya SMCs, although the degree of stimulation does not differ from that in control SMCs. In contrast to the reduction in DNA synthesis induced by PDGF, both PDGF-AA and -BB stimulate the migration of moyamoya SMCs, while only PDGF-BB had a stimulatory effect on control SMCs. Our results concerning the response of control SMCs to PDGF-AA and PDGF-BB are in good agreement with previous reports. Recent evidence suggests that the migration of SMCs is controlled by distinct mechanisms from the cell proliferation and migration of SMCs are induced into the intima. However, we recently suggested the importance of the migration but not replication of SMCs, as well as extracellular matrix deposition, in the later stage of neointimal formation after arterial wall injury. The rapid growth and migration of connective tissue cells (SMCs) promoted by growth factors such as PDGF-BB may be necessary for normal wound repair during the earlier stage after arterial wall injury. The poor proliferative response of moyamoya SMCs to PDGF-BB and -AA may suggest failure in normal repair process of arterial wall injury, and the retained ability in growth response to b-FGF and in migratory response to PDGF-BB and -AA and HGF in moyamoya SMCs might have contributed to the continued neointimal formation in moyamoya disease. Alternatively, our results support the notion by several reports that PDGF is important for the migration but not the replication of arterial SMC during neointimal formation.

The migratory responses of moyamoya and control SMCs differ for PDGF-AA, IL-1β, and IL-6. IL-1β and IL-6 significantly inhibit the migration of moyamoya SMCs but not control SMCs. The difference in the migratory response to IL-1β between moyamoya and control SMCs is striking. IL-1β significantly promoted the migration of every control SMC strain, while it inhibited the migration of every moyamoya SMC strain. IL-1β also lacked a mitogenic effect on moyamoya SMCs, while it caused a significant stimulation of DNA synthesis in control SMCs. IL-1 is a multifunctional cytokine that stimulates cells to secrete PDGF-AA, TGF-β1, and prostaglandins and appears to positively and negatively affect mitogenesis depending on cell types and conditions. IL-1β causes sustained NO production as a consequence of the induction of inducible NO synthetase in vascular SMCs, and IL-1–induced NO production inhibits the proliferation and migration of rat vascular SMCs. However, IL-1β–induced NO production was found to be almost the same in moyamoya and control SMCs. IL-1β may inhibit the migration of moyamoya SMCs through an NO-independent pathway. The reason for the discrepancy between our findings in control SMCs and those in rat vascular SMCs may be explained by the fact that rat SMCs treated with IL-1β can induce higher levels of NO than human arterial SMCs do. Apparently, the differences in cytokine induction of NO exist between species and cell types. Because the mitogenic activity of IL-1 appears to be indirect and mediated by the induction of PDGF-AA, the poor mitogenic response to PDGF-AA in moyamoya SMCs may also contribute to the reduced growth response to IL-1β. IL-1, produced mainly by activated macrophages and monocytes, functions in the generation of systemic and local response to infection, injury, and immunologic challenges. The distinct IL-1 response of moyamoya SMCs may indicate the involvement of a specific inflammatory cascade in moyamoya disease.

Recent evidence has shown that macrophages and T lymphocytes localize in the surface layer of the thickened intima in the intracranial arteries of moyamoya patients, suggesting a role of chronic inflammatory stimuli in SMC proliferation in the thickened intima. We recently reported that moyamoya disease is associated with an HLA-B51 phenotype and that moyamoya patients who have HLA-B51 may be susceptible to a certain form of vasculitis through leukocyte activation. The tissue inflammation or injury of arterial wall involves the activation of leukocytes and macrophages, releasing various cytokines and proinflammatory mediators such as histamine and thereby altering the endothelial barrier, which is normally recovered by the rapid repair process in which the migration and proliferation of SMCs play a key role. The inhibition of migration by IL-1β and the poor mitogenic response to PDGF in moyamoya SMCs might result in the continued increase in vascular permeability, facilitating the prolonged exposure of blood vessels to blood constituents. Lazarov et al recently showed that in an experimental balloon denudation model, the treatment of vascular endothelial growth factor exacerbates neointimal thickening, while that of b-FGF does not. Vascular endothelial growth factor specifically targets endothelial cells to proliferate and alters the vascular permeability. The b-FGF targets a wide variety of cell types, including the proliferation of SMCs and fibroblasts in addition to endothelial cells, but has no effect on vascular permeability. This suggests that the continued increase in vascular permeability is more important in neointimal accumulation than the exposure to excess individual growth factors. Furthermore, IL-1 reportedly stimulates cells to secrete several molecules such as vascular endothelial growth factor and prostaglandins that can alter the vascular permeability. Further investigations focusing on the downstream molecules of IL-1 are essential and may help in elucidating the direct causal relation in this peculiar disease.

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