Increase in Prostaglandin E_2 Production by Interleukin-1β in Arterial Smooth Muscle Cells Derived From Patients With Moyamoya Disease

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Abstract—Moyamoya disease is a progressive cerebrovascular occlusive disease that primarily affects children. The cause is unknown. We examined the production of prostanoids and the expression of cyclooxygenase-2 (COX-2) in cultured arterial smooth muscle cells (SMCs) derived from patients with moyamoya disease. Twelve moyamoya and 8 control cell strains were examined. The steady-state levels of prostanoids in the culture medium did not differ between moyamoya and control SMCs. When the cells were stimulated with interleukin-1β (IL-1β), prostaglandin E_2 (PGE_2) release into the medium was significantly greater from moyamoya SMCs than from control SMCs, whereas the amounts of prostacyclin and thromboxane B_2 did not differ. IL-1β–induced PGE_2 production by moyamoya SMCs was completely blocked by the addition of indomethacin or NS-398. IL-1β significantly stimulated cell migration and DNA synthesis in control SMCs but had an inhibitory effect on moyamoya SMCs. The inhibitory effects on the growth and migration of moyamoya SMCs were caused by excessive secretion of PGE_2 and was reversed with indomethacin treatment. Immunofluorescence studies and Western blot analysis showed greater amounts of COX-2 protein expression in IL-1β–stimulated moyamoya SMCs. These findings suggest that moyamoya SMCs respond to inflammatory stimuli to produce excess amounts of PGE_2 through the activation of COX-2, which increases vascular permeability and decreases vascular tone. This facilitates the exposure of vessels to blood constituents and promotes the development of intimal thickening in moyamoya disease. **(*Circ Res*. 1999;85:912-918.)**

Key Words: interleukin ■ moyamoya disease ■ muscle, smooth ■ prostaglandin

Moyamoya disease is an unusual form of chronic cerebrovascular occlusive disease that is characterized by progressive stenosis or occlusion at the distal ends of the bilateral internal carotid arteries.[1,2] The disease peaks or primarily occurs during the first decade of life, and the neurological symptoms depend on the specific arteries that are occluded.[3] The cause of the disease remains unknown. The findings that the incidence of the disease is highest in, but not confined to, the Japanese[4,5] and that the condition is frequently familial[6,7] suggest the involvement of a genetic factor in its pathogenesis. Previous reports and our findings suggest the involvement of systemic as well as intracranial arteries in moyamoya disease.[8–10]

The migration of medial smooth muscle cells (SMCs) and their proliferation in the intimal layer may occur in response to injury of the vascular wall.[11] Recent evidence[12] suggests a role for chronic inflammatory stimuli in SMC proliferation in the thickened intima of patients with moyamoya disease. The inflammatory response at the sites of injury and infiltration involves the activation of many cytokines in the vascular wall, including interleukin-1 (IL-1), interferon-γ, and tumor necrosis factor-α.[11,13,14] The prostanoids represent a diverse group of autocrine and paracrine hormones that are important mediators of many cellular functions[15–17] In the vasculature, prostacyclin (prostaglandin [PG]I_2) and thromboxane A_2 act in opposite directions in the maintenance of normal homeostasis and vascular tone. PGE_2 may increase vascular permeability and decrease vascular tone.[18] Nitric oxide (NO), which is known to be an endothelium-derived relaxing factor, regulates vascular tone and inhibits SMC migration.[19,20] When stimulated by proinflammatory cytokines such as IL-1, SMCs express cyclooxygenase-2 (COX-2) and produce PGE_2 and express inducible NO synthetase and release NO.[21,22] IL-1, which is produced mainly by induced macrophages and monocytes, functions in the generation of systemic and local responses to infection, injury, and immunologic challenges. The unregulated local production of PGs and NO may be responsible for various pathological processes in the vascular wall.[20,23–25] Based on the assumption that functional alterations in vascular wall cells are involved in the development of intimal thickening in moyamoya disease, we investigated cultured SMCs derived from patients with moyamoya dis-

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TABLE 1. Established Strains of Arterial SMCs Derived From Patients With Moyamoya Disease (HMSMC) and Control Patients (HCSMC)

		Onset of Disease		
Cell Strain	Donor, Age/Sex	Age	Type of Stroke	Donor Artery
HMSMC				
3	8/M	7	TIA	STA
5	11/F	4	TIA	STA
9	9/M	2	TIA	STA
16	8/F	4	TIA	STA
18	8/F	7	INF	STA
22	13/F	13	ICH	STA
23	2/M	2	TIA	STA
30	9/M	9	TIA	STA
32	7/M	2	TIA	STA
36	16/F	8	TIA	STA
38	9/F	9	TIA	STA
50	15/M	6	INF	STA
Mean (SD)	9.6 (3.8)*			
		Primary Disease		
HCSMC				
1	1/F		Head injury	STA
4	18/F		Head injury	STA
5	10/M		Craniosynostosis	STA
7	6/F		Cranial bone tumor	STA
14	1/M		Head injury	STA
16	17/M		Head injury	STA
17	2/F		Cranial bone tumor	STA
18	15/F		ICH	STA
Mean (SD)	8.8 (7.2)			

ICH indicates intracerebral hemorrhage; INF, infarction; TIA, transient ischemic attack; STA, superficial temporal artery.

*NS vs controls (HCSMC).

ease.[26,27] We recently found that moyamoya SMCs show distinct migratory and proliferative responses through an NO-independent pathway when stimulated by IL-1.[28] In the present study, we examined prostanoid production and COX-2 expression in IL-1β–stimulated SMCs derived from patients with moyamoya disease and compared the results with those in SMCs from age-matched control subjects. We show that the distinct responses of moyamoya SMCs to inflammatory stimuli may contribute to the pathological process in the vascular wall of patients with moyamoya disease.

Materials and Methods

Cell Culture

Arterial SMC strains from patients with moyamoya disease (HMSMC) and from control subjects (HCSMC) were established as described previously.[26] We used 12 SMC strains from patients with moyamoya disease and 8 strains from control subjects (Table 1).

The cells were cultured in 5 mL of Eagle's MEM (GIBCO) supplemented with 15% FBS (Biocell) at 37°C under humidified 5% CO_2/95% air and subcultured at a 1:2 split ratio. For the present study, we used cells at 8 to 14 passages.[29]

Determination of Prostanoid Production

Medium was replaced with fresh medium containing 0.5% FBS and 500 U/mL IL-1β (Otsuka Pharmaceutical) with or without 1 μg/mL indomethacin (Sigma Chemical Co), a nonselective COX inhibitor; 1 μmol/L NS-398 (BIOMOL Research Laboratories), a COX-2–selective inhibitor[30]; or 5 μmol/L arachidonic acid (AA; Sigma Chemical Co), and the cells were incubated for 48 hours at 37°C. PGE_2, PGI_2, and thromboxane $(TX)B_2$ secreted into the medium were measured with the use of enzyme immunoassay kits for PGE_2, 6-keto-$PGF_{1\alpha}$, and TXB_2 (Cayman Chemical), respectively.

Migration Assay

SMC migration was monitored in a microchemotaxis chamber (Neuro Probe) with the use of polycarbonate membranes with 8-μm pores, as described previously.[28] Cell suspension was placed in the upper compartment with or without indomethacin (1 μg/mL). The lower compartment contained MEM containing 2% FBS and test reagents (500 U/mL IL-1β and 0.4 to 200 nmol/L PGE_2; Paesel). The samples were incubated in a CO_2 incubator for 18 hours at 37°C.

Incorporation of 5-Bromo-2′-Deoxyuridine Into Cellular DNA

5-Bromo-2′-deoxyuridine (BrdU) incorporation was measured with an immunoperoxidase technique (Amersham Corp), as previously described.[31] Quiescent SMCs were incubated in MEM containing 0.5% FBS, test reagents, and a labeling regent (BrdU) for 48 hours. The test reagents were IL-1β (500 U/mL), indomethacin (1 μg/mL), and PGE_2 (0.4 to 200 nmol/L).

Immunocytochemistry

Quiescent SMCs were incubated with IL-1β (500 U/mL) for 9 hours and fixed in acetone/methanol (1:1) at 4°C for 30 minutes. The cells were preincubated with a 1:250 dilution of normal rabbit serum at 24°C for 30 minutes, with a 1:250 dilution of goat anti–COX-1 or anti–COX-2 antibody (Santa Cruz Biotechnology) at 24°C for 1 hour, and then with a 1:250 dilution of rhodamine-conjugated rabbit anti-goat IgG (ICN Biomedicals) at 24°C for 45 minutes.

Western Blot Analysis

Western blot analysis was performed with 10% acrylamide gels, as described previously.[32] After blocking, membranes were incubated with a 1:250 dilution of goat anti–COX-1 or anti–COX-2 antibody at 24°C for 1 hour and then with a 1:500 dilution of peroxidase-conjugated rabbit anti-goat IgG (ICN Biomedicals) at 24°C for 45 minutes.

Statistical Analysis

Values are given as mean±SD. Differences in data between groups were assessed with the use of an unpaired *t* test. A value of $P<0.05$ was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Prostanoid Production in Culture Medium of Arterial SMCs Derived From Patients With Moyamoya Disease

The steady-state levels of PGE_2, PGI_2, and TXB_2 production in HMSMC strains were low and not significantly different from those in HCSMC strains (Figure 1). Although IL-1β promoted both PGE_2 and PGI_2 production in the culture medium of HCSMC and HMSMC strains, the levels of IL-1β–induced PGE_2 production in HMSMC (44.8-fold) were significantly greater than those in HCSMC (10.2-fold)

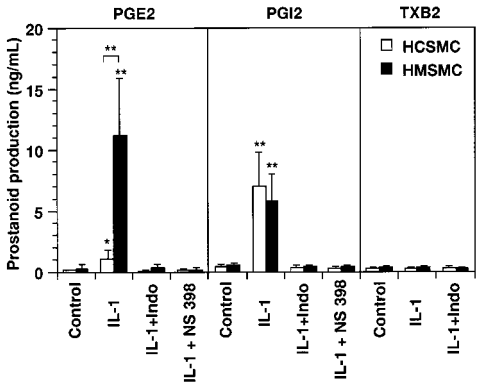

Figure 1. Release of prostanoids into medium of arterial SMCs derived from moyamoya patients (HMSMC) and control subjects (HCSMC). SMCs grown to confluence were washed with MEM containing 0.5% FBS. Medium was replaced with fresh MEM containing 0.5% FBS and IL-1β (500 U/mL) with or without indomethacin (Indo; 1 μg/mL) or NS-398 (1 μmol/L), and cells were incubated for 48 hours at 37°C. PGE_2, PGI_2, and TXB_2 secreted into medium were measured with enzyme immunoassay kits. Columns show mean and SD values for HCSMC (n=8) and HMSMC (n=12). *$P<0.01$ and **$P<0.001$ compared with cells without IL-1β (controls) and with cells from control subjects (HCSMC) by unpaired *t* test.

strains. In contrast, the levels of IL-1β–induced PGI_2 production did not differ significantly between HCSMC and HMSMC strains (Figure 1). IL-1β did not stimulate TXB_2 production by either the HCSMC or HMSMC strain. The addition of indomethacin (1 μg/mL) completely blocked IL-1β–induced prostanoid production by both HMSMC and HCSMC strains (Figure 1). The simultaneous addition of NS-398 (1 μmol/L) with the cytokine completely suppressed PGE_2 and PGI_2 production by both HMSMC and HCSMC strains (Figure 1).

We then compared the conversion of exogenous AA (5 μmol/L) by arterial SMCs from patients with moyamoya disease and from control subjects. As shown in Figure 2, PGE_2 production by HMSMC strains was clearly stimulated by the addition of AA, but the amount did not differ significantly from that produced by control strains (Figure 2). The AA-stimulated PGE_2 production in HMSMC strains was significantly increased by IL-1β compared with that in HCSMC strains.

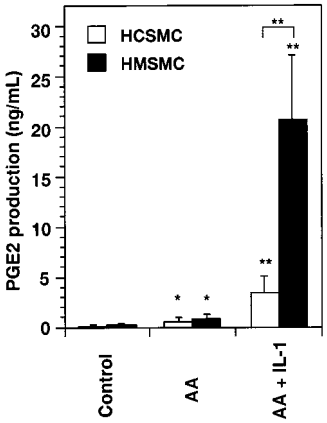

Figure 2. PGE_2 production by HMSMC and HCSMC. SMCs were incubated with fresh MEM containing 0.5% FBS, AA (5 μmol/L), and IL-1β (500 U/mL) for 48 hours at 37°C. PGE_2 secreted into medium was measured with enzyme immunoassay kit. Columns show mean and SD values for HCSMC (n=8) and HMSMC (n=12). *$P<0.01$ and **$P<0.001$ compared with cells without IL-1β (controls) and with cells from control subjects (HCSMC) by unpaired *t* test.

Cell Migration and DNA Synthesis

The number of migrating cells in serum-deficient medium without test mitogens (controls) did not differ between HCSMC (80.4±17.2) and HMSMC (81.1±11.1) strains. IL-1β stimulated cell migration in HCSMC strains, although it significantly inhibited migration in HMSMC strains (Table 2). IL-1β also stimulated BrdU incorporation into cellular DNA in control SMCs but had a rather inhibitory effect on DNA synthesis in moyamoya SMCs (Table 2). The basal labeling indices (controls) were 17.0±4.8% for HCSMC strains and 18.4±5.0% for HMSMC strains (P=NS). When prostanoid synthesis was inhibited with indomethacin, IL-1β significantly stimulated cell migration and BrdU incorporation in both HMSMC and HCSMC strains (Table 2). High concentrations (20 to 200 nmol/L) of exogenous PGE_2 induced the inhibition of IL-1β–stimulated cell migration and DNA synthesis in HCSMC and HMSMC strains, but lower concentrations (0.4 to 4 nmol/L) had no detectable inhibitory effects (Table 2). The higher PGE_2 concentrations correspond to those produced by IL-1β–stimulated HMSMC, and the lower concentrations correspond to those produced by IL-1β–stimulated HCSMC (Figure 1).

COX-2 Expression

We examined the expression of COX-1 and COX-2 proteins in cultured arterial SMCs through immunofluorescence study. No positive immunostaining was found in both moyamoya and control SMCs in the absence of the primary antibody or in the nonimmune goat IgG (Figure 3, A and B). COX-2 protein was hardly detected in moyamoya and control SMCs grown to confluence (Figure 3, C and D). IL-1β stimulated the expression of COX-2 protein in both moyamoya and control SMCs (Figure 3, E and F); however, the increase in the number of COX-2–positive cells was significantly ($P<0.001$) greater in moyamoya SMCs (16.5±2.6%) than in control SMCs (5.3±2.1%). In contrast, COX-1 immunoreactivity did not differ between moyamoya (6.5±3.2%) and control (4.8±2.3%) SMCs in the presence of IL-1β and between moyamoya (5.9±3.7%) and control (5.0±1.8%) SMCs in the absence of IL-1β.

Immunoblot analysis showed that the expression of COX-2 protein was hardly detected in homogenates of moyamoya and control SMCs in the absence of IL-1β (Figure 4). The immunoreactive band to COX-2 increased clearly in both moyamoya and control SMCs in the presence of IL-1β, although the expression of COX-1 protein was not stimulated by IL-1β (Figure 4). The relative density of the COX-2 protein in moyamoya cell strains was significantly ($P<0.001$) higher than that in control cell strains (Figure 4).

Discussion

Previous studies[3,33] have suggested that moyamoya disease is an acquired disorder in which both an immunologic vascular

TABLE 2. Effects of Exogenous PGE$_2$ on Migration of and BrdU Incorporation Into HMSMC and HCSMC Strains

	Migration		BrdU Incorporation	
	HCSMC	HMSMC	HCSMC	HMSMC
Control	1.00	1.00	1.00	1.00
IL-1β	1.42±0.21*	0.55±0.10†§	2.28±0.59*	0.86±0.30§
IL-1β+INDO	1.59±0.21†	1.26±0.19*‡	3.28±0.66†	2.00±0.36†‡
+PGE$_2$ (0.4 nmol/L)	1.54±0.14†	1.20±0.07*‡	3.33±0.41†	2.11±0.45†‡
+PGE$_2$ (4.0 nmol/L)	1.50±0.11†	1.20±0.10*‡	3.12±0.21†	1.98±0.55†‡
+PGE$_2$ (20 nmol/L)	0.56±0.10†	0.53±0.09†	0.56±0.11†	0.65±0.21†
+PGE$_2$ (200 nmol/L)	0.58±0.17†	0.54±0.11†	0.62±0.17†	0.50±0.11†

INDO indicates indomethacin.

Results are given as multiples of the number of migrating cells and multiples of the labeling indices of cultures without test reagents (controls).

*$P<0.01$, †$P<0.001$ compared with cells treated without test reagents (controls).

‡$P<0.01$, §$P<0.001$ compared with control cells (HCSMC).

reaction and subsequent inflammation play important roles. Recent evidence[12] indicates that inflammatory stimuli and the subsequent response of inflammatory cells may produce a proliferative response in SMCs in the thickened intima of patients with moyamoya disease. In the present study, we examined the production of prostanoids, which act as mediators that modulate vascular functions when stimulated by proinflammatory cytokines such as IL-1,[19,34,35] in arterial SMCs derived from patients with moyamoya disease. IL-1β stimulates PGE$_2$ and PGI$_2$ production in both moyamoya and control SMCs. When stimulated by IL-1β, the levels of PGI$_2$ production do not differ between moyamoya and control SMCs. However, the level of IL-1β–stimulated PGE$_2$ production by moyamoya SMCs is significantly greater than that by control SMCs. NO stimulated by IL-1 also acts as a mediator that modulates vascular functions,[19,34,35] but IL-1–induced NO production is found to be almost the same in HMSMC and HCSMC strains.[28]

IL-1 is a multipotent inflammatory mediator that may play a central role in vascular pathophysiology.[36,37] Previous reports indicate that IL-1 stimulates the migration and proliferation of aortic SMCs,[38,39] whereas others have shown it to

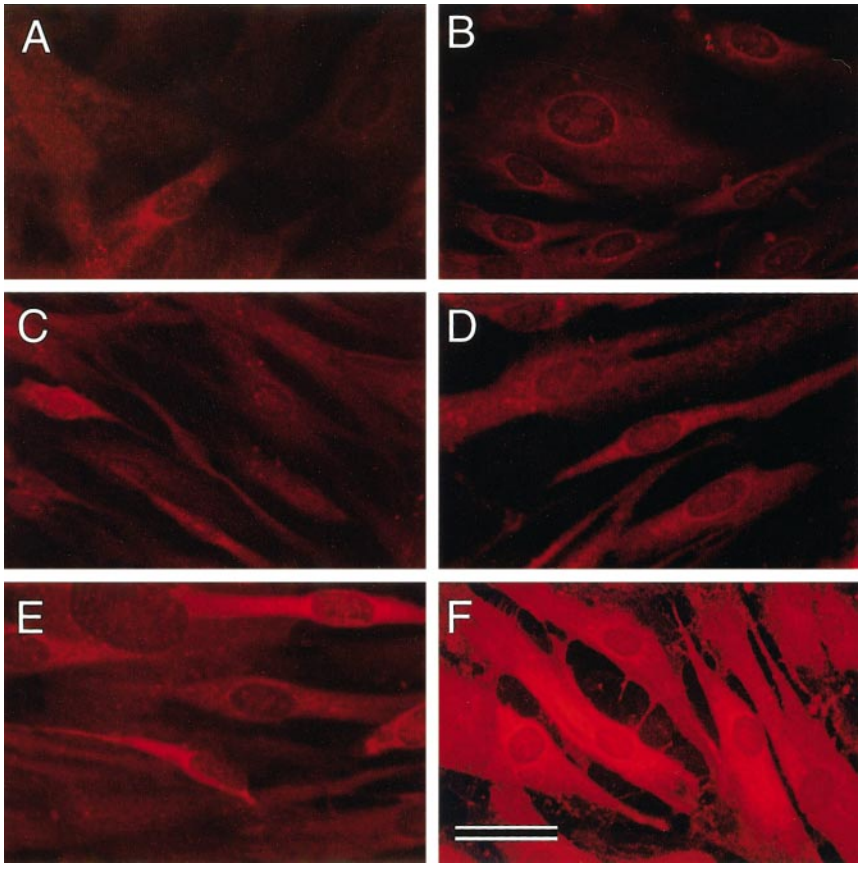

Figure 3. Immunostaining for COX-2 protein on moyamoya (B, D, and F) and control (A, C, and E) SMCs. Cells grown on Lab-Tek chamber glass slides were incubated with fresh medium with (E and F) or without (C and D) IL-1β (500 U/mL) for 9 hours. The cells fixed in acetone/methanol were incubated with (C through F) or without (A and B) an anti–COX-2 antibody and then with rhodamine-conjugated anti-goat IgG. Confocal micrograms were taken with a Bio-Rad MRC 1000 laser scanning confocal imaging system connected to a Zeiss Axiophot. Positive immunostaining for COX-2 protein is observed in cell cytoplasm. Bar, 50 μm.

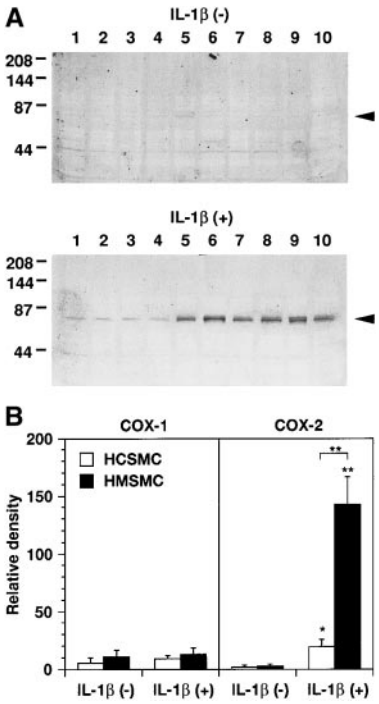

Figure 4. Western blot analysis of COX-1 and COX-2 proteins in moyamoya and control SMCs. Equal amounts of protein were loaded onto 12% slab gels and electroblotted to P membranes. Membranes were incubated with anti–COX-1 or anti–COX-2 antibody and developed with 4CN-plus. A, Expression of COX-2 protein by IL-1β. Lanes 1 through 4 indicate HCSMC-4, -7, -16, and -18 strains; and lanes 5 through 10, HMSMC-3, -18, -23, -30, -32, and -36 strains. B, Densitometric scanning was performed to compare relative protein levels. Experimental varieties were tested in three separate assays. Columns show the mean and SD values for HCSMC and HMSMC strains. *$P<0.002$ and **$P<0.0001$ compared with cells without IL-1β (controls) and cells from control subjects (HCSMC) by independent Student *t* test.

lack mitogenic effects on vascular SMCs.[34,40] IL-1 reportedly stimulates cells to produce platelet-derived growth factor-AA,[39] a positively affecting mitogen, and stimulates cells to produce PGE_2[34] and NO,[19,41] both of which have inhibitory effects on cell mitogenesis. As we reported recently,[28] IL-1β was found to significantly stimulate cell migration and DNA synthesis in control SMCs, whereas it inhibits cell migration and DNA synthesis in moyamoya SMCs. The inhibitory effect of IL-1β on cell migration and DNA synthesis in moyamoya SMCs corresponds to the elevated levels of PGE2, but not NO, production. Indomethacin treatment suppresses PG synthesis and results in stimulatory effects by IL-1β on cell migration and DNA synthesis in moyamoya SMCs. Furthermore, the higher concentrations of exogenous PGE_2, corresponding to the endogenous PGE_2 levels produced by IL-1β–stimulated moyamoya SMCs, in the presence of indomethacin inhibits IL-1β–stimulated migration and DNA synthesis in both moyamoya and control SMCs. Taken together, it is most likely that IL-1β fails to stimulate the migration and proliferation of moyamoya SMCs due to excessive autocrine PGE_2 production from moyamoya SMCs.

The synthesis of PGs that have diverse biological effects on the vasculature is regulated by two successive metabolic steps: the release of AA from membranous phospholipids and its conversion to PGs.[16,17] Two COX isoforms, COX-1 and COX-2, are the key enzymes that convert AA to PGs. COX-1 is constitutively expressed in most tissues. In healthy vessels, PGI_2, which is a protective, "antiatherogenic" mediator, is formed predominantly in the endothelial layer via the actions of constitutive COX-1.[42] In contrast, COX-2 is undetectable under physiological conditions but is markedly induced by several cytokines and growth factors in vascular cells.[43,44] COX-2 limits the proliferation of human vascular SMCs.[45] COX-2 is thought to be involved in the overproduction of prostanoids under pathological conditions such as acute and chronic inflammatory disorders and appears to be expressed only by specific stimulatory events.[17,46] Studies in animals show that vessels damaged by angioplasty or pinch express COX-2, an event that may account for an increased release of protective PGI_2.[47] Bishop-Bailey et al[48] show that IL-1 induces COX-2 in human vessels, with a pattern of prostanoid release of $PGE_2 > PGI_2 > TXB_2$. The formation of PGE_2 is mediated primarily by IL-1β–induced COX-2 in SMCs and macrophages.[21,22] In the present study, protective PGI_2 is released predominantly in IL-1β–stimulated control SMCs. The release of PGI_2 may represent an endogenous defense mechanism against endothelial damage.[48] In contrast, the expression of COX-2 protein and subsequent release of PGE_2 are significantly up-regulated in IL-1β–stimulated moyamoya SMCs compared with control SMCs. The conversion of exogenous AA into PGE_2 by moyamoya SMCs in the presence of IL-1β is significantly greater than that by control SMCs but does not differ between SMCs in the absence of IL-1β. IL-1β–induced prostanoid production by both HMSMC and HCSMC strains is completely blocked by the addition of NS-398 (1 μmol/L), a COX-2–selective inhibitor. Our findings strongly suggest that inflammatory stimuli and the subsequent inflammatory cell response stimulate the overproduction of PGE_2 through an IL-1–induced COX-2 pathway in SMCs in moyamoya disease.

PGs of the E series are thought to modulate vasodilation and vascular permeability.[18,49–51] The excessive amounts of PGE_2 released from moyamoya arterial SMCs through COX-2 activation by inflammatory stimuli may increase vascular permeability and decrease vascular tone, facilitating exposure of the vessels to blood constituents, including growth factors and cytokines that might induce and promote the development of intimal thickening in moyamoya disease. The excessive amounts of PGE_2 also inhibit the migration and proliferation of SMCs that might be necessary for the rapid repair of vascular wall injury, resulting in the continued increase in vascular permeability and facilitating the prolonged exposure of the vessels to blood constituents. The continued increase in vascular permeability may be much more important in neointimal accumulation than the exposure to excess individual growth factors.[52] In addition, PGE_2 and IL-1 are potent stimulators of angiogenesis,[53–55] and they induce the expression of vascular endothelial cell growth factor, which stimulates both angiogenesis and vascular permeability.[56,57] The induction of vascular endothelial cell growth factor by PGE_2 and IL-1 may be an important mechanism in inflammatory angiogenesis.[56] PGE_2 and IL-1

may directly or indirectly play an important role in angiogenesis in moyamoya disease.

The mechanism of the specific response of moyamoya SMCs to IL-1β is presently unknown. The distinct increase in PGE_2, but not NO, production in moyamoya SMCs may indicate altered intracellular signaling pathways by which IL-1 induces NO and PG synthesis. Recent findings demonstrate that COX-2–selective inhibitors have excellent anti-inflammatory properties.[17] Our findings suggest a possible interaction between the immune system and the vessel wall in moyamoya disease and serve as a basis for the clinical use of nonsteroidal anti-inflammatory drugs in the treatment of moyamoya disease.

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Increase in Prostaglandin E_2 Production by Interleukin-1β in Arterial Smooth Muscle Cells Derived From Patients With Moyamoya Disease

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Materials and Methods

Cell Culture

Arterial SMC strains from patients with moyamoya disease (HMSMC) and control subjects (HCSMC) were established as described previously.[26] Arterial specimens were obtained from branches of scrap arteries (superficial temporal arteries) requiring division during indirect bypass or other cranial surgical procedures. We used twelve SMC strains from moyamoya patients and eight from control subjects (Table 1). 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. No associated diseases were found in any of the 12 patients with moyamoya disease, The age of control subjects was 8.8 ± 7.2 years, which was not statistically different from that of the moyamoya patients. 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.

The cells were cultured in 60-mm Falcon dishes in 5 mL of Eagle's minimum essential medium (MEM, GIBCO) supplemented with 15% fetal bovine serum (FBS, Biocell, 62018304) at 37°C under humidified 5% CO_2-95% air. Confluent cultures were treated with 0.25% trypsin-0.02% EDTA for 10 minutes at 37°C and subcultured at a 1:2 split ratio. The number of cells was counted with a hemocytometer after trypsin treatment. For the present study, we used cells at 8-14 passages (within 50% of the final population doubling levels) that showed no signs of senescence in vitro.[29] The cells were carefully examined for mycoplasma contamination by the method described previously.[26]

Determination of Prostanoid Production

SMCs grown to confluence were washed with MEM containing 0.5% FBS. The medium was replaced with fresh medium containing 0.5% FBS and 500 units/mL (1.5 nmol/L) IL-18 (Otsuka Pharmaceutical Co) with or without 1 μg/mL indomethacin (Sigma Chemical Co), a non-selective COX inhibitor, or 1 μmol/L NS-398 (Biomol), a COX-2 selective inhibitor,[30] and the cells were incubated for 48 hours at 37°C. In some experiments, the cells were incubated in the medium with 5 μmol/L arachidonic acid (AA, Sigma). The medium was collected and filtered through a 0.22-μm filter. PGE_2, PGI2, and TXB_2

secreted into the medium were measured with enzyme immunoassay kits for PGE_2, 6-keto-prostaglandin Fla, and TXB2 (Cayman Chemical Co), respectively.

Migration Assay

SMC migration was monitored in a Micro Chemotaxis Assembly (Neuro Probe) using polycarbonate membranes with 8-μm pores, as described previously.[28] Briefly, SMCs grown to confluence were suspended in MEM containing 2% FBS, and 220 μL of cell suspension (1 x 10^5 cells/mL) was placed in the upper compartment of the chamber with or without indomethacin (1 μg/mL). The lower compartment contained 30 μL MEM containing 2% FBS and test reagents. The samples were incubated in a CO2 incubator for 18 hours at 37°C. Nonmigrating cells on the upper surface were scraped off gently, and the membranes were fixed in methanol for 30 minutes at room temperature and stained with Diff-Quick solution. SMCs migrating to the lower surface of the membranes were quantified in five or more randomly selected high fields (x400). The area was measured with an image analyzer (SPICCA-II, Olympus). The test reagents were IL-1β (500 units/ml) and PGE_2 (0.4-200 nmol/L, Paesel GmbH). The assays were performed in a blinded fashion.

Incorporation of 5-Bromo-2'-deoxyuridine (BrdU) into Cellular DNA

BrdU incorporation into cellular DNA was measured using an immunoperoxidase technique (cell proliferation kit; Amersham), as previously described.[31] Briefly, SMCs grown to confluence were arrested in MEM containing 0.5% FBS for 24 hours and then incubated in MEM containing 0.5% FBS, test reagents, and a labeling regent

(BrdU) for 48 hours. The percentage of labeled nuclei was calculated by counting more than 200 cells in each experiment. The test reagents were IL-18 (500 units/ml), indomethacin (1 μg/mL), and PGE_2 (0.4-200 nmol/L). The experiments were performed in a blinded fashion.

Immunocytochemistry

SMCs grown to confluence on Lab-tek chamber glass slides (Nunc) were arrested in MEM containing 0.5% FBS at 37°C for 24 hours and then incubated with IL-18 (500 units/mL) for 9 hours. The cells were fixed in acetone:methanol (1:1) at 4°C for 30 minutes and preincubated with a 1:250 dilution of normal rabbit serum at 24°C for 30 minutes. The cells were incubated with a 1:250 dilution of goat anti-COX-1 or anti-COX-2 antibody (Santa Cruz Biotechnology, Inc) at 24°C for 1 hour and then with a 1:250 dilution of rhodamine-conjugated rabbit anti-goat IgG (ICN Biomedicals, inc) at 24°C for 45 minutes. As negative controls, cells were processed with pre-immune goat serum or without primary antibody. Confocal micrographs were taken with a Bio-Rad MRC 1000 laser scanning confocal imaging system connected to a Zeiss Axiophot.

Western blot analysis

SMCs grown to confluence were arrested in MEM containing 0.5% FBS at 37°C for 24 hours and then incubated with IL-18 (500 units/mL) for 9 hours. Quiescent cells were solubilized in lysis buffer.[32] Equal amounts of protein were loaded on 10% acrylamide slab gels and developed at 25 mA for 2 hours. Samples were transferred from the slab gel to Clearblot-P membranes (ATT0 Co) at 1.5 mA/cm^2 for 2 hours. After blocking with 0.1% casein and 0.1% gelatin for 1 hour,

the membranes were incubated with a 1:250 dilution of goat anti-COX-1 or anti-COX-2 antibody at 24°C for 1 hour and then with a 1:500 dilution of peroxidase-conjugated rabbit anti-goat IgG (ICN Biomedicals, Inc) at 24°C for 45 minutes. Color was developed using 4CN plus (DuPont NEN Research Products). Densitometric scanning was performed to compare relative protein levels using NIH Images for the Macintosh.

Statistical analysis

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