Role of Src Homology 2–Containing Tyrosine Phosphatase 2 on Proliferation of Rat Smooth Muscle Cells

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Objective—Src homology 2–containing phosphotyrosine phosphatase 2 (SHP2) is ubiquitously expressed and believed to function as part of a positive signaling pathway mediating growth factor–induced protein tyrosine phosphorylation. Proliferation of aortic vascular smooth muscle cells (SMCs) is an important contributor to atherosclerosis. We examined the effect of SHP2 expression on SMC proliferative activity.

Methods and Results—SHP2 was abundant in cultured aortic SMCs, and SHP2 staining was markedly increased in the thickened aortic intima in rats with balloon-induced injury. We obtained several SMC clones by using geneticin screening. Endogenous SHP2 expression varied among individual clones. Significant positive relationships were observed between SHP2 expression and bromodeoxyuridine uptake in SMCs stimulated by FBS, platelet-derived growth factor, or insulin-like growth factor-1. In SMCs transiently transfected with SHP2, FBS stimulation significantly increased bromodeoxyuridine uptake beyond the uptake by control SMCs.

Conclusions—Increased SHP2 expression in SMCs may accelerate aortic atherosclerosis by increasing cell growth.

Key Words: src homology 2–containing phosphotyrosine phosphatase 2 ■ smooth muscle cells ■ aorta ■ balloon injury ■ intima ■ media ■ atherosclerosis
Balloon Injury in Rats
After male Wistar-Kyoto rats were anesthetized with sodium pentobarbital (30 mg/kg IP), the left common carotid artery was injured 3 times by inflation of a balloon catheter (2F Fogarty, Baxter) inserted via the external carotid artery. Rats were killed at 1, 2, or 4 weeks after balloon injury, and both common carotid arteries were removed and stored at ~80°C.

Immunohistochemical Study
Frozen sections prepared from carotid arteries were exposed to hydrogen peroxide for 5 minutes at room temperature to block endogenous peroxides. Tissues were incubated with anti-SHP2 antibody (Santa Cruz Biotechnology) with or without blocking peptide (Santa Cruz Biotechnology) for 20 minutes at room temperature and then stained by a streptavidin-biotin complex method by use of a kit from DAKO (LSAB2 kit/HRP). Bound antibodies were visualized by a color reaction. The images were digitized and semiquantified by using software for image analysis.

Plasmid Construction and Transfection of Human SHP2 in Cultured Rat SMCs
Before transient transfection, we examined endogenous SHP2 expression in rat SMCs. A eukaryotic expression vector, PQBI25, was used. It contained a cytomegalovirus promoter, a geneticin (G418) resistance gene, and a red-shift green fluorescent protein (GFP, Takara). Six wells of cultured SMCs were transfected by using a cationic liposome method (TransIT transfection reagent, Mirus). After transfection of the G418 resistance gene, cells were maintained for 2 weeks in medium containing 1 g/L G418. Several clones of SMCs were obtained and were used for Western blotting and transient transfection. Full-length SHP2 cDNA was obtained from RNA of cultured human mesangial cells (purchased from Iwaki Co) by using a reverse transcription–polymerase chain reaction (PCR) method with an oligo(dT) primer. PCR product was digested with the 

\[ \text{Nhe I} \]

 restriction enzyme and inserted into the 

\[ \text{Nhe I} \]

 site of the PQBI25 vector and sequenced. Transient transfection was performed by using the same methods.

Protein Extraction From Rat Aortic SMCs
Rat aortic SMC samples were homogenized in 50 mmol/L HEPES at pH 7.6 containing 1000 U/mL aprotinin, 0.002% phenylmethylsulfonyl fluoride, 20 mg/mL leupeptin, 0.1 mmol/L benzamidine, 1 mmol/L dithiothreitol, 2 mmol/L EDTA, 4 mmol/L orthovanadate, and 1% Triton X-100 at 4°C.

Western Analysis
Western blot analysis was performed according to a method reported previously with some modifications. Equal amount (~10 μg) of each sample were fractionated by electrophoresis in 10% acrylamide resolving gel containing 10% (vol/vol) glycerol and transferred to a nitrocellulose filter. The filter was incubated overnight at 4°C with 0.8 μg/mL of anti-SHP2 antibody (Transduction Laboratories) and then washed. Next, the filter was incubated with 1 μg/mL secondary antibody (anti-mouse IgG-labeled with peroxidase, Amersham International) for 45 minutes at room temperature, washed, and visualized by using an ECL kit (Amersham International).

Cell Proliferation
Bromodeoxyuridine (BrdU) uptake by SMCs was measured by using a kit obtained from Amersham International. SMCs were cultured in a 96-well microplate at 37°C for 24 hours. Then the cells were stimulated for 24 hours by FBS or other growth factors (PDGF, Boehringer-Mannheim Biochemicals; insulin-like growth factor [IGF]-1, Fujisawa) for 24 hours. BrdU was added to incubation media for the last 6 hours. After fixation and blocking, cells were incubated with peroxidase-labeled anti-BrdU for 90 minutes. After the addition of substrate and incubation for 10 minutes, optical density was measured by using an ELISA reader (model 550, Bio-Rad).

Statistical Analysis
Values are expressed as mean±SEM. Sets of data were compared by use of the 2-tailed unpaired Student t test.

Results
Northern Blotting
To better understand the physiological role of PTPases in SMCs, expressions of LAR, LRP, and SHP2 were examined in rat aortic cultured SMCs (Figure 1). The mRNAs encoding rat LRP, LAR, and SHP2 were expressed in SMCs as 3.0-kb, 8.0-kb, and 7.0-kb transcripts, respectively. Transcripts of all 3 phosphatases were abundant in SMCs.

Immunohistochemistry
Left carotid arteries prepared from rats after balloon injury (Figure 2A) showed thickening of the intima compared with the uninjured right carotid arteries (Figure 2B). Expression of SHP2 was observed in the medial layer of both arteries and in the thickening intima of the balloon-injured side. In Figure 2C, the expression of SHP2 was significantly increased in the medial layer of the balloon-injured side compared with the intact side (P<0.05, n=5). Moreover, the expression of SHP2 was significantly increased in the thickening intima of the balloon-injured side compared with the medial layer of the balloon-injured side (P<0.05, n=5).

The staining was abolished by absorbing the antibody with SHP2 amino peptides (data not shown).
Correlation Between SHP2 Expression and BrdU Uptake in SMCs Stimulated With Growth Factors

After cloned SMCs were selected with 1 g/L geneticin, transfected cells were detected by fluorescence of the GFP present in a fusion protein also including the geneticin resistance gene product. In Western blots, SMC clones varied in endogenous SHP2 expression (Figure 3A). BrdU uptake (Figure 3B) was strongly correlated with the amount of SHP2 expression on stimulation with FBS (Figure 4A; \(r=0.818, P<0.05\)). The same was true for stimulation with other growth factors, especially PDGF (Figure 4B; \(r=0.804, P<0.05\)) and IGF-1 (Figure 4C; \(r=0.850, P<0.05\)).

Figure 2. Immunohistochemical analysis of SHP2 in rat arteries after endothelial denudation. A balloon-injured common carotid artery is shown in panel A, and a contralateral control artery is shown in panel B. In both arteries, staining for SHP2 is seen in the tunica media (M). On the balloon-injured side, SHP2 staining was increased in the thickened intima (TI in panel A). This staining was inhibited by absorption of antibody with SHP2 peptides (not illustrated). The images were digitized and semiquantified by using software for image analysis. Semiquantification of staining is shown in panel C. Data are expressed as mean±SEM.

Figure 3. Expression of SHP2 in cloned SMCs. Individual SMC clones show various intensities of expression of native SHP2 (A). BrdU uptake in these SMCs on FBS stimulation is also shown (B). Data are expressed as mean±SEM.
Effects of Transient SHP2 Transfection on BrdU Uptake by SMCs

Immunohistochemical assessment of transient transfection efficacy indicated that ∼10% of cells were stained with anti-GFP antibody. FBS stimulation increased BrdU uptake in SMCs. This effect was augmented in SHP2-transfected SMCs compared with control cells (P<0.05, n=5; Figure 5).

Discussion

PTPases dephosphorylate growth factor receptors and are considered among the most important regulators of signal transduction in tyrosine kinase–type receptors, including the insulin receptor and receptors for various growth factors. PTPases have been shown to regulate the action, maturation, and phosphorylation of substrates of growth factor receptors. PTPases include 2 categories. One consists of LAR and LRP, both of which have a receptor-like transmembrane structure and tandem conserved PTPase domains. The other type, cytosolic-type SHP2 (also known as SHPTP2, PTP1D, PTP2C, and Syp), contains 2 SH2 domains and 1 SH3 sequence. SHP2 binds to tyrosine kinases via its SH2 domains and is activated by phosphorylation of its

Figure 4. Positive correlation between SHP2 expression and BrdU uptake in SMCs stimulated by various growth factors (A, FBS; B, PDGF; and C, IGF-1)

Figure 5. Effect of SHP2 overexpression on BrdU uptake in SMCs. Bars indicate multiples of basal BrdU uptake on FBS stimulation. Data are expressed as mean±SEM.
tyrosine residue. SHP2 also binds to IRS-1, as do other adapter proteins. SHP2 has been thought to positively influence intracellular signal transduction. We have reported previously that SHP2 is expressed prominently in hepatocellular carcinoma induced by 3′-methyl-4-dimethylamino-azobenzene, suggesting that SHP2 may be involved in some situations in which cell proliferation is increased.

Abnormal vascular SMC proliferation and migration participate in the formation of atherosclerotic plaques, in the development of restenosis after percutaneous transluminal angioplasty, and in accelerated arteriopathy after cardiac transplantation. Our Northern analysis disclosed intense expression of mRNA encoding 3 PTPases (LAR, LRP, and SHP2) in rat aortic SMCs; additionally, the expression of SHP2 mRNA in vascular SMCs was demonstrated by use of in situ hybridization. It was reported that SHP2 has a positive role in the angiotensin II pathway, one of the important pathways involved in the proliferation of SMCs. These data indicate that these PTPases may play an important role in the proliferation of SMCs, as has also been demonstrated in liver, muscle, and adipose tissue.

Intimal SMCs are associated with vascular disease and are phenotypically distinct from medial SMCs. The intimal SMC is believed to express a large number of proteins that contribute to cell proliferation and lesion development or stability. It has been reported that some PTPases (SHP1, CD45, PTPβ, PTPα, and PTP1B) are expressed in the balloon-injured vessel wall. In the present study, balloon injury to provoke intimal hyperplasia as a response to endothelial denudation resulted in particularly high arterial SHP2 mRNA expression. The migration of medial SMCs from the media to the intima and the proliferation of intimal cells to form a thickened neointima are sequential events that characterize tissue remodeling in the arterial wall after balloon-induced denudation. Our finding that SHP2 expression was more elevated in thickened intima than in the media suggests that SHP2 expression increases after the SM phenotype changes.

We used cultured rat aortic SMCs to further investigate the role of SHP2 in SMCs. We sought to clone SMCs by using mammalian expression vector containing geneticin resistance and GFP genes, inasmuch as the endogenous expression of SHP2 is likely to vary among SMCs. We obtained several clones of SMCs and examined the expression levels of SHP2 in each cell line. According to our results (Figure 3A and 3B), cultured SMCs have various ranges of SHP2 expression among cell lines. Cultured SMCs are heterogeneous, and we thought that this heterogeneity might affect total cell proliferation. To exclude this influence, we used monoclonal SMCs for our studies. When we examined cell growth by using BrdU uptake, the uptake was positively correlated with SHP2 expression, suggesting that increased SHP2 expression acts to increase cell growth. To confirm this impression, we studied BrdU uptake in cloned SMCs transiently overexpressing SHP2 compared with SMCs transfected with vector only. SHP2-transfected SMCs showed greater BrdU uptake when stimulated by FBS than did vector-transfected SMCs. Various stimuli, particularly growth factors, are known to influence SMC proliferation and migration. In particular, PDGF, IGF-1, and fibroblast growth factor are known to activate the proliferation of SMCs, and the expression of receptors for these ligands is also important in the formation of atherosclerotic lesions. SHP2 binds to IRS-1, IRS-2, and PDGFR receptors and is thought to modulate their signaling. Differences of SHP2 expression in SMCs may conceivably lead to differences in human susceptibility to atherosclerosis.

In conclusion, we have demonstrated that SHP2 is expressed abundantly in SMCs. Increased expression may lead to acceleration of atherosclerosis in the aorta by increasing SMC proliferation. Further studies involving human subjects should provide new clues concerning the pathogenesis of atherosclerosis.

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