Interleukin-1β Inhibits Expression of p21(WAF1/CIP1) and p27(KIP1) and Enhances Proliferation in Response to Platelet-Derived Growth Factor-BB in Smooth Muscle Cells

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Objective—Intimal growth depends on smooth muscle cell (SMC) migration and proliferation and is regulated by thrombotic and inflammatory responses to vascular injury. Platelet-derived growth factor (PDGF)-BB and interleukin (IL)-1β have been shown to contribute to intimal hyperplasia and lesion progression in atherosclerosis. Mitogenic effects of IL-1 on SMCs have been reported and have been attributed to the expression of PDGF-A chain. In some, but not all, studies, IL-1β was found to cooperate with growth factors, including PDGF, in stimulating proliferation. The molecular basis for such cooperative effects is unknown and is the subject of the present study.

Methods and Results—We demonstrate that in baboon aortic SMCs, IL-1β enhances the proliferation induced by PDGF-BB independently of PDGF-A signaling. IL-1β increases the phosphorylation of retinoblastoma protein, a pivotal step in the G1-to-S transition in the cell cycle. Analysis of expression levels of cyclins and cyclin-dependent kinase (CDK) inhibitors suggests that IL-1β stimulates CDKs by downregulating p21 and p27. Consistent with this hypothesis is the finding that CDK2 activity, induced by PDGF-BB, is enhanced 2.3±0.2-fold in the presence of IL-1β.

Conclusions—Our data suggest that IL-1β may promote SMC proliferation after vascular injury and in atherogenesis by suppression of PDGF-BB—induced p21 and p27. (Arterioscler Thromb Vasc Biol. 2002;22:1293-1298.)

Key Words: smooth muscle ▪ platelet-derived growth factor ▪ interleukin-1 ▪ p21(WAF1/CIP1) ▪ p27(KIP1)

In response to vascular injury, medial smooth muscle cells (SMCs) proliferate and migrate into the intima, where they continue to proliferate and produce matrix. In severe circumstances, the resulting neointima narrows the lumen and restricts blood flow. The process of neointimal formation is regulated by the thrombotic and inflammatory response to injury (see reviews1–3). Many growth factors and chemokines may contribute to the activation of SMCs during lesion development, and their nature likely depends on the type of injury as well as the animal model investigated.

It is of interest, with respect to disease in humans, that in baboons, antibodies against the platelet-derived growth factor (PDGF) receptor-β (PDGFRβ) inhibit intimal hyperplasia after arterial balloon injury.4,5 In contrast, blockade of PDGF receptor-α (PDGFRα) has no effect on intimal hyperplasia in that model.5 The PDGF family consists of 4 peptides, PDGF-A, PDGF-B, and the recently described PDGF-C6,7 and PDGF-D chains.8,9 PDGF binds as a homodimer or heterodimer, and the PDGF receptor ligands identified so far are PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and PDGF-AB dimers. PDGF-A, PDGF-B, and PDGF-C bind to PDGFRα, and PDGF-B and PDGF-D bind to PDGFRβ. The principal function of PDGFRβ stimulation in vivo appears to be migration,4,5,10–12 although there is evidence that it also mediates proliferation.13,14

A role for interleukin (IL)-1 in neointimal hyperplasia has been proposed on the basis of the presence of macrophages and cytokines in vascular lesions (see reviews3–5) as well as observations made in animal models, including mice15 and pigs.16 IL-1 might influence vascular lesion development by several different mechanisms, including activation of lymphocytes, induction of monocyte chemoattractant protein-1 expression, upregulation of adhesion proteins in endothelial cells, and activation of SMCs (see reviews17–18).

IL-1 exists in 2 isoforms, IL-1α and IL-1β, and in most studies, their biological effects are identical. [IL-1β] is synthesized as a precursor and is cleaved by IL-1—converting enzyme, also called caspase-1, to yield the mature, biologically active protein (see review20). There are 2 IL-1 receptors (IL-1Rs): type-1 IL-1R mediates the biological responses of IL-1, whereas the type-2 IL-1R is a decoy without signaling capabilities (see review19).

Although IL-1 has been shown to promote the progression of vascular lesions in various animal models,15,16 its effects on cultured SMCs are not clear. IL-1 can be mitogenic, but this activity may be masked by the concomitant production of inhibitory prostaglandins.21 Growth-promoting effects of IL-1 are thought to be mediated, at least in part, by expression of the PDGF-A chain.22,23 In the presence of serum, IL-1 may
actually inhibit proliferation by inducing the expression of cyclooxygenase-2 and inducible NO synthase.\textsuperscript{24,25} In combination with PDGF, inhibiting\textsuperscript{24} as well as activating\textsuperscript{26,27} effects of IL-1 have been reported without further investigation of the underlying molecular mechanisms.

In the present study, we report that in baboon SMCs, IL-1\(\beta\) is cooperative with PDGF-BB in inducing DNA synthesis and proliferation. Our data suggest that IL-1\(\beta\) increases cell cycle progression through G\(_1\)/S by inhibiting expression of the cell cycle–dependent kinase (CDK) inhibitors p21(WAF1/CIP1) and p27(KIP1).

### Methods

#### Materials

IL-1\(\beta\) and IL-1R antagonist were obtained from R&D Systems, and PDGF-BB was kindly provided by Zymogenetics (Seattle, Wash). Protein A agarose and histone H1 were from Roche. Phospho-specific antibodies against extracellular signal–regulated kinase (ERK)1,2, protein kinase B (PKB), and retinoblastoma protein (ser-780) were from Cell Signaling Technology. Anti-phosphotyrosine (clone 4G10) was from Upstate Biotechnology. Blocking murine/human chimeric antibodies to PDGFR\(\alpha\) were from Celltech.\textsuperscript{28} All other antibodies were purchased from Santa Cruz Biotechnology.

#### Cell Culture

Baboon aortic SMCs were prepared by using the explant method as described\textsuperscript{29} and grown in DMEM high glucose supplemented with 10% FBS, 200 U/mL penicillin, and 0.2 mg/mL streptomycin. SMCs between 5 and 16 passages were used for experiments. To achieve quiescence, cells were incubated for 3 days in serum-free MCDB131 supplemented with antibiotics and 2 mM/mL glutamine.

#### DNA Synthesis

Quiescent SMCs at 50% to 80% confluence were treated as indicated in figure legends and incubated in the presence of \textsuperscript{3}H]thymidine (1 \(\mu\)Ci/mL) for 27 to 32 hours. Cells were washed three times in ice-cold PBS before precipitation in 10% trichloroacetic acid overnight at 4\(^\circ\)C. Cells were washed once with 10% trichloroacetic acid, and DNA was solubilized in 0.1N NaOH (0.4 mL per well) by incubating plates for 1 hour at room temperature with constant agitation. Radioactivity was analyzed by scintillation counting. Assays were performed in triplicate.

#### Western Blotting

Quiescent SMCs were stimulated with 10 ng/mL PDGF-BB with or without 0.1 ng/mL IL-1\(\beta\). At the time points indicated, cells were washed twice in PBS and lysed in Laemmli buffer containing 2% SDS. Lysates were boiled and subjected to SDS-PAGE (10 to 30\(\%\)) and protein gel. Samples were subjected to SDS-PAGE. Gels were dried, and bands were visualized (2\(\%\)) and protein gel. Samples were subjected to SDS-PAGE. Gels were dried, and bands were visualized using an enhanced chemiluminescence detection system (Amersham/Pharmacia).

#### CDK2 Assay

After stimulation as indicated, SMCs were washed twice with PBS and harvested in HEB (25 mMOL/L HEPES-NaOH, pH 7.5, 10% glycerol, 5 mMOL/L EDTA, 5 mMOL/L EGTA, 150 mMOL/L NaCl, 50 mMOL/L NaF, 50 mMOL/L pyrophosphate, 1 mMOL/L sodium vanadate, 1 mMOL/L benzamidine, 0.1% 2-mercaptoethanol, 1% Triton X-100, 1 mMOL/L pepstatin A, 2 \(\mu\)g/mL leupeptin, and 20 kallikrein inhibitor units/mL aprotinin). Lysates were cleared by centrifugation for 10 minutes at 14,000 rpm and CDK2-immunoprecipitated (2 \(\mu\)g/mL anti-CDK2). Beads were washed twice in HEB, followed by 2 washes in kinase buffer (20 mMOL/L HEPES-NaOH, pH 7.5, 20 mMOL/L MgCl\(_2\), and 0.1% 2-mercaptoethanol). The kinase reaction occurred in 30 \(\mu\)L on beads in kinase buffer supplemented with 1 \(\mu\)L histone H1 per assay and 0.1 mMOL/L [*P]ATP (7000 cpm/pmol) for 30 minutes at 30\(^\circ\)C. The reaction was terminated by the addition of 10 \(\mu\)L of 4\(\times\) Laemmli buffer. Samples were subjected to SDS-PAGE. Gels were dried, and radioactivity was quantified by phosphorimage analysis (Storm System, Molecular Dynamics).

### Quantification and Statistics

Digitized images obtained by phosphorimaging or by scanning blots were quantified with the use of Image Quant software (Amersham Biosciences, Inc). All experiments were performed at least 3 times. Probability values were calculated by using a 1-tailed paired \(t\) test. Differences were considered significant at \(P<0.05\).

### Results

#### IL-1\(\beta\) Potentiates PDGF-BB–Stimulated Proliferation

To investigate the effect of IL-1\(\beta\) on proliferation in response to PDGF-BB, we measured thymidine incorporation in SMCs after the addition of 10 ng/mL PDGF-BB and various doses of IL-1\(\beta\). The cytokine enhances PDGF-BB–induced DNA synthesis in a wide range of concentrations (from 1 pg/mL to 1 ng/mL), with a maximal stimulation observed at 0.1 ng/mL (Figure 1A). IL-1\(\beta\) alone does not stimulate DNA synthesis (authors’ unpublished data, 2001). The stimulatory effect of IL-1\(\beta\) on PDGF-BB–induced DNA synthesis increases with increasing PDGF-BB concentrations (Figure 1B). Furthermore, we demonstrate that IL-1\(\beta\) increases SMC proliferation induced by 10 ng/mL PDGF-BB.

After 4 days, PDGF-BB alone increased cell number 180\(\pm\)50% compared with 310\(\pm\)70% with IL-1\(\beta\) present (Figure 2). As expected from the failure of IL-1\(\beta\) to stimulate DNA synthesis, the cytokine alone does not induce SMC proliferation.

#### Stimulatory Effect of IL-1\(\beta\) Does Involve IL-1R but Not Occupation of PDGFR\(\alpha\)

To confirm that the effect of IL-1\(\beta\) is mediated by its binding to type-1 IL-1R, we investigated the effect of the IL-1R antagonist (ILRA). ILRA has a slightly activating effect on PDGFR\(\alpha\)–induced DNA synthesis and almost completely abolishes the cooperative effect of IL-1\(\beta\) (Figure 3). In PDGF-AB–stimulated cells, they inhibit 60% of DNA synthesis (Figure 3).

#### IL-1\(\beta\) Does Not Affect Early Signaling of PDGF-BB

To elucidate the molecular mechanism by which IL-1\(\beta\) cooperates with PDGF-BB, we investigated whether IL-1\(\beta\)
IL-1β Enhances PDGF-BB–Induced Proliferation

Figure 1. IL-1β potentiates DNA synthesis induced by PDGF-BB. SMCs were incubated with PDGF-BB and IL-1β in various concentrations as indicated. DNA synthesis was determined by incorporation of [3H]thymidine. A. Open circles indicate that PDGF-BB was present at 10 ng/mL. Data (mean±SD of 3 independent experiments) are presented as percentage of controls, with 100% as the highest activity measured in the absence of IL-1β. *P<0.016. B. Solid circles indicate that IL-1β was present at 0.1 ng/mL; open circles indicate that IL-1β was absent. Data (mean±SD of 3 independent experiments) are presented as the percentage of controls, with 100% as the highest activity measured in the absence of IL-1β. *P<0.037.

IL-1β increases SMC proliferation induced by PDGF-BB. Quiescent SMCs were incubated with 10 ng/mL PDGF-BB and 0.1 ng/mL IL-1β as indicated. IL-1β was added again after 2 days. After 1, 2, 3, and 4 days, cells were released with trypsin and counted by use of a hemocytometer. Data (mean±SD of 3 independent experiments) are presented as percentages, with 100% being the number of cells plated at day 0. *P<0.022.

Figure 2. IL-1β increases SMC proliferation induced by PDGF-BB. Quiescent SMCs were incubated with or without 250 nmol/L IL-1 receptor antagonist (ILRA) and 0.05 mg/mL PDGFRα-blocking antibody (anti-P2) for 30 minutes before stimulation with 10 ng/mL PDGF-BB (B) in the absence or presence of 0.1 ng/mL IL-1β (I). A control included PDGF-AB (AB) with or without PDGFRα antibody. DNA synthesis was determined by incorporation of [3H]thymidine. Data (mean±SD of 3 independent experiments) are presented as percent change of activity in the presence of the blocking agent according to the following formula:

change (%) = \left( \frac{\text{activity with blocking agent}}{\text{activity without blocking agent}} \right) \times 100 - 100

Where IL-1β was present, activity was calculated as difference between activity in the presence of PDGF-BB and IL-1β minus activity in the presence of PDGF-BB alone. *P<0.011 for differences with or without blocking agent; n.s. indicates not significant.

Effects of IL-1β on pRb Phosphorylation and Expression of Cyclins and CDK Inhibitors in Response to PDGF-BB

Because we did not identify a PDGF signaling pathway affected by IL-1β, we investigated the effect of the cytokine on retinoblastoma protein (pRb) phosphorylation, which controls the G1-to-S transition in the cell cycle. In experiments in which we harvested SMCs 2, 4, 8, 16, 24, and 32 hours after PDGF-BB stimulation, pRb phosphorylation was first detectable at 8 hours and reached the maximum between 16 and 24 hours (Figure 5). In the presence of IL-1β, pRb phosphorylation was greatly enhanced at 16, 24, and 32 hours. To investigate the underlying molecular mechanism, we determined the expression levels of cyclins and CDK inhibitors. Surprisingly, we observed reduced levels of cyclin-D1 in the presence of IL-1β, whereas cyclin-E levels remained unchanged (Figure 5). Cyclin-A expression, which depends on E2F activity and thereby on pRb phosphorylation, is apparent at 24 and 32 hours (Figure 5). As expected, given the activating effect of IL-1β on pRb phosphorylation, cyclin-A was also increased by the cytokine. Expression of the CDK inhibitors p21 and p27 was negatively affected by IL-1β. After PDGF-BB stimulation, there were 2 phases of p21 and p27 expression: a slight increase up to 8 hours, which was followed by a strong increase within 8 to 32 hours. This latter phase was markedly suppressed in the presence of IL-1β. p27 levels were prominent in quiescent cells and decreased after stimulation before returning to control levels by 24 hours. In the presence of IL-1β, p27 levels remained low.
Given that IL-1β is inhibitory for cyclin-D1 expression and does not stimulate early pRb phosphorylation (8 hours), we tested the possibility that the IL-1β–mediated downregulation of p21 and p27 increases CDK2 activity. Compared with PDGF-BB alone, costimulation with IL-1β further enhanced CDK2 activity from 100% to 226% (Figure 6). IL-1β alone had no effect. Western blot analysis of cell extracts demonstrated that IL-1β does affect the expression levels of CDK2 (Figure 6).

**Discussion**

Given that PDGF and IL-1 play a role in neointimal growth, we investigated whether IL-1β cooperates with PDGF-BB in the stimulation of SMC proliferation. The cytokine enhances DNA synthesis at a wide range of concentrations, spanning 3 orders of magnitude, with a maximal 3- to 4-fold stimulation at 0.1 ng/mL (Figure 1A). IL-1β is effective at saturating concentrations of PDGF-BB (Figure 1B). This rules out the possibility that IL-1β complements suboptimal levels of PDGF-BB and indicates a synergistic mechanism. Importantly, IL-1β also potentiates the proliferation induced by PDGF-BB (Figure 2). The effect of IL-1β is mediated by binding to type-1 IL-1R, as demonstrated by the inhibitory effect of ILRA. We do not know why ILRA has a slight, but statistically significant, activating effect on PDGF-BB–induced DNA synthesis (Figure 3). Growth-promoting effects of IL-1 on SMCs have recently been ascribed to expression of the PDGF-A chain. Our finding that blockade of PDGFRα, the sole PDGF receptor for PDGF-A, has no effect on the cooperation of IL-1β with PDGF-BB (Figure 3) indicates a different mechanism.

To elucidate the molecular mechanisms underlying the stimulatory effect of IL-1β on growth, we investigated early signaling events of PDGF-BB. Phosphorylation of PDGFRβ, ERK1,2, or PKB by PDGF-BB was not affected by IL-1β (Figure 4). As has been seen in other types of cells, IL-1β transiently activates ERK1,2. Compared with PDGF-BB, however, IL-1β is a weak activator of ERK1,2, and it is unlikely that this mechanism accounts for the 3- to 4-fold activation of PDGF-BB–induced DNA synthesis by the cytokine.

Given the stimulatory effect of IL-1β on proliferation and the lack of effect on early PDGF-BB signaling events, we investigated the phosphorylation of pRb, which is a pivotal...
positive effect of IL-1β on PDGF-BB–stimulated proliferation is consistent with the downregulation of p21 and p27 at times when pRb phosphorylation is enhanced. p21 levels were low in quiescent cells and increased after PDGF-BB stimulation as early as after 2 hours. IL-1β inhibits p21 expression only after 8 hours, which may indicate that early and late expression of p21 by PDGF-BB are controlled by different pathways. Moreover, it has recently been suggested that in PDGF-stimulated SMCs, p21 functions as an assembly factor for cyclin-D1/CDK4 but not cyclin-E/CDK2. Thus, we speculate that early expression of p21 may promote cell cycle progression, whereas late expression is inhibitory. In contrast to SMCs, IL-1β induces p21 in other cell types, clearly indicating that the effect of the cytokine depends on the cellular context. In SMCs, p27 is expressed in quiescent cells, and after PDGF-BB stimulation, expression decreases during G1 and returns to control levels in late S phase. In the presence of IL-1β, p27 levels remain low. Together, these findings suggest to us that PDGF-BB–induced CDK2 activity is enhanced by IL-1β. By measuring CDK2 activities after immunoprecipitation of the kinase, we found that CDK2 was 2.3±0.2-fold more active in cells that have been costimulated with IL-1β and PDGF-BB than in control cells that have been treated with PDGF-BB alone (Figure 6).

In summary, we demonstrate a novel mechanism by which IL-1β may promote proliferation of SMCs in the presence of PDGF-BB. Our data suggest that IL-1β counteracts PDGF-BB–induced expression of the CDK inhibitors p21 and p27, thereby further activating CDK2 and promoting cell cycle progression. This mechanism may contribute to SMC proliferation in vascular lesions, where thrombotic and inflammatory stimuli are concurrently generated.

Acknowledgments

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References


6. Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, Eriksson U. PDG-F–induced proliferation and cyclin-A expression in response to PDGF-BB (Figure 5). Both of these observations are consistent with increased transcriptional activity of E2F, inasmuch as cyclin-A is an E2F-dependent gene. pRb is phosphorylated by 2 CDK complexes, first by cyclin-D–associated CDK4 or CDK6 and thereafter by the cyclin-E/CDK2 complex. These kinases are positively regulated by cyclin subunit and are negatively regulated by CDK inhibitors p21 and p27 (see reviews32,33). In the presence of PDGF-BB, IL-1β does not affect the expression of cyclin-E but suppresses the expression of cyclin-D1, which came as a surprise, considering the positive effect of the cytokine on proliferation. We also measured cyclin-D–associated kinase activity toward the glutathione S-transferase–pRb fusion protein. Activities were low, and we did not observe significant differences between PDGF-BB–stimulated control cells and cells treated with PDGF-BB plus IL-1β (authors’ unpublished data, 2001).

Figure 6. Effect of IL-1β on PDGF-BB–induced CDK2 activity. Quiescent cells were stimulated with 10 ng/mL PDGF-BB with or without 0.1 ng/mL IL-1β. After 20 hours, cells were harvested, and CDK2 was immunoprecipitated from lysates. Kinase activity was assayed on beads, with histone as substrate. The assay mix was subjected to SDS-PAGE, and phosphorylated histone was visualized by autoradiography (A, top). CDK2 protein was determined by Western blot analysis of an extract aliquot before immunoprecipitation (A, bottom). B, Quantification of radioactivity was performed after phosphorimage analysis. Data (mean±SD of 3 independent experiments) are presented as percent stimulation, with 100% obtained with PDGF-BB in the absence of IL-1β. *P<0.005.


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