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Intracellular interleukin 6 mediates platelet-derived growth factor-induced proliferation of nontransformed cells

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ABSTRACT **The functional relevance of interleukin 6 (IL-6) in platelet-derived growth factor (PDGF)-induced cell growth was evaluated in cultures of human fibroblasts, vascular smooth muscle cells, and mesangial cells. The three isoforms of the PDGF—namely, PDGF-AA, -AB, and -BB—induced the expression of the IL-6 gene and proliferation of the nontransformed cells. PDGF-induced transcription, translation, and secretion of IL-6 were diminished in the presence of IL-6 antisense oligonucleotides. While neutralizing anti-IL-6 antibodies failed to affect the growth factor-dependent cell proliferation, IL-6 antisense oligonucleotides inhibited cell division. In addition, IL-6 antisense oligonucleotides abolished PDGF-induced transcription of the genes coding for the cell division cycle 2-related protein (CDC2) and proliferating cell nuclear antigen (PCNA), both of which are regulated in a cell cycle-dependent manner. It is concluded that PDGF-dependent proliferation of nontransformed cells involves the action of intracellular IL-6.**

Interleukin 6 (IL-6), a pleiotropic cytokine with growth- and differentiation-inducing activities, is known to participate in a number of immunological and inflammatory mechanisms (1). Recent *in vitro* studies suggest a novel effect of IL-6 in growth regulation of various tumor cell lines (2–7). Multiple myeloma and AIDS-related Kaposi sarcoma-derived cells have been shown to produce IL-6 and to respond to it with proliferative activity (3, 4). Barut *et al.* (5) and Levy *et al.* (6) reported that the growth-promoting role of IL-6 in tumor necrosis factor-stimulated or fetal calf serum (FCS)-stimulated hairy cell leukemia or myeloma cells is due to the action of intracellular IL-6. Moreover, Lu *et al.* (7) demonstrated that IL-6 undergoes transition from a paracrine growth inhibitor to an intracellular autocrine stimulator during progression of human melanoma. While acquisition of IL-6 production and its possible contribution to autocrine cell growth have been implicated as a key step in certain types of malignant tumor progression, the role of endogenous IL-6 in mitogen-stimulated growth of nontransformed cells is not yet delineated.

Previously, we have shown that platelet-derived growth factor (PDGF) isoform PDGF-BB induces proliferation in various cell types (8, 9) and is capable of stimulating transcription of IL-6 in primary human mesangial cells (MC) (10). Taking into account that PDGF is a potent mitogen that activates the expression of the IL-6 gene, we hypothesized that IL-6 may be an autocrine growth factor that mediates the mitogenic activity of PDGF isoforms in normal, nontransformed cells.

Here we show that all three PDGF isoforms (PDGF-AA, -AB, and -BB) stimulate *de novo* synthesis of mRNA, translation, and secretion of IL-6 in human fibroblasts, vascular smooth muscle cells (VSMC), and MC. While neutralizing anti-IL-6 antibodies failed to affect the PDGF-induced cell division, IL-6 antisense oligonucleotides exhibited a dose-dependent inhibition of PDGF-dependent cell proliferation. The inhibitory effect of IL-6 antisense oligonucleotides was associated with a corresponding diminution of mRNA encoding IL-6, a decreased intracellular expression of IL-6 protein, and consequently a reduced amount of secreted IL-6. In addition, IL-6 antisense oligonucleotides abolished the PDGF-dependent transcription of the genes coding for the cell division cycle 2-related protein (CDC2) and proliferating cell nuclear antigen (PCNA).

METHODS

Cell Cultures. Primary human lung fibroblasts and VSMC were established from sterile lung biopsies and cultivated in RPMI 1640 medium supplemented with 5% FCS and 4 mM L-glutamine, as described (8, 9). Primary cultures of human MC were derived from cortical kidney biopsies (10) and cultivated in serum-free macrophage medium (GIBCO/BRL) supplemented with selenite/insulin/transferrin (Sigma). Subconfluent cell cultures (80%) were used between passages 2 and 6 for all experiments. Cells were growth arrested by starvation for 48 hr in low-serum medium (RPMI 1640 supplemented with only 0.1% FCS), and the medium was replaced every 12 hr. Quiescent cells were challenged with either human recombinant PDGF isoforms at the respective 50% effective dose (ED_{50}) concentration (PDGF-AA, 10 ng/ml; PDGF-AB, 4 ng/ml; and PDGF-BB, 2 ng/ml; GIBCO/BRL).

Measurement of Mitogenicity. Mitogenic effects of the three isoforms of PDGF (PDGF-AA, -AB, and -BB) were determined by incorporation of [^{3}H]thymidine following standard protocols described by Chesterman *et al.* (11). Percentage of inhibition of *de novo* synthesis of DNA was calculated comparing incorporation of [^{3}H]thymidine in the presence of IL-6 antisense phosphorothioate-modified oligonucleotides (PTOs) to incorporation of [^{3}H]thymidine in cells treated with the equimolar concentration of IL-6 sense PTOs. PTOs were added 6 hr before stimulation, at the time of stimulation, and every 48 hr thereafter. Cell counts were performed 7 days after stimulation. All experiments were at least performed in duplicate.

Extraction of Total RNA and Northern (RNA) Blot Analysis. Total RNA was extracted with Trizol reagent (GIBCO/BRL) by following the instructions of the distributor, and 10 μg of heat-denatured RNA per sample was fractionated on a 1% agarose gel with 7% formaldehyde. Northern blotting and hybridization were performed according to standard procedures (12) with 3′ end-labeled oligonucleotides specific to the

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Abbreviations: PDGF, platelet-derived growth factor; IL-6, interleukin 6; IL-6-R, IL-6 receptor; PTO, phosphorothioate-modified oligonucleotides; PCNA, proliferating cell nuclear antigen; VSMC, vascular smooth muscle cells; MC, mesangial cells; CDC2, cell division cycle 2-related protein; FCS, fetal calf serum.

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IL-6 gene (British Biotechnology, Oxford, U.K.), the CDC2 gene (5′-ATG GAA GAT TAT ACC AAA-3′, MWG-Biotechnology, Ebersberg, Germany) (13), and the PCNA gene (5′-ATG TTC GAG GCG CGC CTG-3′, MWG-Biotechnology) (14).

Expression of IL-6 Protein. To quantify secreted amounts of IL-6, cells (1×10^4) were seeded onto 24-well cell culture plates (Costar) and brought to quietude. Six hours prior to stimulation, cells were preincubated with low-serum medium containing either IL-6 antisense, sense PTOs or no PTOs (control). Aliquots (100 μl) of cell supernatants were collected at 0, 6, 12, and 18 hr after the addition of a mitogen. The concentration of secreted IL-6 was determined by enzyme immunoassay (EIA; Advanced Magnetics, Cambridge, MA). Percentage of inhibition of IL-6 secretion was calculated by comparing concentrations of IL-6 in supernatants of cells in the presence of IL-6 antisense PTOs to those of cells treated with the equimolar concentration of IL-6 sense PTOs.

IL-6 Phosphorothioate-Modified Oligonucleotides. A 21-base IL-6 antisense PTO (5′-dGTG GGC TCG AGG GCA GAA TGA-3′), specific for a sequence in the first exon, and a 15-base IL-6 antisense oligonucleotide (5′-dTCC TGG GGG TAC TGG-3′), specific for a sequence in the second exon of the IL-6 gene (15), were used at various concentrations (0.1–10 μM) to inhibit PDGF-induced IL-6 gene expression. The latter PTO has already been successfully used by others to block IL-6 gene expression in human tumor cell lines (4, 6, 7).

To block expression of the gene for the IL-6 receptor (IL-6-R), cells were incubated for 18 hr in the presence of 5 μM IL-6-R antisense oligonucleotides (5′-GCA GCC GAC GGC CAG CAT-3′) (16).

To exclude unspecific effects of PTOs (synthesized by MWG-Biotechnology), sense oligonucleotides complementary to the antisense PTOs and at same concentrations were used as controls in all assays. To rule out toxic effects of PTOs, cell viability was determined by trypan-blue dye exclusion. In the presence of either IL-6 antisense or sense PTOs (10 μM), cell viability was found to be >95% and was not different from controls without PTOs.

Immunofluorescence Analysis. For immunofluorescence analysis of intracellular IL-6 protein, cells were pretreated for 6 hr with either IL-6 antisense or sense PTOs followed by stimulation with PDGF-BB (2 ng/ml) for 8 hr. Cells were washed twice with PBS and fixed with methanol for 15 min (17). After two additional washes with PBS containing 10% FCS (blocking buffer), unspecific binding was blocked by incubating the slides for 1 hr in blocking buffer. Immunostaining was performed with a rabbit monoclonal anti-human IL-6 antibody (British Biotechnology) at 1:1000 dilution and with 2 hr of incubation at room temperature. After two washes with blocking buffer, bound IL-6 antibody was detected with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Boehringer Mannheim, 1:1000 dilution).

RESULTS

PDGF-Induced Transcription of IL-6, CDC2, and PCNA Genes Is Blocked by IL-6 Antisense PTOs. All isoforms of PDGF induced the transcription of IL-6 in all three cell types studied. The PDGF-dependent transcription of IL-6 displayed a characteristic biphasic time course (Fig. 1*a*). A first peak of IL-6 mRNA was detectable between 30 and 60 min after stimulation, declining thereafter to basal level. A second, more prominent peak for IL-6 mRNA was observed 6–8 hr after stimulation. In addition, the transcription of the genes encoding CDC2 and PCNA was stimulated by PDGF isoforms. Conversely, PDGF-AA- and -BB-dependent activation of the genes coding for IL-6, CDC2, or PCNA was abolished in the presence of IL-6 antisense PTOs, whereas IL-6 sense PTOs were ineffective.

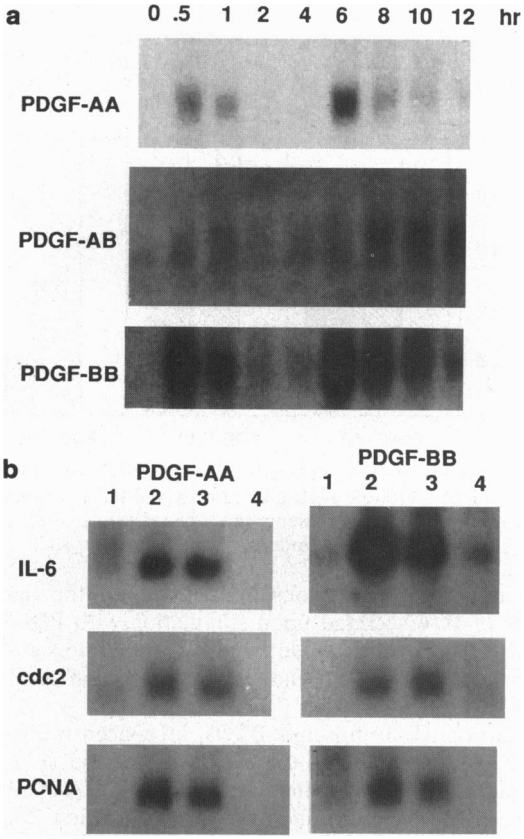

FIG. 1. Effects of PDGF-AA, -AB, and -BB on the transcription of the IL-6 gene and of the genes encoding CDC2 and PCNA. (*a*) Time course of mitogen-induced transcription of the IL-6 gene in human lung fibroblasts. Similar time courses of IL-6 gene transcription were observed in VSMC and MC (data not shown). (*b*) Effects of IL-6 antisense and sense PTOs on PDGF-AA- and -BB-induced transcription of IL-6, CDC2, and PCNA genes in human lung fibroblasts. Total RNA was extracted at time points of maximal transcription (IL-6 at 8 hr; CDC2 and PCNA at 18 hr). Lanes: 1, unstimulated fibroblasts; 2, fibroblasts stimulated with PDGF-AA (10 ng/ml) or -BB (2 ng/ml); 3, fibroblasts preincubated for 6 hr in the presence of IL-6 sense PTOs followed by stimulation with either PDGF-AA or -BB; 4, fibroblasts preincubated for 6 hr in the presence of IL-6 antisense PTOs followed by stimulation with PDGF-AA or -BB.

IL-6 Antisense PTOs Inhibit PDGF-Stimulated Expression of the IL-6 Gene. Enhanced transcription of the IL-6 gene was followed by an increased secretion of IL-6 protein. In PDGF-stimulated cells, we observed a marked increase of secreted IL-6 protein during the first 12 hr, reaching a plateau thereafter. IL-6 antisense PTOs, but not IL-6 sense PTOs, reduced the amount of IL-6 that is secreted upon stimulation with PDGF isoforms as a function of increasing concentration (Fig. 2). At a concentration of 10 μM IL-6 antisense PTOs, the stimulus-induced secretion of IL-6 was abolished. Immunofluorescence staining for IL-6 protein revealed diminished amounts of immunoreactive IL-6 protein in the cytoplasm of cells treated with IL-6 antisense PTOs (Fig. 3). No unspecific autofluorescence of the cells was observed. Furthermore, we confirmed the inhibitory effect of antisense IL-6 PTOs on mitogen-dependent IL-6 synthesis by Western blot analysis of cell lysates (data not shown).

Effect of Anti-IL-6 Antibodies, IL-6, and IL-6-R PTOs on PDGF-Induced Proliferation. Two different monoclonal neutralizing antibodies to IL-6 (Sigma and Advanced Magnetics) were used to block possible autocrine effects of secreted IL-6 on PDGF-induced proliferation, which might be mediated via interaction of IL-6 with its cell surface receptor. By enzyme immunoassay we confirmed that the concentrations of anti-

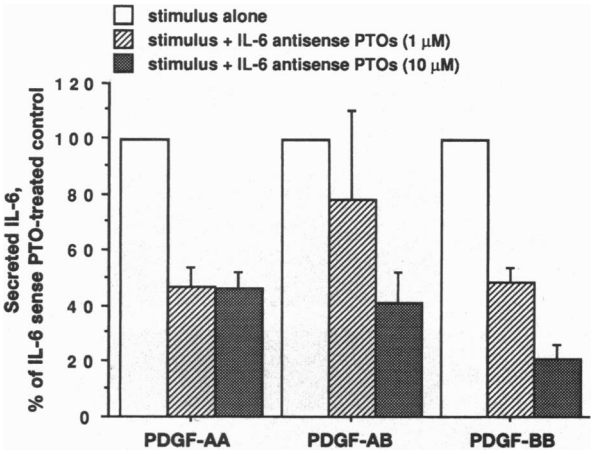

FIG. 2. Effects of IL-6 antisense and sense PTOs on PDGF isoform-dependent secretion of IL-6 by human VSMC as a function of concentration. Each bar represents the mean ± SEM of six experiments. Similar data were obtained from fibroblasts or MC.

bodies used were efficacious in neutralizing the maximal amounts of IL-6 secreted upon stimulation with PDGF isoforms. However, both IL-6 antibodies excerted no significant inhibitory effect on PDGF-induced incorporation of [^{3}H]thymidine.

In contrast, IL-6 antisense PTOs, at concentrations that were effective in reducing the expression of the IL-6 gene, inhibited the mitogen-stimulated synthesis of DNA of all three cell types as a function of increasing concentration (Fig. 4*a*). The mitogenic effect of PDGF was nullified at a concentration of 10 μM IL-6 antisense PTO. All isoforms of PDGF led to an increase in cell counts during an observation period of 7 days (Fig. 4*b*). Consistent with the data obtained for [^{3}H]thymidine incorporation, the mitogen-dependent proliferation was abolished in the presence of IL-6 antisense PTOs.

To exclude an interaction of IL-6 with its corresponding receptor at an intracellular site (18), we suppressed the expression of the IL-6-R with IL-6-R antisense PTOs. However, this did not affect PDGF-dependent cell growth (Fig. 5). The data suggest a mechanism of PDGF-mediated cell growth independent of the IL-6-R.

DISCUSSION

All PDGF isoforms induced the expression of the IL-6 gene. The mitogen-induced activation of fibroblasts, VSMC, and MC resulted in enhanced *de novo* synthesis of DNA and in an increase in cell counts. While IL-6 antisense PTOs diminished both the expression of the IL-6 gene and the proliferation of the cells, neutralizing anti-IL-6 antibodies and IL-6-R antisense PTOs failed to inhibit the growth of cells induced by either isoform of PDGF. In addition, the mitogen-dependent transcription of CDC2 and PCNA was abolished in the presence of IL-6 antisense PTOs.

The three PDGF isomers transiently increased the transcription of the IL-6 gene in all three cell types studied. This finding is consistent with previous reports demonstrating the ability of native PDGF to activate the expression of the IL-6 gene in VSMC (19) and that of PDGF-BB in MC (10). The effect of the PDGF isoforms on expression of the IL-6 gene was observed at concentrations corresponding to the ED_{50} values required for stimulation of growth (8). Recently, IL-6 has been characterized as an autocrine growth factor in various tumor cell lines (2–7). Brach *et al.* (2) reported that interleukin 1-dependent proliferation of leukemic human megakaryoblasts was abolished in the presence of anti-IL-6 antibodies, which suggests a regulatory function of extracellular IL-6 in proliferation of this transformed cell type. In contrast, we observed no inhibitory effect of neutralizing anti-IL-6 antibodies on mitogen-dependent cell growth. However, the missing potency of secreted IL-6 to alter PDGF-dependent cell proliferation *in vitro* does not exclude an intracellular action of IL-6. Our observation of an inhibition of expression of the IL-6 gene in nontransformed cells that results in a loss of the cells' ability to proliferate suggests an essential role for intracellular IL-6 in cell growth. Taking into account that intracellular IL-6 appears to be involved in proliferation of three different nontransformed cell types of mesenchymal origin, we conclude

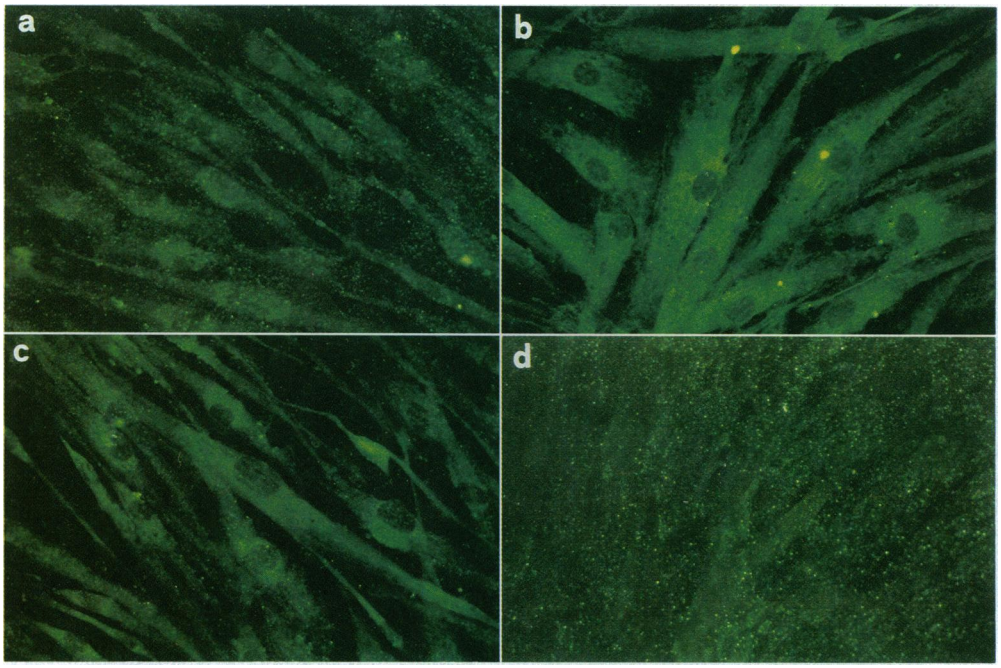

FIG. 3. Effects of IL-6 antisense and sense PTOs on PDGF-BB-dependent intracellular IL-6 protein visualized by immunofluorescence staining in human VSMC. (*a*) Nonstimulated VSMC. (*b*) VSMC stimulated with PDGF-BB at 5 ng/ml. (*c*) VSMC pretreated with 5 μM IL-6 sense PTO for 6 hr followed by stimulation with PDGF-BB. (*d*) VSMC pretreated with 5 μM IL-6 antisense PTO for 6 hr followed by stimulation with PDGF-BB. Similar data were obtained in fibroblasts or MC.

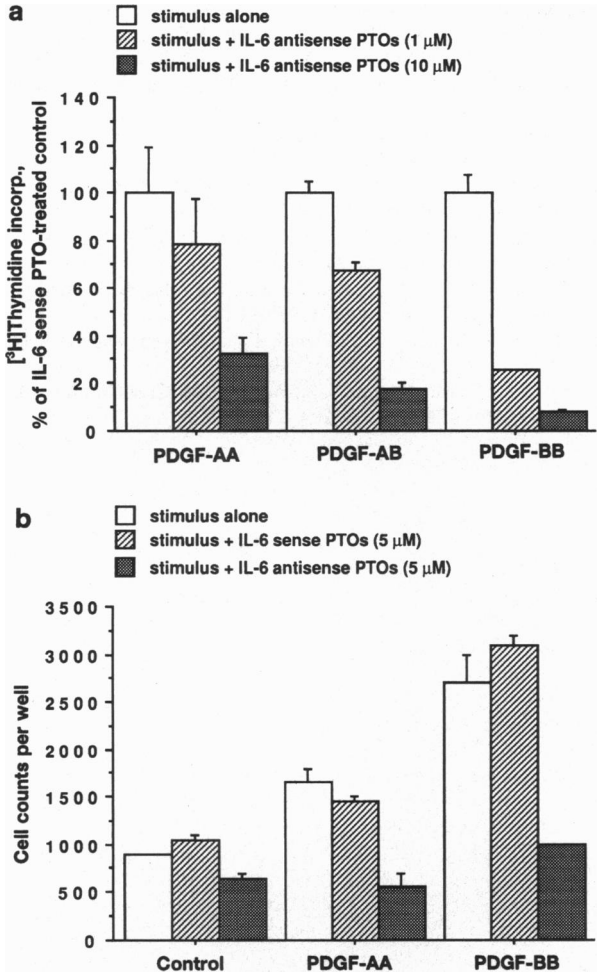

FIG. 4. Effects of IL-6 antisense and sense PTOs on PDGF-dependent mitogenicity as a function of concentration. (*a*) *De novo* DNA synthesis was assessed by [^{3}H]thymidine incorporation (incorp.) in human fibroblasts. Each bar represents the mean ± SEM of six experiments. Similar data were obtained in VSMC or MC. (*b*) Proliferation was assessed by counting MC 7 days after addition of the stimulus. Similar data were obtained in fibroblasts and VSMC.

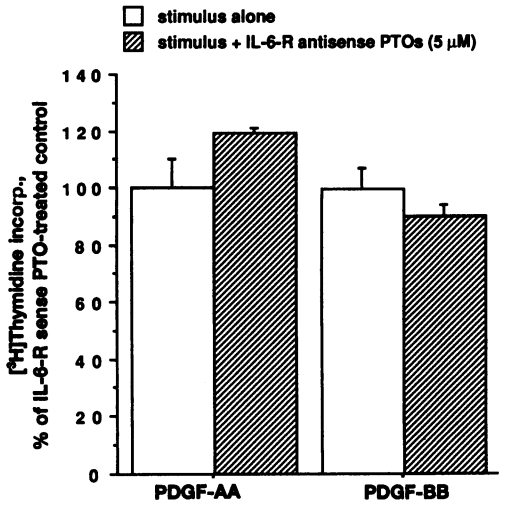

FIG. 5. Effect of 5 μM IL-6-R antisense and sense PTOs on PDGF-induced incorporation (incorp.) of [^{3}H]thymidine in human fibroblasts. Percentage of alteration of *de novo* synthesis of DNA was calculated by comparing incorporation of [^{3}H]thymidine in the presence of IL-6-R antisense PTOs with the incorporation of [^{3}H]thymidine in cells treated with the equimolar concentration of IL-6-R sense PTOs (mean ± SEM).

that the activation of the IL-6 gene serves as a principal mechanism in proliferation of these cells. Similarly, an intracellular signal function of interleukin 1α (IL-1α) has been reported by Stevenson *et al.* (20), demonstrating that IL-1α is synthesized on non-membrane-associated polysomes and the protein associates with microtubules (21). In addition, it was shown that translocation of intracellular truncated forms of IL-1α to the nucleus leads to malignant transformation of mesangial cells (22). These findings support our hypothesis that members of the interleukin family may have intracellular functions that are distinct from the activities of the secreted proteins.

To further elucidate the mode of action of intracellular IL-6 in cell cycle progression, we investigated the effect of IL-6 antisense PTOs on the expression of the CDC2 and PCNA genes, which are strictly associated with the proliferative state of a cell (13, 14, 22). Both genes are activated during transition from G_1 to S phase (13, 22). The CDC2 gene encodes a cell cycle-regulated protein kinase essential for initiation of DNA replication and entry into mitosis (14, 22), while PCNA is a nuclear protein required for DNA synthesis by DNA polymerase δ (22). Consistent with the effects of antisense PTOs on cell proliferation and incorporation of [^{3}H]thymidine, the mitogen-induced expression of the CDC2 and the PCNA gene was diminished in the presence of IL-6 antisense PTOs. This indicates a role for intracellular IL-6 prior to transition from G_1 to S phase of the cell cycle.

Furthermore, the role of the IL-6-R in PDGF-dependent proliferation was investigated (16, 18). In experimental models for cell transformation, internal activation of receptors by autocrine mechanisms has been described in v-*sis*-transformed cells (23) and in hematopoietic cells transfected with an interleukin 3 gene including an endoplasmic retention signal (24). However, proliferation was not altered in the presence of IL-6-R antisense PTOs, which favors a mechanism that is independent of this IL-6-binding protein.

Similar to the PDGF isoforms, *de novo* DNA synthesis and proliferation induced by FCS was blocked by IL-6 antisense PTOs (data not shown). In addition to PDGF, FCS contains a wide number of mitogens. Via interaction with specific cell surface receptors, these growth factors trigger signal pathways that must converge prior to initiation of DNA replication. Regarding this fact, we consider intracellular IL-6 as a factor that is involved in a final common pathway shared by diverse mitogenic signals.

In conclusion, the primary action of IL-6 apparently occurs at the intracellular level during the G_1 phase of the cell cycle and is independent of the expression of the IL-6-R. Further studies are required to ascertain the precise mechanism, by which activation of the IL-6 gene contributes to proliferation of nontransformed cells.

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