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Platelet-derived growth factor synthesis in mesangial cells: Induction by multiple peptide mitogens

(growth factors/*sis* oncogene/gene regulation/human kidney)

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ABSTRACT **Platelet-derived growth factor (PDGF) has been implicated in several nonmalignant pathophysiological processes, including proliferative diseases of the kidney. Glomerular mesangial cells secrete a PDGF-like factor and express the PDGF A-chain and c-sis (or B-chain) mRNAs. We report here that both mRNAs are induced by serum and this effect can be mimicked by recombinant PDGF, which also markedly stimulates DNA synthesis. Other growth factors, such as epidermal growth factor (EGF), transforming growth factor type α, basic fibroblast growth factor (bFGF), and tumor necrosis factor type α (TNF-α) also are mitogenic for human mesangial cells and induce expression of the PDGF mRNAs. EGF, TNF-α, and bFGF also stimulate these cells to secrete a PDGF-like factor. Furthermore, anti-PDGF antibody partially abrogates the mitogenic effect of EGF, suggesting that mitogen-stimulated PDGF synthesis in mesangial cells is at least partly responsible for cell growth induced by other growth factors. In contrast to these results, transforming growth factor type β (TGF-β), while inducing both mRNAs, is not mitogenic, indicating that its effect on message levels can be dissociated from DNA synthesis. These data suggest that several peptide growth factors regulate the growth of mesangial cells and that PDGF may be an effector molecule that plays a role in the mitogenic response to many of these growth stimuli.**

Platelet-derived growth factor (PDGF) is a dimeric peptide mitogen composed of a heterodimer of A and B chains or homodimers of either chain. There is evidence to suggest that some cell types produce only A-chain or B-chain homodimers (1–3), while human platelets contain both chains of PDGF (4). While its exact peptide composition remains to be identified, many tissues and cell lines, mostly of mesenchymal origin, express the mRNAs encoding the A- and/or B-chains of PDGF (3, 5, 6). The B-chain, encoded by the c-*sis* protooncogene (7, 8), is ≈60% similar in nucleotide sequence to the A-chain gene (5). Although located on different chromosomes, the two genes can be expressed independently or coordinately (3, 6, 9, 10).

PDGF-like factors, detectable either by radioreceptor assay or immunoprecipitation, are produced by several diploid cell types, including endothelial cells, smooth muscle cells, macrophages, and placental cytotrophoblasts (9, 11–13). Because of its synthesis in these tissues, and because of its effects on proliferation, chemotaxis, and collagen matrix formation, PDGF may play a role in wound repair and embryogenesis. It may also be involved in pathophysiological states marked by cellular proliferation such as atherosclerosis and bone marrow fibrosis (for review, see ref. 14). In both of these disorders, there is evidence for a role of PDGF in an autocrine or paracrine mechanism of action.

One difficulty in evaluating the role of PDGF in hyperproliferative disease processes is that these entities are marked by infiltration of multiple cell types, making interpretation of PDGF expression and regulation difficult. This is particularly true in the kidney, where glomerular hypercellularity results from infiltrating inflammatory cells as well as the proliferation of intrinsic glomerular cells. Mesangial cells are mesenchymal cells that constitute approximately one-third of glomerular cells (15). There is relatively little information concerning the factors that regulate their normal growth or abnormal proliferation in glomerular diseases (16, 17). We have recently found that cultured mesangial cells produce a PDGF-like factor and respond mitogenically to exogenous PDGF, suggesting a potential autocrine function for this peptide in regulating the growth of these cells (18, 19).

Among the cellular events that occur upon growth factor binding to target cells is the activation of known protooncogenes, such as c-*myc*, and c-*fos*, in addition to other cellular genes (20, 21). In the present study, we demonstrate the induction of PDGF A- and B-chain mRNA levels in mesangial cells by multiple growth factors, including PDGF itself. Many of these same growth factors stimulate release of a PDGF-like factor. Furthermore, we demonstrate that those agonists that stimulate the PDGF mRNAs are also potent mitogens for mesangial cells. The effect of transforming growth factor type β (TGF-β) is an important exception to this observation and indicates that PDGF synthesis may be necessary, although insufficient, for mitogenesis in mesangial cells.

PROCEDURES

Growth Factors and Plasmid Probes. Recombinant PDGF ($PDGF_{v\text{-}sis}$), human tumor necrosis factor type α (TNF-α), and basic fibroblast growth factor (bFGF) were purchased from Amgen Biologicals; purified human TGF-β was from R & D Systems (Minneapolis); purified human transforming growth factor type α (TGF-α) was from Bachem; and purified receptor grade epidermal growth factor (EGF) and anti-PDGF IgG were obtained from Collaborative Research. Radioiodinated human platelet PDGF was purchased from Cambridge Medical Technology Corporation (Billerica, MA). The PDGF A-chain cDNA, provided by C. Betsholtz and C.-H. Heldin (University of Uppsala), was subcloned into M13mp8 (22).

Mesangial Cell Cultures. Human mesangial cells were cultured from glomeruli isolated from human kidneys as described (19, 24). Their identification was confirmed by electron microscopy and by immunohistochemical staining. The cells were positive for the intermediate filaments desmin, myosin, and vimentin, indicative of a mesenchymal origin,

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TGF-α, transforming growth factor type α; TGF-β, transforming growth factor type β; bFGF, basic fibroblast growth factor; TNF-α, tumor necrosis factor type α.

but were negative for keratin and factor VIII antigen, excluding epithelial and endothelial cell contamination, respectively. Mesangial cells also showed no detectable activity for angiotensin-converting enzyme.

Northern Blotting. RNA was prepared from human mesangial cells by lysis in guanidinium isothiocyanate and centrifugation on cesium chloride gradients (25). Total RNA was fractionated on 1% agarose-formaldehyde gels and transferred to GeneScreen (NEN). Prehybridization and hybridization with radiolabeled single-stranded M13 probes were performed as described (26).

RNase Protection Assay. The probe used for these experiments was generously provided by David Goldthwait (Case Western Reserve University). It consists of a 960-base-pair genomic fragment, encompassing portions of the sixth and seventh exons (a *Ban* II/*Ban* II restriction fragment) of the c-*sis* gene (7) that had been subcloned into the *Sma* I site of pT7-2 (United States Biochemical). Probe synthesis was initiated by T7 RNA polymerase in the presence of 40 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$/10 mM NaCl/2 mM spermidine/40 mM dithiothreitol/RNasin (40 units/ml) (Promega Biotec)/0.5 mM each ATP, GTP, and CTP/12 μM UTP/50 μCi of [^{32}P]UTP (800 Ci/mmol; 1 Ci = 37 GBq)/10 μg of linearized template DNA per ml. After incubation at 37°C for 1 hr, the DNA template was digested by DNase I, and the reaction mixture was treated by phenol/chloroform extraction and ethanol precipitation. The newly synthesized RNA probe (1×10^6 cpm) was hybridized to total mesangial cell RNA in 80% formamide/10 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA for 12 hr at 50°C. After hybridization, the reaction mixture was treated with RNases A (50 μg/ml) and T1 (2 μg/ml) and then with proteinase K (0.16 mg/ml). The products were analyzed by electrophoresis through a 7 M urea/6% acrylamide gel, followed by autoradiography.

[^{3}H]Thymidine Incorporation. DNA synthesis was measured as the amount of [^{3}H]thymidine incorporated into trichloroacetic acid-precipitable material as described (19). In some experiments, the proliferative effect of added growth factors was confirmed by cell counting.

PDGF Radioreceptor Assay. Confluent human mesangial cells were maintained in Waymouth's medium without fetal calf serum or insulin for 3 days. After an additional 24 hr in the presence of the growth factor to be tested, the medium was collected, centrifuged to remove cellular debris, and stored at 4°C until assayed for PDGF-like activity. Assays were performed within 14 days of medium collection as described (18, 19). Fibroblasts were cultured from human foreskin tissue and plated at 5×10^4 cells per 2.5-cm^2-well plastic dishes (Costar) in a 1:1 mixture of Dulbecco–Vogt's modified Eagle's medium and Ham's F-12 medium (DVF-12) supplemented with 1% Zeta serum, a low mitogenic serum devoid of measurable PDGF activity.

RESULTS

Regulation of the PDGF A-Chain and c-sis mRNAs by Serum and Purified Growth Factors. We have found that human mesangial cells secrete a PDGF-like molecule and express the mRNAs encoding both the PDGF A-chain and c-*sis* genes (19). In evaluating the autogenous production of PDGF in these cells, we reasoned that if PDGF regulates mesangial cell growth, its synthesis may be controlled by the state of cellular proliferation. PDGF synthesis would be expected to be low in resting or G_0 cells and increase when cells are stimulated by serum if, indeed, endogenous PDGF synthesis is required before cell division can occur. As a first approach, we compared expression of PDGF A-chain and c-sis mRNAs in quiescent serum-starved mesangial cells, which were exposed to serum for up to 24 hr with that in cells maintained in serum-free medium alone. Fig. 1*A* shows that exposure of the cells to 10% fetal calf serum induces steady-state PDGF A-chain mRNA levels with the peak effect occurring at ≈4 hr. As in other cell types, three A-chain transcripts, 2.3, 1.9, and 1.5 kilobases, are detected in mesangial cell mRNA. Their relative abundance does not change after induction. We then measured the effect of a number of purified or recombinant growth factors including PDGF, EGF, TGF-α, TGF-β, TNF-α, and bFGF. Representative Northern blots are shown in Fig. 1 *B* and *C*. Each growth factor stimulated the level of A-chain mRNA 3 to 6 times over that in control cultures (Table 1). We reprobed the same Northern blots with an α-tubulin cDNA (23). Tubulin mRNA does appear to increase in a time-dependent fashion after serum or EGF stimulation, although this increase does not correlate with the kinetics of A-chain mRNA induction. After treatment with the other growth factors, there is little variation in the level of α-tubulin mRNA, demonstrating that the response in A-chain mRNA does not simply reflect a global increase in total RNA synthesis. The response to each growth factor was determined at least twice with similar results.

Because the low level of expression of the c-*sis* gene made detection of its mRNA by Northern blotting difficult, we used

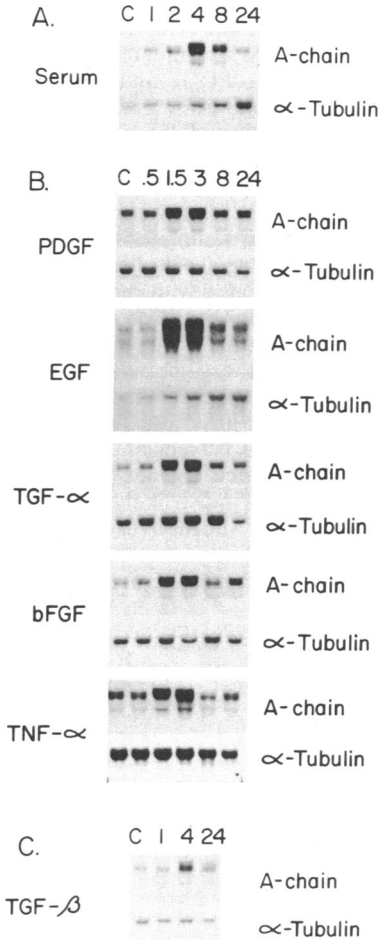

FIG. 1. Regulation of the PDGF A-chain mRNA by serum and growth factors. (*A*) Induction of A-chain expression by 10% fetal calf serum. Lane C (control) represents RNA isolated from cells after incubation in serum-free medium for 24 hr. (*B*) Induction of PDGF A-chain expression by PDGF (10 ng/ml), EGF (10 ng/ml), TGF-α (10 ng/ml), bFGF (10 ng/ml), and TNF-α (50 units/ml). (*C*) Induction of PDGF A-chain expression by TGF-β (2.5 ng/ml). Each lane contains 15 μg of total RNA isolated from mesangial cells treated for the indicated number of hours. The control lanes in *B* and *C* contain RNA isolated at 0 hr. After hybridization with the A-chain cDNA, the probe was removed by boiling and each blot was rehybridized with the α-tubulin cDNA probe.

Table 1. Summary of effects of growth factors on mesangial cells

	[^{3}H]Thymidine	A-Chain mRNA Exp. 1*	A-Chain mRNA Exp. 2	c-sis mRNA Exp. 1†	c-sis mRNA Exp. 2	PDGF-c
Serum (7)	21.8 ± 9.4	17	7	14	ND	ND
PDGF (5)	14.7 ± 3.2	5	10	24	27	ND
EGF (4)	5.4 ± 0.8	7	6	17	9	3.4
TGF-α (7)	6.0 ± 1.4	5	5	28	ND	ND
bFGF (5)	4.4 ± 1.1	4	4	15	ND	4.8
TNF-α (9)	3.5 ± 0.7	3	2	11	ND	5.0
TGF-β (5)	0.4 ± 0.1	3	3	4	ND	ND

Data are expressed as -fold enhancement over serum-starved controls. The number of separate experiments in which incorporation of [^{3}H]thymidine was measured in response to each agonist is in parentheses, followed by the means of the -fold enhancement ± SEM. Relative levels of the A-chain and c-sis mRNAs were determined by scanning densitometry. PDGF-c, PDGF competing activity in the radioreceptor assay, expressed as ng per 10^6 cells. ND, not done.

*From data in Fig. 1.

†From data in Fig. 2.

an RNase mapping technique to determine whether the PDGF B-chain mRNA was coordinately induced with that of the A-chain in response to PDGF and other growth factors. The probe used for these studies was derived from a segment of the c-*sis* gene, consisting of the sixth exon, intervening sequence, and the 5′ portion of exon 7, subcloned into pT7-2, a vector containing the T7 bacteriophage RNA polymerase promoter (see *Procedures*). The complementary RNA probe synthesized from the c-*sis* template was hybridized to total RNA prepared from mesangial cells maintained in serum-free medium and from cells treated with each of the above growth factors. Only those portions of the probe represented in c-sis mRNA are protected from RNase digestion (Fig. 2*F*). As shown in Fig. 2 *A–D*, the same agonists that induce the PDGF A-chain mRNA in quiescent mesangial cells stimulate expression of the c-*sis* transcript. The effect on both the A-chain and c-sis mRNAs is transient, much like the induction of other "competence" genes by PDGF (20). This increase in mRNA levels most likely reflects a short-lived transcriptional response. Alternatively, it could reflect rapid changes in message stability or other posttranscriptional events. These results demonstrate not only the capability of

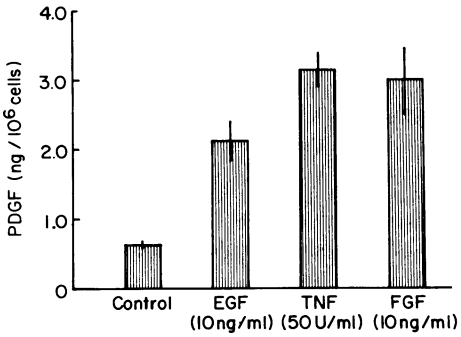

FIG. 3. Effect of peptide growth factors on PDGF release. Confluent human mesangial cell cultures were exposed to each growth factor for 24 hr, at which time cell counts were performed. PDGF-like activity in the medium was measured by a radioreceptor assay. Results are expressed as ng of PDGF released per 1×10^6 cells. None of the growth factors used interfered with the binding of labeled PDGF (data not shown).

exogenous PDGF to induce its own synthesis, but also a common effect on expression of the PDGF mRNAs by multiple other mitogens. Collectively, these data provide supportive evidence that the induction of PDGF synthesis may be part of a normal pathway of cellular proliferation initiated by multiple growth stimuli.

Secretion of a PDGF-like Molecule in Response to Growth Factors. To determine whether the increase in A-chain and c-sis mRNA levels was indicative of increased production and release of PDGF, we assayed conditioned medium from mesangial cell cultures treated with either EGF, bFGF, or TNF-α. After a treatment period of 24 hr, the medium was analyzed for the presence of PDGF by means of a radioreceptor assay. As shown in Fig. 3, these growth factors stimulated the release of a PDGF-like factor that competed with ^{125}I-labeled PDGF for binding to human foreskin fibroblasts. We observed no displacement of ^{125}I-labeled PDGF binding after incubation with any of the purified mitogens themselves (data not shown). The stimulation of the PDGF A-chain and c-*sis* transcripts suggests that the competitive activity in mesangial cell conditioned medium represents secretion of newly synthesized rather than preformed PDGF.

Growth Factor Stimulation of [^{3}H]Thymidine Incorporation. All of the above growth factors stimulate DNA

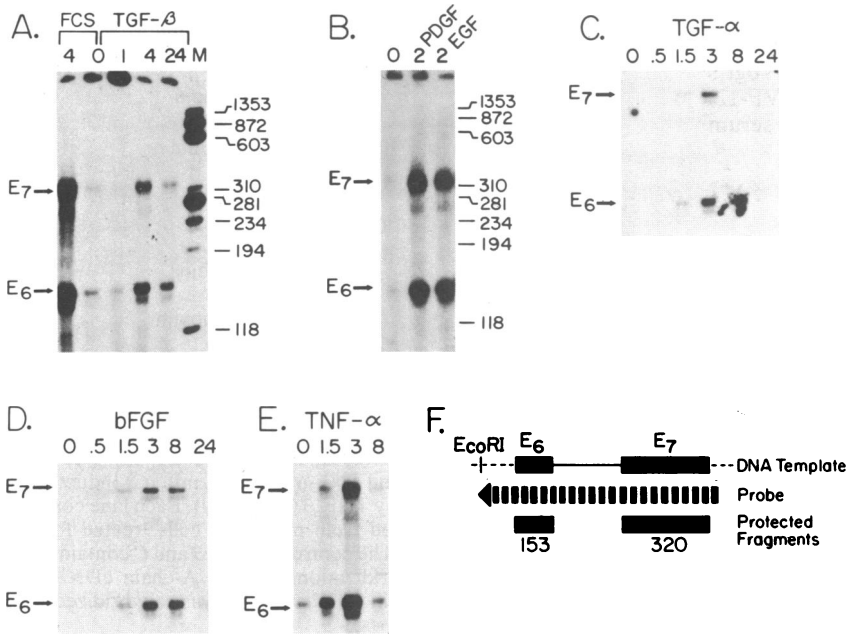

FIG. 2. Regulation of c-sis mRNA levels. Total RNA isolated from cells treated with fetal calf serum (FCS) and TGF-β (*A*), PDGF and EGF (*B*), TGF-α (*C*), bFGF (*D*), and TNF-α (*E*) for the indicated number of hours was assayed by RNase mapping as described in *Procedures*. For TGF-β, TGF-α, bFGF, and TNF-α, RNA from the same experiments shown in Fig. 1 was used for measurement of c-*sis* expression. Size markers (in nucleotides) were derived from *Hae* III fragments of ϕX DNA, end-labeled with T4 DNA polymerase. E_6 and E_7 refer to those portions of the sixth and seventh exons of the c-*sis* gene contained within the RNA probe that are protected from RNase digestion as schematically depicted in *F*. After hybridization to tRNA under identical conditions, the probe was completely digested by RNase (data not shown).

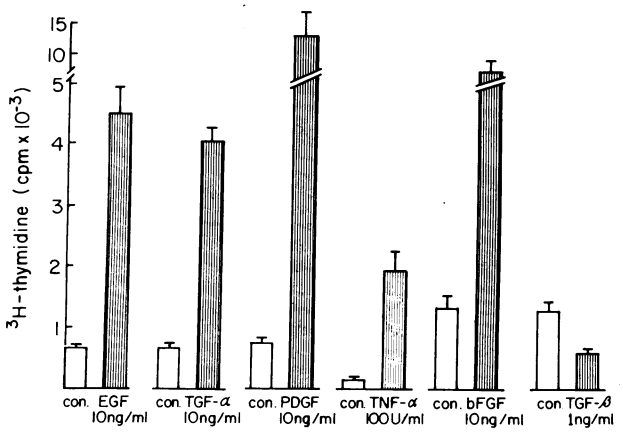

FIG. 4. Regulation of DNA synthesis in mesangial cells. Serum-starved mesangial cells were exposed to each growth factor for a total of 24 hr, followed by measurement of [^{3}H]thymidine incorporation. Each bar represents the mean number of incorporated counts in at least four separate experiments. All conditions were tested in at least triplicate wells in each individual experiment. The SEM is indicated. con., Control.

synthesis in their appropriate target cells, although their effect in mesangial cells is unknown. Because of their effect on the expression of a second growth factor in these cells, we determined whether the induction of the PDGF mRNAs is associated with stimulation of DNA synthesis. Quiescent cultures of mesangial cells were treated with each mitogen using a concentration that yielded maximal [^{3}H]thymidine incorporation in preliminary experiments. As shown in Fig. 4, except for TGF-β, all of the growth factors that induced steady-state levels of A-chain and c-sis mRNAs stimulated [^{3}H]thymidine incorporation into DNA. The proliferative effect of several of the growth factors was further demonstrated by cell counting, as shown in Table 2. TGF-β was the only growth modulator that had a disparate effect, inducing both transcripts (Figs. 1 and 2) but inhibiting DNA synthesis. This inhibitory effect of TGF-β on DNA synthesis was observed with concentrations ranging from 0.25 to 10 ng/ml in both confluent and subconfluent cells (data not shown). These data suggest that the induction of c-sis and PDGF A-chain mRNAs by other growth factors is not merely a consequence of entry into the cell cycle, but rather a specific result of an agonist–receptor interaction. They further indicate that PDGF gene expression may be controlled through different pathways than those governing the initiation of DNA synthesis.

Anti-PDGF Abrogates the Mitogenic Effect of EGF. To demonstrate whether PDGF is at least partially responsible for the mitogenic effect of other growth factors in mesangial cells, we attempted to neutralize the mitogenic effect of EGF by means of an anti-PDGF antibody. Mesangial cells were incubated with EGF alone or EGF plus increasing concentrations of anti-PDGF IgG. As shown in Table 3, the addition of anti-PDGF lowered the level of [^{3}H]thymidine incorporation induced by EGF. It should be noted that this antibody did not neutralize the maximal stimulatory effect of EGF on A-chain mRNA levels (data not shown), suggesting that its effect on DNA synthesis is specific for PDGF and is not due to cross-reactivity with EGF.

Table 2. Effect of peptide growth factors on mesangial cell proliferation

Condition	Cell number × 10^{-4}
Control	14.0 ± 0.5
PDGF	34.0 ± 0.4
EGF	33.0 ± 4.2
bFGF	48.0 ± 7.5
TNF-α	21.0 ± 0.6
TGF-α	45.0 ± 4.4

Subconfluent mesangial cells in 12-well dishes were placed in 1% Zeta serum and treated with each of the indicated growth factors for 3 days. The cells were then removed from the dish and counted using a Coulter cell counter. The data, from a representative experiment, are expressed as the mean ± SEM of three separate wells for each condition. The cells were initially plated at a density of 10 × 10^4 cells per well.

Table 3. Effect of anti-PDGF antibody on EGF-stimulated DNA synthesis in mesangial cells

Condition	cpm per well
Control	1240 ± 59
EGF (5 ng/ml)	3815 ± 202
+ anti-PDGF (10 μg/ml)	3431 ± 178
+ anti-PDGF (25 μg/ml)	3135 ± 144*
+ anti-PDGF (50 μg/ml)	2759 ± 130**

Subconfluent mesangial cells were placed in serum-free medium for 3 days and then incubated under the indicated conditions for an additional 24 hr. [^{3}H]Thymidine incorporation was then measured. The data are expressed as the mean ± SEM from three separate experiments. The conditions were tested in triplicate or quadruplicate wells in each individual experiment. Significant reduction from the value with EGF alone: *, $P < 0.05$; **, $P < 0.01$ (Student's t test).

DISCUSSION

We have demonstrated that multiple growth factors (PDGF, EGF, TNF-α, TGF-α, TGF-β and bFGF) induce the steady-state levels of the PDGF A-chain and c-sis mRNAs in human mesangial cells and have shown that, with the exception of TGF-β, this effect may be related to the induction of DNA synthesis. The previously undescribed mitogenic response of mesangial cells to such a diverse group of peptide mitogens is somewhat surprising, although most of these factors have growth-promoting activity in other cell types. TNF-α, while cytostatic in some tumor cell lines, increases the growth of several types of diploid fibroblasts (27). bFGF is a potent mitogen for several types of mesenchymal cells (for review, see ref. 28). TGF-α and EGF interact with the same receptor, and each alone can stimulate cell growth in diploid fibroblasts (29, 30).

Unlike the case in BALB/c 3T3 fibroblasts, which require serum or PDGF in addition to other mitogens for cell division (31), all of the above growth factors act as complete mitogens in mesangial cells. Their effect on PDGF synthesis raises the possibility that PDGF may be one component of a common mitogenic pathway. This effect may represent, for example, a "cascade" of receptor stimulation, initiated by the primary growth stimulus with subsequent activation of the PDGF receptor through endogenous PDGF production and release, thereby bringing about or amplifying a specific mitogenic response. The partial neutralizing effect of anti-PDGF IgG on the EGF induction of [^{3}H]thymidine incorporation (Table 3) supports such a hypothesis. Whether intracellular PDGF, inaccessible to the antibody, or other non-PDGF mitogens could account for the noninhibitable [^{3}H]thymidine incorporation remains to be determined. The fact that TGF-β has opposite effects on [^{3}H]thymidine incorporation and expression of the PDGF genes in mesangial cells does not exclude a role for PDGF production in mediating DNA synthesis induced by other growth factors. TGF-β has been shown to arrest growth in the G_1 phase of the cell cycle in other cell types (32). One possibility is that this block is generated through distinct pathways that override the stimulatory effect of TGF-β on PDGF synthesis.

While this work was in progress, Paulsson *et al.* (33) observed that PDGF and EGF transiently induce the A-chain, but not the B-chain, mRNA in human foreskin fibroblasts, suggesting that PDGF may be involved in the prereplicative stage of the cell cycle. A major question raised by the data presented here is whether the induction of PDGF synthesis and the initiation of mitogenesis are the result of different intracellular signals. That TGF-β stimulates the PDGF transcripts without inducing DNA synthesis is evidence that the signals that mediate these two effects can be dissociated. TGF-β also stimulates PDGF gene expression in endothelial cells and in mouse embryo AKR-2B cells (10, 34), while it has opposite effects on the growth of these two cell types, inhibiting the growth of endothelial cells and stimulating the growth of AKR-2B cells (35, 36). Collectively, the data indicate that the effects of TGF-β on cell growth may be independent of its effect on PDGF gene expression.

We have previously shown that both rat and human mesangial cell conditioned media contain a factor that shares biologic activity and immunologic cross-reactivity with PDGF purified from human platelets (18, 19). Although other investigators have found only A/A homodimer in the conditioned media of osteosarcoma and other transformed and primary cell lines (1, 5, 6), the peptide composition of the PDGF-like factor in mesangial cells remains to be identified. The inducibility of both PDGF mRNAs in these cells suggests that both homodimers and/or a heterodimer of the two chains may be released upon stimulation. Furthermore, the c-*sis* message appears to be induced to a greater extent than that of the A-chain (Figs. 1 and 2; Table 1). The significance of this differential induction with respect to the structural and functional characteristics of the secreted PDGF-like factor remains unclear. It may indicate that the growth factor-induced PDGF molecule may be different in peptide composition than that expressed in the basal state, or that the c-*sis* translation product is the active moiety in terms of the mitogenic response.

While the *in vivo* biological relevance of our findings remains speculative, our studies suggest that PDGF may play a major role in the pathogenesis of proliferative glomerular diseases, a common cause of permanent glomerular damage. Proliferation of intrinsic glomerular cells, including mesangial cells, as well as the infiltration of platelets and inflammatory cells such as activated monocytes, macrophages, and lymphocytes, are constant features of this pathologic lesion (37). These cells infiltrate the mesangial matrix and reside in direct proximity to mesangial cells. These cell types are also major sources of most known growth modulating factors, providing a setting in glomerular disease for a myriad of interactions among these different growth agonists and inhibitors. We have demonstrated that many of these factors are mitogenic for mesangial cells. It is tempting to speculate that release of polypeptide growth factors, including PDGF itself, by the infiltrating cell population will not only lead to mesangial cell proliferation but also *de novo* synthesis and release of mesangial PDGF, providing a positive signal that initiates or amplifies such a response. The presence of receptors on mesangial cells for these diverse peptide growth factors suggests that they may play a role not only in proliferation, but also in the associated matrix expansion and eventual sclerosis of this important microvascular bed.

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