

Selective Down-Regulation of *c-jun* Gene Expression by Pentoxifylline and *c-jun* Antisense Interrupts Platelet-Derived Growth Factor Signaling: Pentoxifylline Inhibits Phosphorylation of c-Jun on Serine 73

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Received July 3, 2001; accepted March 15, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Platelet-derived growth factor (PDGF) signals through several pathways, including mitogen-activated protein (MAP) kinase, Jun kinase, and C kinase, and stimulates proliferation of fibroblasts. Pentoxifylline inhibits PDGF-driven proliferation of fibroblasts. We have reported that pentoxifylline did not inhibit binding of PDGF to its specific cell-surface receptors or PDGF receptor phosphorylation. In this study, we investigated the effect of PDGF on the expression of *c-fos* and *c-jun*, because *c-fos* and *c-jun* form activator protein-1 complexes that stimulate genes involved in proliferation. We determined whether pentoxifylline would alter the expression of *c-fos* and *c-jun*. Our results indicate that PDGF induced the expression of both *c-fos* and *c-jun*. Pentoxifylline effectively reduced *c-jun* gene expression, which had been up-regulated by PDGF, but did not alter *c-fos* gene expression. The lack of effect on *c-fos* supports other studies from this laboratory, which indicate that pentoxi-

fylline did not inhibit PDGF activation of MAP kinase. Treatment of fibroblasts with a phosphothioate *c-jun* antisense oligodeoxynucleotide reduced the levels of c-Jun protein and blocked PDGF-stimulated proliferation, suggesting a critical role for *c-jun* in PDGF-mediated proliferation. Combination of pentoxifylline and *c-jun* antisense suggested that they were likely inhibiting PDGF-stimulated proliferation at a single site in the PDGF signaling pathway. These results suggest that pentoxifylline inhibits PDGF-stimulated proliferation by selectively decreasing *c-jun* expression. To further define the mechanism of action of pentoxifylline, we assessed the effect of pentoxifylline on c-Jun and phosphorylated c-Jun immunoreactivity in cells treated with PDGF and cells that were transfected with wild-type *c-jun* plasmid using immunocytochemistry and Western blot analyses, and our results indicate that pentoxifylline inhibited phosphorylation of c-Jun on serine 73.

Platelet-derived growth factor (PDGF) is a potent mitogen that has been implicated in the pathophysiology of diseases, including liver fibrosis (Peterson and Isbrucker, 1992), interstitial lung diseases (Uebelhoefer et al., 1995), glomerular nephritis (Gesualdo et al., 1991), nephritis associated with systemic lupus (Nakamura et al., 1992), Graves' ophthalmopathy (Imai et al., 1992), drug-induced ergotism (Pietrogrande et al., 1995), cardiovascular disease (Cimminiello et al., 1994), cancer (Ross et al., 1995), and collagenous colitis (Peterson and Tanton, 1996). We had previously shown that pentoxifylline, a methylxanthine, inhibited PDGF-stimulated proliferation of fibroblasts and liver myofibroblasts (Peterson et

al., 1994; Isbrucker and Peterson, 1995). Pentoxifylline did not alter activation of adenosine receptors (Peterson, 1996), PDGF receptor binding, or PDGF receptor phosphorylation (Slysz and Peterson, 1994), and its effect was independent of an effect on cAMP (Peterson et al., 1998).

A possible mechanism for the inhibitory effect of pentoxifylline on PDGF-stimulated proliferation is inhibition of PDGF postreceptor signaling. Our results indicate that pentoxifylline did not inhibit PDGF-mediated phosphorylation of MAP kinase (Slysz and Peterson, 1994). Several genes involved in proliferation possess AP-1 binding sites and thus would be susceptible to up-regulation by the immediate early genes *c-fos* and *c-jun* (Bamberger et al., 1996). We report that these immediate early genes were up-regulated by PDGF in human fibroblasts. These immediate early genes may play a

This study was supported by an operating grant from the Canadian Institutes for Health Research.

ABBREVIATIONS: PDGF, platelet-derived growth factor; CPSR, controlled process serum replacement factor; MAP, mitogen-activated protein; AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; ODN, oligodeoxynucleotide; CMV, cytomegalovirus; FCS, fetal calf serum; ECL, enhanced chemiluminescence; RDU, relative density units; JNK, Jun kinase; PTX, pentoxifylline.

critical role in the pathogenesis of diseases in which PDGF has been implicated (Gesualdo et al., 1991; Imai et al., 1992; Nakamura et al., 1992; Peterson and Isbrucker, 1992; Ciminiello et al., 1994; Pietrogrande et al., 1995; Ross et al., 1995; Uebelhoer et al., 1995; Peterson and Tanton, 1996; Pinzani et al., 1996). We then determined the effect of pentoxifylline on the PDGF-mediated up-regulation of *c-fos* and *c-jun* gene expression and assessed the involvement of the Jun kinase and C kinase pathways and their modification on PDGF-mediated events. Immunocytochemistry was carried out as described under *Materials and Methods*, using antibodies to c-Jun and serine 73 phosphospecific c-Jun to determine the effect of pentoxifylline on c-Jun phosphorylation, and the results were confirmed by Western blots.

Materials and Methods

Fibroproliferation. The fibroproliferative activity of PDGF was assessed by modification of the tritiated thymidine incorporation method (Dolhman et al., 1984), using normal human skin fibroblasts (Peterson and Isbrucker, 1992). Briefly, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada), antibiotic/antimycotic (Invitrogen), and 0.5% control processed serum replacement-2 (CPSR-2; Sigma-Aldrich, St. Louis, MO.). CPSR-2 is a serum replacement that has low mitogenic activity. Aliquots (200 μ l) of cell suspension (8×10^3 cells) were added to 0.32-cm² flat-bottomed wells of 96-well microtiter plates and incubated for 24 h at 37°C in 5% CO₂ in air. The medium was replaced by 200 μ l of DMEM supplemented with factors and incubated as above for a further 22 h. PDGF B/B (8 ng/ml; R & D Systems, Minneapolis, MN) was used to stimulate proliferation of fibroblasts in the presence or absence of pentoxifylline (Sigma-Aldrich). Methyl-[³H]thymidine (0.5 μ Ci; Amersham Biosciences, Baie d'Urfe, QC, Canada) was added to each well, incubated for an additional 2 h, and then the cells were harvested by aspiration onto glass fiber filters using a Brandel Cell Harvester (Xymotech Biosystems, Mt. Royal, QC, Canada), and the radioactivity was determined by liquid scintillation. All samples were tested in quadruplicate. The [³H]thymidine uptake assay has been validated as a measure of fibroblast number by manual cell count and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Denizat and Lang, 1986) after treatment with proliferative and antiproliferative agents (Peterson et al., 1994).

Assessment of Immediate Early Gene Expression. The effect of pentoxifylline on PDGF-stimulated *c-fos* and *c-jun* gene expression was assessed by Northern analysis using mRNA prepared from dermal fibroblasts that had been treated with PDGF (8 ng/ml) for 30 min in the presence and absence of pentoxifylline before assessment of *c-fos* and *c-jun* mRNA. Measurement of *c-fos* and *c-jun* mRNA was done by Northern analysis using specific oligomers. mRNA was isolated from dermal fibroblasts using Ribosept kits (VWR International, Mississauga, ON, Canada). RNA was subjected to electrophoresis overnight in a denaturing 1% agarose gel, transferred onto a Nytrans⁺ nylon membrane (ICN Biomedicals Inc., Montreal, QC, Canada), and cross-linked with an ultraviolet cross-linker (Stratagene UV Stratalinker 2400; Stratagene, La Jolla, CA). Specific probes (45-mer) for human *c-fos* 5'-CAGCGGGAGGATGACGCTCGTAGTCTGCGTTGAAGCCCGAGACC-3' and human *c-jun* 5'-GCATGAGTTGGCACCCACTGTTAACGTGGTTCATGACTTCTGTT-3' were obtained from Genosys (The Woodlands, TX). Probes were labeled with ³²P using 3'-end labeling kit (Amersham Biosciences). Blots were subsequently stripped and reprobed with ³²P-labeled β -actin as internal control. Initial experiments optimized conditions and showed that *c-fos* mRNA is increased in dermal fibroblasts treated with PDGF for 30 min compared with controls.

Assessment of the Effect of Antisense *c-jun* Oligonucleotide on PDGF-Stimulated Proliferation of Fibroblasts. To verify the

involvement of *c-jun* in the stimulation of proliferation by PDGF, experiments were conducted using antisense oligodeoxynucleotides (ODNs) according to the method of Yoshida et al. (1997). An antisense phosphothioate analog of the oligonucleotides to the 5'-end of *c-jun* was used. The ODN was obtained from Genosys, and the sequence is as follows: 5'-GCAGTCATAGAACAGTCCGTCACCTC-3'. This antisense *c-jun* oligonucleotide specifically decreased cellular levels of c-Jun as assessed on SDS-polyacrylamide gel using a specific c-Jun antibody (Yoshida et al., 1997). A *c-jun* thio missense was used as a control. This ODN was obtained from Genosys, and the sequence is as follows: 5'-CTTCATCATAGCGGAACT-3'. To assess the role of *c-jun* in the stimulation of proliferation, fibroblasts that had been grown in Dulbecco's medium containing 10% CPSR-1 were transferred to Dulbecco's medium containing 10% CPSR-2 24 h before assay. The cells were then trypsinized and plated onto 96-well plates at a density of 40,000 cells per ml in a total volume of 200 μ l. The Dulbecco's medium was removed 24 h later, and cells were incubated with *c-jun* antisense ODN in 150 mM NaCl. The *c-jun* antisense ODN was administered in 20- μ l volume in concentrations ranging from 2 nM to 20 μ M. After this, 80 μ l of Dulbecco's medium supplemented with 0.5% CPSR-2 was added to each well. The plates were then incubated for 3 h at 37°C, after which PDGF was added to the wells in DMEM plus 0.5% CPSR-2 (final concentration of PDGF, 8 ng/ml). Incubation was continued for 24 h, of which tritiated thymidine was added to each well during the last 2 h. At 24 h, cells were harvested as described above for the fibroproliferation assay. Trypan blue exclusion tests were done to test for potential toxicity. Incubation of cells with *c-jun* antisense ODN (up to 20 μ M) did not affect viability as assessed by trypan blue exclusion.

Immunocytochemistry for c-Jun. Human fibroblasts (F8 cells) were plated onto 96-well plates at a density of 8000 cells per well in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. After 24 h, the medium was changed to Dulbecco's modified Eagle's medium with 0.5% fetal calf serum. The cells were then incubated with *c-jun* antisense oligonucleotide (10 μ M) for 24 h and treated with PDGF (8 ng/ml) for 2 h. In preliminary experiments, human fibroblast cells were treated with PDGF for 30 min, and 1, 2, and 3 h, and a peak in the PDGF-induced c-Jun-positive immunoreactivity occurred at 2 h. For all experiments reported here, the 2-h time period was used as the optimum stimulation time with PDGF. After treatment, the cells were fixed with paraformaldehyde (4%), washed extensively with phosphate-buffered saline, and incubated with primary antibody to c-Jun (Oncogene Science, Cambridge, MA) using 50 μ l per well of a 1:500 dilution. After overnight incubation, the primary antibody was removed, and cells were washed and incubated with the secondary antibody overnight. Cells were then washed and incubated with ExtrAvidin peroxidase (1:250; 50 μ l per well; Sigma-Aldrich) for 2 h before addition of the chromagen diaminobenzidine. For the transfection experiments, human fibroblast and PC-12 cells were transfected with a wild-type *c-jun* plasmid that carried a CMV promoter, resulting in high expression of *c-jun* and translation of c-Jun in these cell types after transfection (or mutant *c-jun* plasmid, which could not be phosphorylated in serine 73). All experiments were done using immunocytochemistry and an antibody directed against c-Jun (PC06; Oncogene Science), and the result was verified using another antibody to c-Jun (New England Biolabs, Beverly, MA). An antibody directed at the serine 73 phosphospecific c-Jun (serine 73; New England Biolabs) was used to assess phosphospecific c-Jun. In all instances, and in all cells, the specificity of these antibodies was determined using the mutant serine 73 *c-jun* plasmid and the wild-type *c-jun* plasmid.

Transfection of Cells with *c-jun* Plasmids. PC-12 cells, fibroblast F8 cells, and SK-N-SH neuroblastoma cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium. For PC-12 cells, the medium was supplemented with 6% horse serum, 6% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin and cultured at 7.5% CO₂ at 37°C. For

F8 fibroblast cells, cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, supplemented with 10% antibiotic antimycotic, and grown on tissue culture plastic at 5% CO₂ at 37°C. The PC-12 cells, F8 fibroblast cells, and SK-N-SH neuroblastoma cells were transiently transfected using Fugene 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Using Fugene, these cells were transfected with plasmids containing either *c-jun* (pAAV/CMV-*c-jun*) or mutant *c-jun* serine 73 plasmid, the *c-jun* plasmid construct previously described (Xu et al., 1997). The cells were plated on 96-well plates, and in the case of PC-12 cells, the 96-well plate was coated with collagen. The PC-12 cells were plated in the 96-well plates at a density of 6×10^3 cells per well. The cells were incubated for 24 h, and the medium was replaced with OptiMEM medium (Invitrogen). The F8 fibroblast cells and SK-N-SH neuroblastoma cells were plated in 96-well plates at a density of 8×10^3 cells per well. To carry out the transfection, the Fugene transfection reagent was preincubated with Opti-MEM medium for 5 min at room temperature according to the manufacturer's protocol and was then added, dropwise, to 1 μ g of DNA. The Fugene DNA complex was then incubated for 15 min at room temperature. After this 15-min period, 5 μ l of the Fugene complex was added to each well containing 100 μ l of DMEM and incubated for 24 or 72 h with and without the addition of pentoxifylline as described previously. After the 24- or 72-h incubation, the cells were fixed (4% paraformaldehyde in 0.1 M phosphate buffer for 15 min), and then immunocytochemistry was carried out using antibodies to c-Jun (Oncogene Science) or serine 73-phosphorylated c-Jun (New England Biolabs) to assess protein expression. For observation of neurite process outgrowth in PC-12 cells, the 72-h time point was chosen because previous studies had indicated that 72 h was sufficient to produce neurite outgrowth in cells that were transfected with *c-jun* plasmid.

Western Analysis. To support and confirm the immunocytochemistry results, cell lysates were prepared in Laemmli sample buffer, boiled for 5 min, and 25 μ g of protein resolved on 10% SDS-polyacrylamide gel electrophoresis. In transfection experiments, cell lysates containing 12.5 and 6.25 μ g of protein were used. The proteins were transferred to membranes and probed with antibodies against c-Jun and serine 73-phosphorylated c-Jun, and the protein band was visualized using LumiGlo (New England Biolabs). Protein was measured using a Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hercules, CA), and equal loading was confirmed by Coomassie staining of blots.

Statistical Analysis. An unpaired Student's *t* test was used to compare two variables, and analysis of variance and Student-Newman-Keuls test were used when more than two variables were compared (Zar, 1974).

Results

The expression of *c-fos* and *c-jun* immediate early genes was assessed in skin fibroblasts stimulated to proliferate with PDGF in the presence and absence of pentoxifylline compared with controls (cells stimulated with basal medium alone). The results in Fig. 1 show the effect of exogenous PDGF (8 ng/ml) alone and in combination with pentoxifylline on *c-fos* and *c-jun* mRNA levels in skin fibroblasts. Incubation of fibroblasts with PDGF for 30 min increased *c-fos* and *c-jun* expression in skin fibroblasts. Phosphorimaging analysis showed that PDGF increased *c-fos* and *c-jun* mRNA 20- and 7-fold, respectively. Pentoxifylline did not alter *c-fos* or *c-jun* mRNA levels in control cells and did not reduce the level of *c-fos* mRNA in cells treated with PDGF. However, pentoxifylline markedly reduced *c-jun* mRNA levels in cells treated with PDGF. Phosphorimaging analysis showed that pentoxifylline decreased *c-jun* mRNA (53%) in cells treated

with PDGF compared with cells treated with PDGF alone. Figure 1 shows the same blot probed with a β -actin probe and indicates that there was equal loading in all four lanes. Figure 1B shows the phosphorimaging analysis of results obtained from three independent experiments and indicates that PDGF significantly increased the expression of *c-jun* and *c-fos* and that pentoxifylline significantly reduced the expression of *c-jun* but did not alter *c-fos* expression induced by PDGF. Cells treated in the same manner were also assessed for fibroproliferative activity. The results in Fig. 2 verify that PDGF stimulated proliferation and pentoxifylline blocked proliferation of fibroblasts under the conditions reported in Fig. 1.

The antiproliferative effect of a 30-mer *c-jun* antisense

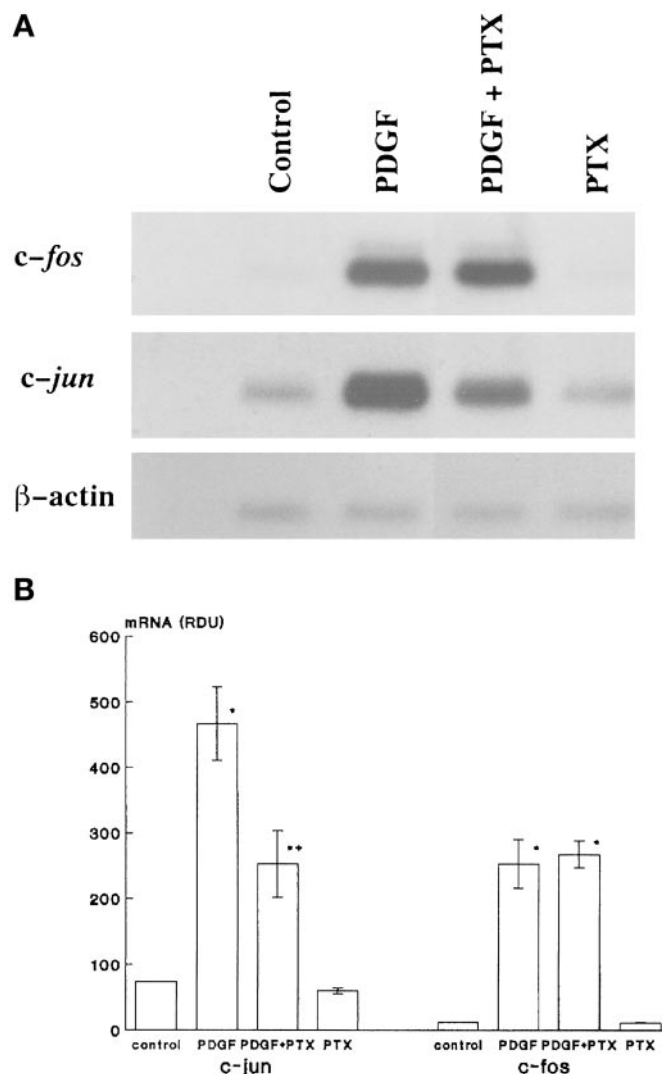


Fig. 1. Effect of PDGF with and without pentoxifylline on the expression of *c-fos* and *c-jun*. A, lane 1 contains mRNA isolated from fibroblasts incubated in basal medium. Lane 2 contains mRNA isolated from fibroblast samples stimulated with PDGF. Lane 3 shows the mRNA for skin fibroblasts stimulated with PDGF plus simultaneous pentoxifylline (3.5 mM). Lane 4 shows the mRNA levels in skin fibroblasts stimulated with pentoxifylline alone. The blot was probed with a 45 mer for *c-fos*, a 45 mer for *c-jun*, and an oligomer for β -actin. The results shown are representative of three independent experiments. B, the results obtained from phosphorimaging analysis, combining the results of the three independent experiments. *, significantly different ($p < 0.05$) compared with control or PTX alone; +, significantly different ($p < 0.05$) compared with PDGF.

ODN was assessed on human dermal fibroblast cells that had been stimulated to proliferate with PDGF (8 ng/ml). Treatment of dermal fibroblasts with *c-jun* antisense ODN significantly reduced PDGF-stimulated proliferation by 85% (Fig. 3A). This almost total block of PDGF-stimulated proliferation by *c-jun* antisense indicates that *c-jun* plays a critical role in PDGF-stimulated proliferation. Our results indicate that treatment of cells with *c-jun* antisense oligonucleotides (2–20 μ M) blocked PDGF-stimulated proliferation but also inhibited basal proliferation. Incubation of cells with a lower concentration of antisense oligonucleotide (1 μ M) inhibited PDGF-stimulated proliferation (35%) but did not affect basal proliferation (Fig. 3B). Treatment of cells with antisense *c-jun* ODN (in concentrations up to 20 μ M) was not toxic, as determined by trypan blue exclusion. Cells were also treated with *c-jun* thio missense, and results indicated that *c-jun* thio missense did not inhibit PDGF-stimulated proliferation (Fig. 3C) compared with an equimolar concentration of *c-jun* antisense, which significantly reduced PDGF-stimulated proliferation.

Cells were incubated with *c-jun* antisense (10 μ M) for 24 h before treatment with PDGF (8 ng/ml) to determine the effect of *c-jun* antisense on c-Jun immunoreactivity in fibroblast cells. In preliminary experiments, cells were incubated with PDGF for 30 min, and 1, 2, and 3 h. In all subsequent experiments, the cells were incubated with PDGF for 2 h, a time point that was sufficient to produce a strong positive immunoreactivity with a specific antibody to c-Jun (Fig. 4B) compared with control (Fig. 4A). The c-Jun-positive immunoreactivity was significantly reduced by preincubation of cells for 24 h with *c-jun* antisense at a concentration of 10 μ M (Fig. 4C). These results indicate that PDGF increases the amount of c-Jun protein and that the *c-jun* antisense oligonucleotide selectively decreases the c-Jun protein, which is produced by incubation with PDGF, suggesting that this antisense oligonucleotide is selective and effective.

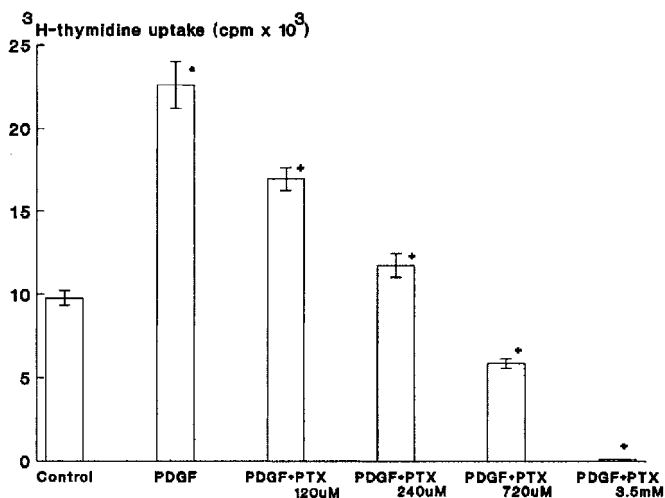


Fig. 2. The effect of pentoxifylline on PDGF-stimulated proliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts in counts per minute. PTX was assessed at the concentrations indicated; PDGF was tested at 8 ng/ml. Results were expressed as mean \pm S.E. Each treatment was done on wells in quadruplicate, and experiments were repeated. Control represents fibroblast proliferation stimulated by DMEM alone. *, significantly different ($p < 0.05$) compared with fibroblasts without PDGF; +, significantly different ($p < 0.05$) compared with fibroblasts stimulated with PDGF in the absence of pentoxifylline.

The results in Fig. 5 show the effect of combination of pentoxifylline and *c-jun* antisense on PDGF-stimulated proliferation. Pentoxifylline (240 μ M) inhibited PDGF-stimulated proliferation by 47%. Preincubation of fibroblasts with *c-jun* antisense (1 μ M) inhibited PDGF-stimulated proliferation by 42%. Combination of pentoxifylline (240 μ M) and *c-jun* antisense (1 μ M) decreased PDGF-stimulated proliferation by 69%.

Treatment of human fibroblast cells with PDGF increased the immunocytochemical staining for c-Jun. PDGF also increased the immunocytochemical staining for serine 73-phosphorylated c-Jun (Fig. 6). Figure 6 shows the effect of 8 ng/ml PDGF (Fig. 6B) compared with 32 ng/ml PDGF (Fig. 6C) compared with control (Fig. 6A) compared with control plus pentoxifylline (Fig. 6D) compared with 8 ng/ml PDGF plus pentoxifylline (Fig. 6E) compared with 32 ng/ml PDGF plus pentoxifylline (Fig. 6F). All PDGF treatment was for 2 h. Serine 73 phosphorylated c-Jun immunoreactivity was detected using the specific antibody to phosphospecific c-Jun described under *Materials and Methods*. These results clearly indicate that PDGF treatment for 2 h at 8 or 32 ng/ml increases the serine 73 phosphorylated c-Jun-positive immunoreactivity in F8 fibroblast cells and that pentoxifylline (3.5 mM) blocks the positive serine 73 phosphorylated c-Jun immunoreactivity in F8 fibroblast cells that have been stimulated with PDGF (8 or 32 ng/ml). These results suggest that pentoxifylline either inhibits the activation of c-Jun to phosphorylated c-Jun or enhances the dephosphorylation of phosphorylated c-Jun.

We then transfected F8 fibroblast cells with a plasmid containing wild-type *c-jun* (pAAV/CMV-*c-jun*). The F8 cells could be transfected, but the transfection efficiency was not as high in human fibroblasts as in the PC-12 cell line. To further explore the effect of pentoxifylline on c-Jun phosphorylation, we used the PC-12 cells transfected with the wild-type *c-jun* plasmid. Results indicate that transfection with wild-type *c-jun* plasmid dramatically increased the immunocytochemical staining for c-Jun. The results illustrated in Fig. 7 show successful transfection with *c-jun* wild-type plasmid and describe the effect of treatment with pentoxifylline and treatment with *c-jun* antisense on serine 73 phosphospecific c-Jun-positive immunoreactivity as assessed using a specific antibody to phosphospecific serine 73 c-Jun. Positive serine 73 phosphospecific c-Jun immunoreactivity is seen in nuclei of cells 72 h after transfection with wild-type *c-jun* plasmid (Fig. 7A) in contrast to a total lack of serine 73 phosphospecific c-Jun immunoreactivity when PC-12 cells were nontransfected (Fig. 7D) and a total lack of serine 73 phosphospecific c-Jun immunoreactivity in PC-12 cells transfected with a serine 73 mutant *c-jun* plasmid (Fig. 7E). These results verify the transfection of PC-12 cells with *c-jun* wild-type plasmid and its detection by immunocytochemistry and verify the specificity of the antibodies. Our results also indicate that treatment of cells with *c-jun* antisense significantly reduced the amount of serine 73 c-Jun immunoreactivity in PC-12 cells. This represents an approximate 40% decrease in the phosphoserine 73 c-Jun-positive immunoreactivity in the *c-jun* wild-type plasmid-transfected PC-12 cells due to treatment with *c-jun* antisense (Fig. 7B). This figure also shows the effect of treatment of cells with pentoxifylline (Fig. 7C). The results indicate that treatment with pentoxifylline completely eliminated the phosphoserine 73 c-Jun-positive im-

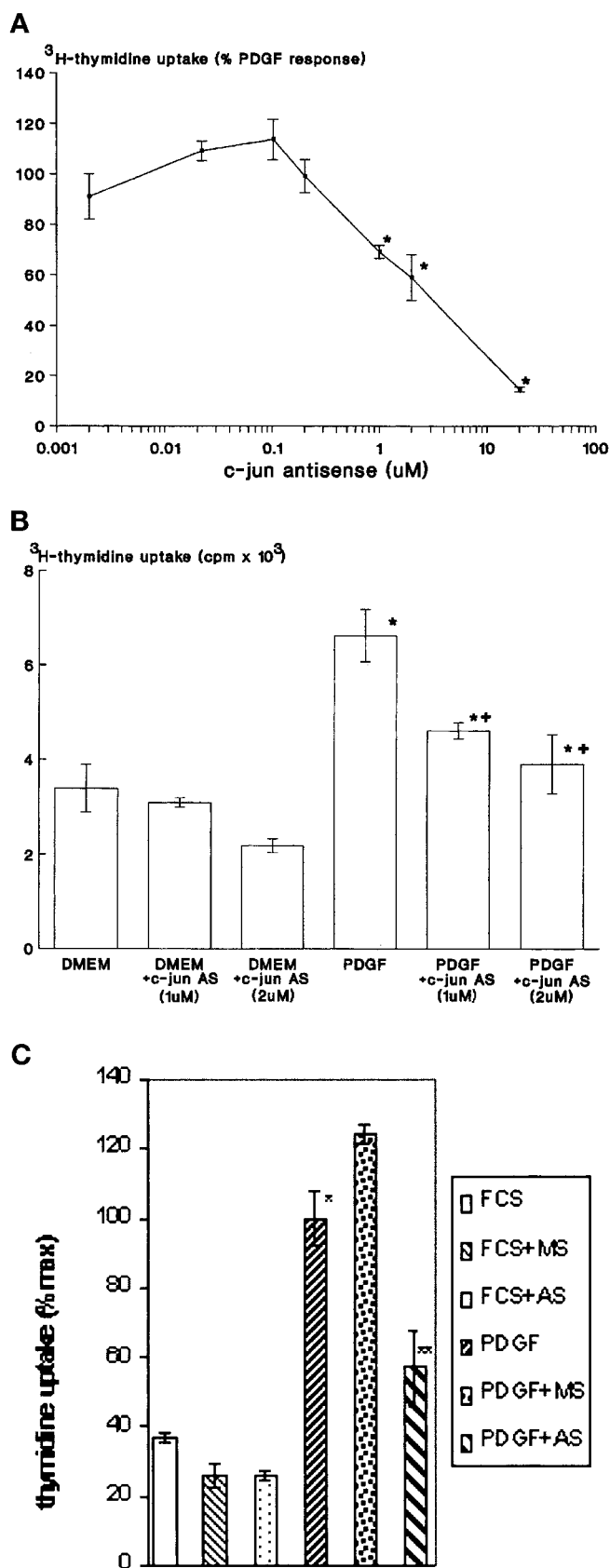


Fig. 3. A, the effect of antisense *c-jun* oligonucleotides on PDGF-stimulated proliferation of human fibroblasts. Fibroproliferation was assessed as tritiated thymidine uptake by human fibroblasts in counts per minute. Human fibroblasts were incubated with antisense *c-jun* oligonucleotides (0.002–20 μM) for 3 h before addition of PDGF (8 ng/ml). The antisense

munoreactivity in PC-12 cells that had been transfected with *c-jun* wild-type plasmid. Cells transfected with serine 73 mutant *c-jun* plasmid demonstrate positive c-Jun immunoreactivity as assessed using a specific antibody to c-Jun (Fig. 7F) and do not demonstrate immunoreactivity with a specific phosphoserine 73 c-Jun antibody (Fig. 7E). The results illustrate that the serine 73 phospho-specific c-Jun antibody identifies only specific cells in which the c-Jun has been phosphorylated after transfection with the *c-jun* plasmid. The results shown in Fig. 7 have been reproduced on three separate occasions, using separate preparations of PC-12 cells and different preparations of transfection agent as well as separate preparations of pentoxifylline and *c-jun* antisense.

Results obtained 24 h after transfection with the wild-type *c-jun* plasmid in another series of transfection experiments using PC-12 cells are illustrated in Fig. 8 and show the phosphospecific serine 73 c-Jun-positive immunoreactivity in PC-12 cells that were transfected with the wild-type *c-jun* plasmid (Fig. 8B). The c-Jun-positive immunoreactivity in cells transfected with *c-jun* wild-type plasmid (Fig. 8B) is compared with PC-12 cells that were not transfected (Fig. 8A), and it is evident that there is marked positive serine 73 phospho-specific c-Jun immunoreactivity in the PC-12 cells (Fig. 8B). This figure also demonstrates the effect of pretreatment with pentoxifylline for 24 (Fig. 8D) or 3 h (Fig. 8F) before transfection with wild-type *c-jun* plasmid. It is evident that there is a marked decrease in phosphoserine 73 c-Jun-positive immunoreactivity in PC-12 cells that have been pretreated for 24 h with pentoxifylline before transfection with wild-type *c-jun* (Fig. 8D). It is also evident that 3 h of pretreatment with pentoxifylline before transfection with wild-type *c-jun* plasmid also dramatically decreases the phosphoserine 73 c-Jun-positive immunoreactivity in PC-12 cells (Fig. 8F). These results were highly reproducible among experiments assessed on three separate occasions, using different preparations of PC-12 cells and separate transfections with *c-jun* wild-type plasmid. Figure 8B shows 110 positive phosphoserine 73 c-Jun immunoreactive PC-12 cells, whereas Fig. 8D shows approximately 10 positive phosphoserine 73 c-Jun immunoreactive PC-12 cells. This represents a >90% decrease in the phosphoserine 73 c-Jun-positive immunoreactivity in cells treated with pentoxifylline for 24 h before *c-jun* wild-type plasmid transfection. The results in Fig. 8F are very similar to the results shown in Fig. 8D and again illustrate a dramatic decrease in the phosphoserine 73-positive immunoreactivity in transfected cells that are pretreated for 3 h with pentoxifylline, such that only 10 positive serine 73 phosphospecific c-Jun immunoreactive

c-jun was in 150 mM NaCl, so controls received the same volume of 150 mM NaCl. Results were expressed as percentage of PDGF response (mean ± S.E.). Each treatment was done on wells in quadruplicate, and experiments were repeated. *, significantly different ($p < 0.05$) compared with corresponding fibroblasts that were not treated with antisense *c-jun* oligonucleotides. B, the effect of antisense *c-jun* oligonucleotides (1 and 2 μM) on PDGF-stimulated proliferation of human fibroblasts is compared with the effect of antisense *c-jun* oligonucleotides on basal (DMEM-stimulated) proliferation. Results were expressed as mean ± S.E. *, significantly different ($p < 0.05$) compared with DMEM control; +, significantly different ($p < 0.05$) compared with PDGF alone. C, the effect of missense *c-jun* (1 μM) on PDGF-stimulated proliferation compared with antisense *c-jun* (1 μM). Results were expressed as percentage of PDGF response (mean ± S.E.). *, significantly different ($p < 0.05$) compared with DMEM control; +, significantly different ($p < 0.05$) compared with PDGF alone.

PC-12 cells are evident in Fig. 8F. Treatment of PC-12 cells with pentoxifylline alone for 24 (Fig. 8C) or 3 h (Fig. 8E) before sham transfection indicates that the background activity is negligible. The graphical representation below the six panels (Fig. 8G) shows the relationship between the phosphoserine 73 *c-Jun*-positive immunoreactivity in PC-12 cells that were transfected (Fig. 8G, T) with wild-type *c-jun* plasmid compared with nontransfected cells (Fig. 8G, non-T) and cells that have been pretreated with pentoxifylline (PTX) for 24 h (T + PTX-24 h) or 3 h (T + PTX-3 h) before transfection as well as cells that were nontransfected but received treatment with pentoxifylline for 24 h (PTX-24 h) or 3 h (PTX-3 h) before sham transfection.

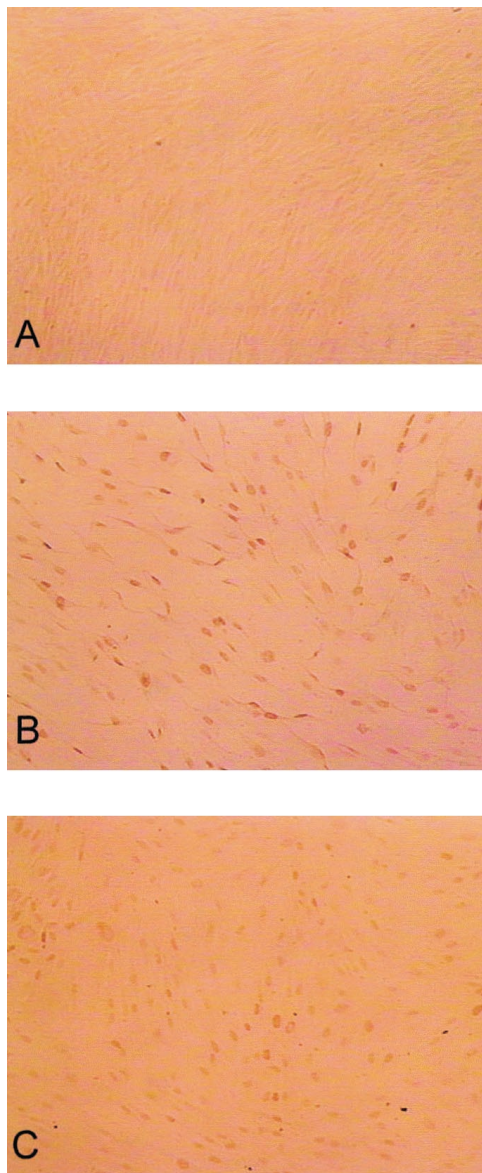


Fig. 4. Immunocytochemistry of human fibroblasts using *c-Jun* antibody. This figure demonstrates the marked positive immunoreactivity to *c-Jun* antibody in fibroblasts treated with PDGF for 2 h (B) compared with untreated cells (A). The figure also shows the effect of *c-jun* antisense treatment on the PDGF-stimulated *c-Jun* immunoreactivity (C). The effect of pretreatment with *c-jun* antisense (10 μ M) plus PDGF (8 ng/ml) for 2 h on *c-Jun*-positive immunoreactivity is assessed in F8 fibroblast cells using an antibody specific for the detection of *c-Jun*. The concentration of *c-jun* antisense ODN was 10 μ M. Original magnification, 100 \times .

Western blot analysis was carried out to support and confirm the results obtained using immunocytochemistry. Cell lysates were prepared from fibroblasts treated with PDGF or PDGF plus pentoxifylline, and Western blots were assessed with selective antibodies to *c-Jun* and serine 73-phosphorylated *c-Jun*. The results shown in Fig. 9 are representative of four experiments and indicate that pentoxifylline treatment decreased the serine 73-phosphorylated *c-Jun*, thereby confirming the immunocytochemical results. Blots were analyzed using ScionImage (Scion Corp., Frederick, MD), and densitometry analysis indicates that pentoxifylline treatment reduced serine 73-phosphorylated *c-Jun* induced by PDGF (Fig. 9B).

In Fig. 10A, we show a representative Western blot, which indicates that serine 73-phosphorylated *c-Jun* is elevated in PDGF-treated fibroblasts (lanes 3 and 5) compared with our control fibroblasts treated only with basal medium, DMEM with 1% FCS (lane 1). Treatment of cells with pentoxifylline reduced the serine 73-phosphorylated *c-Jun* in PDGF-treated fibroblasts (lane 4). Pentoxifylline treatment resulted in a modest reduction in phosphorylated *c-Jun* in control (DMEM with 1% FCS-treated) cells (lane 2). In Fig. 10B, we show the results (means \pm S.E. of four experiments) of densitometry analysis, using Scion image of the ECL from Western blots [expressed as relative density units (RDU)], which show the effect of PDGF and pentoxifylline on serine 73-phosphorylated *c-Jun* and *c-Jun* protein in fibroblasts. The results indicate that serine 73-phosphorylated *c-Jun* was significantly increased (78%) in PDGF-treated fibroblasts compared with fibroblasts treated only with DMEM plus 1% FCS. The results also indicate that pentoxifylline significantly reduced serine 73-phosphorylated *c-Jun* in PDGF-treated fibroblasts by 58% compared with fibroblasts treated only with PDGF. The results in Fig. 10B illustrate that

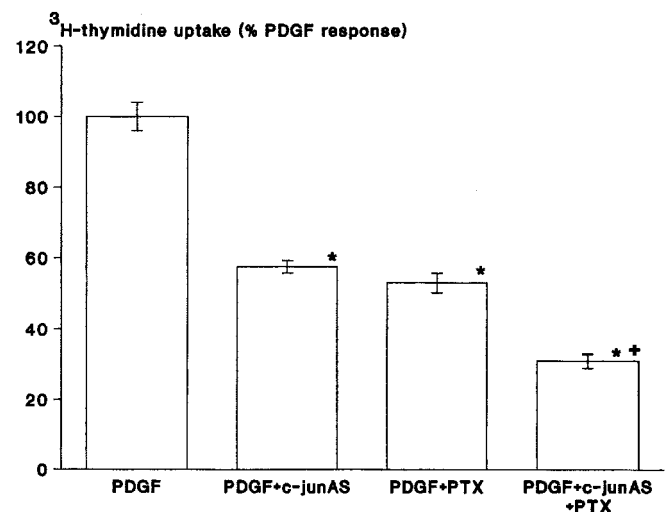


Fig. 5. The effect of combination of pentoxifylline and *c-jun* antisense on PDGF-stimulated proliferation. Fibroproliferation was assessed as tritiated thymidine uptake by fibroblasts in counts per minute. Fibroblasts were preincubated with *c-jun* antisense (1 μ M) for 3 h before addition of other factors. Pentoxifylline was assessed at 240 μ M. Results were expressed as percentage of PDGF response (mean \pm S.E.). Each treatment was done on wells in quadruplicate, and experiments were repeated. PDGF significantly stimulated fibroproliferation compared with fibroblasts incubated with control medium. *, significantly different ($p < 0.05$) compared with fibroblasts stimulated with PDGF; +, significantly different ($p < 0.05$) compared with PDGF plus PTX (240 μ M).

although the effects on c-Jun protein were much less than on the serine 73-phosphorylated c-Jun, PDGF treatment significantly increased c-Jun protein by 13% compared with control (DMEM plus 1% FCS). These results support and corroborate the results obtained using immunocytochemistry (Figs. 4 and 6) where PDGF treatment increased the positive immunoreactivity to c-Jun antibody and serine 73-phosphorylated c-Jun antibody in fibroblasts. These results also support the results in Fig. 1 showing that PDGF increased the *c-jun* mRNA in fibroblasts compared with controls. The results in Fig. 10B also indicate that pentoxifylline significantly reduced the c-Jun in PDGF-treated fibroblasts by 12%. These results support and corroborate the results obtained using immunocytochemistry (Fig. 6) where pentoxifylline treatment inhibited the effect of PDGF treatment on the positive immunoreactivity to c-Jun antibody and serine 73-phosphorylated c-Jun antibody in fibroblasts. These results also support the results in Fig. 1 showing that pentoxifylline inhibited the effect of PDGF on the *c-jun* mRNA in fibroblasts compared with controls. The results in Fig. 10B also show that pentoxifylline did not affect c-Jun in control (DMEM

plus 1% FCS-treated) fibroblasts, and this supports the data shown in Fig. 1 indicating that pentoxifylline did not affect *c-jun* mRNA in controls.

In Fig. 11A, we show a representative Western blot, which indicates that c-Jun protein and serine 73-phosphorylated c-Jun are increased in PC-12 cells that have been transfected with wild-type *c-jun* plasmid (lanes 2 and 5). These results support and corroborate the results obtained using immunocytochemistry (Figs. 7 and 8) where PC-12 cells that were transfected with wild-type *c-jun* plasmid show increased levels of positive immunoreactivity to c-Jun antibody and serine 73-phosphorylated c-Jun antibody. Treatment of cells with pentoxifylline markedly reduced the serine 73-phosphorylated c-Jun in PC-12 cells that were transfected with wild-type *c-jun* plasmid (lanes 3 and 6). In Fig. 11B, we show the results (means of four experiments) of densitometry analysis using Scion image of the ECL from Western blots (expressed as RDU) showing the significant increase in serine 73-phosphorylated c-Jun in PC-12 cells transfected with wild-type *c-jun* plasmid and indicate that pentoxifylline treatment significantly decreased serine 73-phosphorylated c-Jun in

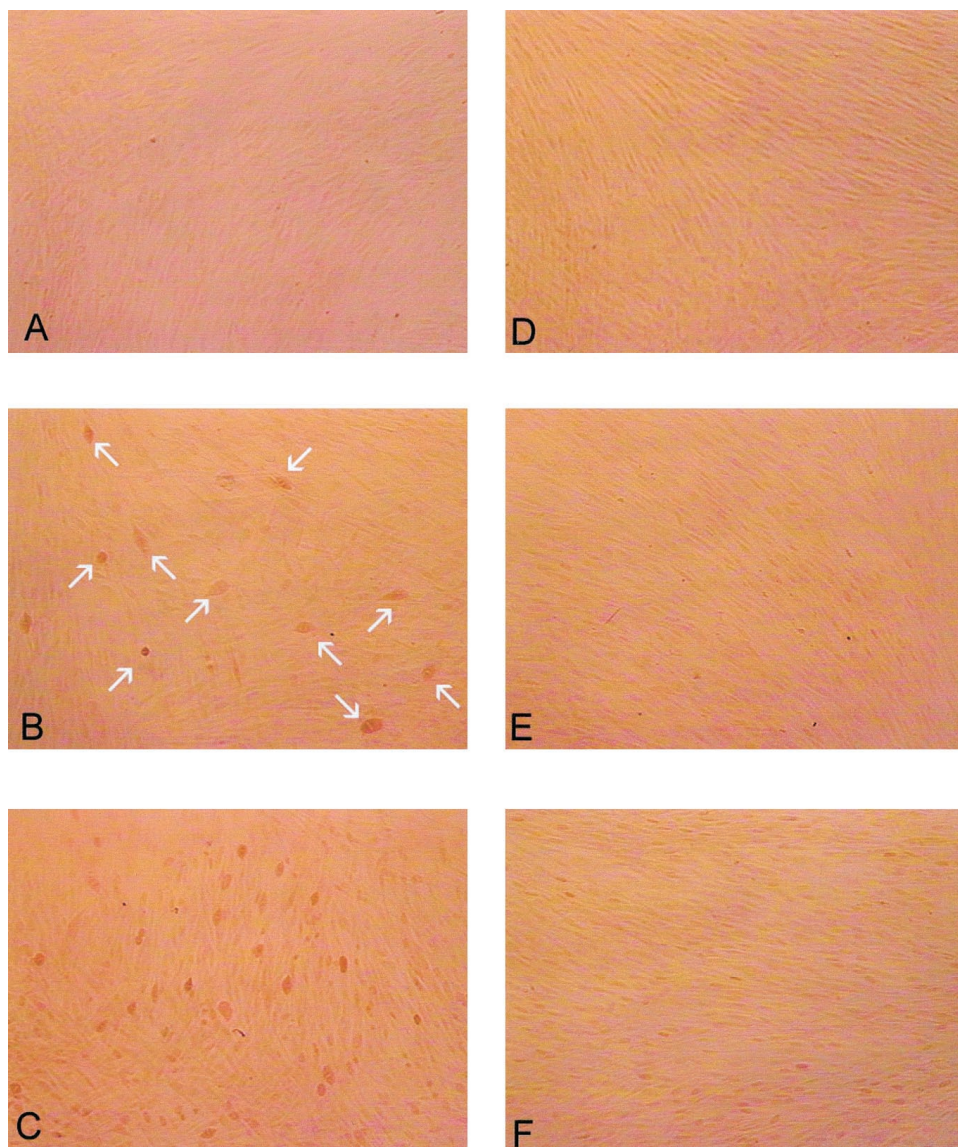


Fig. 6. The effect of PDGF at two concentrations on serine 73 phospho c-Jun-positive immunoreactivity in F8 fibroblast cells. This figure shows the effect of 8 ng/ml PDGF (B) compared with 32 ng/ml PDGF (C), compared with control (A), compared with control plus pentoxifylline (D), compared with 8 ng/ml PDGF plus pentoxifylline (E), compared with 32 ng/ml PDGF plus pentoxifylline (F). All PDGF treatment was for 2 h. Pentoxifylline was studied at a concentration of 3.5 mM. Serine 73 phospho c-Jun immunoreactivity is shown as detected using the phosphospecific c-Jun antibody described under *Materials and Methods*. Original magnification, 100 \times .

PC-12 cells that have been transfected with wild-type *c-jun* plasmid. These results support and corroborate the results obtained using immunocytochemistry (Figs. 7 and 8) where PC-12 cells that were transfected with wild-type *c-jun* plasmid and treated with pentoxifylline show decreased levels of positive immunoreactivity to serine 73-phosphorylated c-Jun antibody compared with transfected cells.

Discussion

The results described here, using immunocytochemistry and Western analysis, indicate that pentoxifylline decreases phosphorylation of c-Jun on serine 73. Previously we reported that PDGF stimulates proliferation of skin fibroblasts (Peterson, 1993; Peterson et al., 1994), myofibroblasts (Isbrucker and Peterson, 1995), and intestinal smooth muscle cells (Peterson and Tanton, 1996). Pentoxifylline inhibited PDGF-stimulated fibroproliferation in a dose-related manner (Peterson et al., 1994) with an IC_{50} value similar to trapidil (Peterson et al., 1994), a drug reported to act at the PDGF receptor (Kuratsu and Ushio, 1990), but pentoxifylline does not compete for the PDGF receptor (Slysz and Peterson, 1994). Under the conditions used in these experiments, the

cells do not show signs of toxicity when exposed to pentoxifylline. If the pentoxifylline is washed off, the cells continue to grow; thus, it appears to be a reversible effect of pentoxifylline on cell proliferation. Interference in the postreceptor signaling of PDGF by pentoxifylline is a reasonable mechanism to investigate because pentoxifylline can increase intracellular cAMP (Meskini et al., 1994), and compounds that elevate cAMP have been reported to inhibit PDGF postreceptor signaling (Wu et al., 1993).

Our present results indicate that PDGF stimulates *c-fos* expression. These results are in agreement with other reports suggesting that PDGF will stimulate *c-fos* gene expression (Rosenwald et al., 1996). The stimulation of *c-fos* mRNA by PDGF is maintained when cells are exposed to pentoxifylline; however, PDGF-stimulated fibroproliferation is abolished when the cells are exposed to pentoxifylline. These results indicate that the action of pentoxifylline on PDGF-stimulated fibroproliferative activity is not via an effect on PDGF receptor signaling through *c-fos* or through *c-fos* by itself. In contrast to the lack of effect of pentoxifylline on *c-fos* in fibroblasts, pentoxifylline may decrease *c-fos* in myofibroblasts (Pinzani et al., 1996). The apparent discrepancy be-

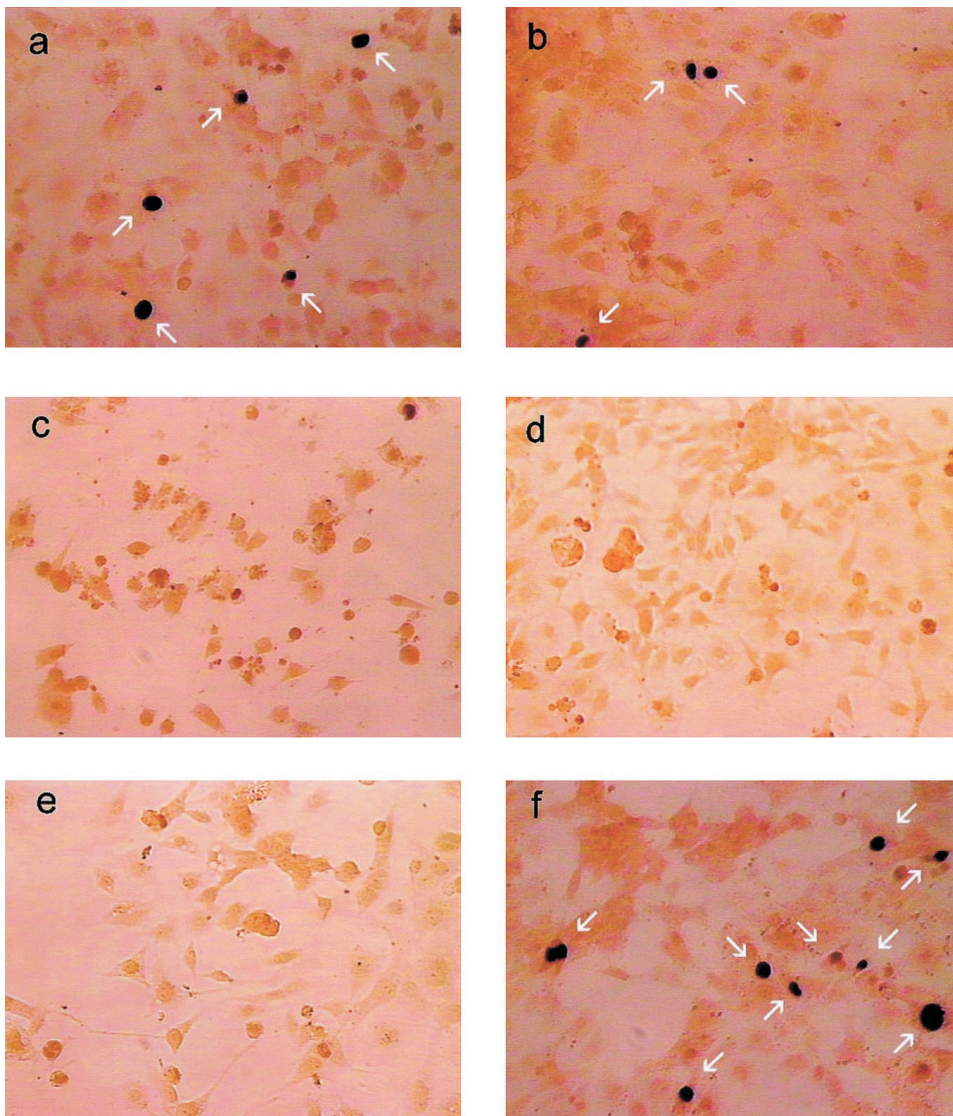


Fig. 7. The effect of pentoxifylline on *c-jun* in PC-12 cells transfected with wild-type *c-jun* plasmid or mutant plasmid. PC-12 cells were transfected with *c-jun* wild-type plasmid or serine 73 mutant *c-jun* plasmid, and the effects of treatment with pentoxifylline and *c-jun* antisense on serine 73 phosphospecific c-Jun-positive immunoreactivity and c-Jun-positive immunoreactivity were assessed using phosphospecific serine 73 c-Jun antibody (A–E) or c-Jun antibody (F). PC-12 cells were transfected with wild-type *c-jun* plasmid (A), transfected with *c-jun* wild-type plasmid and treated with *c-jun* antisense (B), treated with pentoxifylline (C), not transfected (D), transfected with a serine 73 mutant *c-jun* plasmid (E), and transfected with the serine 73 mutant plasmid (F). Pentoxifylline (3.5 mM); *c-jun* antisense ODN (10 μ M). Transfection was carried out as described under *Materials and Methods*. Original magnification, 200 \times .

tween these results relates to inherent differences in these two cell types, particularly in the gene regulatory region (Houglum et al., 1995).

Our results indicate that PDGF increases *c-jun* expression 7-fold over basal expression in human fibroblasts and are in

agreement with results reported in murine fibroblasts (Ryder and Nathans, 1988). Homodimers of the *c-jun* protein product or heterodimeric complexes of *jun-fos* form the AP-1 nuclear transcription factor. An increase in the expression of *c-jun* alone or *c-jun* and *c-fos* would increase the expression of

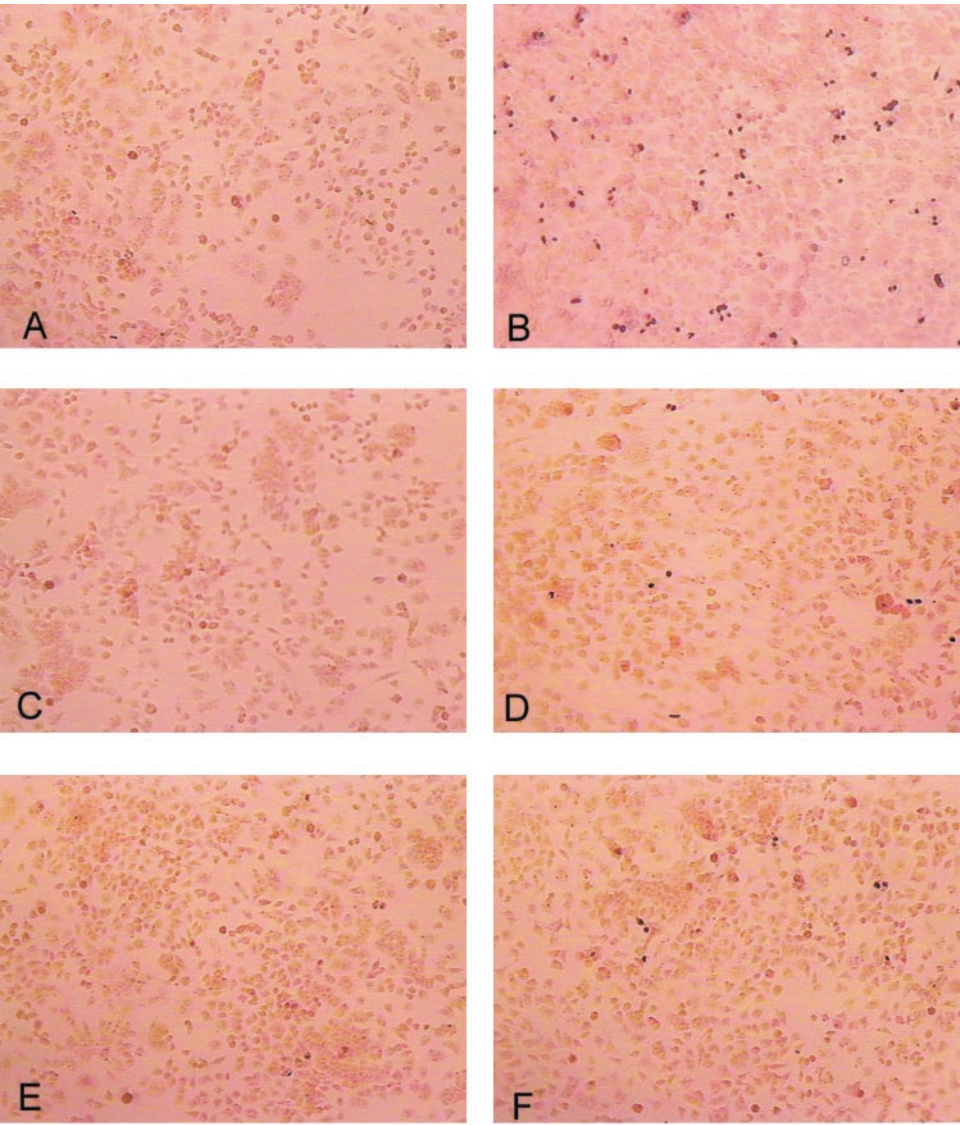
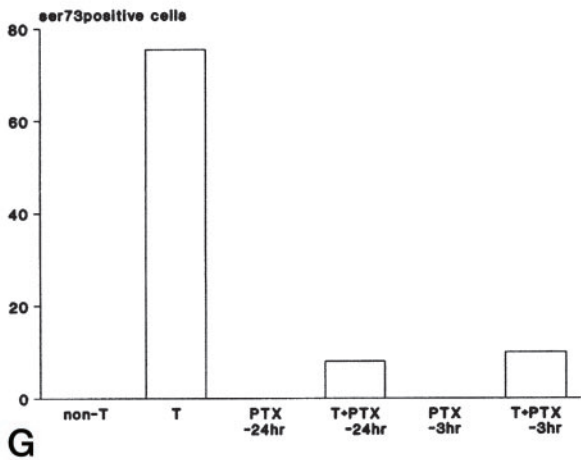


Fig. 8. The effect of pentoxifylline pretreatment on serine 73 *c-Jun* in transfected PC-12 cells. The phosphospecific serine 73 *c-Jun*-positive immunoreactivity is shown in PC-12 cells that were transfected with the wild-type *c-jun* plasmid (B) or not transfected (A), pretreated with pentoxifylline for 24 (D) or 3 h (F) before transfection with wild-type *c-jun* plasmid, or treated with pentoxifylline alone for 24 (C) or 3 h (E) before sham transfection. G, graph. Pentoxifylline was studied at a concentration of 3.5 mM, and cells were treated for either 3 or 24 h before transfection. Transfection was carried out as described under *Materials and Methods*. Original magnification, 100 \times .



genes possessing this regulatory element, including genes involved in proliferation (Slysz and Peterson, 1994). PDGF activates MAP kinase (Coso et al., 1995) and Jun kinase (Bogoyevitch et al., 1995), probably via an effect on Ras (Xie and Herschman, 1995). The results reported here clearly show that PDGF rapidly and reproducibly increased the gene expression of both *c-fos* and *c-jun*.

The elevation of *c-jun* after treatment with PDGF correlates with the increase in proliferative activity due to PDGF and may suggest a role for *c-jun* in the proliferative activity of PDGF but does not in itself reveal the importance of *c-jun* induction for PDGF-dependent cell growth. To establish a role for *c-jun* in the proliferative activity of PDGF, we used antisense technology. Previous studies indicate that this *c-jun* antisense (20 μ M) specifically decreased cellular levels of c-Jun in endothelial cells while not affecting production of other signaling proteins, including nuclear factor κ B and Sp1, as assessed by Western blot analysis (Yoshida et al., 1997). Our results indicate that a phosphothioate *c-jun* antisense oligonucleotide decreased cellular levels of c-Jun in human fibroblasts and effectively blocked PDGF-stimulated proliferation (Fig. 3), indicating that *c-jun* (and c-Jun) plays a critical role in PDGF-stimulated proliferation.

The results in Fig. 5 show that pentoxifylline inhibited PDGF-stimulated proliferation by 47% (to 5159 ± 265 cpm) and that *c-jun* antisense inhibited PDGF-stimulated proliferation by 42% (to 5582 ± 179 cpm). If the two drugs were additive, then one would predict that the combination of these drugs would reduce PDGF-stimulated proliferation by 69% (to approximately 2975 cpm). Our results indicate that the combination of pentoxifylline and *c-jun* antisense decreased PDGF-stimulated proliferation by 69% (to 2997 ± 208 cpm), thereby indicating that these drugs are additive and likely acting at the same site in the PDGF signaling pathway.

Our results show that pentoxifylline specifically inhibits PDGF-stimulated *c-jun* gene expression. An inhibition of *c-jun* would likely affect other cytokines and growth factors, which signal through a *c-jun* pathway. Fibroblast growth factor (Yoshida et al., 1997), insulin-like growth factor-1 (Rhoads et al., 1997), epidermal growth factor (Heinrich and Kraiem, 1997), endothelin-1 (Salamonsen et al., 1997), oncostatin M (Isozaki et al., 1997), nerve growth factor (Courtney et al., 1997), tumor necrosis factor α (Yoshida et al., 1997), and interleukin-1 α and β (Lu et al., 1997) activate Jun kinase and increase *c-jun* gene expression. Modifying the actions of these cytokines and growth factors by targeting *c-jun* suggests other therapeutic uses for pentoxifylline.

The results in Fig. 2 show the effect of pentoxifylline on PDGF-induced cell proliferation. It is impossible to com-

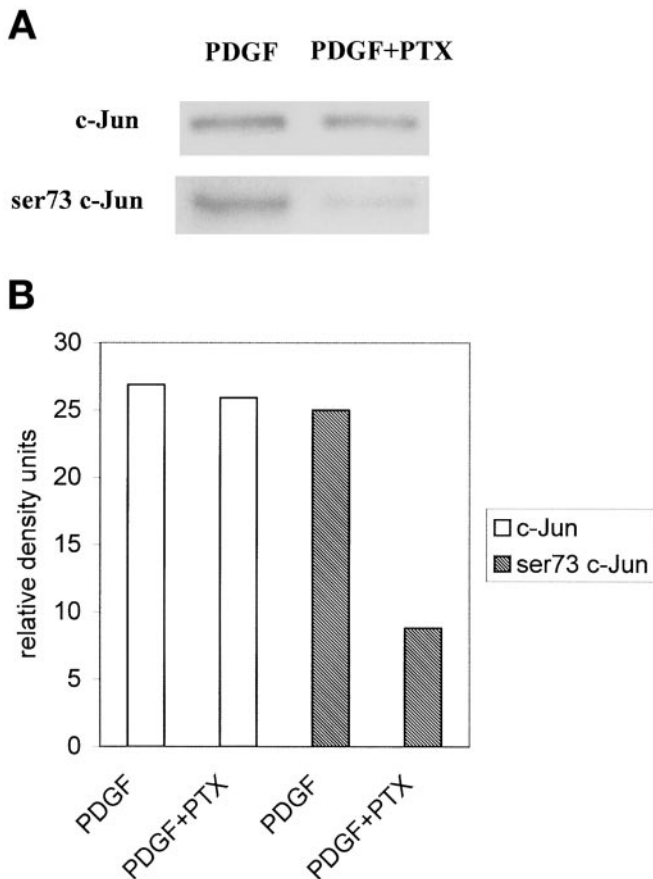


Fig. 9. Effect of PDGF and pentoxifylline on c-Jun and serine 73 phospho c-Jun. A, representative Western blot of cell lysates prepared from fibroblasts treated with PDGF (8 ng/ml for 2 h) with and without pentoxifylline (3.5 mM). This experiment was repeated four times with the same results. B, densitometry of bands analyzed using Scion Image.

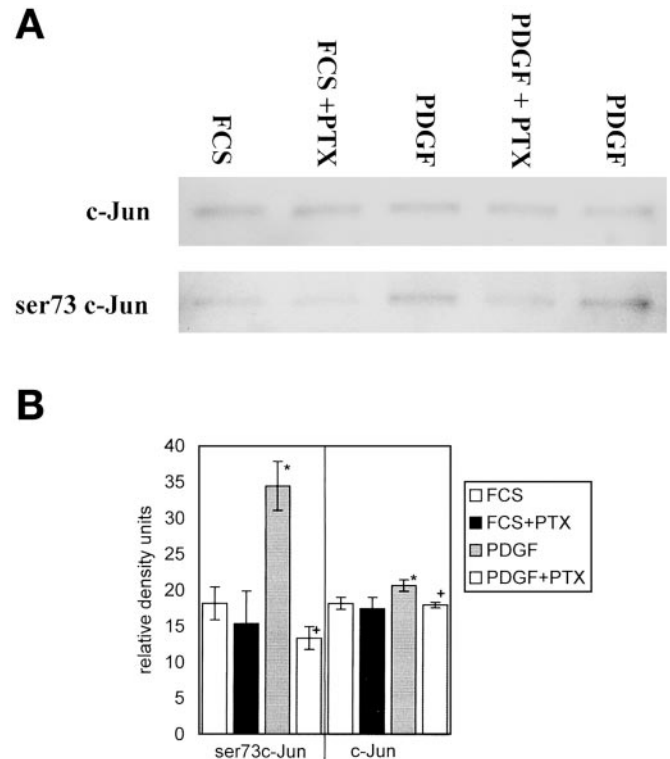


Fig. 10. Effect of PDGF compared with control (1% FCS) on c-Jun and serine 73 phospho c-Jun in fibroblasts with and without pentoxifylline treatment. A, representative Western blot of cell lysates prepared from fibroblasts treated with PDGF (8 ng/ml for 2 h) or 1% FCS with and without pentoxifylline (3.5 mM). This experiment was repeated four times with the same results. B, the effect of PDGF and pentoxifylline on c-Jun and serine 73-phosphorylated c-Jun as assessed by Western blot. The densitometry analysis of the results (means \pm S.E. of four experiments) using Scion image of the ECL from Western blots (expressed as RDU) showing the effect of PDGF (compared with control, 1% FCS) with and without pentoxifylline treatment on serine 73-phosphorylated c-Jun and c-Jun protein in fibroblasts. *, significantly different ($p < 0.05$) compared with control (FCS); +, significantly different ($p < 0.05$) compared with PDGF alone.

pletely eliminate control cell proliferation because it is impossible to grow these cells in a zero sera-supplemented medium. Therefore, baseline proliferation is an inherent component of the total cell proliferation. The data can be expressed as "net" proliferation, i.e., the component of the proliferation that is entirely due to PDGF. We have calculated this and the net proliferation due to PDGF, and the effect of pentoxifylline on the net proliferation clearly indicates that the 240 μ M pentoxifylline caused a significant inhibition of PDGF-induced cell proliferation. Our results indicate that pentoxifylline alone does not affect basal (constitutive) levels of *c-jun* or *c-fos* expression (Fig. 1B), and although incubation of fibroblasts with the phosphothioate *c-jun* antisense oligonucleotide (1 μ M) inhibited PDGF-stimulated proliferation, basal proliferation was not affected (Fig. 3B), suggesting that basal proliferation does not involve PDGF and does not require expression of the immediate early gene *c-jun*.

The results obtained with *c-jun* antisense show that an antisense directed at *c-jun* can selectively inhibit PDGF-

dependent proliferation while not affecting the basal proliferation. Treatment of cells with a *c-jun* missense oligonucleotide did not decrease PDGF-dependent proliferation. These results clearly indicate a role for *c-jun* in PDGF-mediated proliferation and go beyond our initial results (Fig. 1) that suggested a correlation between the actions of PDGF on *c-jun* and the actions of PDGF on proliferation. Preincubation of fibroblasts with *c-jun* antisense resulted in a marked decrease in c-Jun-positive immunoreactivity, indicating that the *c-jun* antisense was specific and selective in blocking the *c-jun* in this cell type.

It has been suggested that compounds that elevate cAMP may act upstream from MAP kinase at the Ras-Raf interaction (Burgering et al., 1993), but such an interaction would be expected to inhibit MAP kinase and alter *c-fos* expression. Earlier studies suggested that pentoxifylline did not inhibit PDGF activation of MAP kinase in fibroblasts and that the effect of pentoxifylline on PDGF-driven proliferation was cAMP-independent (Peterson et al., 1998). Taken together with the current results indicating that pentoxifylline does not inhibit PDGF-induced expression of *c-fos*, our results would suggest that pentoxifylline does not affect the Ras-Raf interaction but interferes with an alternate PDGF signaling pathway that signals from Ras through Jun kinase, or a pathway that regulates cross-talk between MAP kinase and Jun kinase; therefore, pentoxifylline would only alter the expression of *c-jun* and not affect the expression of *c-fos*. A decrease in c-Jun phosphorylation may alter cross-talk between Jun kinase (JNK) and cAMP signaling, as has recently been suggested for extracellular signal-regulated kinase (Houslay and Kolch, 2000).

Our results show that PDGF treatment increased the serine 73 phospho-specific c-Jun immunoreactivity in fibroblasts, and results obtained from Western blot analysis confirm the immunocytochemical results. Our results also indicate that transfection of PC-12 cells with wild-type *c-jun* plasmid (pAAV/CMV-*c-jun*) increased *c-jun* and serine 73-phosphorylated c-Jun immunoreactivity dramatically compared with controls. The increase in phosphorylated c-Jun in the absence of growth factor stimulation in PC-12 cells suggests that transfected Jun is "constitutively" phosphorylated on serine 73, presumably because there is constitutive Jun kinase activity in PC-12 cells (Dragunow et al., 2000). Our results also show that pentoxifylline reduced the serine 73 phospho-specific c-Jun immunoreactivity in F8 fibroblasts, and this was confirmed by Western analysis. Treatment of wild-type *c-jun* plasmid-transfected PC-12 cells with pentoxifylline, for 3 or 24 h before transfection, resulted in a marked 80% decrease in the number of serine 73-phosphorylated c-Jun-positive cells compared with untreated wild-type *c-jun* plasmid-transfected PC-12 cells. When F8 fibroblast cells were pretreated with *c-jun* antisense (10 μ M) before treatment with PDGF, the *c-jun* antisense reduced c-Jun immunoreactivity by 38%. The antisense ODN for *c-jun* also effectively reduced c-Jun immunoreactivity in PC-12 cells that were transfected with the wild-type *c-jun* plasmid. Studies using another cell type, SK-N-SH neuroblastoma cells, showed that transfection with wild-type *c-jun* plasmid enhanced the immunocytochemical staining for c-Jun and that pretreatment with *c-jun* antisense (10 μ M) reduced the c-Jun-positive immunoreactivity by 36%. Our results strongly suggest that pentoxifylline exerts its effect by decreasing the

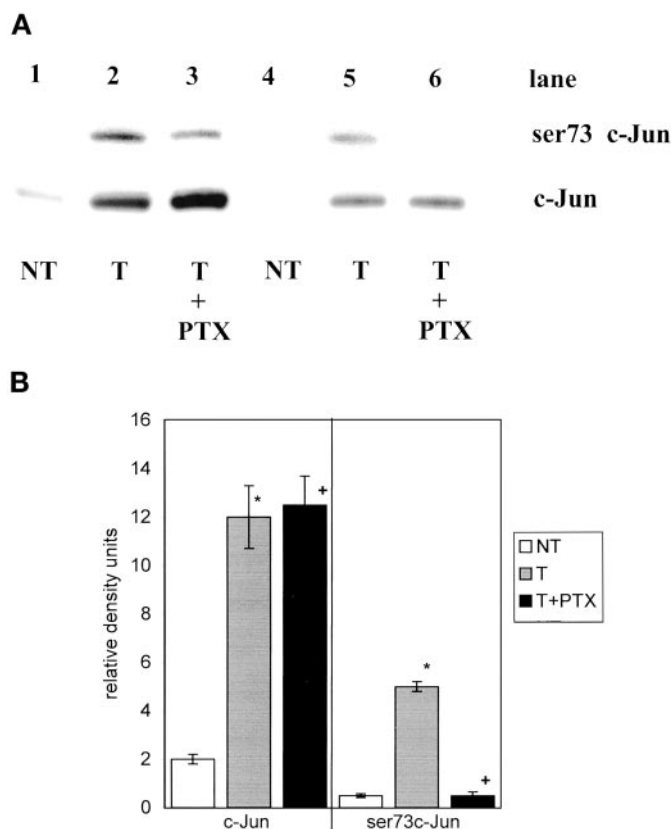


Fig. 11. Western blot analysis of c-Jun and serine 73 phospho c-Jun in PC-12 cells that were transfected with wild-type *c-jun* plasmid. A, representative Western blot of c-Jun and serine 73 phospho c-Jun in cell lysates (12.5 μ g of protein in lanes 1–3 and 6.25 μ g of protein in lanes 4–6) prepared from PC-12 cells transfected with wild-type *c-jun* plasmid. This experiment was repeated four times with the same results. B, the effect of transfection with wild-type *c-jun* plasmid with and without pentoxifylline on serine 73-phosphorylated c-Jun and c-Jun as assessed by Western blot. The densitometry analysis of the results (means of four experiments) using Scion image of the ECL from Western blots (expressed as RDU) showing the effect of transfection with wild-type *c-jun* plasmid with and without pentoxifylline treatment on serine 73-phosphorylated c-Jun and c-Jun in PC-12 cells. *, significantly different ($p < 0.05$) compared with nontransfected (NT); +, significantly different ($p < 0.05$) compared with transfected (T).

phosphorylation of c-Jun on serine 73. The reduction in phosphorylated c-Jun immunoreactivity by pentoxifylline indicates that pentoxifylline decreases the phosphorylated status (i.e., activation) of the c-Jun protein, therefore reducing the amount of activated c-Jun that is available to have an effect further downstream. A decrease in phosphorylated c-Jun would arise as a result of a block of Jun kinase activity or alternatively could arise from an increase in activity of protein phosphatase (PP2A), a phosphatase that is responsible for the dephosphorylation of c-Jun (Black et al., 1991; Lee et al., 1994).

The enhanced expression of c-jun in PC-12 cells has been associated with neurite outgrowth (Heasley et al., 1996). We observed enhanced neurite outgrowth in PC-12 cells that were transfected with wild-type c-jun plasmid compared with cells that were not transfected. When cells were pretreated with pentoxifylline before transfection with the wild-type c-jun plasmid, there was a marked decrease in neurite outgrowth compared with cells that were not treated with pentoxifylline.

In summary, with the aid of antisense oligonucleotides, immunocytochemistry, and Western blot analysis, we showed that c-jun (and c-Jun) plays a critical role in PDGF-dependent proliferation of human fibroblasts. This study provides a mechanism for the inhibitory effect of pentoxifylline on PDGF-stimulated proliferation. Pentoxifylline blocks PDGF postreceptor signaling by selectively inhibiting the up-regulation of c-jun gene expression. The results using the c-jun antisense ODN suggested that Jun kinase might be involved. The results of immunocytochemistry, using specific antibodies to c-Jun and phosphorylated c-Jun in PDGF-stimulated and c-jun plasmid-transfected cells, indicated that pentoxifylline moderately reduced c-Jun protein and markedly inhibited phosphorylation of c-Jun on serine 73, and the results were confirmed by Western blot analysis. The decrease in phosphorylation of c-Jun at serine 73 due to pentoxifylline treatment would decrease the activity of c-Jun and ultimately reduce the transcription factor AP-1 and thereby reduce the AP-1-mediated activation of genes involved in proliferation and collagen synthesis and explain the inhibitory effect of pentoxifylline on proliferation and collagen synthesis.

This novel mechanism could prove to have therapeutic application in diseases where an elevation in PDGF or an up-regulation in c-jun have been implicated in the disease pathogenesis (Gesualdo et al., 1991; Imai et al., 1992; Nakamura et al., 1992; Peterson and Isbrucker, 1992; Cimminiello et al., 1994; Pietrogrande et al., 1995; Ross et al., 1995; Uebelhoefer et al., 1995; Peterson and Tanton, 1996; Pinzani et al., 1996). The mechanism described in this study applies to pentoxifylline but may also apply to compounds very closely related to pentoxifylline, including its metabolite-1 (Isbrucker and Peterson, 1995). The results presented here also suggest that antisense oligonucleotides to c-jun may have a potential application as antifibrotic agents, if these can be targeted to the organ of interest. Diseases showing an up-regulation of c-jun as a major factor in their pathophysiology may be attenuated through the application of a c-jun antisense oligonucleotide.

Other compounds, including JNK interacting protein-1 (Lee et al., 1999), specific JNK inhibitor (Amar et al., 1997), and mitogen-activated protein kinase phosphatase-1 (Lader-

oute et al., 1999), that have been reported to affect c-Jun phosphorylation or inhibit c-Jun kinase have a potential role as antifibrotic agents if they can be targeted to the fibrotic organ of interest. An inhibitory effect on the c-Jun-mediated pathway may explain the antiproliferative and antifibrotic activity of other compounds, including losartin (Varo et al., 1999), pirfenidone (Raghu et al., 1999), enalapril (Peters et al., 1998), octreotide (Fort et al., 1998a), captopril (Uhal et al., 1998), terbinafine (Ricard-Blum et al., 1998), interferon- α (Fort et al., 1998b), and pentifylline (Saika et al., 1996).

The mechanism presented here indicates that pentoxifylline decreases phosphorylation of c-Jun on serine 73. Such a mechanism could have wider implications, because it is known that c-Jun forms heterologous signaling protein combinations with activating transcription factor 2 (Sano et al., 1998), cAMP response element-binding protein (Delgado et al., 1998), and nuclear factor E2p45 related factor (Novotny et al., 1998). In view of the combinations in which c-Jun participates, there is a need to understand the mechanism by which c-Jun and the c-Jun complexes may contribute to fibrotic proliferative disorders, for methods of treatment of such diseases. The results presented here suggest that pentoxifylline blocks c-Jun-mediated proliferation by decreasing c-Jun and markedly inhibiting phosphorylation of c-Jun on serine 73.

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