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Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts

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The effect of various growth factors on the synthesis of hyaluronan in human fibroblasts was investigated. When tested in medium containing 0.5% fetal calf serum, platelet-derived growth factor (PDGF)-BB was found to stimulate hyaluronan synthesis; the maximal response was equal to or higher than that obtained with 10% fetal calf serum. PDGF-AA gave only a limited effect, indicating that the stimulatory effect of PDGF on hyaluronan synthesis was mainly transduced via the B-type PDGF receptor. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β1 also stimulated hyaluronan synthesis; their effects were less than that of PDGF-BB, but combinations of factors produced potent stimulatory effects on hyaluronan synthesis. All factors stimulated hyaluronan synthesis in sparse as well as dense cultures. The effects of the factors on hyaluronan synthesis did not correlate with their mitogenic activities; PDGF-BB, EGF and bFGF are equipotent mitogens, but PDGF-BB had a much more potent effect on hyaluronan synthesis, and TGF-β actually inhibits the growth of fibroblasts under the conditions of the assay.

INTRODUCTION

The growth of cells in culture is regulated by polypeptide factors that stimulate or inhibit cell proliferation. Growth regulatory factors have also been found to stimulate other cellular functions like chemotaxis and matrix production. The functions of such factors *in vivo* are incompletely known, but it is likely that they are of importance in the regulation of growth of the embryo and placenta, in the regulation of haematopoiesis and in tissue repair processes. The most well-characterized of the growth factors that act on connective tissue cells are platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β). The aim of the present study was to determine the effects of these factors on the synthesis of hyaluronan, an important constituent of the extracellular matrix.

PDGF is the major serum mitogen for connective tissue cells *in vitro* (for reviews see Heldin *et al.*, 1985; Ross *et al.*, 1986). Structurally it is composed of two disulphide-linked polypeptide chains, denoted A and B. All three possible isoforms, PDGF-AA, PDGF-AB and PDGF-BB, have been identified and purified from platelets and transformed cells (Stroobant & Waterfield, 1984; Heldin *et al.*, 1986; Hammacher *et al.*, 1988). They have been found to have different functional effects (Nistér *et al.*, 1988), most likely due to the fact that they bind to two distinct receptor types with different affinities; type A receptors bind all three isoforms, whereas type B receptors bind PDGF-BB with high affinity; PDGF-AB with lower affinity, but not PDGF-AA (Heldin *et al.*, 1988; Hart *et al.*, 1988).

EGF, and its homologue TGF-α, stimulate cell proliferation via binding to the EGF receptor which, like the PDGF B-type receptor, is a protein tyrosine kinase (for a review, see Schlessinger, 1986).

Acidic and basic FGF are homologues which bind to the same receptor (reviewed by Gospodarowicz *et al.*, 1986). In addition to their stimulatory effect on the growth of fibroblasts, they act on endothelial cells and stimulate angiogenesis *in vivo*.

TGF-β stimulates or inhibits cell proliferation depending on cell type and culture conditions (for a review, see Sporn *et al.*, 1987). In diploid human fibroblasts, TGF-β inhibits cell proliferation induced by PDGF or EGF, in a cell density dependent manner; the inhibition was found to be more pronounced in dense than in sparse cultures (Paulsson *et al.*, 1988). TGF-β is a potent stimulator of the synthesis of matrix proteins such as fibronectin and collagen (Ignotz & Massagué, 1986; Roberts *et al.*, 1986), and their receptors (Ignotz & Massagué, 1987). At least three isoforms of TGF-β exist (TGF-β1, -β2 and -β3) (Cheifetz *et al.*, 1987; ten Dijke *et al.*, 1988); TGF-β1 is the major form in human platelets, the richest known source of TGF-β. TGF-βs exert their actions via receptors that seem not to be associated with protein tyrosine kinase activities (Massagué & Like, 1985).

Hyaluronan is a linear polysaccharide of generally less than 1000 alternating glucuronic acid and *N*-acetylglucosamine residues (for a review, see Laurent & Fraser, 1986). By interaction with other matrix molecules, e.g. chondroitin sulphate proteoglycans, hyaluronan provides stability and elasticity to the extracellular matrix. Hyaluronan is synthesized by a membrane-bound synthase; monosaccharide units are added to the reducing end of the polysaccharide, as it protrudes through the

Abbreviations used: PDGF, platelet-derived growth factor (A and B denote the two types of disulphide-linked polypeptide chains which can be present in PDGF); EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; DMEM, Dulbecco's modified Eagle's Medium.

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cell membrane (Prehm 1983*a*,*b*). It has been reported that hyaluronan synthesis is related to cell proliferation (Tomida *et al.*, 1975; Hronowski & Anastassiades, 1980; Mian *et al.*, 1986; Matuoka *et al.*, 1987), and a role for hyaluronan in the mitotic process has been suggested (Brecht *et al.*, 1986).

PDGF (Engström-Laurent *et al.*, 1985) and connective tissue-activating peptide III (Castor *et al.*, 1977) have been reported to stimulate hyaluronan synthesis in human fibroblasts in culture. The aim of the present study was to explore further the effect of growth factors on hyaluronan synthesis. We show that PDGF-BB has a higher activity than PDGF-AA, in sparse as well as in dense cultures of human fibroblasts. EGF and bFGF have activities of their own and act synergistically with PDGF-AA or PDGF-BB. TGF-β1 stimulates hyaluronan synthesis under conditions where it inhibits proliferation of human fibroblasts; its effect was additive to that of PDGF-AA or PDGF-BB.

MATERIALS AND METHODS

Growth factors

PDGF-AA and PDGF-BB were purified from supernatants of yeast cells transfected with PDGF A- and B-chain DNA constructs respectively (A. Östman, G. Bäckström, N. Fong, C. Betsholtz, C. Wernstedt, U. Hellman, B. Westermark, P. Valenzuela & C.-H. Heldin, unpublished work). EGF ('receptor grade') and bFGF were purchased from Collaborative Research and Amersham Laboratories respectively. TGF-β was purified from human platelets (G. Bauer, unpublished work); analysis by *N*-terminal amino acid sequencing revealed that it consisted entirely of TGF-β1.

Assay of hyaluronan synthesis

Human foreskin fibroblasts (AG 1523, obtained from the Human Mutant Cell Repository, Camden, NJ, U.S.A.) were grown in 12-well Linbro plates in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. Between one and three days after plating, sparse (about 30000 cells/well) and dense (about 300000 cells/well) cell cultures were rinsed once with DMEM containing 0.5% fetal calf serum and then incubated for 2 days in 1 ml of the same medium. At this time, the medium was changed and the cell cultures were incubated for 2 further days in DMEM containing 0.5% fetal calf serum and various concentrations of growth factors. Under these conditions, a major part of the hyaluronan synthesized is found in the conditioned medium (Engström-Laurent *et al.*, 1985). The hyaluronan concentration in the cell culture medium was determined with a commercial kit (HA Test 50; Pharmacia, Uppsala, Sweden). The intra-assay and inter-assay coefficients of variation in this assay were both $<10\%$ (Brandt *et al.*, 1987).

RESULTS AND DISCUSSION

PDGF purified from human platelets, and containing more than one isoform (Hammacher *et al.*, 1988), has been shown to stimulate hyaluronan synthesis in cultured human fibroblasts (Engström-Laurent *et al.*, 1985). In order to investigate which of the two PDGF receptor classes transduces this effect, the various isoforms of PDGF were investigated with regard to their ability to stimulate hyaluronan synthesis in human fibroblasts maintained in medium containing a low concentration (0.5%) of fetal calf serum. In sparse cell cultures, PDGF-BB had a potent stimulatory effect; in four different experiments the maximal responses were equal to or above that achieved by 10% fetal calf serum. In one representative experiment (Fig. 1*a*), 5 ng of PDGF-BB/ml, which is the dose that gives maximal growth-promoting activity (Heldin *et al.*, 1988), gave the same response as 10% fetal calf serum (about 870 ng of hyaluronan/48 h per 30000 cells). PDGF-AA was considerably less active; only a very small, but significant, stimulatory effect was found (Fig. 1*a*). PDGF-AB gave an intermediary effect (results not shown). PDGF-BB binds to both A-type and B-type PDGF receptors, whereas PDGF-AA binds only the A-type receptors. There are 3–5-fold more B-type than A-type receptors on

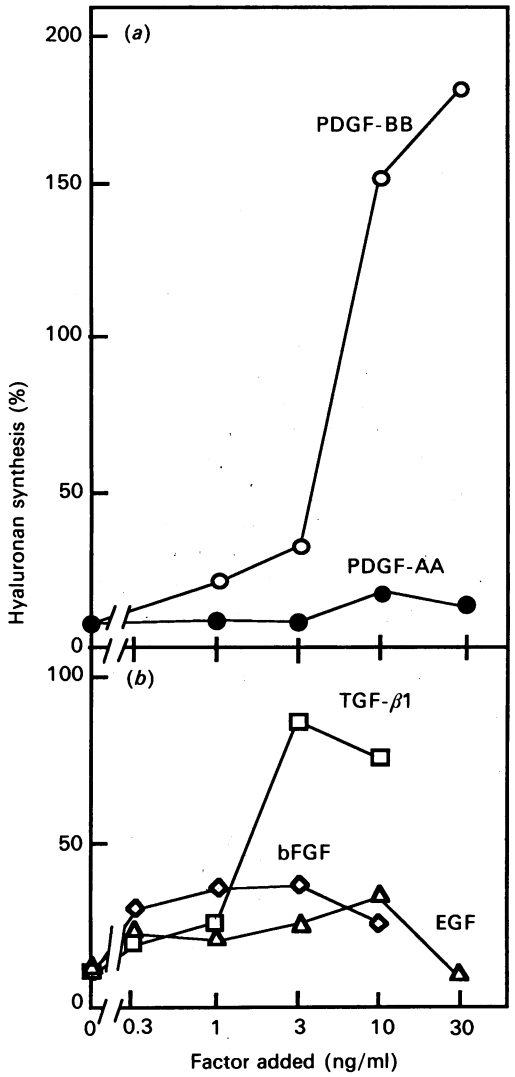

Fig. 1. Stimulation of hyaluronan synthesis in sparse cultures of human fibroblasts

The hyaluronan synthesized in response to various concentrations of PDGF-AA (●), PDGF-BB (○) (*a*) or EGF (△), bFGF (◇) or TGF-β1 (□) (*b*) was determined. Values are given relative to the stimulation given by 10% fetal calf serum (100%; 870 ng of hyaluronan/48 h per 30000 cells).

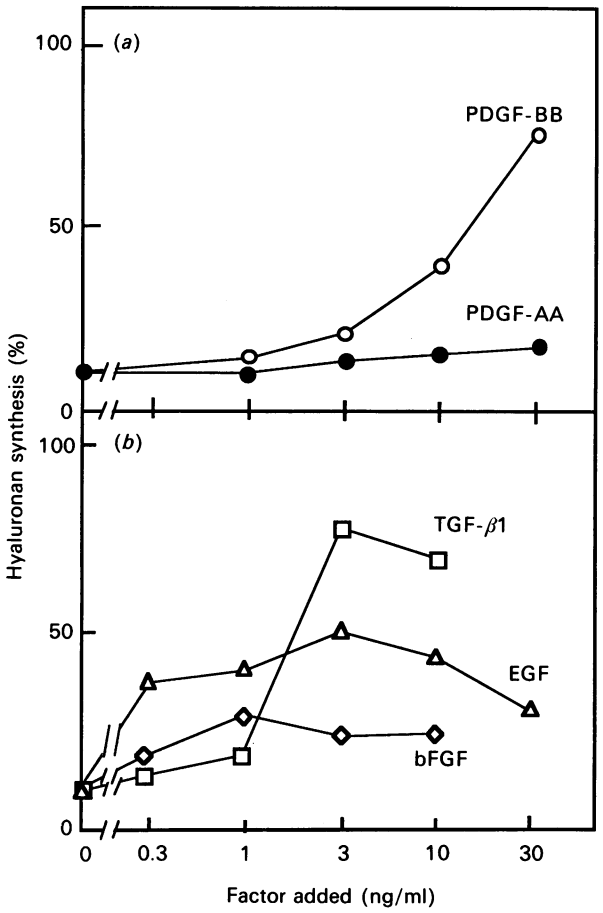

Fig. 2. Stimulation of hyaluronan synthesis in dense cultures of human fibroblasts

The hyaluronan synthesized in response to various concentrations of PDGF-AA (●), PDGF-BB (○) (*a*), EGF (△), bFGF (◇) or TGF-β1 (□) (*b*) were determined. Values are given relative to the stimulation given by 10% fetal calf serum (100%; 1560 ng of hyaluronan/48 h per 300000 cells).

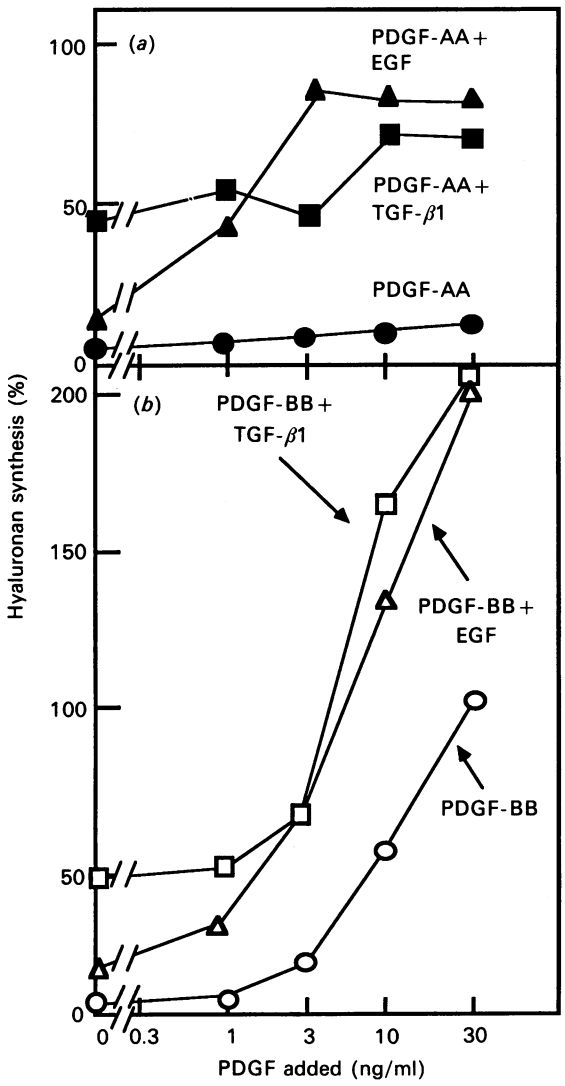

Fig. 3. Stimulation of hyaluronan synthesis by combinations of growth regulatory proteins in sparse cultures of human fibroblasts

The effect on hyaluronan synthesis of EGF (10 ng/ml; ▲, △), TGF-β1 (3 ng/ml; ■, □) or no addition (●, ○), together with various concentrations of PDGF-AA (filled symbols) (*a*) or PDGF-BB (open symbols) (*b*) were determined. Values are given relative to the stimulation given by 10% fetal calf serum (100%; 1300 ng of hyaluronan/48 h per 30000 cells).

this line of human fibroblasts (Östman *et al.*, 1988), but it is unlikely that the dramatic difference in response between PDGF-AA and PDGF-BB is due entirely to the fact that PDGF-AA binds to a lower number of receptors. Rather, the data suggest that the B-type receptor has a more important role than the A-type receptor in mediating the stimulatory effect on hyaluronan synthesis.

EGF and bFGF were also found to have low stimulatory effects on hyaluronan synthesis; maximal effects were obtained at doses similar to those that give maximal effect on growth. TGF-β1 had a larger stimulatory effect, giving a response similar to that of 10% fetal calf-serum at 3 ng/ml (Fig. 1*b*).

The synthesis of hyaluronan is dependent on cell density (Tomida *et al.*, 1975). Furthermore, the cellular response to mitogens decreases with increasing cell density, whereas the inhibitory effect of TGF-β on growth of human fibroblasts increases (Paulsson *et al.*, 1988). In order to correlate the stimulatory effect on hyaluronan synthesis with the mitogenic response, we also investigated the effect of the growth regulatory factors on dense cultures of human fibroblasts.

As expected, the synthesis of hyaluronan on a per cell basis was lower in the dense cultures compared with the sparse cultures (10% fetal calf serum gave 1557 ng/48 h per 300000 cells. Compared to the stimulatory effect of 10% fetal calf serum, the response to PDGF-BB was lower in dense cultures compared to sparse cultures, whereas the relative effects of PDGF-AA, EGF, bFGF and TGF-β1 were similar in sparse and dense cultures (Fig. 2). It is notable that TGF-β1 stimulated hyaluronan synthesis both in dense cell cultures, where it inhibits cell growth, and in sparse cell cultures, where it has no effect on cell growth.

Combinations of different growth regulatory factors were also investigated. Increased stimulatory effects on hyaluronan synthesis were found when EGF (10 ng/ml) or TGF-β1 (3 ng/ml) were tested together with PDGF-AA (Fig. 3*a*) or PDGF-BB (Fig. 3*b*). Interestingly,

PDGF-AA and EGF, both of which gave very limited responses when tested separately, gave a powerful stimulation together, approaching that of 10% fetal calf serum (Fig. 3*a*). In a separate experiment, the effect of 3 ng of bFGF/ml was also found to act synergistically with PDGF-AA and PDGF-BB in concentrations up to 30 ng/ml (results not shown).

In conclusion, we have shown that PDGF-BB and TGF-β1 have potent stimulatory effects on hyaluronan synthesis in sparse as well as in dense cultures of human fibroblasts. PDGF-AA, EGF and bFGF were also found to stimulate hyaluronan synthesis, but were less efficient. No correlation with the mitogenic effects of the factors was found; EGF and bFGF, which are as potent mitogens as PDGF-BB, had much weaker effects than PDGF-BB on hyaluronan synthesis, and TGF-β, which inhibits the growth of human fibroblasts, had a clear stimulatory effect on hyaluronan synthesis.

The role of hyaluronan in the embryonic development has recently been brought into focus (for a review, see Toole *et al.*, 1984). Furthermore, it was recently shown that TGF-β and bFGF are present during stages of limb development which are characterized by active hyaluronan synthesis (Toole *et al.*, 1989). Our data suggest that hyaluronan synthesis in embryonic tissues may be regulated by growth promoting factors.

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REFERENCES

Brandt, R., Hedlöf, E., Åsman, J., Bucht, A. & Tengblad, A. (1987) Acta Oto-Laryngol. Suppl. **442**, 31–35

Brecht, M., Mayer, U., Schlosser, E. & Prehm, P. (1986) Biochem. J. **239**, 445–450

Castor, C. W., Ritchie, J. C., Scott, M. E. & Whitney, S. L. (1977) Arthritis Rheum. **20**, 859–868

Cheifetz, S., Weatherbee, J. A., Tsang, M. L.-S., Anderson, J. K., Mole, J. E., Lucas, R. & Massagué, J. (1987) Cell (Cambridge, Mass.) **48**, 409–415

Engström-Laurent, A., Feltelius, N., Hällgren, R. & Wasteson, Å. (1985) Ann. Rheum. Dis. **44**, 614–620

Gospodarowicz, D., Neufeld, G. & Schweigerer, L. (1986) Mol. Cell. Endocrinol. **46**, 187–204

Hammacher, A., Hellman, U., Johnsson, A., Östman, A., Gunnarsson, K., Westermark, B., Wasteson, Å. & Heldin, C.-H. (1988) J. Biol. Chem. **263**, 16493–16498

Hart, C. E., Forstrom, J. W., Kelly, J. D., Seifert, R. A., Smith, R. A., Ross, R., Murray, M. & Bowen-Pope, D. F. (1988) Science **240**, 1529–1531

Heldin, C.-H., Wasteson, Å. & Westermark, B. (1985) Mol. Cell. Endocrinol. **39**, 169–187

Heldin, C.-H., Johnsson, A., Wennergren, S., Wernstedt, C., Betsholtz, C. & Westermark, B. (1986) Nature (London) **319**, 511–514

Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M. & Westermark, B. (1988) EMBO J. **7**, 1387–1393

Hronowski, L. & Anastassiades, T. P. (1980) J. Biol. Chem. **255**, 10091–10099

Ignotz, R. A. & Massagué, J. (1986) J. Biol. Chem. **261**, 4345–4347

Ignotz, R. A. & Massagué, J. (1987) Cell (Cambridge, Mass.) **51**, 184–197

Laurent, T. C. & Fraser, J. R. E. (1986) Ciba Found. Symp. **124**, 8–23

Massagué, J. & Like, B. (1985) J. Biol. Chem. **260**, 2636–2645

Matuoka, K., Namba, M. & Mitsui, Y. (1987) J. Cell Biol. **104**, 1105–1115

Mian, N. (1986) Biochem. J. **237**, 333–342

Nistér, M., Hammacher, A., Mellström K., Siegbahn, A., Rönnstrand, L., Westermark, B. & Heldin, C.-H. (1988) Cell (Cambridge, Mass.) **52**, 791–799

Paulsson, Y., Beckmann, M. P., Westermark, B. & Heldin, C.-H. (1989) Growth Factors, in the press

Prehm, P. (1983*a*) Biochem J. **211**, 181–189

Prehm, P. (1983*b*) Biochem J. **211**, 191–198

Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. M., Roche, N. S., Wakefield, L. M., Heine, U. I., Liotta, L. A., Falanga, V., Kerhl, J. M. & Fauci, A. S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4167–4171

Ross, R., Raines, E. W. & Bowen-Pope, D. F. (1986) Cell (Cambridge, Mass.) **46**, 155–169

Schlessinger, J. (1986) J. Cell Biol. **103**, 2067–2072

Sporn, M. B., Roberts, A. B., Wakefield, L. M. & de Crombrugghe, B. (1987) J. Cell Biol. **105**, 1039–1045

Stroobant, P. & Waterfield, M. D. (1984) EMBO J. **3** 2963–2967

ten Dijke, P., Hanssen, P., Iwata, K. K., Pieler, C. & Foulkes, G. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 4715–4719

Tomida, M., Koyama, H. & Ono, T. (1975) J. Cell. Physiol. **86**, 121–130

Toole, B. P., Goldberg, R. L., Chi-Rosso, G., Underhill, C. B. & Orkin, R. W. (1984) in The Role of Extracellular Matrix in Development (Trestad, R. L., ed.), pp. 43–66, Alan R. Liss, New York.

Toole, B. P., Munaim, S. I., Welles, S. & Knudson C. B. (1989) Ciba Found. Symp. **143**, in the press

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