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Characterization of the molecular mechanism involved in the activation of hyaluronan synthetase by platelet-derived growth factor in human mesothelial cells

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The molecular mechanism involved in the stimulation of hyaluronan synthetase in normal human mesothelial cells was investigated. Exposure of mesothelial cells to platelet-derived growth factor (PDGF)-BB stimulated hyaluronan synthetase activity, measured in isolated membrane preparations, as well as hyaluronan secretion into the medium. The effect on hyaluronan synthetase was maximal after 6 h of treatment. In contrast, the stimulatory effect of transforming growth factor-β1 reached a maximum after 24 h. The stimulatory effect of PDGF-BB was inhibited by cycloheximide. The phosphotyrosine phosphatase inhibitor vanadate was found to stimulate hyaluronan synthetase activity, and to potentiate the effect of PDGF-BB. The protein kinase C (PKC) stimulator phorbol 12-myristate 13-acetate (PMA) also stimulated hyaluronan synthetase; furthermore, depletion of PKC by preincubation of the cells with PMA led to an inhibition of the PDGF-BB-induced stimulation of hyaluronan synthetase activity. Thus the PDGF-BB-induced stimulation of hyaluronan synthetase activity is dependent on protein synthesis and involves tyrosine phosphorylation and activation of PKC.

INTRODUCTION

Mesothelial cells line the pleura, pericardium and peritoneum. Normal human mesothelial (NHM) cells can be maintained *in vitro* in a rich medium supplemented with epidermal growth factor (EGF) and cortisol (Connell & Rheinwald, 1983). EGF, platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1) and fibroblast growth factor (FGF) have been found to be mitogenic for NHM cells (Connell & Rheinwald, 1983; Laveck *et al.*, 1988; Gabrielson *et al.*, 1988).

Hyaluronan is an important constituent of the extracellular matrix, and is implicated in many biological processes (for a review see Laurent & Fraser, 1986). A transiently increased production of hyaluronan coincides with periods of cellular migration, both in embryonic tissue (for a review see Toole *et al.*, 1989) and in adult regenerating tissue (Hasty *et al.*, 1981). Furthermore, an increased level of hyaluronan is found in association with invasive tumours (for a review see Knudson *et al.*, 1989). Specifically, in highly malignant mesothelioma, the high hyaluronan concentration in pleural fluid, pericardial fluid and ascites has been used as a biochemical marker in the diagnosis of this disease (Arai *et al.*, 1979; Roboz *et al.*, 1985; Dahl & Laurent, 1988; Dahl *et al.*, 1989). The synthesis of hyaluronan has been found to be stimulated by PDGF-BB and certain other growth factors in cultured fibroblasts (Engström-Laurent *et al.*, 1985; Heldin *et al.*, 1989), mesothelial cells (Honda *et al.*, 1991) and Ito cells of the liver (Heldin *et al.*, 1991).

Hyaluronan is synthesized by a membrane-linked enzyme, hyaluronan synthetase, which catalyses the polymerization of the sugar residues in UDP-glucuronic acid and UDP-*N*-acetylglucosamine to a linear polysaccharide which, concomitant with its synthesis, is translocated out into the extracellular space (Prehm, 1983*a*,*b*, 1984; Philipson & Schwartz, 1984). Hyaluronan synthetase activity has been related to many cellular functions, such as cell growth (Tomida *et al.*, 1975; Hronowski & Anastassiades, 1980; Matuoka *et al.*, 1987), metastasis (Toole *et al.*, 1979) and mitosis (Brecht *et al.*, 1986). However, our knowledge of the mechanism of regulation of hyaluronan biosynthesis at the molecular level is still incomplete.

The primary aim of the present work was to investigate the molecular mechanisms involved in the regulation of hyaluronan synthetase in NHM cells. We show by direct measurement of enzyme activity that PDGF-BB, phorbol 12-myristate 13-acetate (PMA), vanadate and TGF-β1 stimulate hyaluronan synthetase activity. Furthermore, we demonstrate that the stimulation is dependent on active protein synthesis, and that phosphorylations on tyrosine and serine or threonine residues are likely to be involved.

MATERIALS AND METHODS

Materials

PDGF isoforms were recombinant material obtained from C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden; Östman *et al.*, 1989). TGF-β1 was a gift from K. Miyazono (Ludwig Institute for Cancer Research, Uppsala) and EGF was a gift from A. Thomason (Amgen, Thousand Oaks, CA, U.S.A). Mouse monoclonal antibodies against human cytokeratin 19 and vimentin were obtained from Dakopatts. Cortisol, vanadate, PMA (dissolved in dimethyl sulphoxide at 1.6 mM), leupeptin and uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) were purchased from Sigma. Radiolabelled UDP glucuronic acid (UDP-[^{14}C]GlcUA; specific radioactivity 303.0 mCi/mmol) was obtained from New England Nuclear. F-10 medium and the other cell culture reagents were from GIBCO, Paisley, Scotland, U.K. Streptomyces hyaluronidase was obtained from Seikagaku Kogyo, Tokyo, Japan.

Abbreviations used: NHM, normal human mesothial; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-β1; FGF, fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; GlcUA, glucuronic acid; PKC protein kinase C.

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Isolation and culture conditions of NHM cells

NHM cell cultures were initiated from biopsies of patients undergoing surgery for coronary artery disease, and were obtained from the anterial or medial part of the pleural cavity (the project was approved by the Ethical Committee, University Hospital, Uppsala). The biopsy samples were cleared of excess fat, providing transparent samples of tissue, and rinsed three times in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS-D). Then the tissue was subjected to enzymic disaggregation. A few droplets of trypsin/EDTA [0.25% (w/v) trypsin, 0.02% (w/v) EDTA] were applied directly onto a small piece of mesothelial tissue which was spread out in a 35 mm × 10 mm Costar cell culture dish. After 30 min of incubation at 37 °C, 1 ml of F-10 medium supplemented with 50% fetal calf serum (FCS), 100 units of penicillin/ml and 100 μg of streptomycin/ml was gently squirted on to the tissue. The mesothelial tissue was then transferred to another cell culture dish and shaken gently in 0.5 ml of fresh medium; after a few seconds the medium was removed and combined with the first one. After overnight incubation, the medium from trypsin-treated cells was changed to F-10 medium supplemented with 15% FCS, cortisol (0.4 μg/ml), EGF (10 ng/ml) and antibiotics. The cells were routinely cultured in this medium, unless otherwise stated, at 37 °C in a humidified atmosphere of air/CO_2 (19:1). Medium was changed twice weekly, and cells were passaged at confluence using trypsin/EDTA. The cells had a culture lifespan of about 15 population doublings.

Characterization of cell cultures

Cell cultures were examined by light microscopy for mesothelial characteristics. Cells grown on cover slips were cultured in F-10 medium containing 15% FCS but deprived of EGF and cortisol for 6–9 days, essentially as described by Connell & Rheinwald (1983). Expression by these cells of keratin and vimentin was demonstrated by staining with monoclonal antibodies to human cytokeratin 19 and vimentin (diluted 1:20 v/v with PBS) in a moist chamber for 2 h at room temperature, using standard immunofluorescence procedures.

Determination of hyaluronan synthesis in cultured NHM cells

NHM cells were grown in 135 mm tissue culture dishes in F-10 medium containing 15% FCS, EGF and cortisol until semiconfluent cultures were obtained (about 1×10^6 cells/dish). The medium was then removed and replaced with 30 ml of F-10 medium containing 0.5% FCS. After about 40 h, the quiescent cells received fresh F-10 medium (12 ml) containing 0.5% FCS and supplemented with PDGF-BB (50 ng/ml), 100 nM-PMA, 30 μM-vanadate, TGF-β1 (10 ng/ml) or 1 μg of cycloheximide/ml, or combinations thereof. After different time periods, hyaluronan in the culture medium was determined with a commercial kit (hyaluronan Test 50; Pharmacia, Uppsala, Sweden). Under similar conditions, about 80% of the hyaluronan synthesized by cultured human foreskin fibroblasts was found in the conditioned medium (Engström-Laurent *et al.*, 1985). Each data point was analysed in duplicate or triplicate; the data shown are from representative experiments or are the mean values of two or more different experiments. The variation between the determinations was about 10%.

Determination of hyaluronan synthetase activity in membrane preparations of NHM cells

For determination of hyaluronan synthetase activity, cells stimulated with different agents, as described above, were harvested from the dishes with a rubber policeman in 6 ml of 50 mM-Hepes, pH 7.0, containing 0.24 M-sucrose, 2 mM-EDTA, 1 mM-dithiothreitol, 10 μg of leupeptin/ml and 100 kallikrein-inhibitory units of trasylol/ml (buffer A). This and the following steps were performed at 4 °C. The cell suspensions (obtained from 2×10^6 cells for each sample) were subjected to three cycles of freezing at −70 °C followed by thawing, and then homogenized with 20 strokes in a Dounce homogenizer. The cell homogenates were centrifuged at 95000 *g* for 20 min (70 Ti rotor; Beckman Instruments). The cell pellets were then resuspended in 150 μl of buffer A and Dounce-homogenized with two strokes. Samples of 80 μl of the homogenate were tested for hyaluronan synthetase activity as described by Tomida *et al.* (1974) and Prehm (1983*a*), with some modifications. The reaction mixture (100 μl) contained 400 μM-UDP-GlcNAc, 20 μM-UDP-[^{14}C]GlcUA, 6 mM-$MgCl_2$ and 50–100 μg of membrane protein [determined by the method of Hartree (1972)] in buffer A. After incubation at 37 °C for 1 h, the reaction was terminated by adding 10 μl of 20% SDS and heating the samples at 95 °C for 3 min. Then 100 μg of hyaluronan [M_r 40000 (Healon Pharmacia, Uppsala); autoclaved for 150 min (Bothner *et al.*, 1988)] was added as carrier and the mixture was applied to a Sephadex G-50 Superfine column (12.5 cm × 1 cm), eluted with 0.5 M-NaCl. The radioactivity obtained in the void volume of the chromatogram was sensitive to Streptomyces hyaluronidase digestion and was considered to represent ^{14}C-labelled hyaluronan. The variation between individual determinations was generally less than 20%.

RESULTS

NHM cells isolated from pleural biopsy samples were cultured in medium supplemented with EGF and cortisol according to Connell & Rheinwald (1983). The cells rapidly adhered to plastic, appearing oval or flattened with numerous granules around the nucleus and with frilled and folded cell margins. Rapidly growing cells in the presence of EGF, cortisol and 15% FCS adopted an elongated morphology. In the presence of 0.5% FCS, without EGF and hydrocortisol, the cells were thinner and more spread out. Cell cultures could be stained with anti-(cytokeratin 19) (a major mesothelial keratin; Moll *et al.*, 1982; Wu *et al.*, 1982) and anti-vimentin antibodies, depending upon their state of growth, as described by Connell & Rheinwald (1983), indicating their mesothelial cell origin (results not shown).

The activity of hyaluronan synthetase in membrane preparations of NHM cells was determined in the presence and the absence of PDGF-BB and compared with hyaluronan release into the cell culture medium (Fig. 1). Hyaluronan synthetase activity, determined in membrane fractions of PDGF-BB-stimulated cells, increased with time; maximal stimulation of hyaluronan synthetase activity was obtained 6 h after the addition of PDGF-BB and lasted for at least 8 h (Fig. 1*b* and results not shown). The increase in enzyme activity was accompanied by an increase with time in the amount of hyaluronan in the medium (Fig. 1*a*).

To investigate whether the stimulatory effect of PDGF-BB was dependent on active protein synthesis, the effect of cycloheximide on hyaluronan synthetase activity was studied with or without simultaneous addition of PDGF-BB (Table 1). A dramatic inhibition of hyaluronan synthetase activity was observed after addition of cycloheximide into this medium. The result is consistent with previous observations that hyaluronan synthetase undergoes rapid turnover (Tomida *et al.*, 1974; Mian, 1986*a*; Bansal & Mason, 1986). In the presence of cycloheximide, PDGF-BB was unable to cause an increase in hyaluronan synthetase activity. These results indicate that active protein

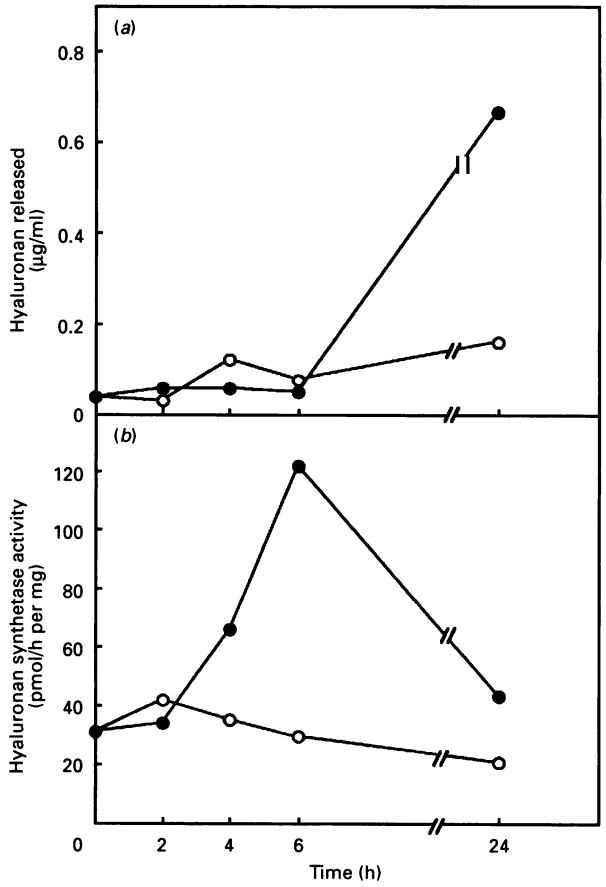

Fig. 1. Time-dependence of the effect of PDGF-BB on hyaluronan release from and hyaluronan synthetase activity in NHM cells

Hyaluronan release into the medium of NHM cells (*a*) and hyaluronan synthetase activity in membrane preparations of these cells (*b*) were determined in the presence (●) or absence (○) of PDGF-BB (50 ng/ml) for different periods of time. The data shown are representative of four separate experiments.

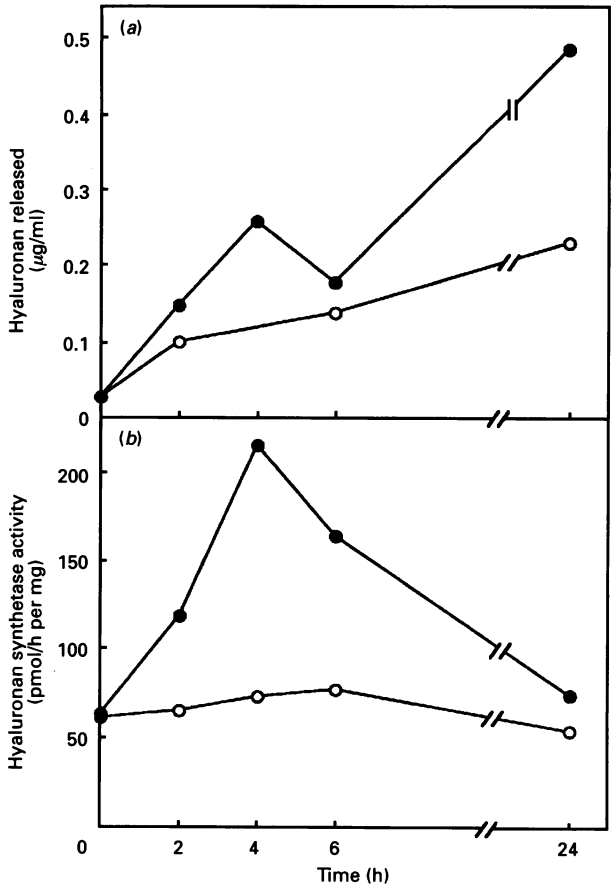

Fig. 2. Effect of vanadate on hyaluronan release from and hyaluronan synthetase activity in cultured NHM cells

The amount of hyaluronan released into the cell culture medium (*a*) and the hyaluronan synthetase activity in membrane preparations (*b*) were determined after incubation of NHM cells for different time periods in the presence (●) or absence (○) of 30 μM-vanadate. Each data point represents the mean value of two separate experiments. The variation was less than 10% in (*a*) and less than 20% in (*b*).

Table 1. Effects of cycloheximide and vanadate on PDGF-BB-stimulated hyaluronan synthetase activity

PDGF-BB (50 ng/ml), 1 μg of cycloheximide/ml or 30 μM-vanadate was added alone or in combination to quiescent NHM cell cultures. After 2 h of incubation, membranes were prepared and tested for hyaluronan synthetase activity, measured as pmol of glucoronic acid transferred from UDP-GlcUA/h per mg of protein.

Addition	Hyaluronan synthetase activity (pmol/h per mg)
None (control)	130±10
PDGF-BB	216±36
Cycloheximide	45±5
PDGF-BB+cycloheximide	46±3
Vanadate	133±30
PDGF-BB+vanadate	230±9

synthesis is required for PDGF-BB to stimulate hyaluronan synthesis in NHM cells.

PDGF-BB mediates its cellular effects by activation of intrinsic protein tyrosine kinases in its α- and β-receptors (Heldin & Westermark, 1990). Vanadate is a known inhibitor of protein phosphotyrosine phosphatases (Swarup *et al.*, 1982). In order to explore the possibility that tyrosine phosphorylation is involved in the stimulation of hyaluronan synthetase activity, the effects of vanadate on the synthetase activity was tested. Vanadate itself was found to induce about a 3-fold stimulation of hyaluronan synthetase activity compared with control cultures (Fig. 2*b*), and to increase further the stimulatory effect of PDGF-BB (Table 1). The stimulatory effect of vanadate on hyaluronan synthetase activity reached a maximum after 4 h (Fig. 2*b*). A time-dependent increase in hyaluronan synthesis and release into the medium was also found in response to vanadate (Fig. 2*a*).

TGF-β1 stimulated hyaluronan synthetase activity with different kinetics than PDGF-BB and vanadate (Fig. 3). Maximal stimulation of hyaluronan synthetase (almost 2-fold) was obtained after about 24 h (Fig. 3*b*), followed by increased hyaluronan release into the culture medium (Fig. 3*a*).

In order to investigate whether hyaluronan synthetase is a target for protein kinase C (PKC), the effect of the PKC activator PMA on hyaluronan synthetase activity was examined (Fig. 4). PMA was found to induce a 10-fold increase in hyaluronan synthetase activity after 6 h of treatment (Fig. 4*b*). There was also a concomitant increase in the concentration of hyaluronan released into the medium (Fig. 4*a*).

To investigate whether the observed effect of PDGF-BB on hyaluronan synthetase activity could be ascribed to activation of

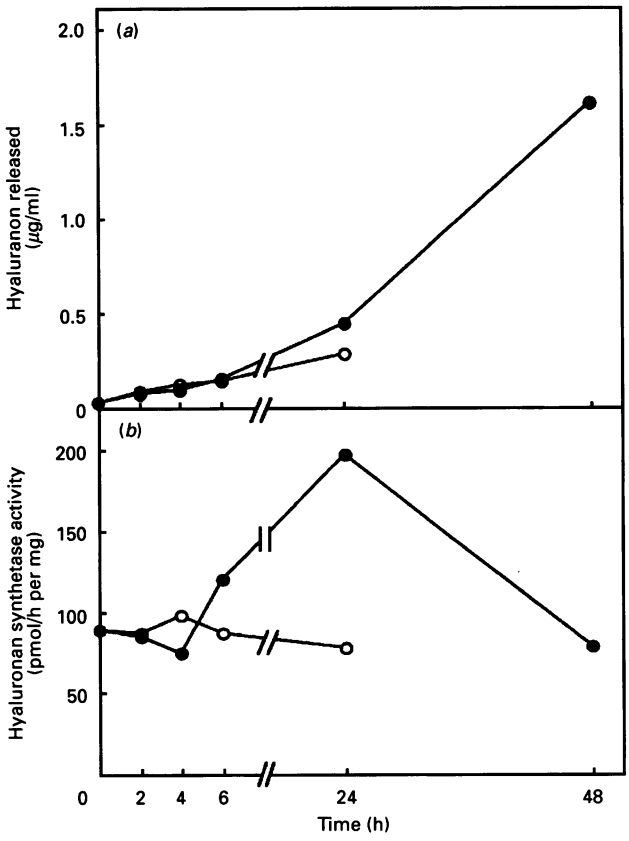

Fig. 3. Time-dependence of the effect of TGF-β1 on hyaluronan release and hyaluronan synthetase activity

Semiconfluent cultures of NHM cells were preincubated for 40 h in F-10 medium containing 0.5% FCS, and then cultured without (○) or with (●) 10 ng of TGF-β1/ml. The amount of hyaluronan released into the medium (*a*) and the hyaluronan synthetase activity in membrane preparations (*b*) are shown. The values shown are mean values from four different experiments.

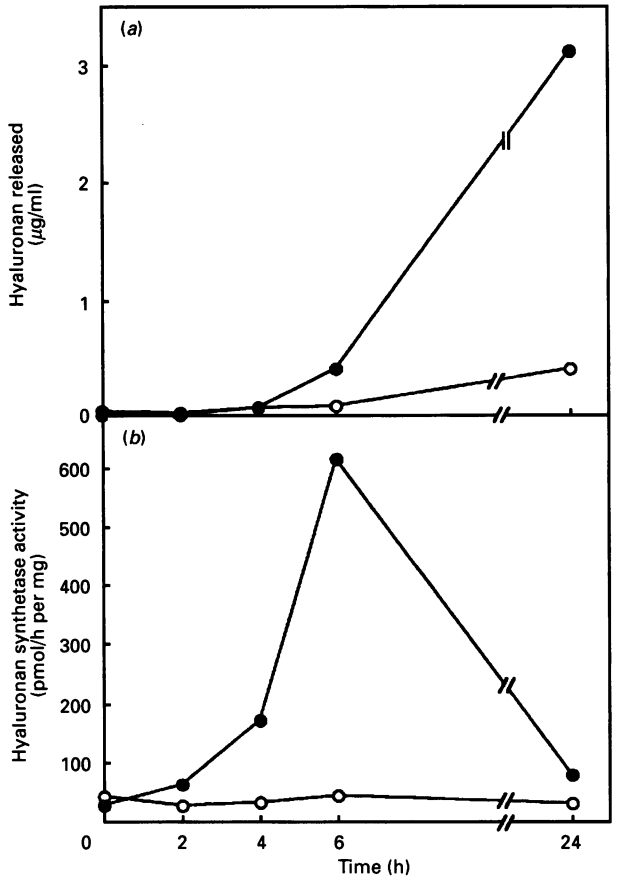

Fig. 4. Time-dependence of the effect of PMA on hyaluronan release and hyaluronan synthetase activity

Quiescent NHM cells were treated with (●) or without (○) 100 nM-PMA for the indicated time periods. The hyaluronan release into the cell culture medium (*a*) and hyaluronan synthetase activity in membrane preparations (*b*) were determined. The data shown are representative of two different experiments.

PKC, the effect of depletion of PKC by an overnight incubation of cells with PMA was investigated. Cells were cultured in F-10 medium containing 0.5% FCS overnight, and then incubated for an additional 24 h in the presence or absence of 100 nM-PMA. As expected, in the PMA-treated cells further addition of PMA had no effect on hyaluronan synthesis. In addition, treatment with PDGF-BB gave a lower stimulation of hyaluronan synthetase activity in PKC-depleted cells compared with control cells which retained PKC (Table 2), suggesting that PDGF-BB mediates its effect on hyaluronan synthetase activity in part via activation of PKC. In contrast, the stimulatory effect of vanadate on hyaluronan synthetase activity was hardly affected by PKC depletion (Table 2).

Table 2. Effect of PKC depletion on stimulation of hyaluronan synthetase activity by PDGF-BB or vanadate

NHM cells were cultured for 16 h in F10 medium containing 0.5% FCS. Cultures were then incubated for an additional 24 h in the absence or presence of 100 nM-PMA. Cells were then washed and given fresh medium containing 100 nM-PMA, 50 ng of PDGF-BB/ml, 30 μM-vanadate or no addition, and 6 h later, hyaluronan synthetase activity in membrane preparations was determined (pmol of glucoronic acid transferred from UDP-GlcUA/h per mg of protein). The values are means ± S.E.M. of four separate experiments.

	Hyaluronan synthetase activity (pmol/h per mg)	
Addition	Control cells	PMA-treated cells
None	82±12	76±14
PMA	554±12	84±13
PDGF-BB	148±29	86±25
Vanadate	86±21	114±30

DISCUSSION

In previous studies it has been demonstrated that hyaluronan synthetase activity in various biological systems can be stimulated with a variety of compounds, such as cyclic AMP and calf serum (Tomida *et al.*, 1977), EGF (Lembach, 1976), vanadate (Ohashi *et al.*, 1988), PMA (Ullrich & Hawkes, 1983), insulin-like growth factor-I (Honda *et al.*, 1989, 1991), PDGF-BB, FGF, EGF and TGF-β1 (Heldin *et al.*, 1989). In the present work we have explored the molecular mechanism behind the stimulation of hyaluronan synthetase by PDGF-BB in NHM cells. Analysis of membrane preparations from mesothelial cells pretreated with different agents revealed that hyaluronan synthetase activity increased during the first 6 h of treatment with PDGF-BB, PMA or vanadate; in contrast, TGF-β1 caused an increase in hyaluronan synthetase activity only after 24 h.

The PDGF-BB-induced increase in hyaluronan synthetase activity was found to be dependent on the synthesis of new protein (Table 1), suggesting that the activity of hyaluronan synthetase is regulated in part at the translational level. Tyrosine phosphorylation of as yet unidentified substrates is likely to be involved in the stimulation of translation, since the phosphotyrosine phosphatase inhibitor vanadate enhanced the effect of PDGF-BB on the enzyme activity (Table 1). PDGF-BB exerts its effects on cells via binding to two structurally related receptors, denoted α and β, both of which are protein tyrosine kinases (Heldin & Westermark, 1990). That tyrosine phosphorylation is involved in stimulation of hyaluronan synthetase activity was also suggested by the finding that the stimulatory effects of EGF and insulin-like growth factor-I on hyaluronan synthesis were blocked by the tyrosine kinase inhibitor genistein (Honda *et al.*, 1991). It seems likely that PDGF-BB exerts part of its stimulatory effect on hyaluronan synthetase via activation of PKC, since the effect of PDGF-BB was lowered in cells depleted of PKC (Table 2). PDGF has been shown to activate PKC (Raines *et al.*, 1990), but the exact mechanism by which the activation is exerted is not known. It is possible that activation of phospholipase C-γ is involved; this enzyme catalyses the degradation of phosphatidylinositol bisphosphate to inositol trisphosphate and diacylglycerol, an activator of PKC. Phospholipase C-γ has been found to be a substrate for the PDGF receptor kinase (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989), and the phosphorylation of the enzyme on tyrosine residues stimulates its catalytic activity (Kim *et al.*, 1991). Whether the effect of PDGF-BB on hyaluronan synthetase activity is exerted by phospholipase C-γ-mediated activiation of PKC remains to be elucidated. An additional possibility, which requires further investigation, is that the activity of hyaluronan synthetase is regulated by direct phosphorylation of the enzyme, e.g. on tyrosine residues by PDGF receptors or on serine/threonine residues by PKC. Evidence supporting the notion that the activity of hyaluronan synthetase is regulated by protein phosphorylation has been presented (Mian, 1986*b*; Prehm, 1989).

TGF-β1 stimulated hyaluronan synthetase activity with much slower kinetics than PDGF-BB, vanadate and PMA. It is possible that the effect of TGF-β1 is indirect and involves the stimulation of PDGF-BB, or other factors, which then stimulate hyaluronan synthetase in an autocrine manner. TGF-β1 has been found to induce the synthesis of PDGF-BB in AKR-2B cells (Leof *et al.*, 1986) and in endothelial cells (Daniel *et al.*, 1987).

It is known that PDGF is mitogenic for NHM cells (Gerwin *et al.*, 1987; Laveck *et al.*, 1988; Gabrielson *et al.*, 1988), and that mesotheliomas frequently produce PDGF (Gerwin *et al.*, 1987; Versnel *et al.*, 1988, 1991). It is thus possible that autocrine growth stimulation involving PDGF-BB is an important step in the formation of malignant mesothelioma (Versnel *et al.*, 1991). In addition, large amounts of hyaluronan often accumulate in mesothelioma patients (Waxler *et al.*, 1979; Arai *et al.*, 1979). It is thus possible that PDGF, or other factors that are produced by mesothelioma cells, stimulate the production of hyaluronan by the surrounding mesothelial cells or fibroblasts, or by the mesothelioma cells themselves.

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