Inhibition of Platelet-derived Growth Factor-BB-induced Receptor Activation and Fibroblast Migration by Hyaluronan Activation of CD44°‡§

Received for publication, June 12, 2006 Published, JBC Papers in Press, June 28, 2006, DOI 10.1074/jbc.M605607200

Lingli Li¹, Carl-Henrik Heldin¹, and Paraskevi Heldin‡§¹

From the ¹Ludwig Institute for Cancer Research, Uppsala University, Biomedical Center, Box 595, S-751 24 Uppsala and ²Department of Medical Biochemistry and Microbiology, Uppsala University, Biomedical Center, Box 582, S-751 23 Uppsala, Sweden

The extracellular matrix molecule hyaluronan was found to suppress platelet-derived growth factor (PDGF) β-receptor activation and PDGF-BB-induced migration of primary human dermal fibroblasts. The suppressive effect of hyaluronan was neutralized by a monoclonal antibody that specifically inhibits hyaluronan binding to its receptor CD44. Moreover, co-immunoprecipitation experiments showed that the PDGF β-receptor and CD44 can form a complex. Interestingly, the inhibitory effect of hyaluronan on PDGF β-receptor activation was not seen in the presence of the tyrosine phosphatase inhibitor pervanadate. Our observations suggest that hyaluronan suppresses PDGF β-receptor activation by recruiting a CD44-associated tyrosine phosphatase to the receptor.

Hyaluronan is the most common glycosaminoglycan present in the extracellular matrix of mammalian tissues, for example in epidermis and dermis. It is synthesized by both normal and malignant cells, such as fibroblasts (2) and breast cancer cells (3), and is involved in a variety of physiological events, such as cell proliferation, differentiation, and migration (4). The ability of hyaluronan to affect cell migration has been attributed partly to its physicochemical properties; hyaluronan molecules form continuous and porous biological meshworks that exhibit visco-elastic properties. This property makes tissues resilient and malleable, which is important to facilitate cell motility during development, tissue remodeling, and cancer. Furthermore, several studies have provided evidence that hyaluronan exerts signaling effects through interactions with certain cell surface receptors, such as CD44 and RHAMM (5, 6). The predominant receptor for hyaluronan is the cell surface adhesion receptor CD44 that is expressed in most cell types transferring signals from the outside of the cell to the inside in response to hyaluronan binding (7–9). CD44 exists in several isoforms because of alternative splicing of the gene, which can further undergo extensive post-translational modifications by glycosylation and addition of polysaccharide chains. This diversity in its structure accounts for its diverse biological functions and ability to interact with numerous other macromolecules in addition to hyaluronan, including collagen, fibronectin, growth factors, cytokines, and metalloproteinases (10, 11). The most common form, CD44s (CD44 standard form), is expressed by a wide variety of cells, including hematopoietic cells and dermal fibroblasts. Studies have shown that CD44s facilitates lymphocyte homing and fibroblast migration during wound healing (12–14). Longer variant isoforms, termed CD44v, are found on endothelial cells, epithelial cells, activated lymphocytes, and some tumor cells. The cytoplasmic domain of CD44 interacts with ERM proteins and ankyrin and thus indirectly with the cytoskeleton (10).

Notably, among the three hyaluronan-synthesizing enzymes (Has1, -2, and -3), the Has2 isoform seems to be most important in mediating cell migration. For example, Has2−/− mouse cardiac endothelial cells display reduced ability to migrate, a process that can be reversed by gene rescue or by the addition of exogenous hyaluronan (15). In addition, studies on various tumor cells, such as mesotheliomas, colon carcinomas, and breast cancer, revealed that cells lacking Has2 expression migrated much slower compared with Has2-expressing cells (16–18). Interestingly, not all types of cells respond to hyaluronan by increased locomotion; Chinese hamster ovary cells (19) and low metastatic murine T cell lymphoma cells (20) reduced their motility in response to hyaluronan.

Growth factors also play a central role in cell migration during both normal and pathological conditions, including embryogenesis, wound healing, and tumor invasion. In this regard, platelet-derived growth factor (PDGF)²-BB is a powerful stimulator of the migration of mesenchymal cells, such as fibroblasts (21–23). PDGF-BB mediates its cellular effects through binding to α- and β-tyrosine kinase receptors (PDGFRα and PDGFRβ, respectively), resulting in receptor dimerization and activation through autophosphorylation; this leads to recruitment of Src homology 2 domain signal transduction molecules to the receptor (24). Recent studies indicate that PDGFRβ and other tyrosine receptor kinases can be dephosphorylated

---

1 To whom correspondence should be addressed. Tel.: 46-18-471-4261; Fax: 46-18-471-4975; E-mail: Paraskevi.Heldin@imbim.uu.se.

2 The abbreviations used are: PDGF, platelet-derived growth factor; PDGFRβ, PDGF β-receptor; HABP, hyaluronan-binding protein; PTP, protein-tyrosine phosphatase; WGA, wheat germ agglutinin; PBS, phosphate-buffered saline without Ca²⁺ and Mg²⁺; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium.

---

This work was supported in part by Swedish Cancer Society Grant 3446-803-10XBC and Uppsala University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
by a number of protein-tyrosine phosphatases (PTPs), which thereby affect downstream signaling of the receptors (25).

The effects of growth factors and matrix components on cells are interdependent, because growth factors affect the synthesis of extracellular molecules and their degrading enzymes, whereas the extracellular macromolecules modulate growth factor-mediated cellular functions. Previous studies have demonstrated a synergism between αβ integrin and PDGF-BB in mediating endothelial cell migration (26, 27). In this study, we investigated the possibility that hyaluronan affects PDGF-BB-induced cell migration of human dermal fibroblast cultures.

MATERIALS AND METHODS

Cell Culture—Biopsies were taken from elective breast reduction surgery, after the approval of the patient (Department of Plastic Surgery, University Hospital, Uppsala, Sweden), and transported to the laboratory in chilled phosphate-buffered saline without Ca2+ and Mg2+ (PBS) but containing antibiotics (100 IU penicillin/ml and 100 μg of streptomycin/ml; Veterinary Institute, Uppsala, Sweden). Following a quick wash in 70% ethanol, the biopsy was washed with PBS and minced to 1–2-mm3 pieces with a razor blade. Dermis and epidermis were separated, using fine forceps, after an overnight incubation in 2.5 mg of dispase per ml of Dulbecco’s modified Eagle’s medium and 1–2-mm3 pieces with a razor blade. Dermis and epidermis were separated, using fine forceps, after an overnight incubation in 2.5 mg of dispase per ml of Dulbecco’s modified Eagle’s medium (grade II, 0.5 units/ml; Roche Applied Science) (DMEM; Invitrogen) at 4 °C. Fibroblasts from the dermal sheets were then cultured from explants in complete medium (DMEM supplemented with 10% fetal bovine serum (Biowest) and antibiotics). Skin fibroblasts (passages 6–10) from the explants were supplemented with 10% fetal bovine serum (Biowest) and antibiotics.

Cell Migration Assay—Human dermal fibroblasts (8 × 104 cells per well in 6-well plates) were cultured in complete medium for 24 h; thereafter, the medium was replaced with starvation medium. Then a wound was made by scraping the confluent cell cultures, crosswise, with the tip of a 1-ml pipette. The floating cells were washed away with DMEM supplemented with 0.1% FCS (starvation medium). The wound closure was monitored by an inverted microscope (Nikon) at four different positions along the wound, immediately and after different time periods up to 24 h in untreated cultures and in cultures treated with various concentrations of PDGF-BB. To investigate the effect of hyaluronan on fibroblast migration induced by 1 ng/ml PDGF-BB, the cultures were preincubated for 2 h with hyaluronan (1–100 μg/ml, Mw 0.5–1.5 × 106; Enzyme Hylumed) in the absence or presence of rat monoclonal Hermes-1 antibodies (used as serum-free supernatants from the myeloma culture; generously provided by Professor Sirpa Jalkanen, Turku, Finland), which specifically inhibits the binding of hyaluronan to the CD44 receptor. The cell migration was quantified by NIH Image 1.63 software. The data were exported to Excel and statistically analyzed by Student’s t test.

Analysis of Hyaluronan Synthesis—Fibroblasts (8 × 104 cells/well in 6-well plates) were grown for 24 h in complete medium, followed by a 24-h starvation. The quiescent cells received fresh starvation medium supplemented with various concentrations of PDGF-BB, and at various time points the hyaluronan content in the conditioned media was quantified, essentially as described previously (16). The assay is based on the formation of a complex between hyaluronan and the hyaluronan-binding protein (HABP) domain of aggrecan, the binding of which essentially is irreversible. The HABP was isolated from crude bovine cartilage extract by affinity chromatography on a hyaluronan-EAH-Sepharose 4B (Amersham Biosciences) (28). A part of this HABP was biotinylated with biotin-aminocaproic acid sulfo-NHS (catalog number B1022, Sigma). The assay was carried out on MaxiSorp 96-well Nunc-ImmuNo Plates (catalog number 439454, Nunc) that were precoated overnight with 1 μg of HABP/ml in 50 mM carbonate buffer, pH 9.5, washed in PBS containing 0.5% Tween 20, and blocked in PBS containing 1% BSA. The hyaluronan standards (0–100 ng/ml) and samples of conditioned media, at appropriate dilutions in blocking solution, were then added, and the plates were incubated for 1 h at 37 °C. Following washing, 100 μl of biotinylated HABP (b-HABP; 1 μg/ml) was added, and samples were incubated for 1 h. After excess washing, the b-HABP specifically bound to the immobilized hyaluronan was determined by incubation for 1 h with streptavidin-biotinylated horseradish peroxidase complex (catalog number RNP1015V, Amersham Biosciences), followed by the addition of 100 μl of 3,3',5,5'-tetramethylbenzidine substrate solution (catalog number T4444, Sigma) for 15 min. 50 μl of 2 M H2SO4 was then added, and the absorbance was measured at a wavelength of 450 nm. The hyaluronan content was calculated by comparing with a standard curve made from known concentrations of hyaluronan, and the values were statistically analyzed by Student’s t test.

Generation and Purification of Hermes-1 Fab Fragments—Eight mg of Hermes-1 IgG was extensively dialyzed against 20 mM sodium phosphate and 10 mM EDTA, pH 7.0, followed by concentration to ~0.5 ml using an Aminco Ultra-15 centrifugal filter device (Mw 10,000 cutoff, Millipore). Fab fragments were then generated using ImmunoPure Fab preparation kit (catalog number 44885, Pierce) by 5 h of digestion at 37 °C with immobilized papain followed by purification according to the manufacturer. In addition, an UltraLink immobilized protein G column (catalog number 53127, Pierce) was used to remove Fc fragments and undigested IgG from Hermes-1 Fab fragments.

Cell Adhesion Assay—Glass coverslips in 12-well culture dishes were coated with 0.5 mg/ml hyaluronan or 0.5% BSA (negative control for general attachment), in 10 mM Hepes, pH 7.4, containing 137 mM NaCl, 4.7 mM KCl, 0.65 mM MgSO4, and 1.2 mM CaCl2 overnight in a cold room. Dermal fibroblasts were incubated for 24 h in starvation medium, trypsinized, washed, and diluted to 1 × 105 cells/ml with starvation medium. The cells were then incubated for 30 min in the cold room without or with Hermes-1 IgG, Hermes-1 Fab fragments, or Hermes-3 IgG (ammonium sulfate precipitate from serum-free myeloma culture medium; generously provided by Professor Sirpa Jalkanen, Turku, Finland). Cell samples of 1 × 105 cells per coverslip were allowed to adhere for 30 min at 37 °C. Nonadherent cells were removed with two washings in the above-described Hepes buffer, and the number of adhered cells was measured by the hexose-aminidase assay (29).

PDGFRB Precipitation, Co-immunoprecipitation, and Immunoblotting—Normal human dermal fibroblasts (precipitation, 2 × 105 cells/6-cm culture dish; co-immunoprecipitation, 4 × 105 cells/10-cm culture dish) were starved as described above,
exposed to hyaluronan, Hermes-1, and/or pervanadate, before stimulation with PDGF-BB for 15 min as indicated. Cultures were washed once with cold PBS and lysed on ice for 15 min in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate), supplemented with phosphatase and protease inhibitors (0.5 mM Na3VO4, 1 μg/ml aprotinin, 0.5 mg/ml Pefabloc, 10 μl leupeptin). Lysates were collected in 1.5-ml Eppendorf tubes, homogenized with a 20-gauge needle on ice; subsequently insoluble debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C. PDGFRβ, in the cleared lysates, was absorbed to 12 μl of wheat germ agglutinin (WGA)-Sepharose (Sigma) for 2 h end-over-end at 4 °C.

For co-immunoprecipitation, the cleared lysates were incubated for 2 h with 2 μg of polyclonal antiserum against PDGFRβ (CTβ; a generous gift from Dr. Carina Hellberg, Ludwig Institute for Cancer Research, Uppsala, Sweden) or 5 μg of anti-CD44 IgG (Hermes-1, purified by ammonium sulfate) before the addition of 50 μl of protein A-Sepharose (Amersham Biosciences) per sample. As controls, rabbit IgG (I-5006, Sigma) and rat IgG (I-4131, Sigma) were used. PDGFRβ bound to WGA beads, or immunoprecipitates collected on protein A-Sepharose beads, were spun down, washed twice with lysis buffer, and resuspended in 30 μl of sample buffer with dithiothreitol, and heated 5 min at 99 °C to release the proteins from the beads; they were then subjected to 7.5% SDS-PAGE and transferred to a membrane.

After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Hybond™-C Extra, Amersham Biosciences) for immunoblotting. Nonspecific binding sites on the membrane were blocked with 5% defatted milk in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, supplemented with 0.1% Tween 20), at room temperature for 1 h or overnight at 4 °C, followed by two washes in TBS-T. The membranes were then incubated with a monoclonal phosphotyrosine antibody (PY99, 1 μg/ml; catalog number SC-7020, Santa Cruz Biotechnology), PDGFRβ antibody (CTβ, 2 μg/ml), or CD44 antibody (1 μg/ml; Hermes-1) in TBS-T buffer containing 1% BSA overnight at 4 °C. After five washes in TBS-T, the membranes were incubated with anti-mouse horseradish peroxidase IgG (1:3000; Amersham Biosciences), anti-rabbit horseradish peroxidase IgG (1:3000; Amersham Biosciences), or anti-rat horseradish peroxidase IgG (1:1000; Santa Cruz Biotechnology catalog number SC-2006) for the detection of phosphorylated PDGFRβ, total PDGFRβ, and hyaluronan receptor CD44 immune complexes, respectively. Immunoreactive bands were then detected by enhanced chemiluminescence. The membranes were stripped with Stripping buffer (catalog number 0100-01, Immunokemi) and photographed with a Leica Microsystems microscope.

RESULTS

PDGF-BB Induces Migration and Stimulates Hyaluronan Production by Human Dermal Fibroblasts—Because both PDGF-BB and hyaluronan have been implicated in cell migration (4, 22), we investigated the importance of these molecules for migration of human dermal fibroblasts. First, we examined the effects of different concentrations of PDGF-BB both on migration and hyaluronan synthesis. In a wounding experiment in confluent cultures of dermal fibroblasts, PDGF-BB was found to stimulate in a dose-dependent manner the wound closure over a 24-h period. Maximum effect was seen at 10 ng/ml PDGF-BB; at this concentration, the wound in the cell culture healed about 3-fold faster than in unstimulated cell cultures (Fig. 1A). Fig. 1B shows that the wound closure was almost complete in PDGF-BB-stimulated cultures after 24 h. Notably, PDGF-BB-stimulated cells exhibited an elongated and polarized morphology with membrane sheets, i.e. membrane ruffles or lamellipodia, at the leading edge of migrating fibroblasts. These membrane sheets were stained with specific antibodies for the hyaluronan receptor CD44, demonstrating an accumulation of CD44 preferentially at the leading edge of the migrating cells (Fig. 1C). The nonstimulated fibroblasts exhibited less polarization and membrane ruffle formation than PDGF-BB-stimulated cells, but yet expressed CD44 at the leading edge of migrating cells (Fig. 1C).

A 24-h stimulation of fibroblasts with 10 ng/ml PDGF-BB also induced an approximate 3-fold increase of hyaluronan amount (1.1 μg/ml) relative to unstimulated cells; 1 ng/ml...
PDGF-BB resulted in a 1.8-fold increase (about 0.7 μg/ml) (Fig. 1D). One ng of PDGF-BB per ml was the lowest concentration that gave significant effects on cell migration, as well as on hyaluronan production; this concentration was therefore chosen to investigate if endogenously produced hyaluronan affected PDGF-BB-mediated fibroblast migration. Culturing of the dermal fibroblasts in the presence of Streptomyces hyaluronidase, which specifically degrades hyaluronan to tetrasaccharides, did not affect the PDGF-BB-induced cell migration (Fig. 1E). Thus, PDGF-BB-induced dermal fibroblast motility in vitro appeared not to be affected by endogenously produced high molecular weight hyaluronan.

Binding of Hyaluronan to CD44 Suppresses PDGF-BB-induced Fibroblast Migration—Given the fact that the hyaluronan amount is high in skin (about 0.5 mg/ml), we investigated the effect of exogenously added hyaluronan on PDGF-BB-induced fibroblast migration. As shown in Fig. 2, the stimulation of cell motility induced by 1 ng/ml PDGF-BB was significantly inhibited by addition of exogenous hyaluronan at 25 μg/ml or higher concentrations of hyaluronan (Fig. 2A). Lower concentrations of exogenously added hyaluronan had no effect on growth factor-stimulated cells (data not shown).

To investigate the role of CD44 for the inhibitory effect of hyaluronan on PDGF-BB-induced migration, Hermes-1 monoclonal antibodies that bind to CD44 and block the binding of hyaluronan (31) were used. First, we verified that Hermes-1 antibodies could inhibit attachment also of the dermal fibroblasts used in this study to a hyaluronan-coated substratum (Fig. 2B). Furthermore, Fab fragments of anti-CD44 Hermes-1 blocked cell attachment, whereas the nonhyaluronan blocking anti-CD44 Hermes-3 antibodies did not (Fig. 2B). Importantly, addition of Hermes-1 restored the hyaluronan-mediated suppression of PDGF-BB-induced motility of fibroblasts (Fig. 2C; p < 0.05). These results suggest that hyaluronan-CD44 complexes, formed when cells are surrounded with high amounts of hyaluronan, can function as negative modulators of PDGF-BB-mediated cell motility.

PDGFRβ Activation Is Inhibited by CD44-Hyaluronan Complexes—To gain insights into the mechanism behind the inhibitory effect of the CD44-hyaluronan complex on PDGF-BB-mediated fibroblast migration, we examined the effect of exogenously added hyaluronan on PDGFRβ activation, in the absence or presence of Hermes-1 (Fig. 3). Interestingly, incubation of PDGF-BB-stimulated fibroblasts with increasing hyalu-
Hyaluronan-inhibited PDGFRβ Activation and Fibroblast Motility

**FIGURE 2.** PDGF-BB-induced fibroblast migration is inhibited by exogenous hyaluronan and restored by Hermes-1. A, wounded human dermal fibroblast cultures (8 × 10^5 cells per well in 6-well plates) were incubated for 2 h in starvation medium alone or in medium containing 25–100 μg of hyaluronan/ml; 1 ng of PDGF-BB/ml was then added, and incubation was continued another 24 h; thereafter the wound size was measured. B, the attachment of dermal fibroblasts to hyaluronan-coated substratum was analyzed in the presence of the IgG and Fab fragment of the blocking CD44 antibody Hermes-1, as well as the nonblocking antibody Hermes-3 (25–50 μg/ml). C, Hermes-1 antibodies (0–50 μl serum-free supernatant/ml) were added into wounded fibroblast cultures 1 h before exposure to 100 μg of hyaluronan/ml and 1 ng of PDGF-BB/ml, as described in A. The wound closure was quantified by NIH Image 1.63 software, at wound gaps for 2 h in starvation medium alone or in medium containing 10–100 μg of hyaluronan/ml and then stimulated with 5 ng of PDGF-BB/ml for 15 min; thereafter cells were lysed. B, in A but blocking CD44 antibodies, Hermes-1 (50 μl/ml), were added 1 h before exposing the cultures to hyaluronan (100 μg/ml) and PDGF-BB (5 ng/ml) followed by cell lysis. C, in A but the effect of approximately equimolar amounts of chondroitin sulfate A (CS-A) were compared with hyaluronan (10 μg/ml CS-A corresponds to 100 μg/ml hyaluronan). A–C, PDGF-BB-receptors were adsorbed to WGA-agarose, and the precipitated proteins were subjected to SDS-PAGE followed by transfer to nitrocellulose membrane. 

**FIGURE 3.** PDGF-BB-induced phosphorylation of PDGFRβ receptor is inhibited by hyaluronan and restored by blocking CD44 antibody. A, quiescent fibroblast cultures (2 × 10^5 cells per 6-cm culture dish) were incubated for 2 h in starvation medium alone or in medium containing 10–100 μg of hyaluronan/ml and then stimulated with 5 ng of PDGF-BB/ml for 15 min; thereafter cells were lysed. B, in A but blocking CD44 antibodies, Hermes-1 (50 μl/ml), were added 1 h before exposing the cultures to hyaluronan (100 μg/ml) and PDGF-BB (5 ng/ml) followed by cell lysis. C, in A but the effect of approximately equimolar amounts of chondroitin sulfate A (CS-A) were compared with hyaluronan (10 μg/ml CS-A corresponds to 100 μg/ml hyaluronan). A–C, PDGF-BB-receptors were adsorbed to WGA-agarose, and the precipitated proteins were subjected to SDS-PAGE followed by transfer to nitrocellulose membrane. 

Ronan amounts led to a marked decrease of PDGFRβ phosphorylation, achieved at 50 μg/ml and higher concentrations of hyaluronan; densitometric analysis showed that this reduction in the phosphorylation of the PDGFRβ was statistically significant (p < 0.05). Importantly, the presence of Hermes-1 mAbs neutralized the inhibitory effect of hyaluronan on PDGF-BB-induced β-receptor phosphorylation (Fig. 3B; p < 0.05). The presence of Hermes-1 Fab fragments that inhibit the binding of hyaluronan to CD44 (Fig. 2B), but are unable to induce CD44 dimerization, also neutralized the inhibitory effect of hyaluronan on PDGF-BB-induced β-receptor phosphorylation (supplemental Fig. 1). These findings suggest that hyaluronan-CD44 complexes inhibit PDGFRβ activation. The effect was specific for hyaluronan because equimolar concentrations of chondroitin sulfate did not inhibit PDGFB-BB-induced receptor autophosphorylation (Fig. 3C).

**PDGFRβ Receptor Forms a Complex with CD44**—The hyaluronan-mediated reduction of PDGF-BB-induced β-receptor phosphorylation and its restoration by blocking mAb Hermes-1 suggested a close proximity of PDGFRβ and CD44. Therefore, we examined the possibility that these molecules form a complex by performing co-immunoprecipitation expen-
Immunoprecipitation of PDGFR β/H9252 using the CT β/H9252 antiserum followed by immunoblotting of CD44 using the Hermes-1 antibody yielded a band of 90–105 kDa, i.e. the expected size of the standard isoform of CD44; no CD44 band was seen when a nonimmune serum was used for precipitation (Fig. 4A). The co-immunoprecipitation of PDGFR β/H9252 and CD44 was seen in unstimulated as well as in PDGF-BB-stimulated cells. Re-probing the blot with anti-PDGFR β/H9252 antibodies (CT β/H9252) revealed the expected 190-kDa band, and re-probing with anti-phosphotyrosine antibodies (PY99) revealed that the receptor was phosphorylated and activated after, but not before, PDGF-BB stimulation.

When the reverse experiment was done, i.e. immunoprecipitation of CD44 followed by blotting with PDGFR β antiserum, a band of 190 kDa, i.e. the size of PDGFR β, was seen with or without PDGF-BB stimulation (Fig. 4B). Re-probing of the blot with Hermes-1 and PY99 antibodies resulted in bands with molecular masses corresponding to CD44 and phosphorylated β-receptor, respectively. Replacement of Hermes-1 with Hermes-3 (another CD44-specific antibody) gave similar results (data not shown). These findings suggest that the PDGFR β and CD44 form a complex in dermal fibroblasts, before as well as after stimulation with PDGF-BB. Notably, although the PDGFR β, as expected, was tyrosine-phosphorylated after PDGF-BB stimulation, CD44 was not recognized by anti-phosphotyrosine antibodies, suggesting that CD44 is not a substrate for the PDGFR β.

We also investigated the localization of CD44 and PDGFR β receptor in dermal fibroblasts using immunostainings. In unstimulated cells, both CD44 and PDGFR β were detected diffusely all over the cell with a preference at ruffle structures, where a partial co-localization was observed (Fig. 5A). In
Hyaluronan-inhibited PDGFRβ Activation and Fibroblast Motility

PDGF-BB-stimulated cells, the fibroblasts exhibited more ruffles and filopodial extensions, and CD44 was preferentially localized at these ruffle structures, where also the PDGF β-receptors were localized (Fig. 5A). PDGF-BB stimulation induced internalization of β-receptors into endosomes already after 15 min, noticed as dot-like structures; notably, CD44 was not present in these structures, suggesting that CD44 is not internalized together with the PDGF β-receptor. Double staining for hyaluronan and CD44 in PDGF-BB-treated cultures revealed a partial co-localization of hyaluronan and CD44 at ruffle- and filopodia-like extensions of the plasma membrane (Fig. 5B). Exogenously added hyaluronan also co-localized partially with CD44 (Fig. 5B); the staining was lost after Streptomyces hyaluronidase digestion (data not shown).

Protein-Tyrosine Phosphatase(s) Is Involved in the Hyaluronan-induced Inactivation of PDGFRβ—To investigate whether the hyaluronan-induced inhibition of PDGF β-receptor activation involved dephosphorylation of the receptor, we used the PTP inhibitor pervanadate in fibroblast cultures. As shown in Fig. 6, hyaluronan caused an approximate 40% reduction in the PDGF-BB-stimulated phosphorylation of the β-receptor. Incubation with pervanadate induced an increase in the phosphorylation of β-receptors, and interestingly, in the presence of pervanadate there was no difference between cells incubated with or without hyaluronan. These data suggest that the hyaluronan-CD44-mediated negative regulation of PDGFRβ activation in human dermal fibroblasts involves the recruitment of a PTP to the receptor.

DISCUSSION

We show in this study that hyaluronan inhibits PDGF-BB-induced activation of PDGFRβ and cell motility. The monoclonal antibody Hermes-1, which blocks the binding of hyaluronan to CD44, restored PDGF β-receptor activation and motility, indicating that CD44 mediates the inhibiting effect on PDGFRβ. Furthermore, we demonstrate that PDGFRβ and CD44 form a complex and that the inhibitory effect of hyaluronan is neutralized by inhibition of tyrosine phosphatases. Our data suggest that hyaluronan-activated CD44 modulate PDGFβ signaling by recruiting tyrosine phosphatase(s) to the receptor.

Our finding that PDGFRβ and CD44 are associated into a complex adds to the examples of interactions between PDGFRβ and receptors for matrix molecules. Thus, PDGFRβ has been shown previously to interact with the integrin αvβ3 (26, 27). However, the interaction with αvβ3 enhances PDGF signaling, whereas the interaction with CD44 suppresses PDGF signaling.

An important task for future studies will be to identify the molecular mechanisms whereby CD44 inhibits PDGFRβ. CD44 does not exhibit any intrinsic enzymatic activity, but from earlier studies it has been evident that CD44 interacts with other proteins, both in a hyaluronan-dependent and -independent manner and thereby modulates cellular responses (10). For example, fibroblast growth factor signaling is promoted by binding of fibroblast growth factors to heparan sulfate chains in the extracellular part of the CD44v3 isoform, whereby the ligand is presented to the signaling tyrosine kinase receptor (32). CD44 has also been shown to act as a co-receptor for the ErbB family of transmembrane tyrosine kinases (33, 34) and for the transforming growth factor-β type 1 serine/threonine kinase receptor (35). In these cases, CD44 interaction enhances growth factor signaling.

We found that hyaluronan-activated CD44 inhibits PDGFR β tyrosine kinase activity and suppressed signaling. Because pervanadate neutralized the inhibitory effect, it is likely that hyaluronan binding to CD44 activates a PTP that acts on the autophosphorylated PDGFRβ. Although several PTPs have been shown to have phosphorylated PDGFRβ as substrate (25), the PTP(s) involved in CD44-mediated PDGFRβ dephosphorylation has not been identified. Interestingly, the C termini of CD44 possess potential PDZ domain-binding sites (36); it is an interesting possibility that CD44 interacts with PDZ domain-containing protein phosphatases (37, 38). Notably, the PTP CD45, which dephosphorylates Lck and Fyn and other members in the Src family of tyrosine kinases, has been shown to have a negative regulatory role in CD44- and α5β1 integrin-mediated cell adhesion (39, 40).

The concentrations of hyaluronan needed to obtain the negative effects on PDGFRβ activation were rather high, i.e. >25 μg/ml. However, the concentration of hyaluronan in normal skin has been estimated to be 0.5 mg/ml, suggesting that the modulating effects of hyaluronan on PDGF signaling that we have demonstrated may have physiological relevance.
The effect of hyaluronan on PDGF signaling may also be of relevance in pathophysiological situations, such as wound healing. During wound healing, fibroblasts from the surrounding connective tissue become activated by the action of cytokines and growth factors released by platelets and infiltrating macrophages, and migrate and infiltrate the wound provisional matrix that is composed of fibrin and fibronectin. Inside the wound, fibroblasts synthesize large amounts of hyaluronan (2), fibronectin, and collagen types I and III (41). PDGF-BB stimulates hyaluronan synthesis and promotes the assembly of hyaluronan-containing pericellular matrices around mesenchymal cells (1). The findings of the present study suggest that hyaluronan networks may suppress the activity of PDGF-BB and infiltration of fibroblasts into the provisional matrix before matrix remodeling occurs. Thus, the observations by us and others suggest that growth and motility of cells in tissues are regulated by growth factors and extracellular molecules together in a synergistic as well as antagonistic manner (30).

Acknowledgments—We thank Drs Sirpa Jalkanen and Carina Hellberg for the generous gifts of antibodies.

REFERENCES

1. Heldin, P., and Pertoft, H. (1993) Exp. Cell Res. 208, 422–429
2. Heldin, P., Laurent, T. C., and Heldin, C.-H. (1989) Biochem. J. 258, 919–922
3. Heldin, P., de la Torre, M., Ytterberg, D., and Bergh, J. (1996) Oncol. Rep. 3, 1011–1016
4. Toole, B. P. (2004) Nat. Rev. Cancer 4, 528–539
5. Bourguignon, L. Y., Singleton, P. A., and Diedrich, F. (2004) J. Biol. Chem. 279, 29654–29669
6. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. (2002) J. Biol. Chem. 277, 4589–4592
7. Knudson, W. (1998) Front. Biosci. 3, D604–D615
8. Hamada, J., Sawamura, Y., and Van Meir, E. G. (1998) Front. Biosci. 3, D657–D664
9. Cichy, J., and Pure, E. (2004) FEBS Lett. 556, 69–74
10. Ponta, H., Sherman, L., and Herrlich, P. A. (2003) Nat. Rev. Mol. Cell Biol. 4, 33–45
11. Thorne, R. F., Legg, J. W., and Isacke, C. M. (2004) J. Cell Sci. 117, 373–380
12. Jalkanen, S., Bargatze, R. F., de los Toyos, J., and Butcher, E. C. S. (1987) J. Cell Biol. 105, 983–990
13. Turley, E. A. (1992) Cancer Metastasis Rev. 11, 21–30
14. Greiling, D., and Clark, R. A. (1997) J. Cell Sci. 110, 861–870
15. Camenisch, T. D., Spicer, A. P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M. L., Calabro, A., Jr., Kubalak, S., Klewer, S. E., and McDonald, J. A. (2000) J. Clin. Investig. 106, 349–360
16. Jacobson, A., Rahmanian, M., Rubin, K., and Heldin, P. (2002) Int. J. Cancer 102, 212–219
17. Li, Y., and Heldin, P. (2001) Br. J. Cancer 85, 600–607
18. Udagawa, L., Brownlee, G. R., Waltham, M., Blick, T., Walker, E. C., Heldin, P., Nilsson, S. K., Thompson, E. W., and Brown, T. J. (2005) Cancer Res. 65, 6139–6150
19. Brinch, J., and Heldin, P. (1999) Exp. Cell Res. 252, 342–351
20. Kubens, B. S., Nikolai, G., and Zanker, K. S. (1997) Cancer Lett. 118, 189–200
21. Heldin, C. H., and Westermark, B. (1999) Physiol. Rev. 79, 1283–1316
22. Yu, J., Moon, A., and Kim, H. R. W. (2001) Biochem. Biophys. Res. Commun. 282, 697–700
23. Ronnstrand, L., and Heldin, C. H. (2001) Int. J. Cancer 91, 757–762
24. Heldin, C. H., Ostman, A., and Ronnstrand, L. (1998) Biochim. Biophys. Acta 1378, F79–F113
25. Persson, C., Savenhed, C., Bourdeau, A., Tremblay, M. L., Markova, B., Bohmer, F. D., Haj, F. G., Neel, B. G., Elson, A., Heldin, C. H., Ronnstrand, L., Ostman, A., and Hellberg, C. (2004) Mol. Cell. Biol. 24, 2190–2201
26. Schneller, M., Vuori, K., and Ruoslahti, E. (1997) EMBO J. 16, 5600–5607
27. Woodard, A. S., Garcia-Cardenas, G., Leong, M., Madri, J. A., Sessa, W. C., and Languino, L. R. (1998) J. Cell Sci. 111, 469–478
28. Tengblad, A. (1979) Biochim. Biophys. Acta 578, 281–289
29. Landegren, U. (1984) J. Immunol. Methods 67, 379–388
30. Elcei, B. P. (2001) Circ. Res. 89, 1104–1110
31. Teder, P., Bergh, J., and Heldin, P. (1995) Cancer Res. 55, 3908–3914
32. Sherman, L., Wainwright, D., Ponta, H., and Herrlich, P. (1998) Genes Dev. 12, 1058–1071
33. Bourguignon, L. Y., Zhu, H., Zhou, B., Diedrich, F., Singleton, P. A., and Hung, M. C. (2001) J. Biol. Chem. 276, 48679–48692
34. Wobus, M., Rangwala, R., Sheyn, I., Hennigan, R., Coila, B., Lower, E. E., Yassin, R. S., and Sherman, L. S. (2002) Appl. Immunohistochem. Mol. Morphol. 10, 34–39
35. Bourguignon, L. Y., Singleton, P. A., Zhu, H., and Zhou, B. (2002) J. Biol. Chem. 277, 39703–39712
36. Hung, A. Y., and Sheng, M. (2002) J. Biol. Chem. 277, 5699–5702
37. Saras, J., Claesson-Welsh, L., Heldin, C. H., and Gonez, L. J. (1994) J. Biol. Chem. 269, 24082–24089
38. Bass, M. D., and Humphries, M. J. (2002) Biochem. J. 368, 1–15
39. Sheni, H., Sevitt, J., Zanker, K. S., Thomas, M. L., and Brown, E. J. (1999) J. Immunol. 162, 7120–7127
40. Li, R., Wong, N., Jabali, M. D., and Johnson, P. (2001) J. Biol. Chem. 276, 28767–28773
41. Singer, A. J., and Clark, R. A. (1999) N. Engl. J. Med. 341, 738–746