Syndecan-2 Is Involved in the Mitogenic Activity and Signaling of Granulocyte-Macrophage Colony-stimulating Factor in Osteoblasts*

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We previously showed that granulocyte-macrophage colony-stimulating factor (GM-CSF) binds to heparan sulfate proteoglycans expressed at the surface of osteoblastic cells and that the mitogenic activity of this cytokine is dependent on the presence of fully sulfated proteoglycans. In this study, we determined if GM-CSF interacts with syndecans, a family of cell surface heparan sulfate proteoglycans. Human primary osteoblasts were found to express syndecan-2 and -4 but few syndecan-1 transcripts and proteins. Recombinant human GM-CSF coupled to biotin was found to bind to syndecan-2. Immunocytochemical transmission electron microscope analysis showed co-localization of syndecan-2 and GM-CSF at the cell membrane surface. Syndecan-2 also co-localized at the cell surface and co-immunoprecipitated with the GM-CSF receptor a chain, suggesting a strong interaction between the cytokine, its receptor, and syndecan-2. Phosphorylation of tyrosine residues in syndecan-2 associated with the a chain of the GM-CSF receptor was increased after cell stimulation by GM-CSF. Antisense oligonucleotides that reduced specifically the expression of syndecan-2 inhibited the mitogenic activity of GM-CSF and the activation of extracellular signal-regulated kinase-1 induced by the cytokine. Our results indicate functional interactions between syndecan-2 and GM-CSF in osteoblasts, and we propose that syndecan-2 plays a role as a co-receptor for this cytokine.

Matrix and cell surface heparan sulfate proteoglycans (HSPG)† are complex and versatile molecules that display various functions in the cellular environment. HSPGs interact with many different ligands and thereby participate to cell adhesion, proliferation, and differentiation (1). Among HSPGs expressed on the surface of most types of cells, syndecans form a family that includes four members, three of them (syndecan-1, -2, and -4) being cloned in human cells (2–5). Syndecans are expressed in cell-, tissue-, and development-specific patterns. In mineralized tissues, as in other mesenchymal tissues, syndecan-1 is only expressed transiently at particular stages of morphogenesis and cell differentiation (6). For example syndecan-1 is expressed during mesenchymal condensation in the developing tooth (7). In adult tissues, syndecan-1 is mainly expressed in epithelial cells (8). In contrast, the major source of syndecan-2 appears to be mesenchymal cells. Notably, syndecan-2 occurs during mouse bone development and osteoblast differentiation and persists in differentiated hard tissues (9). Syndecan-3 was first identified in neural tissues but also seems to play a role in limb development (10). Syndecan-4 displays a more ubiquitous distribution in different tissues (11). These transmembrane PGs appear to display two main functions. One is to bind extracellular ligands such as matrix adhesive proteins, cell-cell adhesion molecules, enzymes, and growth factors (11–13). All known ligand binding sites are localized on glycosaminoglycan (GAG) chains that syndecans bear on the extracellular domain of their core protein. These GAGs are mostly of the heparan sulfate type, although chondroitin sulfate chains could also be associated with syndecan-1 and -4 (11). The second function of syndecans may be to promote signaling events that are associated with the ligand-dependent activation of high affinity receptors. For example, syndecans were found to stimulate fibroblast growth factor receptor (FGFR)-1 occupancy and signaling by FGF (14). This may result from an increased availability of the ligand retained at the cell surface or from a more efficient presentation to the receptor (15, 16). Moreover, syndecans display a highly conserved intracytoplasmic domain that includes several phosphorylatable residues. This may indicate an important function of the cytoplasmic domain that may be kinase substrates. Indeed, syndecan-4 can be phosphorylated on a serine residue, this phosphorylation being regulated by the protein kinase C and a FGF-dependent serine/threonine phosphatase (17). On the other hand, the cytoplasmic tail of syndecans was shown to be associated with molecules involved in intracellular signal transduction. Thus, the cytosolic part of syndecan-3 can bind a protein complex including Src family kinases and their substrates (18). The COOH-terminal FYA sequence of syndecans was reported to interact with syntenetin, a PDZ protein (19). Another PDZ domain-containing protein, the CASK/LIN-2, was found to bind the COOH-terminal motif of syndecan-2 (20, 21). These proteins may connect syndecans to the cytoskeleton and different signaling pathways and allow them to participate to signaling events induced by PG ligands.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an heparin binding factor (22, 23) implicated in the control of hematopoietic cell proliferation and differentiation (24, 25). The biological activities of GM-CSF are mediated by a transmembrane high affinity receptor that is a heterodimeric complex composed of an a chain, which binds GM-CSF specifically with low affinity, and a b chain, which does not bind the

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§ The abbreviations used are: HSPG, heparan sulfate proteoglycans (PG); GAG, glycosaminoglycan; FGFR, fibroblast growth factor (FGF) receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; rh, recombinant human; mAb, monoclonal antibody; hOB, human osteoblastic; PCS, fetal calf serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; AS, antisense oligomer; R, random; ERK, extracellular signal-regulated kinase.
cysteine but is essential for its signal transduction (24). Both α and β chains are members of the hematopoietin receptor superfamily and lack intrinsic tyrosine kinase activity. Signaling by GM-CSF depends on tyrosine kinases associated with the receptor, such as Jak2, which is associated with the β chain of the GM-CSF receptor (26). Stimulation of GM-CSF-dependent cell lines with the cytokine has been found to induce a variety of immediate cellular responses including rapid tyrosine phosphorylation of cellular substrates, activation of components of the Ras signaling pathway such as mitogen-activated protein kinase (MAPK), and induction of early genes transcription (26, 27). In the skeleton, GM-CSF is a potential regulator of bone cells. This hematological factor was found to be produced by different osteoblastic cell lines in response to stimuli (28, 29). Exogenous GM-CSF stimulates human osteoblast-like cell growth and antagonizes the induction by 1,25-dihydroxyvitamin D of osteocalcin and alkaline phosphatase activity (30). We previously showed that GM-CSF is an autocrine growth factor for normal human osteoblastic cells (31). We subsequently found that GM-CSF not only binds to HSPG expressed at the osteoblastic cell surface but that the mitogenic activity of GM-CSF is dependent on the presence of fully sulfated PGs in these cells (32). This suggested to us that GM-CSF could be a ligand for syndecans that may be involved in the control of the mitogenic activity of the cytokine. In this study we therefore determined if GM-CSF is able to interact with syndecans in osteoblasts. Our results provide evidence for functional interactions between syndecan-2 and GM-CSF, and we propose that syndecan-2 may play a role as a co-receptor for this cytokine in osteoblastic cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unglycosylated recombinant human (rh) GM-CSF was kindly provided by Novartis. Heparin, heparinase III, chondroitinase ABC, protein A-Sepharose, dianaminobenzamide and biotinylaminocaproic acid N-hydroxysuccinimide (N-biotin) were purchased from Sigma. Hitrap Q cartridges were purchased from Amersham Pharmacia Biotech. Monoclonal anti-syndecan-1 (MCA681), mouse monoclonal antibodies 10H4 and 8G3 recognizing syndecan-2 and -4, respectively (5, 9), mouse monoclonal antibodies recognizing the human common β chain receptor for interleukin-3, interleukin-5, and GM-CSF, and mouse monoclonal anti-human GM-CSF α chain receptor. The secondary antibodies were anti-mouse IgG linked to colloidal gold particles (Amersham Pharmacia Biotech), which were enlarged by precipitation of metallic silver before microscopic visualization using an inverse condenser (Olympus BH-2). For transmission electron microscopy analysis, cells were incubated with 10 ng/ml rhGM-CSF for 5 min at 37 °C, then washed in PBS and fixed in 4% paraformaldehyde. A double immunostaining using mouse anti-syndecan-2 and polyclonal rabbit anti-GM-CSF or anti-GMR Rs was then performed. Labeling was revealed using anti-mouse and anti-rabbit secondary antibodies linked to 10-nm and 5-nm beads, respectively. After the final immunolabeling, cells were postfixed with 2% glutaraldehyde, dehydrated in graded ethanol series, and embedded in epoxy resin. Ultrathin sections (60–70 nm) were performed, treated with uranyl acetate and lead citrate, and observed in transmission electron microscopy (Jeol 100 CXII) at high magnification. Isolated and coupled gold particles (total >1,000 particles) localized at the outer surface of the cells were counted.

**Preparation of Proteoglycans**—A protein fraction enriched in proteoglycans was obtained as described (35). AHTO-7 cells were lysed in 4 mM guanidine-HCl extraction solution (pH 6) containing 50 mM sodium acetate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM 6-aminohexanoic acid, 20 mM benzamidine-HCl, and 2% Triton X-100. Extraction solution was then exchanged on Sephadex G-25 for a chromatography buffer (20 mM Tris (pH 7.2), 7 mM urea, 10 mM EDTA, 5 mM N-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, 2% Triton X-100). The protein extract was then deposited on a Q-Sepharose column (Hitrap). After washing with chromatography buffer containing 0.1 mM NaCl, proteoglycans were eluted with the same buffer containing 1 mM NaCl. The proteoglycan fraction was desalted, and the protein content was assayed by UV spectrophotometry.

**Binding Assay of Biotin-rhGM-CSF to PG**—Biotin was linked to rhGM-CSF using a previously described method (36). To do that, 10 μg of rhGM-CSF were reacted with 1 μg of NS-biotin in PBS at pH 8.3. The reaction was performed in the presence of 100 μg of heparin to protect potential heparin-binding sites on GM-CSF. At the end of the reaction, 2% containing 2 mM NaCl was added to dissociate heparin from proteins, and rhGM-CSF linked to biotin (biotin-rhGM-CSF) was separated from free reagents (0.2 mM Sepharose G-25). Proteoglycans (biotin-rhGM-CSF) obtained as described above were subjected to 4–15% SDS-PAGE under nonreducing conditions and were electrotransferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech). The membrane was cut in different strips; one part was used for binding assay, and the other part for immunoblotting as described below, except that dianaminobenzamide was used as peroxidase substrate. Precise marks on the membrane allowed its reconstitution at the end of the experiments. Before the binding assay, strips of the membrane were digested at 37 °C with 10 milliliters/ml heparinase III or 33 milliliters/ml chondroitinase ABC diluted in Tris-HCl (pH 7.2) containing 1 mM CaCl2, 0.5 mM mg/ml bovine serum albumin, and a mixture of protease inhibitors (35). Digested and nondigested strips of the membrane were blocked for nonspecific binding in 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl (Tris-buffered saline) containing 0.5% gelatin in 1 h at room temperature and then exposed to biotin-rhGM-CSF for 90 min at 37 °C. At the end of this incubation, after rapid washes, strips were reacted with avidin-peroxidase for 30 min. Recombinant hGM-CSF bound to proteoglycans on the membrane was revealed using dianaminobenzamide as peroxidase substrate.

**Immunoprecipitation and Immunoblotting**—Confluent AHTO-7 cells were serum-starved for 24 h. The medium was then changed for Dulbecco’s-modified Eagle’s medium containing 1% bovine serum albumin, and after a 1-h incubation at 37 °C, the cells were treated with rhGM-CSF for 0, 5, 10, or 30 min. The cells were then transfected onto ice, washed with PBS, and lysed in 10 mM Tris (pH 7.5) with 5 mM EDTA, 200 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1

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**TABLE I**

| Syndecan | Sense | Antisense | Internal |
|----------|-------|-----------|----------|
| 1        | 5′-TGAAGAAGAAGGACGAAGGC-3′ | 5′-TTCAAGAGAGGACAGAGTG-3′ | 5′-CAGGAGAATTCATTGCTGCAG-3′ |
| 2        | 5′-GACGATGACTACGCTTCTG-3′ | 5′-TTGATATCCCTGCTGCTGG-3′ | 5′-CTCGTATATAGCTCGCTGG-3′ |
| 4        | 5′-TGAAAGACCTCATGATCGG-3′ | 5′-GACGATGACTACGCTTCTGC-3′ | 5′-CATCTCCTCTCCTCAAGGG-3′ |

| Length   |
|----------|
| 204 bp   |
| 262 bp   |
| 319 bp   |

Aliquots of the amplified cDNA were size-fractionated in 2% agarose gel, and the PCR products were then identified by Southern hybridization using specific internal oligonucleotide probes (Table I) that were 5′ end-labeled.

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**Syndecan-2 Modulates GM-CSF Activity**
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mM activated sodium orthovanadate, 10% glycerol, and 0.5% Triton X-100. The cell lysates were clarified by incubation with 10 μg of protein A-Sepharose for 1 h and centrifugation at 12,000 × g for 10 min. Protein content was then quantified. One mg of each protein extract was incubated overnight with polyclonal anti-GMRα or anti-syndecan-2 (10H4) and 10 μl of protein A-Sepharose. Immune complexes were washed 3 times with lysis buffer, boiled 10 min in Tris containing 2.3% SDS, 10% glycerol, and 5% 2-mercaptoethanol, then resolved on SDS-polyacrylamide gel and electrotransferred onto a polyvinylidene fluoride membrane. Immunoblotting was performed by incubating the membrane with primary antibodies at 4 °C overnight and then with horseradish peroxidase-conjugated anti-immunoglobulin (Amersham Pharmacia Biotech) as the secondary antibody. Signals were detected using a chemiluminescent peroxidase substrate (Amersham Pharmacia Biotech).

Antisense Oligonucleotide Experiments—The antisense oligomer (AS) used was complementary to the first 25 bases of the translation start site of rat syndecan-2 mRNA (37) and had the following sequence: CCAGCGACGTGCGGTGCAC. A random (R) oligonucleotide composed of the 25 randomly arranged bases of the AS was used as control and had the following sequence: AAGTCGGCGCGCCGCTTCAAGC. The R sequence was checked on a computed data base not to be complementary to any known sequence. The oligonucleotides were protected from endonucleases by phosphorothioate modification at the 3′ end. Before oligonucleotide treatment, hOB cells were plated at 5,000 cells/cm² in multiwell chambers and cultured for 2 days in the presence of 10% FCS to allow homogenous cell adhesion and with 30 mM sodium chlorate to reduce the presence of sulfated glycosaminoglycans at the cell surface (32). The medium was then changed for serum-free Dulbecco’s modified Eagle’s medium containing 2 μM AS or R oligonucleotides or the diluent. After 2 days of culture in the presence of oligonucleotides, the medium was replaced, and the culture was continued for 3 days in the presence or absence of 10 μg/ml rhGM-CSF or 1% FCS and in the presence or absence of 10 ng/ml heparin. At the end of the culture, the cells were washed, fixed in 2.5% glutaraldehyde, stained with hematoxylin, and the number of cells/cm² was counted using an ocular integrator mounted on a microscope (Olympus, BH2). To ensure that AS affected only syndecan-2, the effect of AS and R oligonucleotides on the expression of syndecans was determined by immunocytochemistry in the same culture conditions as the cell proliferation assay. Cell cultures were cultured for 2 days in the presence of AS or R oligonucleotides and stopped before rhGM-CSF treatment, and immunocytochemistry of syndecan-1, -2, and -4 was performed as described above. The effect of syndecan-2 inhibition on the expression of GM-CSF receptor was also determined by examining the activation of MAPK induced by rhGM-CSF (Fig. 2A). As expected, the amplified cDNAs have the predicted sizes for the syndecans. These products hybridized with the specific probes. hOB cells expressed high levels of mRNA for syndecan-2 and -4 and lower mRNA levels for syndecan-1. B, hOB cells were stained with purified mouse anti-human syndecan-1 (a), anti-syndecan-2 (b), or anti-syndecan-4 (c). Negative control cells were reacted with mouse Ig to detect nonspecific staining (d). Staining was revealed using anti-mouse IgG link to gold particles that were enlarged by precipitation of metallic silver. A low level of syndecan-1 was detected compared with syndecan-2 and -4. Ig, IgG, Ig, light chain.
Syndecan-2 Modulates GM-CSF Activity

**Fig. 2.** Syndecan-2 binds biotin-GM-CSF. A, 200 μg of a PG fraction obtained from AHTO-7 cells were separated on SDS-PAGE and transferred onto a hydrophobic membrane. Intact (lane 1), heparinase III-digested (lane 2), or chondroitinase ABC-digested (lane 3) strip of the membrane was incubated with rhGM-CSF bound to biotin. B, a strip of the membrane was immunoblotted with anti-syndecan-2 (lane 4) or anti-syndecan-4 (lane 5). After incubation with avidin-peroxidase, staining was revealed using diaminobenzamidine. Different PGs bound rhGM-CSF (arrowheads), and one of these is recognized by anti-syndecan-2 (double arrow).

**Fig. 3.** GM-CSF and GMRα colocalize with syndecan-2 at the cell surface. Human osteoblastic cells were grown until confluence, fixed with paraformaldehyde, double-immunostained with polyclonal rabbit anti-GM-CSF, and monoclonal mouse anti-syndecan-2 (A) or polyclonal rabbit anti-GMRα and monoclonal mouse anti-syndecan-2 (B). Stainings were revealed with anti-rabbit Ig bound to 5-nm beads and anti-mouse Ig bound to 10-nm beads, respectively. Cells were then post-fixed, and 60–70-nm sections were performed and observed with a transmission electron microscope at high magnification \(\times 60,000\). About 25% of the beads present at the cell surface were associated with beads of different size (arrow).
rhGM-CSF was abolished in AS-treated cells (Fig. 6). This effect was not due to a toxic effect of oligonucleotides since AS-treated cells were still able to respond to 1% FCS to the same level as control cells (Fig. 6). Moreover, we checked that the number of AS-treated cells was higher after 3 days of culture compared with the number of cells present when GM-CSF treatment was initiated (not shown), indicating that the lower number of AS-treated cells at the end of the culture resulted from a reduced proliferation rate and not from cell death. These results indicate that syndecan-2 is involved in the mitogenic activity of GM-CSF in osteoblastic cells.

We previously showed that low doses of heparin are able to recover the GM-CSF-induced proliferation in cells that express PGs with under-sulfated GAGs (32). In the present study, we found that the addition of heparin did not rescue the inhibition of GM-CSF mitogenic activity induced by syndecan-2 AS (Table II). The inability of heparin to rescue the inhibitory effect of AS on cell growth further suggests that, in addition to its heparan sulfate compounds, the core protein of syndecan-2 participates to the control of GM-CSF-induced cell growth.

Effect of Syndecan-2 Suppression on GM-CSF Signaling—
Binding of GM-CSF to its high affinity receptor leads to the activation of the MAPK signaling pathway (27) and rapid phosphorylation of p44 and p42 MAPK (also referred as extracellular signal-regulated kinase-1 (ERK1) and ERK2, respectively). To determine the effect of syndecan-2 suppression on GM-CSF signaling, we examined the effect of AS oligonucleotides on activation of ERK1 and ERK2 by rhGM-CSF. AHTO-7 cells were treated with AS or R oligonucleotides and stimulated with rhGM-CSF. Total protein extract was then separated on SDS-PAGE, and the level of activated ERK1 and ERK2 was determined by Western blot. We found that rhGM-CSF enhanced the level of activated ERK1 and that suppression of syndecan-2 inhibited ERK1 activation by rhGM-CSF (Fig. 7). These results together with our data on syndecan-2 phosphorylation and heparin effect strongly suggest a role for syndecan-2 in intracellular signaling induced by GM-CSF binding.

FIG. 5. Specific inhibition of syndecan-2 expression by AS oligonucleotides. A, immunostaining of syndecan-2, GMRa and -b in hOB cells grown for 2 days in the absence (Control) or in the presence of random or antisense syndecan-2 oligonucleotides. Cells were incubated with mAb 10H4, CDw116, or anti-GMR§ in the presence of random or antisense syndecan-2 oligonucleotides. Cells were incubated using anti mouse-Ig linked to gold particles that were enlarged by precipitation of metallic silver. Control cells reacted with mouse Ig showed no specific staining. Original magnification, ×250. B, quantitative determination of syndecan-2- and GMR§-positive cells. AS oligonucleotides reduced the fraction of syndecan-2-positive cell, but not GMRa- or GMR§-positive cells compared with untreated and R-treated cells. The data are the mean of 9–4 replicates. The asterisks indicates a significant difference with untreated cells (white bars) and random-treated cells (p < 0.001).

FIG. 6. Inhibition of syndecan-2 expression decreases cell proliferation and inhibits the mitogenic activity of GM-CSF. Human osteoblastic cells treated with or without AS or R syndecan-2 oligonucleotides were cultured for 3 days in the presence or absence of rhGM-CSF or 1% FCS and then counted. The basal cell growth was reduced in AS-treated cells. Recombinant hGM-CSF-stimulated cell proliferation in control and R-treated cells but not in AS-treated cells. Asterisks indicate a significant difference with cells cultured in the absence of rhGM-CSF, and pound signs indicate a significant difference with cells cultured in the absence of AS oligonucleotides or R-treated cells (p < 0.05).
Syndecan-2 Modulates GM-CSF Activity

Heparin does not rescue the inhibition of GM-CSF mitogenic activity induced by syndecan-2 antisense

Human osteoblastic cells treated with or without AS oligonucleotides (2μM) were cultured for 3 days in the presence or absence of 10 ng/ml rhGM-CSF and/or 10 ng/ml heparin and then counted. The initial cell number/cm² at day 0 was 4709 ± 159. For comparison, random oligonucleotides had no effect in this assay (see Fig. 6). Data are the mean ± S.E. of 3–4 replicates.

| Number of cells/cm²          | Untreated cells | rhGM-CSF | Heparin | rhGM-CSF + Heparin |
|------------------------------|-----------------|----------|---------|--------------------|
| Control cells                | 6291 ± 213      | 878 ± 285 | 6412 ± 97 | 8662 ± 82         |
| AS-treated cells             | 5448 ± 201      | 5406 ± 253 | 5599 ± 128 |

* A difference with cells cultured in the absence of rhGM-CSF.
* A difference with untreated control cells (p < 0.05).

**Fig. 7.** Inhibition of syndecan-2 inhibits MAPK activation induced by rhGM-CSF. AHTO-7 cells were grown until confluence and treated for 2 days with R or AS syndecan-2 oligonucleotides. The cells were then incubated in the presence or absence of 10 ng/ml rhGM-CSF for 5 min and lysed. 100 μg of total protein extract were Western-blotted with anti-activated MAPK that recognizes both ERK1 and -2. rhGM-CSF stimulation increased ERK1 in R-treated cells but not in AS-treated cells.

**Table II**

| Oligonucleotides | ERK-1 | ERK-2 | rhGM-CSF | - | + | - | + |
|-------------------|-------|-------|----------|---|---|---|---|

**Discussion**

We recently showed that the mitogenic activity of GM-CSF in osteoblasts depends on interactions with cellular sulfated heparan sulfate chains (32). The results presented in this paper provide the first evidence for functional interactions between GM-CSF and syndecan-2 and identify this transmembrane HSPG as a potential co-receptor for this cytokine.

We first examined the binding capacity of PGs isolated from osteoblastic cells and showed that different HSPGs display affinity for GM-CSF. Heparinase treatment reduced homogenously the binding of the cytokine to PGs, showing that binding of GM-CSF to the cellular osteoblastic PGs depends on heparan sulfate GAGs. Identification of PGs that bear GAGs responsible for the interactions with GM-CSF is an important step to understanding how these interactions are involved in the regulation of GM-CSF activity. Syndecans appeared to be good candidates for the interactions with GM-CSF. Indeed, this protein family is the major form of membrane-associated HSPG expressed by many cells (12, 13) and is able to bind growth factors including FGF (39) and heparin binding growth-associated molecule (40). We therefore examined the expression of syndecans in human osteoblasts. Our findings demonstrate that osteoblastic cells derived from adult human bone express low levels of syndecan-1 and high levels of syndecan-2 and -4. The low pattern of syndecan-1 expression is consistent with reports showing that syndecan-1 is expressed in mesenchymal tissues only at particular stages of the development and is almost restricted to epithelial cells in adult tissues (6, 7). In contrast, syndecan-2 was found to be expressed exclusively by mesenchymal cells in different tissues, whereas syndecan-4 is seen in the most ubiquitous distribution in different tissues (9, 11). The distinct expression of syndecan-1, -2, and -4 in normal human osteoblastic cells is in accordance with their expression in osteogenic cells during mouse development and rat neonatal ossification (9, 41).

Immunoblotting analysis performed in parallel with the binding assay on PGs extracted from osteoblastic cells allowed identification of syndecan-2 as a major HSPG that binds GM-CSF. To determine if this interaction between blotted syndecan-2 and GM-CSF also occurs at the cell surface, we performed a double immunological staining of syndecan-2 and the cytokine in hOB cells. Our data showed that labeled molecules of syndecan-2 and GM-CSF were associated and co-localized on the cell surface, which supports our results showing binding of GM-CSF to syndecan-2. We therefore examined this interaction to determine if it is functional and may play a role in the mitogenic activity of the cytokine. We used an antisense strategy to reduce syndecan-2 expression. AS oligonucleotides reduced specifically immunoreactive syndecan-2 levels, whereas syndecan-1 and -4 levels were not modified. Although not striking, the efficiency of AS to reduce syndecan-2 expression was comparable with that previously observed in human osteoblastic cell cultures (31). Interestingly, the reduction in syndecan-2 levels induced by AS oligonucleotides was associated with decreased basal cell proliferation. This may be related in part to reduction of endogenous GM-CSF activity, since we previously found that this cytokine is an autocrine growth factor in human osteoblasts (31). Moreover, the mitogenic activity of rhGM-CSF was abolished by syndecan-2 AS, and this effect was associated with inhibition of ERK1 activation. The inhibition of GM-CSF mitogenic activity in AS-treated cells was not due to a reduction of GM-CSF receptor expression. These data indicate that syndecan-2 plays a role in the control of GM-CSF signaling and activity in osteoblasts.

We previously showed that reduction of GAGs sulfation by chlorate treatment, which results in lower binding capacity of GAGs at the cell surface, also inhibits the mitogenic activity of GM-CSF (31). Thus, a major role of syndecan-2 in GM-CSF sequestration at the cell surface may account for the involvement of this HSPG in the cytokine activity. Interaction with GAGs has been shown to protect soluble factors from degradation (42). Such interactions are also thought to increase the concentration of the ligand at the proximity of high affinity receptors (15, 16, 43). The number of GM-CSF receptors expressed by responding cells is very low, typically 800 to 1000 sites/cell in hematopoietic cells and 120 sites/cell in endothelial cells (24). Binding of GM-CSF to GAGs of syndecan-2 may increase the probability for this ligand to interact with its high affinity receptors. Interaction with HSPG may also promote ligand-receptor binding. Such a role was demonstrated for perlecain, a HSPG present in the basement membrane that binds FGF-2 and allows FGF-2-mediated mitogenic response by promoting the binding of this factor to its high affinity receptor (44). Syndecans were also found to increase FGF-2 binding to its receptor when transfected in cells that express low levels of cell-surface HSPG (14). In the present study, the co-localization of syndecan-2 and GMRe at the surface of osteoblastic cells suggests that syndecan-2 may play a role in the presentation of GM-CSF to its receptor. In addition, syndecan-2 co-immunoprecipitated with GMRe, indicating a strong association between syndecan-2 and the receptor. Based on these results, direct interactions between this HSPG and GMRe can be postulated similar to those observed between heparin and other
Syndecan-2 Modulates GM-CSF Activity

dependent growth factor receptors such as FGFR. Indeed, a ternary functional interaction between heparin, FGFR, and FGFR has been demonstrated (45). A heparin binding domain was found in the FGFR receptor, and suppression of this site by mutation was shown to inhibit heparin and FGFR binding to the receptor and to result in inhibition of the tyrosine kinase activity of this receptor (45). Interestingly, the α and β chains of the GM-CSF receptor belong to the hematopoietin receptor superfamily (46). These glycoproteins comprise structures related to fibronectin type III domain, and heparin-binding sites have been localized in these fibronectin domains (46, 47). Thus, functional interactions between these potential heparin-binding sites in GM-CSF receptor and heparan sulfate chains in syndecan-2 may participate to the control of GM-CSF signaling.

The interacting capacity of heparan sulfate chains of syndecans may not be alone responsible for all syndecan functions. In a previous study, we found that the inhibitory effect of chlorate treatment on GM-CSF mitogenic activity was reversed by low concentrations of heparin (32). In contrast, the inhibitory effect of AS oligonucleotides on cell growth was not rescued by the addition of heparin. This suggests that the core protein is also required and may play a role in the control of GM-CSF activity. The cytoplasmic tail of syndecans contain four conserved tyrosine residues (11). Recently, tyrosine residues of syndecan-1 and -4 were shown to be phosphorylated in adherent fibroblasts (48). This phosphorylation seems to depend on the cell type and to be tightly controlled by both kinases and phosphatases, suggesting that tyrosine phosphorylations may be a key event in syndecan activity. We show here that phosphorylation of tyrosine residues of syndecan-2 associated with GMRs in osteoblastic cells is increased in the presence of rhGM-CSF. This indicates that syndecan-2 is involved in intracellular signaling events that are activated by the cytokine. These results are consistent with recent findings showing that the cytoplasmic domain of syndecans are associated with signaling molecules. For example, syndecan-3, a cell surface receptor for heparin binding growth-associated molecule, binds tyrosine kinases of the Src family and substrates such as cortactin (18). Moreover, this signaling pathway appears to be activated during heparin binding growth-associated molecule-dependent neurite outgrowth (18). Highly conserved cytoplasmic domains of syndecans were also shown to interact with PDZ domains of CASK, a membrane-associated guanylate kinase (20, 21). This type of multidomain scaffold proteins interacting with the cytoskeleton is thought to organize specific signaling networks and to connect extracellular signals to downstream signaling pathways. These observations together with the results presented in this paper suggest that syndecan-2 core protein may participate in the modulation of GM-CSF mitogenic activity by activation of proper signaling pathways run in parallel or connected to intracellular signaling events that depend on the GM-CSF high affinity receptor.

Interactions between syndecan-2 and GM-CSF may have functional biological implications in osteoblast P. It may result in fine tuning of osteoblastic cell growth and differentiation. Indeed GM-CSF was shown to be an autocrine/paracrine regulator of osteoblastic cell growth in vitro (28–31) and in vivo in mouse calvaria osteoblastic cells. This cytokine was also found to modulate alkaline phosphatase activity and osteocalcin (30) and reduces type I collagen expression. It can also be postulated that syndecan-2 may be involved in the control of other heparan sulfate binding factors than GM-CSF in osteoblasts. For example, FGF-2 that binds to and is modulated by syndecans in various cell types (6) is known to affect osteoblast proliferation and differentiation through interaction with FGFRs (49). We recently found that syndecan-2 is co-expressed with FGFRs in osteoblasts during rat calvaria osteogenesis (41). Syndecan-2 may therefore play an important regulatory role, controlling the biological activity of local heparan sulfate binding growth factors in osteoblasts.

In summary, our results provide evidence that syndecan-2 interacts with GM-CSF and its receptor at the surface of human osteoblastic cells. Both the heparan sulfate chains that are responsible of GM-CSF binding and the core protein that seems involved in intracellular signaling events form a co-receptor for the cytokine. Thus, different domains in syndecan-2 are involved in the control of GM-CSF mitogenic activity and signaling in human osteoblastic cells.

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Syndecan-2 Modulates GM-CSF Activity

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