Granulocyte-Macrophage Colony Stimulating Factor Activates Proteoglycan, Type II Collagen, and cAMP Production by Rat Articular Chondrocytes Through Specific Binding Sites

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ABSTRACT. *Objective.* To evaluate the effects of granulocyte-macrophage colony stimulating factor (GM-CSF) on rat articular chondrocyte (AC) with respect to DNA synthesis, collagen type II and proteoglycan (PG) synthesis and expression, and cAMP production; to examine these cells for the presence of GM-CSF-specific binding sites; and to study their regulation by growth factors and cytokines.

Methods. First passage monolayers of rat AC were incubated with various concentrations of recombinant human GM-CSF, and then [^{3}H]-thymidine, [^{3}H]-proline, and [^{35}S]SO$_4$ incorporation and cAMP production were measured. The density of GM-CSF-specific binding sites, the effects of growth factors and cytokines on receptor density, and the activation of certain post-receptor signaling pathways were also examined by labeling the cell monolayers with [^{125}I]-GM-CSF.

Results. GM-CSF (6–100 U/ml) inhibited (30%) [^{3}H]-thymidine incorporation into DNA, and, in contrast, stimulated up to 3.6- and 2-fold [^{35}S]SO$_4$ and [^{3}H]-proline incorporation into glycosaminoglycan side chains and collagen molecules, respectively. GM-CSF also increased aggrecan and type II collagen (Coll II) transcripts by 2- to 3-fold, respectively. These effects were associated with a concentration-dependent increase in cAMP production. A single class of high affinity (K_d = 98 pM; B_{max} = 7.08 pM/μg DNA) binding sites of about 220 kDa were found. The [^{125}I]-GM-CSF binding to the cells was slightly increased with phorbol 12-myristate 13-acetate (PMA), insulin-like growth factor-I, platelet derived growth factor, basic fibroblast growth factor, and tumor necrosis factor-α, and decreased with pertussis toxin, cholera toxin, and interleukin-1ß.

Conclusion. These results suggest that GM-CSF may play a role in the regulation of chondrocyte metabolism as an anabolic agent and may stimulate cartilage healing under pathological conditions. (J Rheumatol 2001;28:2075–84)

Key Indexing Terms:

PERTUSSIS TOXIN   CHOLERA TOXIN   CYTOKINES
PHORBOL 12-MYRISTATE 13-ACETATE (PMA)   GROWTH FACTORS

Articular cartilage is an avascular connective tissue in which the cells or chondrocytes are embedded in an abundant extracellular matrix composed mainly of type II collagen (Coll II) fibers, proteoglycans (PG), glycoproteins, and other minor components. Chondrocytes maintain the structure and composition of the extracellular matrix by synthesizing and degrading matrix macromolecules. These 2 processes are normally in equilibrium, as tissue mass of healthy cartilage remains practically unchanged throughout life in skeletally mature individuals. However, in aging and diseases, like osteoarthritis, articular cartilage frequently undergoes degeneration, which may result in tissue destruction. The factors that regulate chondrocyte metabolism *in vivo* are not very well known. Factors such as growth hormone, thyroxin, sex hormones, and corticosteroids seem to be important during skeletal growth[1], but in skeletally mature individuals their role is clearly limited. Insulin-like growth factor-I (IGF-I), which is produced in response to growth hormone[2], is the principal cartilage-stimulating factor during development and growth[3]. Several growth factors, including IGF-I and II, basic fibroblast growth factor (bFGF), epidermal growth factor, platelet derived growth factor (PDGF), and transforming growth factor-ß

(TGF-ß), have mitogenic effects on cultured articular chondrocytes. IGF-I and TGF-ß have been reported in many studies to stimulate proteoglycan synthesis in cell and organ cultures[4,5], whereas certain cytokines such as interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) that are secreted and released into the joint cavity during inflammation[6,7] may adversely affect articular cartilage metabolism[8].

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a member of the hematopoietic factor family that stimulates the proliferation, maturation, and function of granulocyte-macrophage lineages from bone marrow cells[9–11]. In addition to its proliferative actions on granulocyte-macrophage progenitor cells, GM-CSF also stimulates mature cells. It enhances phagocytosis by neutrophils and macrophages and their ability to increase superoxide production, leukotriene synthesis, and arachidonic acid release, and the release of several cytokines, in response to secondary stimuli[9-11]. GM-CSF is produced by a variety of cell types, including osteoblasts[12] and synovial fibroblasts[13] in response to cytokine, immune, and inflammatory stimuli[6,7]. GM-CSF is also found in increased amounts in rheumatoid synovial effusions[14,15] and is produced by human articular chondrocytes (AC), in response to stimulation by IL-1ß and TNF-α[16,17], suggesting its involvement in synovial inflammation. To our knowledge, the effects of GM-CSF on AC have not yet been described. We report the *in vitro* responses of cultured rat AC to GM-CSF, the presence of functional specific binding sites for this cytokine, and the regulation of their density by growth factors and cytokines.

MATERIALS AND METHODS

Cell culture. Rat AC were isolated by collagenase digestion of articular cartilage fragments obtained from the humeral and femoral heads of 4 one-month-old Wistar rats, as described[18,19]. Pooled cells were cultured under standard conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U penicillin and 50 μg streptomycin per ml). Confluent cells were released by 5 min digestion at 37°C with Trypsin-EDTA solution (Eurobio, Paris, France), rinsed with Ca^{2+}/Mg^{2+}-free Hanks' buffer, resuspended in DMEM + 10% FCS, and seeded in 24 well culture plates at 2×10^4 cells/well. Only the first passage cells were used in all experiments.

[^{3}H]-thymidine incorporation. Mitogenic effect of GM-CSF was evaluated by [^{3}H]-thymidine incorporation into DNA. In order to arrest cell growth, subconfluent cultures were incubated in DMEM containing 0.2% FCS for 24 h. The medium was then replaced with DMEM containing 2.5% FCS, and various concentrations (6–100 U/ml) of recombinant human GM-CSF (specific activity > 6×10^{-7} U/mg protein; Boehringer, Mannheim, Germany). Incubation was continued for 24 h and [^{3}H]-thymidine (NEN, Boston, MA, USA specific activity 20 Ci/mM) was added (1 μCi/ml) for the last 6 h of culture. [^{3}H]-thymidine incorporation was stopped by rinsing the cells twice with phosphate buffered saline (PBS), twice with cold 5% trichloroacetic acid, and once with 80% ethanol. The air-dried cell layer was lysed at 37°C for 2 h with 0.5 ml 10 mM EDTA, pH 12.3. Samples (0.2 ml) were dissolved in 10 ml Ecosint H (Prolabo Co, Paris, France) and counted in an LKB ß-scintillation counter. The results are expressed as cpm per culture. Mean values of 4 replicate cultures ± standard deviation (SD) were calculated and used as a measure of [^{3}H]-thymidine incorporation.

Proteoglycan synthesis. Growth-arrested confluent cultures were incubated in Hams F-12 medium plus various concentrations (6–100 U per ml) recombinant human (rh) GM-CSF and 5 μCi/ml of [^{35}S]-Na$_2$SO$_4$ (NEN, carrier-free). After 48 h, the media were removed and cleared by centrifugation. Cell layers were washed twice with PBS and digested with 0.2% papain at 55°C for 2 h. Papain digests were cleared by centrifugation and the supernatants of the corresponding medium cell digest counterparts pooled. The clear digests plus media were adjusted to 5% Na acetate and 50 μg/ml carrier chondroitin sulfate from bovine trachea (Sigma) was then added. The glycosaminoglycans were precipitated overnight at –20°C, as sodium salts, with 2 volumes of absolute ethanol. Each precipitate was collected by centrifugation (10 min, 4°C, $15,000 \times g$), dissolved in 0.5 ml distilled water, and precipitated again, as above. The precipitate was finally dissolved in 0.5 ml distilled water and counted. Preliminary studies (not reported) have shown that virtually all unincorporated [^{35}S]SO$_4$ was removed, whereas more than 95% [^{35}S]-PG (from rat articular cartilage) were precipitated under these conditions. The results are expressed as cpm per culture. Mean values of 6 replicate cultures ± SD were calculated and used as a measure of ^{35}SO$_4$ incorporation.

In order to characterize PG chromatographically, confluent passage 1 cell monolayers in 75 cm^2 culture flasks were labeled during 48 h with 50 μCi/ml carrier-free [^{35}S]-Na$_2$SO$_4$. The culture media + cell layers were adjusted to 4 M guanidinium chloride dissolved in a 0.05 M Tris-HCl buffered solution, pH 7.2, containing protease inhibitors, as described[20], and extraction carried out at 4°C overnight. The extracts were enriched (1 mg/ml) with carrier PG from human articular cartilage, then extensively dialyzed at 4°C against 0.05 M Tris-HCl buffer containing 0.15 M NaCl and protease inhibitors. [^{35}S]-PG were precipitated and recovered as above.

Collagen synthesis. Collagen synthesis was measured as described for PG, except that the cells were labeled with 1 μCi/ml [^{3}H]-proline. The media and corresponding pepsin digests of cell monolayers were pooled and adjusted to pH 3.0 with acetic acid. Type I collagen from bovine skin (100 μg/ml) was added as carrier and collagen precipitated by adding an equal volume of 4 M NaCl as described[21]. The precipitate was dissolved in 0.5 ml 3% (v/v) acetic acid, incubated at 20°C for 2 h with pepsin (2 mg/ml), then precipitated again as above. Finally the precipitate was dissolved in 3% acetic acid and counted. Preliminary study using this method as described to purify collagen molecules[22] showed that the recovery of ^{3}H-collagen from bovine articular cartilage was > 96% and intraassay variation < 7% (unpublished data).

RNA extraction and Northern blot analysis. Confluent rat AC monolayers cultured in 75 cm^2 flasks were rinsed twice with PBS and incubated at 37°C in DMEM + 2.5% FCS without or with 100 U/ml rhGM-CSF for 6 or 24 h. At the end of the incubation time, total cellular RNA was extracted using the Extract-All (Eurobio) solution according to the manufacturer's protocol. Twenty micrograms of RNA per lane were separated on 1.2% formaldehyde/agarose gel and transferred to hybond N+ nylon filters in 0.05 M NaOH for 3 h. Blot was prehybridized 30 min at 68°C in ExpressHyb buffer (Clontech, Palo Alto, CA, USA). The same membrane was first probed with 545, then 228, and finally with 646 base pair (bp) fragments encoding rat aggrecan[23], type II collagen[24], and GAPDH[25], respectively. Specific cDNA probes were labeled with [α-^{32}P]dCTP using a nick translation kit (Gibco-BRL, Glasgow, Scotland). Hybridization was carried out overnight at 68°C in the same buffer with the addition of 10^6 cpm of each radiolabeled probe per ml. Next day, the filter was washed twice at 68°C in $2 \times$ SSC/0.1% sodium dodecyl sulfate (SDS) for 15 min, then twice at 68°C in $0.1 \times$ SSC/0.1% SDS for 45 min. Hybridizing bands were visualized and quantified using a phosphor imager (Molecular Dynamics, Sunnyvale, CA, USA).

Radiolabeled probes. The rat aggrecan, type II collagen, and GAPDH probes were cloned by polymerase chain reaction from rat chondrocyte cDNA[18]. The cloned probes were confirmed by restriction enzyme mapping.

Sepharose-CL 2B column chromatography. [^{35}S]-PG samples (200,000 cpm) were deposited on the column (90 × 1.5 cm) and equilibrated and

eluted (5 ml/h) with 0.05 M Tris-HCl buffer containing 0.1 M NaCl and protease inhibitors[20]. Fractions (2.5 ml each) were collected using an automatic LKB fraction collector and 0.2 ml samples counted. To evaluate the capacity of [^{35}S]-PG to form aggregates, 20 μg hyaluronic acid (Sigma, from human umbilical cord) was added to each sample and aggregation allowed to proceed overnight at 4˚C under gentle shaking. The samples were run on the same column as described above. The column void volume (Vo) and total volume (Vt) were determined using human articular cartilage [^{35}S]-PG aggregates and [^{35}S]SO$_4$ (20,000 cpm each), respectively.

Intracellular cAMP determination. Confluent monolayers in 24 well plates were washed twice with PBS and incubated at 37˚C for 1 h with PBS containing 0.2% bovine serum albumin (BSA), and 1 mM isobutyl-methylxanthine (IBMX) to inhibit phosphodiesterases. rhGM-CSF (25–200 U/ml) was added in triplicate cultures to each well, and the reaction was stopped at the indicated times. cAMP was then measured using a commercial radioimmunoassay (RIA) kit (Immunotech, Marseille, France) as recommended by the manufacturer. The results were expressed as nmol/μg DNA. The cell DNA was measured by fluorometry using Hoechst dye 33258, essentially as described[26].

[^{125}I]-rhGM-CSF binding. Growth-arrested confluent cells cultured in 24 well plates were washed with PBS and incubated for 1 h in PBS/0.2% (w/v) BSA. Recombinant human [^{125}I]-GM-CSF (Amersham, Little Chalfon, UK; specific activity 900 Ci/mmol) was added (70,000 cpm) to each well, and plates were gently shaken at room temperature for the indicated times. Cell monolayers were then carefully rinsed with PBS/BSA, solubilized in 0.4 ml 1 N NaOH, and counted in an LKB gamma counter.

Binding sites were saturated by incubating cell monolayers for 2 h with increasing concentrations (up to 350,000 cpm) of [^{125}I]-GM-CSF. Specific binding was determined by subtracting values obtained with 200-fold excess of GM-CSF over [^{125}I]-GM-CSF from the corresponding value obtained with [^{125}I]-GM-CSF alone. Hill coefficient, K_d, and B_{max} values were calculated by computing the binding data, using the Ebda software (Biosoft, Miltown, NJ, USA)[19,21].

To evaluate the effects of adenylcyclase on GM-CSF binding, cells were rinsed and preincubated with IBMX. After 1 h, pertussis toxin (100 ng/ml) or cholera toxin (100 ng/ml) was added to 70,000 cpm [^{125}I]-GM-CSF and the radioactivity was determined, as above. Similarly, the effect of protein kinase C (PKC) activation was studied following addition of 50 ng/ml phorbol 12-myristate 13-acetate (PMA).

The effect of growth factors and cytokines on the density of binding sites in the cell monolayers was evaluated following 24 h cell incubation at 37˚C with each of the following factors: 100 ng/ml IGF-I, 100 ng/ml IGF-II, 100 U/ml rhGM-CSF, 1 ng/ml TGF-ß, 100 ng/ml PDGFbb, 2 ng/ml bFGF, 100 ng/ml IL-1ß, 100 ng/ml TNF-α. The cells were then rinsed with PBS/BSA and labeled as above.

Cross-linking of [^{125}I]-rhGM-CSF to its binding sites. Growth arrested confluent monolayers grown in 25 cm^2 culture flasks were washed twice and incubated for 1 h at 4˚C under gentle shaking with 5 ml PBS/0.2% BSA and containing 2×10^6 cpm [^{125}I]-GM-CSF per ml, with or without 200 ng cold rhGM-CSF. The cell layers were rinsed with PBS and the ligand-receptor complexes cross-linked with disuccinimidyl suberate reagent as reported[19]. The cell monolayers were rinsed with 0.05 mM Tris-HCl buffer, pH 7.5, containing protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 10 mM ethylenediaminetetraacetic acid (EDTA)], detached from the flask, then sedimented by centrifugation for 1 min at 10,000 × g. Each cell pellet was solubilized by incubation for 10 min in cold 10 mM Tris-HCl, pH 7.5, containing 0.5% (v/v) Nonidet P-40, 10 mM EDTA, and 0.5 mM PMSF. The cell lysates were centrifuged as above and aliquots from the supernatant counted in the gamma counter.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Samples (15 μl) of cell lysates were mixed with electrophoresis sample buffer containing protease inhibitors and solubilized material was subjected to SDS-PAGE (10% acrylamide gel) under nonreducing conditions, as described[19]. The gel was fixed and dried under vacuum and radio-labeled material revealed by autoradiography using Kodak radiographic films.

RESULTS

[^{3}H]-thymidine incorporation. To examine the mitogenic effect of rhGM-CSF on cell growth, rat AC were treated with various doses of this factor and labeled with [^{3}H]-thymidine for the last 6 h of culture. Treatment of cells with rhGM-CSF resulted in 30% inhibition of [^{3}H]-thymidine incorporation into DNA (Figure 1A). The concentration required for significant inhibitory effect was 25 U/ml.

PG and collagen synthesis. In contrast to cell growth, treatment with GM-CSF induced concentration-dependent stimulation (3.6- and 2-fold) of [^{35}S]SO$_4$ (Figure 1B) and [^{3}H]-proline (Figure 1C) incorporation into proteoglycan and collagen molecules, respectively. The minimal effective concentration was 12 U/ml for both matrix macromolecules.

Aggrecan and Coll II mRNA expression. Next, we examined with Northern blot whether the GM-CSF induced stimulation of PG and collagen production can be detected at the gene level. Growth-arrested cultures were incubated with 100 U/ml rhGM-CSF for 6 and 24 h and the gene expression was studied as described in Materials and Methods. As shown in Figure 2A, GM-CSF increased the expression of aggrecan (Ag) and Coll II transcripts after 6 h of treatment. At 24 h of continuous stimulation with rhGM-CSF the amount of aggrecan transcripts decreased whereas that of Coll II further increased (Figure 2A). The mRNA integrity and the quantity of materials deposited in the gel were controlled by hybridization with the GAPDH housekeeping gene. The quantitative densitometric analysis of radioautographs (Figure 2A) showed that the levels of expression of Ag and Coll II genes were 2–3-fold higher in cells treated with rhGM-CSF than in control cultures (Figure 2B). These data are in agreement with the above biochemical findings.

Chromatographic analysis of [^{35}S]-PG. The profiles of newly synthesized [^{35}S]-PG are shown in Figure 3. As shown in Figure 3A, cell monolayers synthesize mainly small PG. GM-CSF slightly stimulated the proportion of large PG monomers and aggregates (Figure 3A). It also augmented the proportion of aggregatable PG-monomers that partially eluted under the first aggrecan peak, but also under the second peak containing large monomers (Figure 3B).

cAMP production. To determine whether GM-CSF coupling to the receptor results in activation of adenylate cyclase, we measured the intracellular cAMP accumulation in control and GM-CSF treated cells. A time-course study showed an early (1 min) rise in cAMP production, which reached a plateau after 10 min following addition of 100 U/ml GM-CSF (Figure 4, insert). When the cells were incubated for 10 min with various concentrations of rhGM-CSF, intracellular cAMP accumulation was increased in a concentration-dependent manner, with a minimal effective concentration at 25 U/ml (Figure 4).
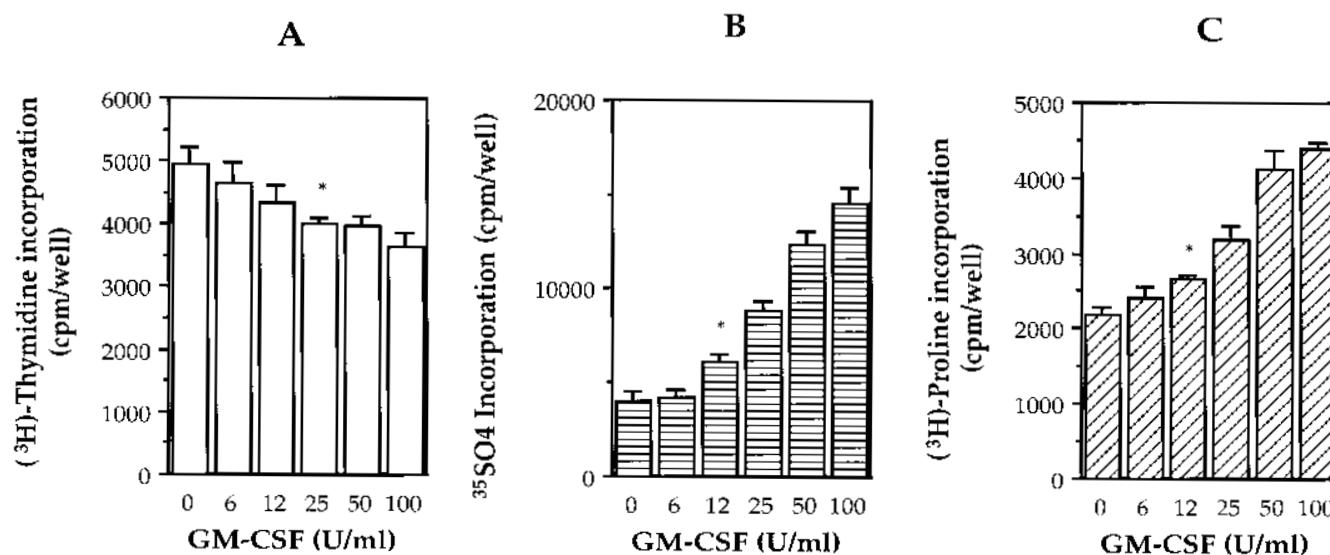
Figure 1. Effects of rhGM-CSF on [³H]-thymidine, [³⁵S]SO₄, and [³H]-proline incorporations. Growth arrested monolayers were incubated for 24 h (A) or 48 h (B and C) in culture medium containing 2.5% FCS (A) or 0.2% FCS (B and C) and the indicated concentrations of rhGM-CSF. The cells were then exposed to [³H]-thymidine for the last 6 h of culture or continously labeled with [³⁵S]SO₄ or [³H]-proline (B, C) for 48 h. The radioactivity in media and cell monolayers was determined as described in Materials and Methods. The values are the means ± SD of 4 (A) or 6 (B and C) replicate cultures. Similar results were observed in 2 independent experiments: *first significant difference *0.05 > p > 0.02 (Student's t test) between GM-CSF treated and control cultures.

[¹²⁵I]-GM-CSF binding. The presence of specific [¹²⁵I]-GM-CSF binding sites on unstimulated and stimulated rat AC was evaluated by incubating cell monolayers with [¹²⁵I]-GM-CSF. In the preliminary experiments, it was shown that [¹²⁵I]-GM-CSF (70,000 cpm/ml) binds at room temperature to AC monolayers in a linear fashion at early time, reaching a plateau after 2 h (Figure 5a; insert). This time was subsequently used in other binding studies. Incubation of the cells with increasing concentrations of [¹²⁵I]-GM-CSF (0–340,000 cpm) resulted in a concentration-dependent increase in the total binding (Figure 5). The bulk of the total binding was due to the specific binding. The nonspecific binding, obtained with 200-fold excess of unlabeled over labeled rhGM-CSF, represented less than 25% of the total binding. A Scatchard plot (Figure 5b; insert) of the binding data and a Hill coefficient of 0.97 indicate a single class of binding sites, and the K_d of 98 pM indicates a high affinity. The B_{max} for these receptors was 7.08 pM/μg DNA, which would represent about 13,000 binding sites per cell in a confluent monolayer.

A single band of 220 kDa corresponding to binding sites cross-linked to [¹²⁵I]GM-CSF was present on the autoradiograph of the gel used to separate cell extract under nonreducing SDS-PAGE conditions (Figure 6). The upper band represents complexes that have not entered the gel. The binding was specifically inhibited with unlabeled ligand.
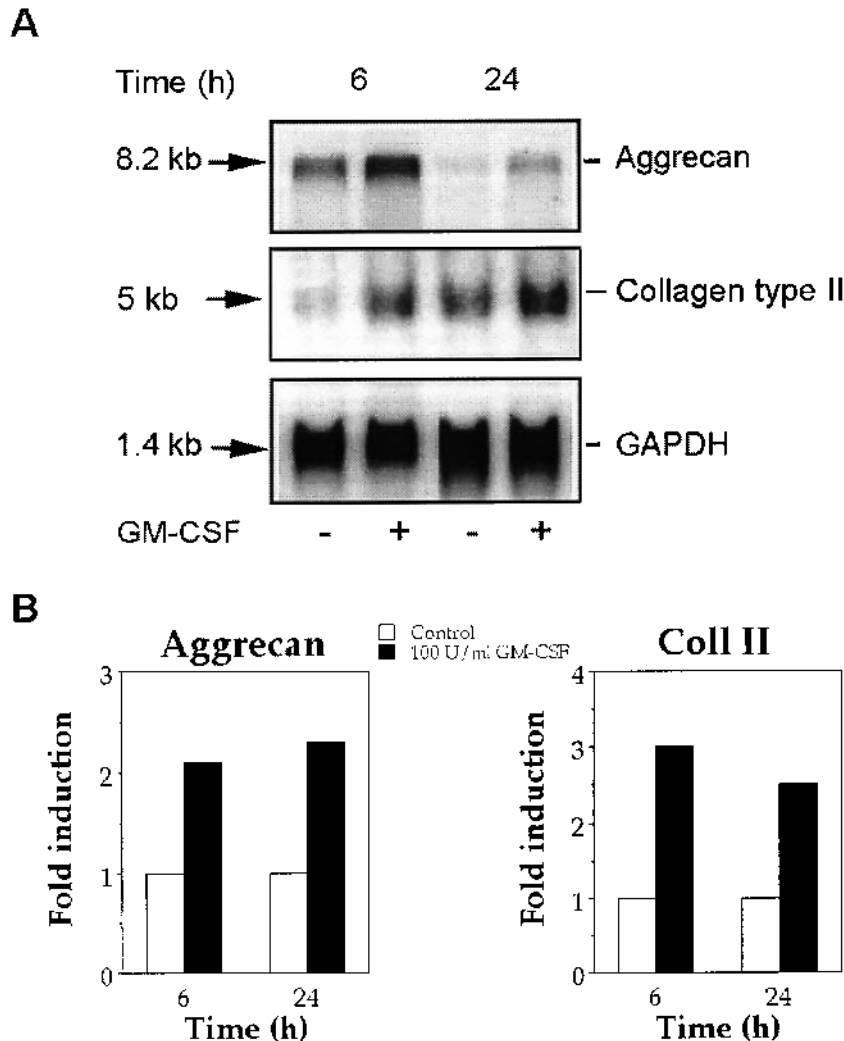
Effects of regulatory factors on [¹²⁵I]-GM-CSF binding. In the presence of phorbol ester the binding was slightly increased (12.5%), whereas it was significantly decreased in the presence of pertussis toxin (35%) or cholera toxin (17%) (Figure 7). Three growth factors when added into the culture medium 24 h before labeling increased [¹²⁵I]-GM-CSF binding to its sites. In the presence of PDGF, bFGF, and IGF-I we observed 16, 26, and 21% increase of the binding, respectively. In contrast, IGF-II and TGF-ß had no effect. Of the 2 cytokines tested TNF-α stimulated (19.4%) whereas IL-1ß decreased (19%) GM-CSF binding (Figure 7).

DISCUSSION

To our knowledge, this is the first report describing the responses of rat AC to rhGM-CSF. An important and novel finding of this study is the GM-CSF induced stimulation of PG and collagen synthesis and aggrecan and Coll II gene expression in articular chondrocytes. In contrast with previous reports on the GM-CSF induced effects on proliferation and differentiation of granulocyte-macrophage precursor cells from bone marrow[9-11] and of human osteoblasts[12], GM-CSF is not a mitogen for rat AC and slightly depresses DNA synthesis in these cells. Its effect on proteoglycan and collagen synthesis is not species-specific, since recombinant human GM-CSF was active on rat articular chondrocytes.

A few preliminary remarks are necessary. Articular chondrocytes cultured as monolayers tend to lose, with time in culture, a capacity to produce macromolecules characteristic for hyaline cartilage, such as aggrecan and type II collagen[27]. The cells used in this study express mRNA specific for these markers as shown previously[18,19] as well as in the present study, but a certain degree of "dedifferentiation" is attested by the synthesis of a large proportion of small PG as shown by Sepharose-CL 2B chromatography. Serum starved cells are used in order to minimize serum

A

Time (h)   6   24

8.2 kb → ▬ Aggrecan

5 kb →   ▬ Collagen type II

1.4 kb → ▬ GAPDH

GM-CSF   −   +   −   +

B

□ Control
■ 100 U/ml GM-CSF

Aggrecan

Coll II

Fold induction

Time (h)

Figure 2. Effects of rhGM-CSF on aggrecan and collagen type II mRNA expression. Confluent AC cultures were incubated at 37°C for 6 or 24 h in serum-free culture medium without (−) or with (+) 100 U/ml rhGM-CSF. A: 20 μg of total RNA were analyzed by Northern blot hybridization for aggrecan and Coll II expression, as described in Materials and Methods. Bottom band, GAPDH was rehybridized as a control for RNA quantification. B: densitometric quantification (Abs aggrecan or/Coll II)/GAPDH) of the corresponding spots shown in A. A blot representative of 2 independent experiments is shown.

effects. In this way we obtain more consistent and more reproducible results. FCS induces powerful stimulatory or even inhibitory effects (depending on origin or batches from the same manufacturer) on cultured cells and may potentiate or mask the effects of specific factors.

Since the production of GM-CSF and the expression of GM-CSF-specific mRNA are augmented in inflamed joints[14,15] and in IL-1ß and TNF-α stimulated articular chondrocytes[16] and synovial fibroblasts[13], this factor is usually considered to be a proinflammatory cytokine[13]. However, our results indicate that this cytokine may also take part in joint repair by stimulating chondrocytes to produce more PG and collagen type II matrix macromolecules. The rat AC monolayers responded to rhGM-CSF with clear cut increases in PG and collagen synthesis. These effects of

GM-CSF were more consistent and potent than the effects of other growth factors, including IGF-I and TGF-ß tested in our laboratory under identical conditions (article in preparation). In addition, the cells stimulated by GM-CSF produced significantly more cartilage-specific large and aggregatable PG monomers, as shown by Sepharose-CL 2B column chromatography under associative conditions. These findings suggest that GM-CSF promotes phenotypic differentiation of cultured chondrocytes and may similarly act on these cells *in vivo*. This is not surprising, since this factor promotes phenotypic differentiation of granulocyte and macrophage precursor cells and also stimulates specific functions of mature cells[9-11]. The chromatographic profiles obtained in the presence of hyaluronan (HA) (Figure 3) indicate that a fraction of the small PG monomers (3rd peak,
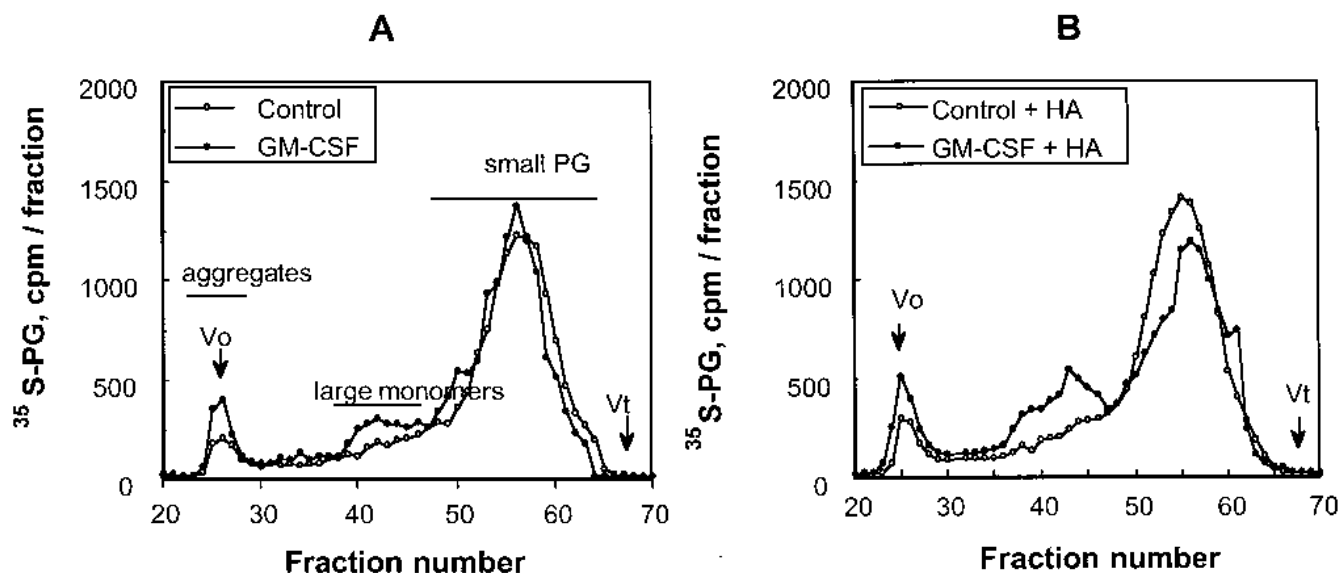
A

B

Figure 3. Sepharose-CL 2B column chromatography of [^{35}S]-PG. Growth arrested confluent cells were incubated for 24 h at 37°C without or with 100 U/ml rhGM-CSF. Two hours later, cells were continuously labeled with 50 μCi/ml carrier-free [^{35}S]SO$_4$. [^{35}S]-PG were extracted and purified as described in Materials and Methods. The 200,000 cpm aliquots were incubated at 4°C overnight without (A) or with 20 μg hyaluronate (HA) (B) and laid on the top of the column. The [^{35}S]-PG were eluted from the column and 2.5 ml fractions collected and counted as described. From left to right: 1st peak = aggrecan; 2nd peak = large PG-monomer; 3rd peak = small PG. Vo = void volume; Vt = total volume of the column.
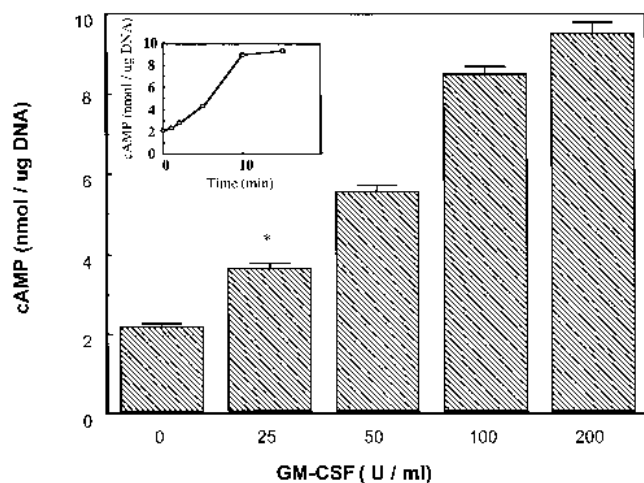

Figure 4. GM-CSF induced cAMP production. Growth arrested confluent rat AC monolayers were incubated 1 h in PBS buffer containing 0.2% BSA and 1 mM IBMX. rhGM-CSF was then added for 1 to 15 min (insert) or 10 min and cAMP determined using a RIA kit, as described in Materials and Methods. Insert: time-course study of cAMP production in the presence of 100 U/ml GM-CSF. The values are the means ± SD of triplicate cultures. *p < 0.02 (Student's t test) indicates first significant difference between GM-CSF treated and control cultures.

Figure 3) were in fact aggregatable PG that bind to HA and form relatively small aggregates that eluted under the second large monomer peak. The GM-CSF concentration that induces a stimulation of PG synthesis by rat articular chondrocytes [about 12 U (2 ng)/ml] is in the range of those found in cultures of human articular chondrocytes (1.5

ng/ml) or human synovial fibroblasts (1–2 ng/ml) following their incubation with IL-1ß[13,16].

Cultured rat articular chondrocytes possess functional binding sites for GM-CSF. Their interaction is accompanied within minutes by an increase in intracellular cAMP, which indicates that adenylate cyclase activation is a part of GM-CSF postreceptor signaling mechanisms. In agreement with this statement are the results obtained with regulators of adenylate cyclase activity such as pertussis toxin or cholera toxin, which both partially inhibited [^{125}I]-GM-CSF binding. Pertussis toxin stimulates the inhibitory G-protein subunit of adenylate cyclase and usually decreases (but may increase in some cells) cAMP production. Cholera toxin activates the stimulatory G-protein subunit of the enzyme and increases cAMP. An increase in cellular cAMP may account, at least partially, for the observed effects of GM-CSF. Exogenous cAMP was reported to increase keratan sulfate synthesis in canine articular chondrocytes[28] and sulfate incorporation in porcine[29], rabbit[30], and fetal calf[31] cartilage PG and to inhibit DNA synthesis in porcine articular chondrocytes[29]. The effect of GM-CSF on PG and collagen synthesis may be secondary to slowed cell growth[32]. This is unlikely because GM-CSF induces weak inhibition of chondrocyte proliferation and strong stimulation of PG and collagen synthesis (this study). In addition, many factors that strongly slow down chondrocyte growth (IL-1ß, TNF-α, interferon-γ) are potent inhibitor of PG synthesis[33].

Some evidence was also supported in this study in favor of protein kinase C activation as a postreceptor GM-CSF
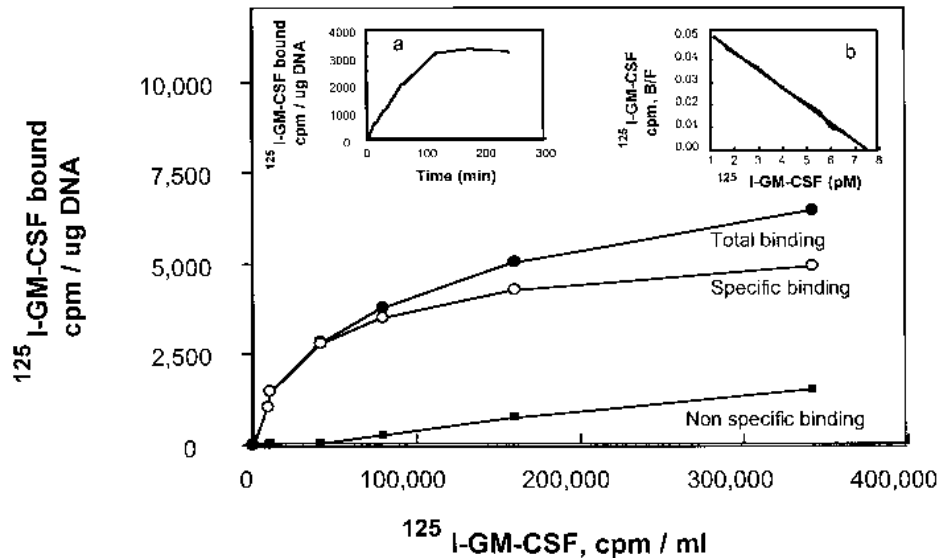
Figure 5. [^{125}I]-GM-CSF binding. Confluent monolayers were treated and labeled with [^{125}I]-GM-CSF as described. Specific and nonspecific binding were determined with 200-fold excess of rhGM-CSF over [^{125}I]-rhGM-CSF. Inserts: (a) time-course of binding (70,000 cpm) at room temperature; (b) Scatchard plot of the binding data. The values are the means of triplicate cultures. For clarity, standard deviations were not indicated.
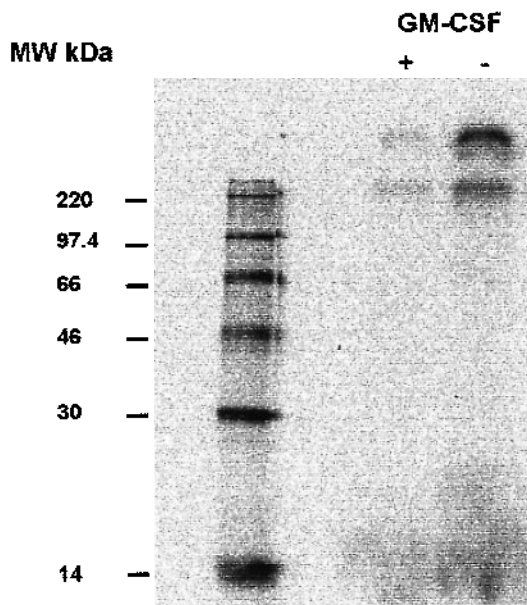

Figure 6. Autoradiography of [^{125}I]-GM-CSF receptor complexes. Confluent monolayers were treated and labeled with 2×10^6 cpm [^{125}I]-rhGM-CSF/ml as described. The labeled ligand/receptor complexes were cross-linked with disuccinimidyl suberate and extracted. Extracts were analyzed under nonreducing conditions by SDS-PAGE using 10% acrylamide gel, as described. On the left lane the positions of MW markers are indicated and on the 2 right lanes the extracted material labeled with [^{125}I]-rhGM-CSF in presence (+) or absence (–) of unlabeled GM-CSF.

signaling pathway. Indeed, PMA, a direct activator of PKC, caused a small but significant increase in [^{125}I]-GM-CSF binding. Studies have shown that GM-CSF binding to the receptor results in activation of PKC and in the phosphory-lation of tyrosine residues of its intracellular tyrosine kinase domain[34]. Although information on the intracellular second messengers that mediate the biological actions of GM-CSF is available mainly from studies on cells of myeloid origin, this cytokine is reported to increase intracellular cAMP and cGMP in human T lymphocytes[35], and to activate phospholipase A_2[36], resulting in an increase in arachidonic acid[37] and leukotriene[34] production and release by human neutrophils.

The GM-CSF receptor is composed of cytokine-specific N-glycosylated 80 kDa α chain and a 130 kDa ß chain that is also shared by IL-3 and IL-5[34]. The interaction of GM-CSF with the ligand-specific α chain results in a close association of the α and ß chains, leading to the formation of a high affinity receptor[38]. The results of our cross-linking experiment have shown a single binding protein of about 220 kDa that is in good agreement with the above cited data. On the 10% SDS-polyacrylamide gel, we can see 2 bands, one entering the gel of roughly 220 kDa and the second remaining at the origin of loading. This latter band may represent larger labeled complexes that have not entered the gel. Both bands disappeared when the labeling was done under competitive conditions in the presence of unlabeled ligand. Similarly, only a single class of high affinity (K_d 98 pM) receptors was detected in our cell model after analyzing the binding data. The B_{max} for these receptors was 7.08 pM/μg DNA, which would give about 13,000 binding sites per cell of a confluent monolayer, presuming even distribution of the receptor.

Other studies on mainly murine and human hematopoietic cells of myeloid lineage or their cancerous counterparts have produced a variety of results. Most reported a small number of high affinity (K_d 20–50 pM) receptors and a large
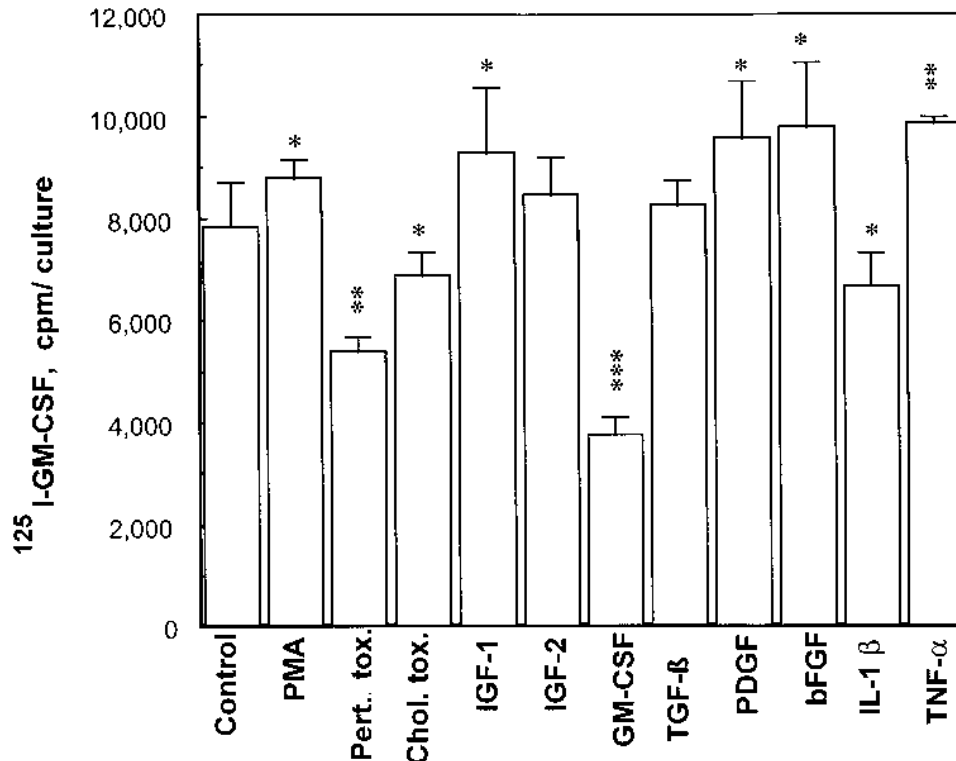
Figure 7. Effects of regulatory factors on [^{125}I]-GM-CSF binding. Confluent cell monolayers were incubated with PMA, pertussis toxin, or cholera toxin for 2 h or with growth factors and cytokines for 24 h prior to labeling with [^{125}I]-GM-CSF. Concentrations are given in Materials and Methods. Values are means ± SD of 6 different cultures. Significant differences (Student's t test) between treated and control cultures: *$0.05 > p > 0.02$; **$0.02 > p > 0.01$; ***$p < 0.01$.

number of low affinity (K_d 2–10 nM) receptors[39,40]. However, relatively small numbers of specific binding sites for GM-CSF (± 5000 per cell) have been detected on most cell lines. Often there is good agreement between the presence of high affinity cell surface receptors and cell responsiveness to GM-CSF. However, receptor activation can be independent of high affinity binding. A single alanine substitution, by site directed mutagenesis, in the N-terminal helix of mouse GM-CSF abrogates the high affinity binding of the ligand to mouse myeloid NFS-60 and 3E6 cell lines, but does not change the wild-type biological activity of the cytokine[41].

Several growth factors and cytokines positively or negatively regulate the GM-CSF receptor density on cultured rat articular chondrocytes. These factors are often found in increased amounts in the synovial fluid or synovial tissue in arthritis[14,15] and thus may regulate GM-CSF activity through modulating either receptor density or GM-CSF production. Certain of these factors, such as bFGF, IGF-I, PDGF, and TNF-α, increase GM-CSF receptor density, whereas others, like IL-1ß, decrease it (this study). The partial suppression of [^{125}I]-GM-CSF binding by GM-CSF reported in this study may be due to a competition for the binding sites. In other studies IL-1ß and TNF-α induce GM-CSF-specific

mRNA expression and gene product production in cultured human articular chondrocytes[16,17] and synovial fibroblasts[13]. Similarly, GM-CSF production is upregulated by TGF-ß and downregulated by bFGF in human articular chondrocytes[16]. The significance of these findings for chondrocyte biology is not clear.

Elevated levels of GM-CSF were found in synovial effusions[14,15] and synovial cells[13,42] from patients with arthritis and, to a lesser degree, osteoarthritis, suggesting its involvement in the pathogenesis of these conditions. As GM-CSF enhances neutrophil mediated cartilage damage *in vitro*[43], it is thought to be a proinflammatory cytokine. However, aside from this last proinflammatory effect, there is little experimental evidence in favor of its proinflammatory role. GM-CSF is an anabolic factor for certain bone marrow cells. It accelerates differentiation and maturation of precursor cells of granulocyte-macrophage lineages and also enhances specific functions of mature differentiated cells, such as human osteoblasts[12].

We suggest that GM-CSF may also be an anabolic factor for articular chondrocytes under physiological, and possibly pathological conditions. Produced in abundance by bone marrow cells, GM-CSF may, under physiological conditions, contribute to maintain cartilage phenotype and, by

increasing PG and collagen II production in inflammation, accelerate cartilage repair. Further studies are needed to elucidate the exact role of this cytokine in joint pathophysiology.

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