CD44, a Cell Surface Chondroitin Sulfate Proteoglycan, Mediates Binding of Interferon-γ and Some of Its Biological Effects on Human Vascular Smooth Muscle Cells

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Several cytokines and growth factors act on cells after their association with the glycosaminoglycan (GAG) moiety of cell surface proteoglycans (PGs). Interferon-γ (IFN-γ) binds to GAG; however, the relevance of this interaction for the biological activity of IFN-γ on human cells remains to be established. Human arterial smooth muscle cells (HASMC), the main cells synthesizing PG in the vascular wall, respond markedly to IFN-γ. We found that treatment of HASMC with chondroitinase ABC, an enzyme that degrades chondroitin sulfate GAG, reduced IFN-γ binding by more than 50%. This treatment increased the affinity of 125I-IFN-γ for cells from a Kd value of about 93 nM to a Kd value of about 33 nM. However, the total binding was reduced from 9.3 ± 0.77 pmol/μg to 3.0 ± 0.23 pmol/μg (n = 4). Interestingly, pretreatment with chondroitinase ABC reduced significantly the cellular response toward IFN-γ. The interaction of IFN-γ with chondroitin sulfate GAG was confirmed by affinity chromatography of isolated cell-associated 35S, 3H-labeled PG on a column with immobilized IFN-γ. The cell-associated PG that binds to IFN-γ was a chondroitin sulfate PG (CSPG). This CSPG had a core protein of approximately 110 kDa that was recognized by anti-CD44 antibodies on Western blots. High molecular weight complexes between IFN-γ and chondroitin 6-sulfate were observed in gel exclusion chromatography. Additions of chondroitin 6-sulfate to cultured HASMC antagonized the antiproliferative effect and expression of major histocompatibility complex II antigen induced by IFN-γ. These results indicate that IFN-γ binds with low affinity to the chondroitin sulfate GAG moiety of the cell surface CSPG receptor CD44. This interaction may increase the local concentration of IFN-γ at the cell surface, thus facilitating its binding to high affinity receptors and modulating the ability of IFN-γ to signal a cellular response.

IFN-γ is a glycoprotein produced by activated T-lymphocytes and is released during the immune response and in inflammatory conditions (1, 2). IFN-γ elicits antiviral, antiproliferative, and immunomodulatory activities in different cells (3–5). IFN-γ causes its pleiotropic effects partially through interaction with a specific plasma membrane receptor (6, 7). In addition, the biologically active IFN-γ-receptor complex requires the species-matched interaction of the complex with at least one additional accessory factor that conveys different cellular responses (8, 9). However, the structural basis of these interactions is not completely understood.

Proteoglycans (PGs) are ubiquitous components of cell membranes. They consist of a core protein to which one or more glycosaminoglycan (GAG) chains are covalently attached. GAGs are linear, sulfate-substituted carbohydrates. The main GAG types in PG are as follows: chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, and heparin. This last one is restricted to mast cell granules (10–12). Because of the high sulfate and carboxyl group content in their GAG moieties, PGs are the most negatively charged polymers in living tissues. This property allows them to interact with proteins with clusters of positively charged amino acids (13, 14). Thus, apart from being structural elements for extracellular matrix and basement membrane assembly, PGs/GAGs also serve as cell surface receptors for a wide range of proteins, including growth factors, enzymes, cytokines, chemokines, lipoproteins, and viruses (11, 12, 15–22).

Human recombinant IFN-γ is a 146-amino acid polypeptide where three positive charged clusters of basic amino acids are localized in the carboxyl terminus: 1) SnKKKRDFF, residues 87–96, total charge +2; 2) AKTKRRKRS, residues 127–135, total charge +5; and 3) LFRGRRAS, residues 138–145, total charge +3. These sequences do not correspond completely to the consensus GAG binding sequences reported for other proteins (13). However, experimental data indicate that these regions may provide IFN-γ with the capacity to interact with negatively charged GAG (18, 23). Crystallographic analysis of human IFN-γ indicates that these sequences of basic amino acids are exposed on the surface of the protein (24, 25). Furthermore, these sequences are similar to the nuclear localization signal, a consensus of basic amino acids required for efficient transport of proteins, including IFN-γ, from the cytosol to the nucleus (26). Our previous experiments with synthetic peptides suggested that the cluster of basic amino acid residues 127–135 (AKTKRRKRS) of human IFN-γ is involved in the binding with chondroitin-sulfate PGs from the extracellular albumin; FCS, fetal calf serum; chABC, chondroitinase ABC; Hep I, heparitinase I; PAGE, polymerase chain reaction; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; CSPG, chondroitin sulfate PG; CS-GAG, chondroitin sulfate GAG.
matrix of human arterial smooth muscle cells (HASMC) (18). In addition, results have shown that IFN-γ immobilized in CSPG generates a higher response than soluble IFN-γ from HASMC in culture. Sudir et al. (27) recently showed that this basic cluster of amino acids was involved both in heparin and IFN-γ-receptor recognition. Furthermore, results from studies with site-directed mutagenesis and controlled proteolysis suggest that part of this region (residues 131–135, KRKRS) is critical for receptor binding and biological activity (28–31). Together, these results indicate that the basic clusters in IFN-γ carboxy-terminal structure are important for the biological activity of IFN-γ.

Arterial smooth muscle cells in culture respond markedly to IFN-γ by expressing class II MHC such as HLA-DR (32). These genes are up-regulated by smooth muscle cells in experimentally injured arteries and human atherosclerotic plaques, probably induced by secretion of IFN-γ by a subset of T-cells present in the arterial wall (33). IFN-γ is present in human atherosclerotic plaque (34). This cytokine is also a potent inhibitor of both cell proliferation and collagen synthesis in HASMC (35, 36). Based on these in vitro effects, Libby et al. (37) suggested that chronic activation of these cells by IFN-γ might contribute to mechanistic instability of atherosclerotic plaques. Such a hypothesis is supported by results from double knockout mice lacking both IFN-γ receptor and apoE that develop much less atherosclerosis than the apoE single knockout mice do (38). Taken together, these results imply that IFN-γ signaling may promote atherogenesis and plaque disruption. Therefore, inhibition of IFN-γ signaling in arterial cells may be a possible therapy against acute coronary syndromes. This hypothesis, however, requires more knowledge about mechanisms involved in signaling the presence of IFN-γ in human cells. The interaction between IFN-γ and GAG has been reported previously (18, 39). However, characterization of the interaction and its relevance for the biological activity of IFN-γ on human cells is not completely understood. In the present study, we explored whether cell surface GAG in HASMC may have a function in binding IFN-γ and if this interaction may modulate the biological activity of IFN-γ to induce a biological response.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hepes, Triton X-100, N-ethylmaleimide, e- amino caproic acid, benzamidine HCl, phenylmethylsulfonyl fluoride, heparan sulfate (7,500 kDa), cetylpyridinium bromide, and ethyleneminoxetic acid were purchased from Sigma. Chondroitin 6-sulfate (CS6) (40–80 kDa), protease-free chondroitinase ABC (EC 4.2.2.4), and heparitinase I (EC 4.2.2.8) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Molecular weight standards for exclusion chromatography, cyogen bromide-activated Sepharose, Hi-Trap Q, and Superose 6 PC 3.2/3.0 columns were bought from Amersham Pharmacia Biotech. Collagen-1 was purchased from Collaborative Biomedical Products, Becton Dickinson, Labware (Bedford, MA). Cell culture media, antibiotics, nonessential amino acids, fetal calf serum (FCS), Dulbecco’s phosphate-buffered saline (DPBS) with and without calcium and magnesium, and culture vessels were purchased from Life Technologies, Inc. Cell culture-tested BSA and trypsin-EDTA were purchased from Sigma. Na151[35S]SO4 (25–40 Ci/mg), l-4,5-3H-leucine (120–190 mCi/mmol), unlabeled recombinant human IFN-γ (10 units/ng), recombinant human 125I-labeled IFN-γ (1000 Ci/mol, M, 17,000, 10 units/ng), and hyperfilms for autoradiography were from Amersham Pharmacia Biotech. Liquid scintillation mixture, Ready Safe, for aqueous samples was from Beckman Instruments Inc. Monoclonal mouse anti-human HLA-DR, CR3/43, negative control mouse IgG1, biotinylated rabbit anti-mouse immunoglobulins, normal rabbit serum, and ABCComplex/AP for determination of HLA-DR expression in human cells were purchased from Dako (Dako- patts AB, Sweden). Mitochondrial activity kit (XTT) and BclU ELISA kit for nonradioactive quantification of cell activation and proliferation, respectively, were purchased from Roche Molecular Biochemicals (Bromma, Sweden). Salts, buffer substances, and detergents used in this work were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Bio-Rad.

**Cell Culture**—Primary cultures of HASMC from inner media of human uterine arteries were established using a previously described explantation technique (40). The cells were harvested by trypsinization and cultured at a cell density of 5 × 104 cells/cm2 in 6-, 12-, 24-, and 96-well plates for binding experiments and biological assays of IFN-γ activity and in 80-cm2 bottles for inhibition experiments with the cells. HASMC were cultured in bottles coated with a film of collagen-1 (41). The cells were allowed to proliferate in Waymouth’s medium plus 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mmol/liter sodium pyruvate, 4 mmol/liter glutamine, and nonessential amino acids (growing medium). After 2 days, the medium was removed, and cells were washed three times with DPBS, and then cultured in Waymouth’s medium containing the supplements indicated above but only 0.5% FCS (sera-poor medium) in order to synchronize the cells by stopping proliferation. After 3 days, the medium was removed, and cells were washed 3 times with DPBS and cultured in growing medium for 3–4 days until the cells were confluent. The experiments were carried out with cells between passages 3 and 12. HASMC were tested for mycoplasma contamination during each passage by using a mycoplasma test kit from Gen-Probe Inc. Endotoxin levels were regularly tested in cell culture media and cell culture reagents with Coatest/ endotoxin (Chromogenix AB). Levels detected were 0.01 enzyme units/ml.

**Binding Assay**—The ability of 125I-labeled IFN-γ to bind to HASMC was measured using binding assays carried out in 12-, 24-, or 96-well plates. The plates were washed three times with ice-cold DPBS, 0.2% BSA and incubated for 10 min at 4 °C with ice-cold bicarbonate-free Waymouth’s medium, 0.2% BSA. The cells were incubated with 125I-labeled IFN-γ (100–1000 cpm/pg) at concentrations indicated in the figures at 4 °C for 4 h. The plates were then placed over ice and washed three times with ice-cold Waymouth’s medium, 0.2% BSA and three times with ice-cold DPBS. The cells were then washed three times with DPBS and then twice with 2 mol/liter NaCl in 20 mol/liter/leucine (pH 7.4) to induce a maximal release of 125I-labeled IFN-γ bound to GAGs (42). These washes were collected. The cells were next dissolved with 2 × 0.2 mol/liter NaOH, and their protein was measured. The radioactivity measured in the 2 mol/liter NaCl, pH 7.4, wash represented the fraction of 125I-labeled IFN-γ bound extracellularly to sulfated GAGs through ionic interactions. The radioactivity measured in cells dissolved with 0.2 mol/liter NaOH represented the fraction of 125I-labeled IFN-γ bound through nonionic interactions to cell membrane, probably to the receptor and 125I-labeled IFN-γ internalized by the cells. The amount of radioactivity was counted in a Compu gamma counter (LKB, Wallac, Sweden), and aliquots were used for protein determination (43). The data from binding experiments were analyzed to determine maximum binding (Bmax) and association constant (Kd) by nonlinear regression analysis (44) using the program GraphPad PRISM Software, Inc. (San Diego, CA).

**Digestion of Cell Surface Glycosaminoglycans with Chondroitinase ABC/Heparitinase I—HASMC were treated with chondroitinase ABC (chABC) and heparitinase I (Hep I) in order to digest cell surface chondroitin sulfate and heparan sulfate, respectively. Cell media were collected from the HASMC (3 days, 5 × 105 cells/cm2) and washed three times with DPBS, 0.2% BSA and incubated for 2 h at 37 °C with or without 0.01 unit/ml of the enzymes in DPBS, 0.2% BSA. Cell viability in the DPBS and glycosidase-treated cells was greater than 90%, as judged by trypan blue exclusion and cell morphology. Dishes without cells were run in parallel as control. After incubation, the cells were washed three times with DPBS, 0.2% BSA. The cells were then incubated with 125I-labeled IFN-γ to perform binding experiments or with unlabeled IFN-γ to study cellular response toward IFN-γ as described below. In order to keep cells depleted from pericellular GAGs, the incubation with IFN-γ were done in the presence of chABC and/or Hep I (0.01 U/ml). Control cells were treated and incubated in similar conditions but without enzyme(s).

**Isolation of Cell-associated Proteoglycans**—HASMC (5,000 cells/cm2) were cultured as described above in Waymouth’s medium plus 10% (v/v) FCS (complete medium) for 2 days. After this period, the medium was changed to sera-poor formulation. After 2 days, medium was removed, and the cells were washed three times with DPBS and then cultured in diploid medium (sulfate-free) supplemented with 10% FCS, penicillin/streptomycin, sodium pyruvate, and nonessential amino acids (diploid medium A). After 1 day, the medium was removed, and cells were washed three times with 1 mol/liter sodium chloride (10% FCS, 4 mol/liter glucose (10–50 μCi/ml) and 17 μCi/ml [3H]leucine (120 Ci/mmol). The cells remained in this medium for 3 days. After this period, the cell culture medium was harvested. The cells were washed three times with DPBS and then incubated for 30 min at room temperature with sera-free culture medium containing 50 μg/ml heparin. This step was included in order to remove peripheral, extrinsically associated PG (45). Heparin-
IFN-γ Interaction with Glycosaminoglycans

containing medium was discarded, and the cells were washed three times with DPBS. Cell-associated proteoglycans were isolated by incubating HASMC with 15 ml of extraction buffer for 30 min with gentle shaking. The extraction buffer contained 0.15 mol/liter NaCl, 5 mmol/liter MgCl₂, 2 mmol/liter EDTA, 0.255 mmol/liter dithiothreitol, 1 mmol/liter phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% Triton X-100, 0.25 mol/liter a-aminooacetic acid, 5 mmol/liter N-ethylmaleimide, 5 mmol/liter benzamidine HCl, 10 mmol/liter Tris-HCl, pH 7.2. The cell extracts were then dialyzed against ion exchange chromatography buffer (8 mol/liter urea, 2 mmol/liter EDTA, 0.5% Triton X-100, and 20 mmol/liter Tris-HCl, pH 7.5, containing protease inhibitors) for 2 days with two medium changes before dialysis. After dialysis, the elute was passed through HiTrap Q ion exchange columns equilibrated in binding buffer. After the samples were loaded, the columns were washed with binding buffer containing 0.25 mol/liter NaCl to remove glycoproteins. The bound material was finally eluted with a gradient from 0.25 mol/liter to 3 mol/liter NaCl in binding buffer, and the radioactivity in each collected fraction was measured. The fractions rich in [35S]-labeled PGs eluted around 1.5 mol/liter NaCl. These PG-containing fractions were pooled, dialyzed against water, and lyophilized. These samples were used for IFN-γ affinity chromatography as described below. GAG composition was analyzed by agarose gel electrophoresis (46). The [35S], [3H]-labeled PG samples were analyzed by SDS-PAGE in a 4–12% linear gradient gel. The positions of the radioactive bands were visualized by autoradiography of dried gels. [14C]-methylated protein molecular weight standards (Amersham Pharmacia Biotech) were used to estimate the average sizes of [35S], [3H]-labeled cell PGs.

Affinity Chromatography on Sepharose-IFN-γ Column—A Sepharose-IFN-γ column (5 × 1-cm diameter) was prepared from IFN-γ bound to cyanogen bromide-activated Sepharose according to the manufacturer’s procedure. The column was equilibrated in binding buffer: 5 mmol/liter Hepes, pH 7.4, 20 mmol/liter NaCl, 5 mmol/liter CaCl₂, and 2 mmol/liter MgCl₂. A similar column containing no IFN-γ and blocked with ethanolamine served as a control column for unspecific binding. The cell extract containing the [35S]sulfate- and [3H]leucine-labeled PGs synthesized by HASMC were equilibrated in binding buffer and passed half through the IFN-γ column and half through the control column. The columns were washed with 25 ml of the same buffer. Bound material was eluted with a gradient from 20 mmol/liter to 500 mmol/liter NaCl, and fractions of 1 ml were collected. The peaks containing the cell PGs retained by the immobilized IFN-γ were dialyzed and lyophilized. Thereafter, the samples were dissolved in 1.5 ml of 20 mmol/liter Tris-HCl, pH 7.5, and divided in three aliquots. One aliquot was treated with chondroitinase ABC (20 units/ml), the second aliquot with heparitinase I (20 units/ml), and the third without enzyme (control).

The samples were incubated overnight at 37 °C. The reaction was stopped by rapidly freezing the samples at −20 °C. The aliquots were equilibrated in binding buffer and passed again through the IFN-γ affinity column as described above.

Western Blot Analysis of Cell-associated Proteoglycans—Total and cell-associated proteoglycans with affinity for IFN-γ were isolated as described above. The preparations of cell-associated proteoglycans were incubated with or without 0.10 units/ml chondroitinase ABC over-night at 37 °C. The reaction was stopped by rapidly freezing the samples at −20 °C. The aliquots were equilibrated in binding buffer and passed again through the IFN-γ affinity column as described above.

RESULTS

125I-IFN-γ Binding to Cell Membrane-associated Glycosaminoglycans—The possibility that cell membrane-associated GAGs in HASMC contributes to the binding of IFN-γ was addressed by removal of pericellular GAGs with degradative enzymes that cleave GAGs. Fig. 1 shows that treatment of HASMC with chABC reduced drastically the binding of 125I-IFN-γ. Treatment with Hep I, an enzyme that hydrolyzes heparan GAG, did not affect the binding of 125I-IFN-γ. Fig. 2A shows the binding isotherms for the fraction of 125I-IFN-γ that can be dissociated by raising the concentration of NaCl to 2 mol/liter. It appears that 18 HASMC treated with chABC/Hep I the binding isotherm of 125I-IFN-γ reached saturation at lower concentration than in the untreated cells (control). The binding data fitted best a two-site binding model. This analysis showed that, as a consequence of pericellular GAG removal, the apparent dissociation constant (Kd) for the binding was decreased from 93 ± 11 nm to 32 ± 5.5 nm (n = 4), and the maximum binding (Bmax) was reduced from 9.30 ± 0.77 to 3.0 ± 0.23 pmol/μg cell protein (n = 4). Fig. 2B shows that

FIG. 1. Binding of 125I-IFN-γ to chondroitinase- and heparitinase I-treated and untreated HASMC. Confluent HASMC cultured in 24-well plates were treated 2 h with chABC (0.01 unit/ml) or Hep I (0.01 unit/ml) in DPBS containing 0.2% BSA. Control cells without enzymes were incubated in parallel. The cells were then incubated with 125I-IFN-γ (2 ng/ml) in Waymouth’s cell medium containing 10% fetal bovine serum. After a 2-h incubation, cell-associated radioactivity counted, and cell protein were measured. The values are the average and S.D. of four determinations.

Evaluation of IFN-γ/Chondroitin Sulfate Interaction by Analytical Gel Exclusion Chromatography—To study the formation of complexes between chondroitin sulfate and IFN-γ, we evaluated the elution behavior of the IFN-γ in a Superose 6 PC, 2.3/0.3 column in the presence and absence of chondroitin 6-sulfate GAG in the elution buffer. The column was operated with an automated SMART system (Amersham Pharmacia Biotech). Flow was 0.032 ml/min. In control experiments, the column was equilibrated in PBS, and 20 nl of the IFN-γ (17,000 kDa) at 5 mol/liter, dissolved in the same buffer, was injected. In other runs, the column and the solution of IFN-γ were equilibrated in PBS containing 1 mg/ml C6S (40–80 kDa). The absorbance of the fractions eluted was followed at 254 nm. The same column was calibrated with standard proteins indicated in the legend to Fig. 8.

Competition with Soluble Glycosaminoglycans: Effect on HLA-DR Antigen Expression and Cell Proliferation—HASMC were cultured in 96-well plates as described above. Cells were incubated with IFN-γ (0, 10, and 1,000 ng/ml) with or without C6S (11 and 110 μg/ml). After 3-day incubations, the cellular response toward IFN-γ was studied by measuring cell surface expression of major histocompatibility complex II or HLA-DR, cell proliferation by bromodeoxyuridine incorporation, and mitochondrial dehydrogenase function in cells. HLA-DR expression was measured by an enzyme-linked immunosassay with monoclonal antibody HLA-DR, CR3/43, and mouse IgG1-negative control antibody as described (18). Briefly, the optimal dilution of the antibodies and ABCcomplex were determined previously by checkerboard titration. The absorbance values obtained with the negative control antibody were subtracted from the absorbance values obtained with the antibody against HLA-DR. Effects of IFN-γ on cell proliferation and mitochondrial activity were measured with BrdU ELISA and XTT colorimetric kits according to the manufacturer’s procedure.
digestion with chABC plus Hep I affected much less the amount of $^{125}$I-IFN-γ that remained cell-associated after treatment with 2 mol/liter NaCl and that was dissolved in 0.2 mol/liter NaOH. This fraction contains $^{125}$I-IFN-γ bound to cells through nonionic interaction. This fraction may also contain some $^{125}$I-IFN-γ internalized by the cells despite performing the binding experiments at 4 °C.

**Effect of Cell Surface Glycosaminoglycan Removal on the Cellular Response toward IFN-γ.** The results described above indicate that IFN-γ binds to cell surface-associated CSPG and that the interaction contributes to the total binding of IFN-γ to HASMC. We then explored if the removal of CSPG could affect the cellular response toward IFN-γ. One of the anti-collagenous effects ascribed to IFN-γ is its ability to inhibit cell proliferation (1). Recombinant IFN-γ has been reported to inhibit human smooth muscle cell proliferation in vitro in a dose-response manner (35). Therefore, we studied if the removal of cell surface-associated CSPG could affect the antiproliferative effect of IFN-γ on HASMC in culture. The experiments were performed with HASMC preincubated with protease-free chABC or without enzyme (control cells), as described under “Experimental Procedures.” HASMC control cells and chABC-treated ones were incubated with IFN-γ, as indicated in the legend to Fig. 3. HASMC proliferation was measured using a XTT colorimetric assay for cell proliferation (51). The results, illustrated in Fig. 3, show that removal of cell surface CS-GAG by treatment with chABC decreased significantly the antiproliferative effect of IFN-γ on HASMC when compared with control cells. These results indicate that interaction of IFN-γ with cell surface CSPG enhances the cellular effect of IFN-γ on HASMC.

**IFN-γ Affinity Chromatography of Cell-associated Proteoglycans.** To further characterize the interaction of IFN-γ with cell surface PG from HASMC, we used affinity chromatography of metabolically labeled cell-associated PG on immobilized IFN-γ. Fig. 4A shows that only one peak of biolabeled material was obtained. This peak, as expected for PGs, contained more $^{35}$S- than $^{3}$H-labeled leucine. The peak size retained by IFN-γ eluted at 150 mmol/liter of NaCl. This indicates that the interaction of IFN-γ with GAGs takes place at physiological ionic strength. In the IFN-γ column, 80% of the radioactive PG-rich fraction was bound, whereas only 3% of the radioactivity was retained in a similar control column. Fractions 5–13 from the NaCl gradient containing the cell-associated PG that was bound to IFN-γ were pooled, dialyzed, and lyophilized. This IFN-γ-bound PG preparation was divided into three equal parts. One part was treated with chABC, and another was treated with Hep I; the third was a control without enzyme, as described under “Experimental Procedures.” The samples were passed again through the Sepharose IFN-γ affinity column. It can be observed in Fig. 4B that pretreatment with chABC, which hydrolyzes chondroitin sulfate GAG, abolished completely the binding of cell-associated $^{35}$S-, $^{3}$H-labeled PGs to the immobilized IFN-γ. Hep I had no effect in the binding of cell-associated $^{35}$S-, $^{3}$H-labeled PGs to IFN-γ. These results show that IFN-γ binds to cell-associated CSPGs synthesized by HASMC.

**Characterization of Total and IFN-γ-bound Cell-associated Proteoglycans—SDS-PAGE analysis.** SDS-PAGE analysis of cell-associated $^{35}$S-, $^{3}$H-labeled PGs was used to further characterize the cell-associated PG expressed by HASMC and to immunologically probe the chondroitin sulfate PG that binds to IFN-γ. Fig. 5 shows the results obtained. The gels showed three bands of PG with different relative molecular mass: one class size that remains on the top of the separating gel (Fig. 5, band I, ≥400 kDa), a second class around 200 kDa (band II), and a third class of low molecular mass, between 100 and 46 kDa (band III). Digestion with chABC and Hep I of the IFN-γ-bound $^{35}$S-, $^{3}$H-labeled PGs, prior to SDS-PAGE analysis, indicated that the macro-
molecules of band II and III are chondroitin/dermatan-containing PGs, while those of band I represent heparan sulfate containing PGs, probably perlecan, since these cells express this PG (52). Versican (CSPG, > 400 kDa) was not detected in these preparations. These analyses support the results obtained with affinity chromatography and confirmed that cell-associated PGs contain mainly CS-GAG with minor amounts of heparan sulfate-GAG.

In an effort to identify the PG that binds to IFN-γ, we performed Western blot analysis. Fig. 6 shows representative Western blots of total preparation of cell-associated PG (Fig. 6A) and the cell-associated PG eluted from an IFN-γ affinity column (Fig. 6B). These membranes were probed with a monoclonal antibody against CD44 and a polyclonal antibody against biglycan. Western blot analysis of the total preparation of cell-associated PG indicated the presence of biglycan and CD44 (Fig. 6A, lanes 1 and 2, respectively). These were chondroitin sulfate PGs. Western blot analysis of the cell-associated PG that binds IFN-γ showed a positive reaction with antibody against cells surface receptor CD44 (Fig. 6B, lane 1 (control) and lane 2 (chABC-treated)). However, no immunoreactivity was observed with anti-biglycan (Fig. 6B, lane 3) or anti-decorin antibodies (data not shown). Treatment with chABC increased the reaction with the antibodies and shifted downward the molecular mass of PGs, from ~250 to ~42 kDa for biglycan and from ~150 to ~110 kDa for CD44 approximately (Fig. 6B, lane 1 (control) and lane 2 (chABC-treated)). These changes in molecular weight before and after chABC digestion indicate that the CS-GAG moiety in biglycan is markedly larger than in CD44. The Western blot results indicate that in the SDS-PAGE analysis of the total preparation of cell-associated PG (Fig. 5) the CSPGs in band II contain biglycan and that band III contains CD44. These results suggest that HASMC express the majority of their cell-associated PG as CSPG and that the core protein of CSPG that binds to IFN-γ is immunologically related to CD44, a cell surface CSPG receptor.

Analysis of CD44 RNA Transcript in Human Arterial Smooth Muscle Cells—RT-PCR was carried out to investigate the presence of RNA transcript encoding CD44 in HASMC. RT-PCR was performed on RNA isolated from HASMC culture in the presence of 10% fetal bovine serum (proliferative condition) and in sera-free medium (nonproliferative condition). Total RNA isolated from human arterial tissue was also analyzed. The primers used are located in the common exons E3 (forward primer) and E16 (reverse primer) shared by all CD44 isoform genes. Fig. 7 shows analysis of the RT-PCR products from total RNA from HASMC and human arterial tissue. The agarose gels showed a single size DNA band of approximately 453 base pairs, the predicted size for this CD44 product. No differences in the levels of CD44 mRNA were observed between prolifera-
Antagonizing Effect of Soluble Chondroitin 6-Sulfate to the Cellular Responses toward IFN-γ—The results described showed that CS-GAG contributes to the total binding of IFN-γ to cell surface PG in HASMC and that C6S is able to form complexes with IFN-γ at physiological salt concentrations. The next question was if this interaction could modulate the biological activity of IFN-γ. We studied if soluble C6S added to HASMC in culture could modify the cell expression of class II MHC HLA-DR induced by IFN-γ. Induction of class II MHC HLA-DR antigens is a unique biological property of IFN-γ. This cytokine can also inhibit serum- or growth factor-induced proliferation of vascular smooth muscle cells; therefore, we chose as markers of cellular response toward IFN-γ the HLA-DR expression and cell proliferation. The addition of C6S to the cell culture medium together with IFN-γ antagonized the antiproliferative effect of IFN-γ, inducing an increase in the incorporation of bromodeoxyuridine by the cells (Fig. 9A). Furthermore, soluble C6S antagonized the expression of HLA-DR cell surface antigens by the cells (Fig. 9B). The effects were directly related to the molar ratio between C6S and IFN-γ. These results indicate that the extracellular addition of C6S glycosaminoglycans inhibits the biological effects of IFN-γ on HASMC.
with chABC decreased by more than 50% the total binding of IFN-γ to HASMC. Analysis of the binding data for the fraction probably associated by ionic bonds indicates the existence of two binding components for IFN-γ in HASMC: a low affinity component with an apparent Kd about equal 93 nM and a component with higher affinity that was unmasked after CS-GAG digestion with an apparent Kd about equal to 33 nM. The low affinity binding, due to the CS-GAG, provided the cells with a larger total binding. Similar interpretation was given to results from analogous experiments performed to study the binding of basic fibroblast growth factor and low density lipoproteins to pericellular GAG in fibroblasts (20, 42). These studies reported that basic fibroblast growth factor and low density lipoproteins also showed low affinity sites and high affinity sites that were unmasked after removal of the pericellular GAG by treatment with chABC. Interestingly, the affinity constants of these three different proteins, basic fibroblast growth factor, low density lipoproteins, and IFN-γ, for the low affinity GAG sites were in the nanomolar range: 2 nM for basic fibroblast growth factor, 31 nM for low density lipoproteins, and 93 nM for IFN-γ. The changes in affinity and maximal binding values, after removal of CS-GAG, are probably due to an increase in the exposure of less abundant, high affinity-specific IFN-γ receptors in the cell surface or to the elimination of abundant high capacity, low affinity cell surface components, the CS-GAG. Although our experiments do not allow us to select between these alternatives, they indicate that cell surface CS-GAG contribute significantly to the total binding of IFN-γ in HASMC. These results agree with previous reports describing the presence of two different molecular forms of human IFN-γ receptors in human cells: one with a Kd of about 10⁻¹⁰ M (high affinity binding) and another with a Kd of 10⁻⁹ to 10⁻⁸ M (low affinity binding) (53, 54). Furthermore, antibodies that block the binding of IFN-γ to the high affinity receptor do not inhibit the binding of IFN-γ to the low affinity receptor. The low affinity receptor thus seems to be a different molecular structure (55).

Experiments performed with GAG-degrading enzymes and affinity chromatography indicate that chondroitin sulfate is the main GAG through which IFN-γ binds to cell surface PGs in HASMC. Chondroitin sulfate appeared to be also the main type of GAG synthesized by HASMC. The degradation of CS-containing PGs completely abolished the binding to IFN-γ. These results also showed that the binding of IFN-γ to cell CSPG was reversible at near physiological concentrations of NaCl, between 150 and 200 mM/liter NaCl. This reversibility may contribute to modulate the action of the cytokine in response to changes in concentration.

HASMC in culture expressed CSPG as the predominant cell-associated proteoglycan, and the core protein of the CSPG that binds to IFN-γ was immunologically related to CD44. Western blot analysis showed one band of immunoreactivity at ~150 to <200 kDa in undigested cell-associated PG and CSPG isolated by affinity chromatography on IFN-γ columns. Digestion of GAGs lowers the molecular mass to ~110 kDa. This data suggests that the cell-associated PG that binds IFN-γ is a CD44-related CSPG. A similar CD44-related CSPG is expressed in activated endothelial cells and melanoma cells. CD44-related CSPG mediates migration of these cells and invasion into matrix (19, 45, 56). CD44 is a transmembrane glycoprotein with extracellular, membrane, and cytoplasmic domains. CD44 appears to mediate cell-cell and cell-matrix interactions (57), and it also has multiple proinflammatory functions (58). In addition, CD44 is the receptor for hyaluronate and mediates T-lymphocyte homing. Ligand binding to CD44 promotes T-cell activation and interleukin-2 release and, on monocytes, induces cytokine release (59). The CD44 gene contains 19 exons, 12 of which may be alternatively spliced, leading to the existence of multiple isoforms of CD44 (60). The diversity of CD44 isoforms is further amplified by the presence of different GAG attachment sites on its extracellular domain. These GAG attachment sites appear to contribute to the multiple functions of CD44 isoforms (61). For example, CD44 decorated with HS, but not CS, were reported to interact with growth factors (50), and CD44 in the form of a CSPG mediates migration (57) and binding to collagen type I (45). We believe that our observations show for the first time that HASMC express the cell surface receptor CD44-related CSPG and that this is involved in the cell surface binding of IFN-γ.

The removal of cell surface CS-GAG decreased the antiproliferative effect of IFN-γ on HASMC. Furthermore, competition experiments showed that purified C6S added extracellularly antagonized the antiproliferative effect of IFN-γ and the induction of class II MHC molecules. These results suggest that the interaction with CS-GAG modulates the ability of IFN-γ or the IFN-γ:IFN-γ receptor complex to generate a biological response. The results from affinity chromatography experiments showing the binding of IFN-γ to CSPGs isolated from cells as well as the exclusion chromatography results showing the formation of high molecular weight complexes at physiological salt concentrations between IFN-γ and soluble C6S support this interpretation. According to Schlessinger and collaborators, the primary function of these low affinity GAG receptors on cell surfaces is to reduce the dimensionality of ligand diffusion from three-dimensional volume of the extracellular space to two dimensions (62). As a consequence, the probability of IFN-γ interaction with the high affinity receptor will be enhanced. Since most surface receptors diffuse in the cell membrane, lateral mobility will allow encounters between high density low affinity CS-GAG receptors and unoccupied, less abundant high affinity IFN-γ receptors, thus transmitting signals. This interpretation is supported by data from kinetic analysis with surface plasmon resonance showing a high dissociation constant rate of the interaction between IFN-γ and C6S (data not shown). This result suggests that a high dissociation from low affinity CS-GAG may lead to complex formation with high affinity receptors for IFN-γ. This is in agreement with the proposal that extracellular proteoglycans in general serve as a reservoir of cytokines and growth factors (63, 64).

The active form of IFN-γ is a homodimer consisting of two intertwined copies (monomers) of a single protein (25). The biologically active IFN-γ:receptor complex requires the species-specific interaction of IFN-γ with at least one additional accessory factor (65, 66). Studies with a covalently linked IFN-γ mutant suggest that each domain of IFN-γ may function independently to trigger a biological signal. Structurally, this corresponds to each domain of IFN-γ binding one receptor and one accessory factor (67). The potential interaction of different combinations of accessory factors with the complex of IFN-γ with its receptor has been suggested as a mechanism that modulates its pleiotropic activities (9, 25). The results from the present work suggest that the cell surface CSPG identify as CD44 may be added to the list of possible accessory factors involved in the binding and regulation of IFN-γ activity on cells. The competing action of soluble C6S on the biological effect of IFN-γ can be interpreted within the frame of two models. In one model, excess soluble C6S in the extracellular compartment could displace the IFN-γ from cell surface CSPG diminishing the pericellular concentration of IFN-γ from which the high affinity receptor, responsible for signal transduction, picks up the cytokine. In a second model, soluble C6S could antagonize the potential accessory factor function of the cell surface GAG.
tachments may allow for discrimination between these models. Recent findings indicate that fragments of hyaluronan, a non-sulfated GAG, potentiate the effect of IFN-γ on macrophages (68). This may be related to the fact that CD44 is a receptor for hyaluronan. Together with our results, these data suggest the importance of the interaction of GAGs with IFN-γ in mediating its effects on cells.

The interaction of IFN-γ with GAGs may be a mechanism to control the concentrations of the cytokine in plasma and tissues. Unfortunately, there are not data on the control the concentrations of the cytokine in plasma and tissues. Increases of the IFN-γ possibly be higher than the concentrations measured in plasma (68). This may be related to the fact that CD44 is a receptor for GAG, the local concentration of active IFN-γ in tissue may in mediating the cytokine at specific local places, thus making unnecessary increases of the IFN-γ levels in plasma or tissue fluids that could compromise the functionality of other cells or tissues.

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