Proteoglycan Core Protein Is Accumulated in Cultured Chondrocytes in the Presence of the Ionophore Monensin*

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The nature of the proteoglycan antigen which accumulates in chick embryo chondrocytes that had been incubated in monensin was examined. Both the culture media and cell lysates were immunoprecipitated using antibody which primarily is directed against core protein. Gel filtration and electrophoresis of the immunoprecipitates showed that molecules corresponding to completed proteoglycan, core protein, and link protein were present in the immunoprecipitates. Monensin caused the cellular accumulation of core protein which was both underglycosylated and undersulfated. These results suggest that monensin affects early events of proteoglycan biosynthesis and that it may be useful for elucidating those events.

The monovalent ionophore monensin interferes with a variety of eucaryotic cell functions. Monensin impairs the secretion of macromolecules and they accumulate intracellularly (1-6). Monensin inhibits receptor recycling and the transport of membrane proteins from the Golgi to the plasma membrane (7-9). The ionophore also perturbs the normal post-translational processing of secretory proteins. The post-translational processing steps altered by monensin include protein glycosylation and sulfation of proteoglycans (10-12). The available evidence has implicated the Golgi complex as a primary site of action of monensin (2, 5, 6), although the rough endoplasmic reticulum has also been implicated (6, 13).

Recent studies have shown that monensin inhibits the secretion of [3H]glucosamine-labeled chordroitin sulfate from rat chondrosarcoma cells, and inhibits the incorporation of [3H]glucosamine into chondroitin sulfate, but not hyaluronic acid (14). Radioimmunoassays specific for type II collagen and cartilage proteoglycan have shown that the monensin inhibition of secretion of these macromolecules is accompanied by their accumulation within cultured chondrocytes (13).

We have utilized an antibody for cartilage proteoglycan to determine that the proteoglycan antigen accumulated by the cells is due to an increase in an underglycosylated, undersulfated, 450,000-dalton proteoglycan core protein.

EXPERIMENTAL PROCEDURES

Materials—Monensin and goat-antirabbit y globulin were purchased from Calbiochem-Behring. 2-Amino-3-hydroxymethyl-1,3-propanediol base, guanidinium chloride, 6-amino capric acid, benzamidine, N-ethylmaleimide, phenylmethylsulfonyl fluoride, and SDS were purchased from Sigma. Omnitfluor, Protosol, ENHANCE, 1-[14C]glucosamine HCl and L-[3H]serine were from New England Nuclear, while H35SO4 was from ICN. Culture medium and horse serum were from Gibco. Nonidet-P40 was purchased from Particle Data Laboratories, Ltd. Purified chicken cartilage proteoglycan type 1 (A,D fraction, termed PGS-1) was kindly provided by Dr. P. Goetzlinc, University of Connecticut.

Condrocyte Preparation and Incubation Conditions—Sterna were dissected from 17-day-old chick embryos and prepared and cultured as previously described (11). After monensin had been added at 1 x 10^-6 M, culture tubes were gassed with 5% CO2 and capped. A preincubation was carried out for 1 h at 37 °C on a rotary shaker bath, after which the chondrocytes were harvested and resuspended in fresh medium containing the previous concentration of monensin and radioactive precursors as needed. The tubes were gassed as before, and incubated at 37 °C on a rotary shaker bath until the experiment was terminated after 3 h by separation of cells and medium by centrifugation.

The medium was frozen after addition of the protease inhibitors phenylmethylsulfonyl fluoride, N-ethylmaleimide, EDTA, 6-amino capric acid, and benzamidine HCl at final concentrations of 1, 2, 10, 100, and 5 mM, respectively. Cell pellets were washed twice in phosphate-buffered saline and then lysed by suspension for 5 min in 1 ml of 1% Nonidet-P40, 0.02 M Tris, 0.15 M NaCl, pH 7.4, with the above concentrations of protease inhibitors. Nuclei were pelleted by centrifugation at 15,000 rpm on a Brinkmann 8000 Eppendorf centrifuge for 2 min. The supernatant was removed and aliquoted for either (a) direct analysis (b) immunoprecipitation with anti-proteoglycan antiserum, or (c) precipitation with nonimmune serum as a control.

Immunoprecipitation of Cell Lysate and Culture Medium—Immunoprecipitation was performed using antiserum from rabbits inoculated with purified PGS-1 proteoglycan or with nonimmune rabbit serum. 20 μl of serum was added to 500 μl of cell lysate or 2-3 ml of culture medium. Incubation was at 4 °C for 12-15 h at which time 5 units of goat-antirabbit globulin are added. After 2 h at 4 °C, the precipitated radioactivity was pelleted by centrifugation at 3000 g for 5 min. The pellets were washed twice with buffer containing protease inhibitors, 0.05% Nonidet-P40, 0.15 M NaCl, 0.02 M Tris, pH 7.4, then suspended in either 6 M guanidinium chloride with protease inhibitors for gel filtration on Sepharose 2BCL or in 2% SDS, 0.025 M Tris, 10% glycerol, pH 6.8, for SDS-polyacrylamide electrophoresis. Control studies showed the specificity and completeness of immunoprecipitation.

Analytical Procedures—Gel filtration was performed on a Sepharose 2BCL column (1.5 x 96 cm) equilibrated with 4 M guanidinium chloride, 0.05 M Tris, pH 7.0, with protease inhibitors. Fractions of 3 ml were collected and analyzed for radioactive content. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (15). Polyacrylamide gels were either 5% polyacrylamide lower gels or 12.5% polyacrylamide as needed. Upper (stacking) gels were always 4% polyacrylamide. For gel slicing experiments, the gels were cut into uniform slices which were incubated overnight at 37 °C in a scintillation fluid of 0.4% Omnitfluor, 3.0% Protosol in toluene, then counted in a scintillation counter. Autoradiography was performed using ENHANCE and Kodak XR-5 x-ray film.

RESULTS AND DISCUSSION

Gel Filtration of Secreted Proteoglycan—Fig. 1 shows the elution profile of the labeled proteins which had been immu

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n-oprecipitated from culture media. The elution position of the major peak of radioactivity from control medium is identical to the elution position of purified chicken proteoglycan monomer (16) run on the same column. Immunoprecipitates containing incorporated [35S]SO4 also elute in the position of the chicken proteoglycan standard. Thus, the apparent molecular weight of the major [3H]serine-labeled material in these immunoprecipitates is consistent with authentic chick proteoglycan, and probably represents completed proteoglycan which has been secreted from chondrocytes.

The largest labeled protein which was immunoprecipitated from monensin-treated culture medium elutes later than the control protein (Fig. 1). Its elution position is likely due to the known undersulfation of the glycosaminoglycan chains of the proteoglycans (11).

The peak of [3H]serine label which elutes at fractions 47-48 in both control and monensin samples is probably link protein. Total immunoprecipitates were subjected to electrophoresis on SDS-polyacrylamide gels and showed a major band with an apparent Mr = 45,000, which is identical to the molecular weight established for link protein (17). This labeled band also migrated in the same position as an identical band obtained from immunoprecipitates of cell lysates (see below).

As described in other studies (11, 13, 14), less proteoglycan is secreted in the presence of monensin. This was found in the current studies as well. In a typical experiment, 4 x 104 cpm were precipitated in the control sample, while 1.5 x 104 cpm were precipitated in the monensin sample.

Gel Filtration of Chondrocyte Cell Lysate—The elution profile of immunoprecipitated cell lysates revealed 2 major protein peaks for both control and monensin samples (Fig. 2). The first peak centered on fraction 41 and a second peak centered on fraction 48 (Fig. 2). Appropriate fractions for the first and second peaks were pooled, dialyzed extensively against distilled water containing protease inhibitors, and analyzed by SDS-polyacrylamide gel electrophoresis. The first peak is a protein of approximately 450,000 daltons (Figs. 3 and 4), while the second peak is approximately 45,000 daltons (Figs. 4 and 5).

Since equal numbers of chondrocytes were processed for cell lysis, there is a relative accumulation of [3H]serine label in the immunoprecipitates of monensin-treated cell lysates. In a typical experiment, 5.5 x 104 cpm were precipitated in the control samples while 7 x 104 cpm were precipitated in the immunoprecipitates is consistent with authentic chick proteoglycan, or of [3H]serine in control medium immunoprecipitate (---), [3H]serine in monensin medium immunoprecipitate.

monensin sample. This result is consistent with an earlier study utilizing a specific radioimmunoassay for proteoglycan which showed an increase in the intracellular concentration of proteoglycan antigen in monensin-treated chondrocytes (13).

In several experiments, [35S]SO4 was also added to the culture medium. Fig. 2B illustrates the elution profile of the control sulfated molecules obtained by immunoprecipitation. Surprisingly, the [35S]SO4 label elutes in 2 peaks. The first peak elutes in the exact position of the [3H]serine-labeled proteoglycans from the control culture medium (Fig. 1, arrow). The second peak elutes in the position of the undersulfated proteoglycan from the monensin-treated culture medium. The presence within cells of a sulfated proteoglycan with a decreased apparent molecular weight may mean that limited proteolysis may be occurring despite the presence of five protease inhibitors during immunoprecipitation. Very little [35S]SO4 label is immunoprecipitated from monensin-treated cell lysate (Fig. 2C). This is not surprising, due to the dramatic effect of monensin on sulfation of proteoglycans. Monensin at 1 x 10-6 M inhibits proteoglycan sulfation to about 5% of control levels (11, 14).

SDS-Polyacrylamide Gel Electrophoresis of Immunoprecipitates from Cell Lysates—The results obtained from the gel filtration experiments indicate that four main components can be isolated from cell lysate immunoprecipitates: two types of sulfated proteoglycan, a 450,000-dalton protein, and a 45,000-dalton protein (Figs. 2 and 3). In order to further characterize the four species, we have used SDS-5% polyacrylamide gel electrophoresis together with double-label culture conditions.

The fluorogram of the immunoprecipitate of control and
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Fig. 3. SDS-5% polyacrylamide gel electrophoresis of immunoprecipitates and pooled fractions from Fig. 2. Cell lysate immunoprecipitates and the pooled and dialyzed fractions from the Sepharose 2BCL gel filtration experiment in Fig. 2 were analyzed by electrophoresis and autofluorography as described under "Experimental Procedures." 1, control cell lysate; 2, pooled fractions 46-50, Fig. 2B; 3, pooled fractions 38-43, Fig. 2B; 4, monensin cell lysate; 5, pooled fractions 46-50, Fig. 2A; 6, pooled fractions 38-43, Fig. 2A. FN, unred human plasma fibronectin, M₆ = 440,000; FN, reduced human plasma fibronectin, M₆ = 220,000; OA, ovalbumin, M₆ = 43,000.

Fig. 4. Comparison of the electrophoretic mobility of immunoprecipitated proteins and standards of known molecular weight. The electrophoretic mobility (Rₑ) of proteins on a SDS-5% polyacrylamide gel is plotted versus the logarithm of their known molecular weights. (O), electrophoretic mobilities of immunoprecipitate bands; (●), standards. FN, unred human plasma fibronectin; FN, reduced human plasma fibronectin; M₆, myosin; P, phosphorylase b; BSA, bovine serum albumin; H, rabbit IgG heavy chain, OA, ovalbumin.

Fig. 5. SDS-12.5% polyacrylamide gel electrophoresis of immunoprecipitates of cell lysates. Cell lysate immunoprecipitates were analyzed by electrophoresis and autofluorography as described under "Experimental Procedures." Lane 1, monensin cell lysate; lane 2, control cell lysate. P, phosphorylase b; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase; STI, soybean trypsin inhibitor; L, lysozyme. Only the lower molecular weight region is shown.

results of the experiments shown in Figs. 2, 3, and 6, it is clear that the 450,000-dalton protein accumulates within monensin-treated chondrocytes.

Fig. 7 shows the results of an experiment in which [¹⁴C] glucosamine and [³⁵S]serine were used. The majority of the labeled glucosamine migrates at the top of the gel while the 450,000-dalton protein contains primarily [³⁵S]serine. Only the data from a control experiment are shown; the monensin-treated samples showed very similar results, with the bulk of the [¹⁴C]glucosamine label at the top of the gel and a small amount of [³⁵S]label co-migrating with the 450,000-dalton protein. However, the distribution of [³⁵S]serine in labeled

Fig. 6. SDS-5% polyacrylamide gel electrophoresis of chondrocyte lysate immunoprecipitates. Chondrocyte cultures were labeled with [³⁵S]sulfate and [³⁵S]serine and analyzed after electrophoresis by counting radioactivity in gel slices. A, monensin cell lysate immunoprecipitate. B, control cell lysate immunoprecipitate. ---, [³⁵S]serine; ---, [³⁵S]sulfate. Arrow indicates the border between the stacking and running gels. The bromphenol blue dye front is 12 cm from the sample wells.
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Fig. 7. SDS-5% polyacrylamide gel electrophoresis of immunoprecipitates of cell lysates. Chondrocyte cultures labeled with [3H]glucosamine and [14C]glucosamine were analyzed after electrophoresis by counting radioactivity in gel slices; ---, [3H]glucosamine; --.-, [14C]glucosamine. Arrow indicates the border between the stacking and running gels. The bromphenol blue dye front is 12 cm from the sample wells.

core protein was similar to Fig. 6, for monensin and control samples.

The identical electrophoretic mobility of authentic purified proteoglycan with the majority of the 35S0 4 and [14C]glucosamine label (Figs. 6 and 7) identifies the stacking gel band as proteoglycan of the cell lysate. The 450,000-dalton protein is poorly glycosylated and poorly sulfated. It probably is core protein as defined by immunochemical criteria and by its similar electrophoretic mobility compared to core protein described elsewhere (17). The 45,000-dalton protein (Fig. 5) is probably link protein based upon its molecular weight and its presence in immunoprecipitates of proteoglycans (17). This protein also co-precipitates from the culture medium of the chondrocytes (Fig. 1).

These composite results confirm our earlier immunochemical studies utilizing a specific radioimmunoassay which proved that monensin causes an accumulation of proteoglycan antigen within chondrocytes (13). We have now shown that most of the material which accumulates is a 450,000-dalton putative core protein of proteoglycans. Further experiments are underway to determine in which organelle it accumulates. Morphological evidence indicates that chondrocytes may be blocked by monensin at an earlier stage in the process of intracellular translocation, since the rough endoplasmic reticulum of chondrocytes appears distended (13). A block at the region of the rough endoplasmic reticulum would be consistent with the observation that the accumulated protein antigen is under-glycosylated and under-sulfated. In summary, monensin may prove useful for accumulating core protein precursors in the pathway of proteoglycan synthesis.

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