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Neoplastic Modulation of Extracellular Matrix: Stimulation of Chondroitin Sulfate Proteoglycan and Hyaluronic Acid Synthesis in Co-Cultures of Human Colon Carcinoma and Smooth Muscle Cells

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Previous studies have shown that human colon carcinomas contain elevated amounts of chondroitin sulfate proteoglycan (CS-PG) and hyaluronic acid, and that the major site of synthesis of these products is the host mesenchyme surrounding the tumor. These findings have led to the proposal that the abnormal formation of the tumor stroma is modulated by the neoplastic cells. The experiments of this paper were designed to explore further this complex phenomenon in an in vitro system using co-cultures of phenotypically stable human colon smooth muscle (SMC) and carcinoma cells (WiDr). The results showed a 3–5-fold stimulation of CS-PG and hyaluronic acid biosynthesis in the co-cultures as compared to the values predicted from the individual cell type cultured separately. The increase in CS-PG was not due to changes in specific activity of the precursor pool, but was rather due to a net increase in synthesis, inasmuch as it was associated with neither a stimulation of cell proliferation nor with an inhibition of intracellular breakdown. These biochemical changes were corroborated by ultrastructural studies which showed a marked deposition of proteoglycan granules in the co-cultures. Several lines of evidence indicated that the SMC were responsible for the overproduction of CS-PG: i) SMC synthesized primarily CS-PG when cultured alone, in contrast to the WiDr, which synthesized exclusively heparan sulfate proteoglycan; ii) only

Abbreviations used: CS-PG, chondroitin sulfate proteoglycan; DMEM, Dulbecco's modified Eagle's medium-high glucose; GAGs, glycosaminoglycans; HBSS, Hanks' balanced salt solution-calcium and magnesium free; HS-PG, heparan sulfate proteoglycan; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SFM, serum free defined medium; SMC, human colon smooth muscle cells; TCM, tumor conditioned medium; WiDr, human colon carcinoma cells.

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the SMC in co-culture stained with an antibody raised against the amino terminal peptide of a CS-PG (PG-40), structurally and immunologically related to that synthesized by the SMC; iii) the stimulation of CS-PG in SMC could be reproduced, though to a lesser extent, using medium conditioned by WiDr, whereas medium conditioned by SMC had no effects on WiDr. In conclusion this study has reproduced in vitro a tumor-associated matrix with a proteoglycan composition similar to that observed in vivo and provides further support to the concept that production of a proteoglycan-rich extracellular environment is regulated by specific tumor-host cell interactions.

Key words: glycosaminoglycans, hyaluronic acid, colon carcinoma cells

The active interplay between malignant and host mesenchymal cells leads to the formation of a tumor stroma which provides the infrastructure required by the tumor cells for successful growth and infiltration [1]. Often this newly synthesized tumor stroma is associated with the abnormal expression of extracellular matrix gene products [1–3]. For instance, chondroitin sulfate protoglycan (CS-PG) is found in elevated concentration in tumors of the breast [4], lung [5], pleura [6], liver [7], colon [8,9], and prostate [10] but is present in only trace amounts in the normal tissue [2]. A positive role of CS-PG on tumor cell proliferation is provided by its growth-promoting activity on mammary carcinoma cells in vitro [11] and on Ehrlich ascites tumor cells in vivo [12], and by the observation that enzymes that degrade chondroitin sulfate can inhibit the growth of the latter tumor cells [13].

The central hypothesis of our research is that the formation of the tumor matrix is a specialized process, and that changes in proteoglycan composition derive from a phenotypic modulation of normal connective tissue cells induced by the presence of cancer cells and/or tumor cytokines. This hypothesis is based in part on our original observation that extracts of human colon carcinoma tissue contain elevated amounts of chondroitin sulfate [8] and that the major biosynthetic product of colon carcinoma maintained in short-term organ culture is a small CS-PG, whereas normal colon synthesizes primarily heparan sulfate and dermatan sulfate proteoglycans and only trace amounts of CS-PG [9]. We have further shown, using both cytochemistry and autoradiography, that the connective tissue cells, primarily fibroblasts and smooth muscle cells, in the peritumoral stroma are the major sites of synthesis and accumulation of CS-PG [8]. These studies are in agreement with the fact that human colon carcinoma cells cultured alone synthesize exclusively heparan sulfate proteoglycan (HS-PG) [14], and that normal human colon fibroblasts are stimulated to synthesize CS-PG when exposed to medium conditioned by colon carcinoma cells [15].

The concept that neoplastic cells can modulate the formation of their own stroma is also supported by our in vivo study on the interaction between rabbit V2 carcinoma cells and mesentery [16]. The mesentery becomes markedly hypertrophic within 7–10 days following intraperitoneal implantation of V2 carcinoma cells, but it remains unchanged after implantation of normal hepatocytes. This increase in mesenteric mass is associated with a marked quantitative elevation in chondroitin sulfate, hyaluronic acid and collagen [16]. In support of our findings are the reports that in co-cultures of tumor and mesenchymal cells or in fibroblasts exposed to tumor-conditioned medium, there is a marked stimulation of the synthesis of hyaluronic acid and sulfated glycosaminoglycans [17–19].

The present study was designed to explore further this complex phenomenon of tumor matrix production. We have isolated smooth muscle cells from human colon

and investigated the effect of co-culturing these cells with human colon carcinoma cells on the synthesis and turnover of proteoglycans. The results demonstrate that in co-cultures of the two cell types, there was a marked increase in the synthesis of hyaluronic acid and CS-PG. This increase was a true stimulation of synthesis since co-cultures affected neither cell proliferation nor intracellular turnover of CS-PG. These biochemical changes were associated with marked ultrastructural changes in extracellular matrix which became enriched in proteoglycan granules.

MATERIALS AND METHODS

Materials

All the reagents were of analytical grade unless otherwise specified. Heparitinase, chondroitinase ABC and AC II, protease-free chondroitinase ABC, *Streptomyces* hyaluronidase, carrier-free [^{35}S]sulfate as sodium salt (43 Ci/mg S), and L-[^{35}S]methionine (1,111 Ci/mmol) were obtained from ICN Biochemicals (Irvine, CA). ^{125}I-Na and L-[3,4,5 ^{3}H(N)]leucine (140–150 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Type II cytochrome c, calf thymus DNA, papain, Pronase, collagenase, elastase, ribonuclease, deoxyribonuclease, and neuraminidase were from Sigma Chemical Co. (St. Louis, MO). All the chromatographic gels were from Pharmacia P-L Biochemicals (Piscataway, NJ). Media and fetal calf serum were obtained from GIBCO (Grand Island, NY). The source of other materials was the same as described previously [15,20,21].

Cell Culture

Human smooth muscle cells (SMC) were isolated from the tunica muscularis of colectomy specimens from patients undergoing surgery for colon cancer. Samples (1–2 cm^3) were removed within minutes from freshly excised specimens and transferred to the culture laboratory in sterile, high glucose, Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/ml penicillin, 10 μg/ml gentamycin, 100 μg/ml streptomycin sulfate, and 2 μg/ml fungizone (amphotericin B). Following removal of the mucosa, SMC were isolated using a modification [22] of a procedure previously described [23]. Briefly, the samples were cut into smaller pieces with sharp blades and incubated for 1–2 h at 37°C in a mixture of collagenase (1 mg/ml) and elastase (0.25 mg/ml) in Hanks' balanced salt solution (HBSS). The resulting cell-tissue suspension was filtered through a 85-μm stainless steel mesh, and the filtrate was collected in 20% fetal calf serum to inhibit further digestion. The 1,000-g cellular pellets were resuspended in DMEM supplemented with 10% serum and seeded on 75-cm^2 Falcon flasks which were precoated with fibronectin, 100 μg/ml. Human colon carcinoma cells (WiDr) were obtained from American Type Culture Collection (Bethesda, MD) and were cultured as previously described [14].

Morphologic Studies

Several morphological and immunohistochemical criteria were used to establish the nature and phenotypic stability of the cells isolated from the tunica muscularis of human colon. First, the cells were grown in glass wells (Lab-Tek Products, ICN) and were studied by light and transmission electron microscopy. Specifically, we searched by light microscopy for the characteristic growth in multilayers and the formation of "hills and valleys," and by electron microscopy for the presence of cytoplasmic

microfilaments and dense bodies. Detailed ultrastructural analysis of the proteoglycan-rich extracellular matrix was performed using ruthenium red [24] or cuprolinic blue at the critical electrolyte concentration [25] as described before [14]. Secondly, the cells were reacted with a battery of monoclonal antibodies directed against smooth muscle specific isoactin, designated CGAT [26] and HHF35 [27], and monoclonal antibodies against various intermediate filament proteins, including vimentin and desmin (DAKO Co., Santa Barbara, CA) and isokeratins (AEI-3 from Boehringer Manneheim Biochemicals, Indianapolis, IN). Cells were fixed for 10 min in Carnoy's solution, washed and reacted with the various monoclonal antibodies [27]. Sections of normal human colon were also reacted with the antibodies. The antibodies against smooth muscle specific actin were derived from hybridoma ascites and were therefore diluted to 1:4,000 and 1:8,000 before use [27]. It has been previously shown [27] that the HHF35 antibody reacts specifically with actin isotypes of virtually all muscle cells, but it is unreactive with epithelial, endothelial, neural, and other connective tissue cells, all potential sources of contamination of our primary culture. Reactivity was visualized by using avidin-biotin immunoperoxidase [27] with the various control combinations as described before [14]. Immunoreactivity was tested periodically to monitor the phenotypic stability of the SMC.

Co-Cultures and Metabolic Labeling

To determine whether there was any interaction between human smooth muscle and colon carcinoma cells, a series of experiments was performed in which cells were either cultured separately or in combinations at various ratios. In a typical experiment, about 10^5 cells were seeded into 35-mm dishes either separately or together at WiDr/SMC ratios of 1:1 (i.e., equal proportion of the original dilution), 1:2, 1:3, and 1:4. In all the experiments, the initial density of the various cultures was identical so all the cells were exposed to the same amount of nutrients, growth factors and surface area. Typically, the cells were grown in DMEM with 10% serum for 1–4 days and then incubated for various periods of time in serum-free defined medium (SFM) as described before [28] with minor modifications. The SFM contained equal amounts of DMEM and Ham's F-12 supplemented with insulin, transferrin, and ascorbate (5 μg/ml each) and sodium selenite (5 ng/ml). This medium has been shown to maintain cultures of SMC in a quiescent, viable state for extended periods of time [28] and is thus suitable for studying the effects of tumor-SMC interactions in the absence of growth factors and hormones which are present in serum.

Metabolic labeling with [^{35}S]sulfate (20–40 μCi/ml) was performed as previously described [14]. Additional experimental details are provided under Results and in the figure legends.

Isolation and Quantification of Sulfated and Unsulfated Glycosaminoglycans

Triplicate cultures were metabolically labeled with radiosulfate for 48 h in SFM. After removing the media, the cell layers were washed with HBSS and extracted overnight at 4°C with 4 M guanidine HCl containing 2% Triton X-100 and various protease inhibitors [29]. To remove unincorporated radioactivity, the media and cell extracts were chromatographed separately on disposable 10-ml columns of Sephadex G-50 (fine) which had been equilibrated in the same buffer containing 0.2% Triton X-100, and aliquots of the excluded volumes were counted for total incorpo-

ration. The remaining excluded volumes were pooled and precipitated overnight at −20°C with 5 volumes of ethanol/1.3% potassium acetate. The suspensions were centrifuged at 10,000*g* for 2 h, and aliquots of the supernatants were counted to calculate recovery of the ^{35}S-labeled GAGs. The pellets were digested overnight with papain (22 U/ml) at 65°C in 3 ml of 0.5 M sodium acetate (pH 6.0), containing 10 mM L-cysteine and 25 mM EDTA, and the digests were precipitated and centrifuged as above. The supernatants were counted for recovery, and the 10,000-g pellets were sequentially digested for 4 h each with ribonuclease (200 μg/ml) and deoxyribunuclease (200 μg/ml) in 0.5 M sodium acetate, the latter buffer containing 12 mM $MgCl_2$. Finally, the solutions were digested with Pronase (270 μg/ml) for 18 h at 40°C, precipitated, and centrifuged as above. The final recovery of the ^{35}S-labeled GAGs 89% ± 9% (n = 15).

The pellets were resuspended in 200 μl of H_2O, and to identify the GAGs 20-ul aliquots were subjected to the following treatments in the appropriate buffer: 1) neuraminidase, 10 U/ml, at 37°C for 4 h; 2) *Streptomyces* hyaluronidase, 20 U/ml, at 60°C for 16 h [8]; 3) heparitinase, 1.6 U/ml, at 45°C for 18 h [30]; 4) HNO_2 at pH 1.5 for 15 min [31]; 5) chondroitinase ABC and AC II, 0.2 U/ml each, at 37°C for 90 min [32]. Aliquots of 0.1–2 μl (2–20 ng GAG) from either control (buffer alone) or treated samples were applied to cellulose acetate strips (Sepraphore III, Gelman) which were presoaked with 30% methanol for 30 min followed by 0.5 M lithium chloride (pH 8.4) containing 10 mM EDTA, and subjected to electrophoresesis in the same buffer at a constant 9 mA for 15 min at 4°C. After electrophoresis, the strips were reacted with ^{125}I-labeled cytochrome c as previously described [33] with minor modifications. Briefly, the strips were immersed in absolute ethanol containing 1% Tween 20 and dried. The dried strips were immersed for 5 min in 6 M urea (pH 3.0) containing 0.05% Tween 20, 2.5% polyethyleneglycol 6000, 40 μg/ml cold cytochrome c, and about 5×10^6 cpm of ^{125}I-labeled cytochrome c. The strips were washed with urea and formate buffer, pH 3.0, dried, and exposed at −70 °C to X-ray film (Kodak Ortho-GO) for 6–12 h in Lanex intensifying screens (Kodak). For quantification, duplicate autoradiograms were evaluated in an Ultrascan XL densitometer (LKB Products AB, Bromma, Sweden), and their absorption was compared to that of standard GAGs run in parallel. In order to eliminate the possible interference of radiosulfate with the ^{125}I-induced autoradiographic bands, all the samples used were decayed for at least one half-life before subjecting them to electrophoresis and autoradiography with ^{125}I-labeled cytochrome c. In any event, less than 10^2 cpm of ^{35}S-labeled GAGs were applied, an amount that did not generate any band during the relatively short (6–12 h) exposure of the autoradiograms [33].

Isolation and Characterization of Proteoglycans

The methodology for the isolation and characterization of newly synthesized proteoglycans has been reported in detail previously [14,15]. Briefly, the media and the cell extracts were chromatographed separately on disposable Sephadex G-50 (fine) columns equilibrated and eluted in 8 M urea, 0.2% Triton X-100, 0.1 M NaCl. Aliquots of the excluded volumes were counted and the remaining samples were analyzed by DEAE-Sephacel chromatography in 8 M urea with a linear NaCl gradient between 0.1 and 0.8 M. [14]. The proteoglycan peaks were pooled and further studied by analytical Sepharose CL-2B or CL-4B chromatography [21]. Following their release by Pronase [21] or alkaline borohydride [34], the radiolabeled GAGs were

further studied by analytical Sepharose CL-6B or Sephadex G-50 columns [20], before and after various digestions and chemical treatments as outlined before [14,35,36].

Effects on Cell Proliferation and Proteoglycan Turnover

To determine whether co-cultures of WiDr and SMC had any effect on cellular growth, we measured the amount of DNA and the incorporation of [^{3}H]thymidine into DNA in cells cultured separately or in various combinations. At the end of each incubation, generally 4–5 days, the cells were trypsinized and the 1,000-g pellets were homogenized in 50 mM sodium phosphate buffer, pH 7.4, containing 2 M NaCl and 2 mM EDTA [36]. The DNA content was measured using a fluorimetric assay [37] with calf thymus DNA as standard. The data obtained with radiosulfate incorporation were then normalized to the amount of DNA. Cells were also labeled for 4–6 h with 0.2 μCi/ml [^{3}H]thymidine, and the amount of ^{3}H-activity incorporated into trichloroacetic acid-precipitable material was determined [15].

To assess whether the effects on proteoglycan synthesis could be due to an inhibition of intracellular turnover, cells cultured separately or in a 1:1 combination were labeled with [^{35}S]sulfate for 24 h; carefully washed in isotope-free medium supplemented with 1 mM SO_4; and subsequently chased for 4, 8, 24, and 32 h. After each chase interval, macromolecular and free radioactivity associated with the cell layers and media were calculated as before [15].

Analysis of the Protein Core of the Major Proteoglycan Synthesized by SMC

To study the protein core of the major proteoglycan synthesized by SMC, two sets of experiments were performed. In the first, SMC were labeled for 48 h with [^{3}H]leucine and [^{35}S]sulfate, and the proteoglycan were purified by DEAE-Sephacel chromatography as described above. Aliquots of proteoglycans from the medium and cell layer were treated with protease-free chondroitinase ABC, 0.15 U/ml, at 37°C, for 90 min in enriched Tris-HCl, pH 8.0, containing 10 μg/ml of chondroitin 4- and 6-sulfate standard. Control or treated samples were analyzed by SDS-PAGE followed by fluorography [14]. The mobility of the bands was assessed by running a mixture of standard proteins (Pharmacia) stained with Coomassie brilliant blue.

In the second set of experiments, WiDr or SMC cells were incubated with 100 μCi/ml [^{35}S]methionine for 1 h to label both the precursor as well as the completed proteoglycans. At the end of the incubation, the media were removed and the cell layers were extracted and immunoprecipitated as described before [38], using an affinity purified anti-PG-40 antiserum [39]. The anti-PG-40 antiserum was raised against the amino terminal peptide (Asp-Glu-Ala-Ser-Gly-Ile-Gly-Pro-Glu-Val-Pro-Asp-Asp-Arg-Asp) deduced from the nucleotide sequence of a ubiquituous proteoglycan with a 40-kDa protein core, and thus called PG-40 [39]. At the end of the immunoprecipitation, the samples were analyzed by SDS-PAGE as above.

Cellular Source of Proteoglycan Synthesis

To determine the cells responsible for the synthesis of proteoglycans, SMC or WiDr cells or co-cultures at 1:1 ratio were reacted with the anti-PG-40 antiserum or with an affinity-purified antiserum raised against the HS-PG of EHS tumor [40]. We have previously shown that the latter antibody recognizes the cell surface HS-PG synthesized by the WiDr [3,14]. In these experiments, cells were fixed in methanol

for 10 min, washed in phosphate buffered saline, and processed for immunohistochemistry as described above. The anti-PG-40 antiserum was also tested against normal human colon (the tissue source of the SMC) and against human skin; the latter is enriched in fibroblasts and would thus serve as internal positive control for the anti-PG-40, an antibody raised against a fibroblast CS-PG [39].

Effects of Tumor Conditioned Medium

To determine whether the effects induced by the presence of colon carcinoma cells could be mediated by soluble factors (cytokines) released by the tumor cells, serum-free medium conditioned by the tumor cells (TCM) was prepared as described [15], with the exception that it was dialyzed (MW cutoff = 1,000) against SFM and supplemented with 2 mM L-glutamine and 50 μg/ml ascorbate before use. Confluent cultures of SMC were incubated for 24 h in the presence or absence of TCM and concurrently labeled with [^{35}S]sulfate as above. Reciprocal experiments were also performed by testing the effects of medium conditioned by SMC on the WiDr.

RESULTS

Morphologic Studies

The cytoplasm of the cells isolated from the tunica muscularis of human colon was stained with monoclonal antibodies [26,27] against SMC isoactin (Fig. 1A) or against desmin (Fig. 1B). Over 90% of the SMC were positive with either antibody, indicating that these cells expressed a phenotype characteristic of smooth muscle cells [22,23,26,27]. The anti-actin antibodies reacted intensely with SMC of small arterioles, venules and the tunica muscularis of normal human colon (Fig. 1C), thus confirming the specificity of these antibodies for smooth muscle proteins [26]. In contrast, SMC were unreactive with antibodies against vimentin or keratin (not shown), two intermediate filament proteins typically expressed by fibroblasts and epithelial cells, respectively [41]. By electron microscopy the cells showed numerous cytoplasmic bundles of microfilaments and several dense bodies (Fig. 1E), and displayed an arrangement in "hills and valleys," features characteristic of SMC in vitro [23]. These immunological and ultrastructural features remained constant for up to 12 passages.

When the two cell types were co-cultured at a 1:1 ratio, the colon carcinoma cells (WiDr) grew as small neoplastic aggregates which were intensely stained by monoclonal antibodies against keratin (Fig. 1D, arrowheads). Within 2 days in co-culture, there was deposition of an abundant extracellular matrix (Fig. 1F) which contained large amounts of ruthenium red-positive material (Fig. 1G). The density of the proteoglycan granules progressively increased, as visualized by cuprolinic blue staining (Fig. 1H), and by 5 days in co-culture the proteoglycan granules had largely filled the extracellular space (Fig. 1I). When SMC were cultured alone the amount of polyanionic material visualized by cationic dyes was markedly less than that of co-cultures (not shown). In contrast, the WiDr cells cultured separately did not assemble any detectable matrix, a result consistent with our previous studies [14].

These results established the smooth muscle nature of the cells isolated from human colonic wall and suggested a stimulation of matrix production and deposition when these cells were co-cultured with colon carcinoma cells.

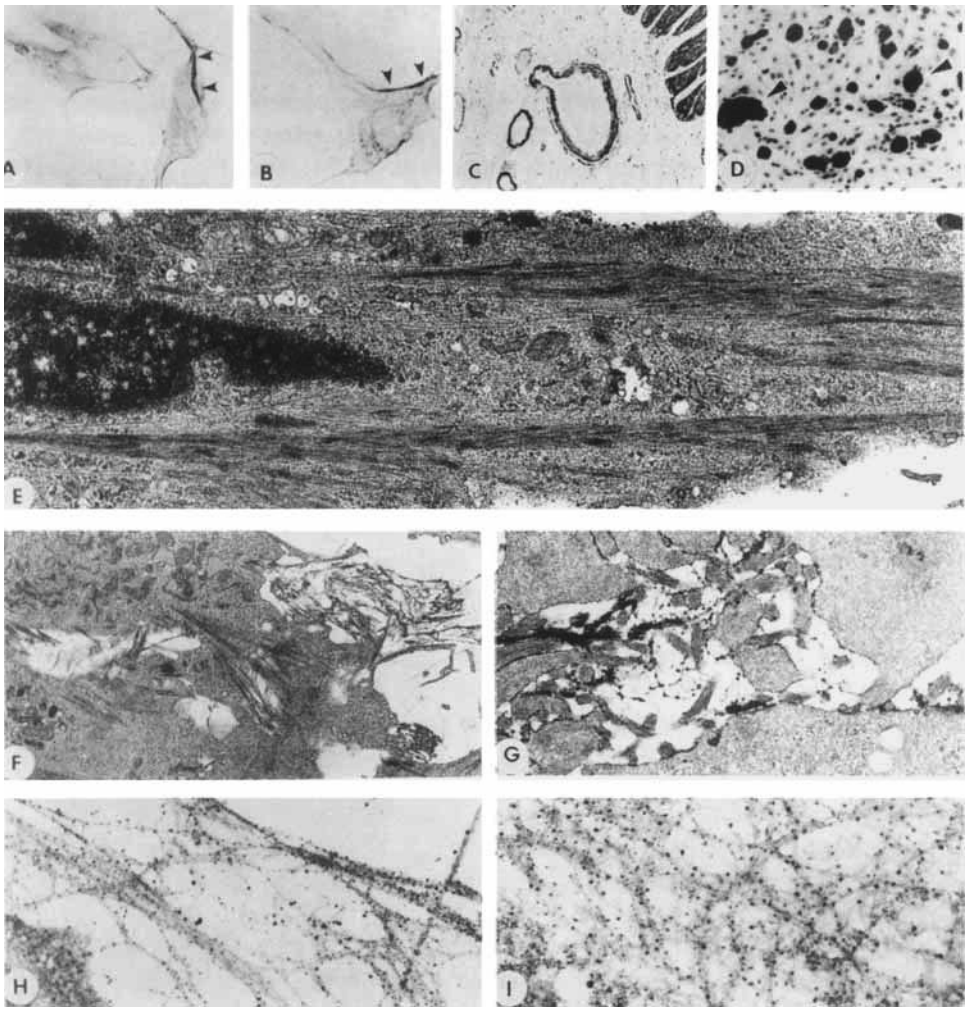

Fig. 1. Immunohistochemical and cytochemical studies. Human SMC stain with monoclonal antibodies against smooth muscle isoactin (**A,** arrowheads) and against human desmin (**B,** arrowheads) using avidin-biotin immunoperoxidase technique. The former antibody reacts intensely with blood vessels and tunica muscularis of normal human colon **(C).** In co-culture of WiDr and SMC, only the former cells, which grow as coarse neoplastic aggregates, react with antibodies against keratin (**D,** arrowheads). The nuclei of the SMC (D) are dark because they are counterstained with hematoxylin. By electron microscopy, the SMC contain bundles of cytoplasmic microfilaments and numerous dense bodies (**E,** arrowheads) disposed along the length of the cells. The co-cultures show a progressive accumulation of extracellular matrix betwwen SMC (**F,** arrowhead). This matrix is stainable by ruthenium red **(G)** and cuprolinic blue after 2 **(H)** and 4 days **(I)** in culture. A, B, ×800; C, ×150; D, ×320; E, ×10,000; F, ×3,500; G, H, ×15,000; I, ×22,000.

Stimulation of [^{35}S]Sulfate Incorporation

To investigate the molecular nature of the morphological changes in extracellular matrix described above, SMC and WiDr, either alone or in co-culture, were metabolically labeled with radiosulfate for various periods of time and the newly synthesized macromolecules were quantitated. Figure 2 shows the time-course incorporation of [^{35}S]sulfate into total macromolecules (media plus cell extracts) of the two cell types cultured separately or at a 1:1 ratio (equal proportion of the original cell dilution, i.e., 50% of SMC plus 50% of WiDr). The amount of [^{35}S]sulfate incorporated by the co-cultures (Fig. 2B, closed circles) at all time intervals was significantly greater than the predicted value (Fig. 2B, dashed line). The latter was calculated from the incorporation of radiosulfate by SMC (Fig. 2A, open squares) and WiDr (Fig. 2A, closed squares) cells cultured separately. The increase in ^{35}S activity occurred within the first 8 h and by 48 h had reached about 300% above the predicted values. The proportion of radiolabeled macromolecules associated with the medium and cell layer remained constant throughout the labeling period (not shown), suggesting that there was no preferential increase in secretion.

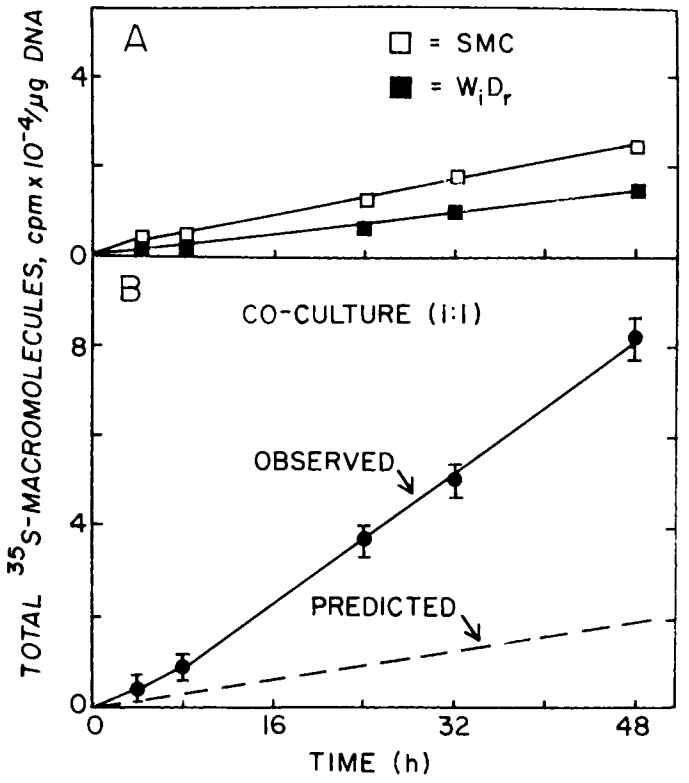

Fig. 2. Time course of [^{35}S]sulfate incorporation by SMC (□) and WiDr (■) cultured either separately (A) or at 1:1 ratio (●,B). Each time point represents duplicate dishes for the SMC and WiDr cultured alone, and triplicate dishes (±SD) for the co-cultures. The predicted values for the incorporation by co-cultures (dashed line, B) were obtained by summing 50% of SMC plus 50% of WiDr value in A for each time interval.

These results indicate that there was a synergistic, rather than additive, effect between the WiDr and SMC in co-culture on the synthesis of sulfate macromolecules (essentially all proteoglycans, see below) and that this effect was protracted in time for at least 48 h in culture.

Stimulation of Glycosaminoglycan Synthesis

To determine whether the increase in [^{35}S]sulfate incorporation was associated with an absolute increase in sulfated and unsulfated GAGs, the total (media plus cell extracts) macromolecules accumulated over 48 h of culture were purified and analyzed by electrophoresis as described before [33]. Recovery of total GAGs was calculated by quantifying aliquots of ^{35}S-labeled GAGs during the various purification steps. Figure 3 shows typical autoradiograms of electrophoretograms of purified total GAGs as visualized by ^{125}I-labeled cytochrome c before (Figure 3A) and after (Fig. 3B–D) enzymatic or chemical treatments. When cultured alone, SMC synthesized

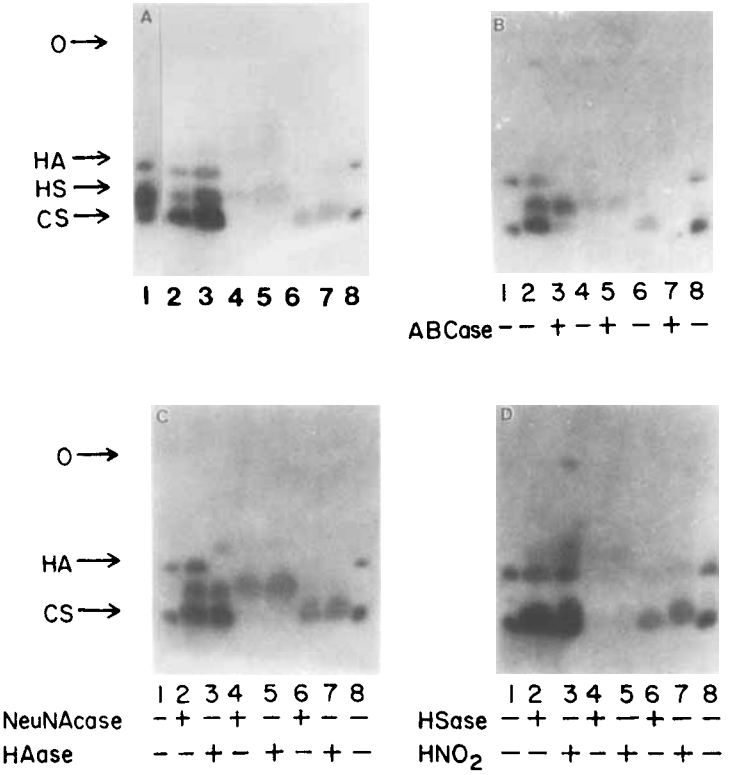

Fig. 3. Autoradiograms of total (media plus cell extracts) GAGs electrophoresed on cellulose acetate and visualized by ^{125}I-cytochrome c before **(A)** and after enzymatic and chemical treatment **(B–D)**. The GAGs were purified from cells cultured alone or together for 48 h in serum-free medium, and were subjected to chondroitinase ABC (ABCase), neuraminidase (NeuNAcase), *Steptomyces* hyaluronidase (HAase), heparitinase (HSase), or nitrous acid at pH 1.5 (HNO_2), as described under Materials and Methods. Standard GAGs include 5–10 ng hyaluronic acid (HA), 20 ng heparan sulfate (HS), and 2.5-5 ng chondroitin sulfate (CS); 0 = origin. In each panel, **lanes 1,8** represent standard GAGs, **lanes 2,3** the co-cultures, **lanes 4,5** the WiDr, and **lanes 6,7** the SMC. The final recovery of ^{35}S-labeled GAGs was 89% ±9% (n = 15). For quantitative data see Table I.

primarily two components comigrating with hyaluronic acid and chondroitin sulfate standard, respectively, and only trace amounts of material migrating at the position of heparan sulfate (Fig. 3A, lanes 6,7). In contrast, WiDr synthesized exclusively a band comigrating with heparan sulfate and only small amounts of hyaluronic acid (Fig. 3A, lanes 4,5). When the two cell types were co-cultured, there were three major bands (Fig. 3A, lanes 2,3) corresponding to hyaluronic acid, heparan sulfate, and chondroitin sulfate, respectively. A band migrating before hyaluronic acid was present in the WiDr and in the co-cultures; this was identified as a sialomucin because it was degraded by neuraminidase (Fig. 3C, lane 2) but was resistant to hyaluronidase (Fig. 3C, lane 3). The identity of the various GAGs was determined by subjecting parallel aliquots of purified GAGs to either chondroitinase ABC (Fig. 3B), *Streptomyces* hyaluronidase (Fig. 3C), and heparitinase or HNO_2 treatments (Fig. 3D).

Quantification of GAGs by scanning densitometry showed an approximate threefold increase in hyaluronic acid and chondroitin sulfate in the co-cultures, and only minimal change in heparan sulfate (Table I). The specific activity of sulfated GAGs in the co-cultures (calculated as ^{35}S cpm/μg sulfated GAGs, cf. footnote to Table I) was nearly identical to that calculated from the specific activity of the SMC and WiDr GAGs cultured separately.

These results indicate that the co-culture of SMC and WiDr induced an absolute increase in total hyaluronic acid and that the increase in [^{35}S]sulfate incorporation was due primarily to an increase in chondroitin sulfate, a molecule synthesized only by the SMC. Furthermore, the concurrent absolute increase in chondroitin sulfate and in [^{35}S]sulfate incorporation, without significant changes in specific activity of the precursor pool of sulfate, strongly indicates that the observed difference was due to a true increase in synthesis and/or a decreased turnover of CS-PG.

Effects on Cell Proliferation and Proteoglycan Turnover

Since the net increase in matrix production described above could be due to a stimulation of cell proliferation, separate experiments were performed in which cells were cultured for 4 days either separately or mixed at various ratios. The cells were

TABLE I. Quantification of Autoradiograms of Sulfated and Unsulfated Glycosaminoglycans Detected by Electrophoresis and ^{125}I-Cytochrome c Labeling*

	Total glycosaminoglycans (μg/μg DNA)		
Glycosaminoglycan type	SMC	WiDr	Co-culture (1:1)
Hyaluronic acid	0.66 (0.60–0.72)	tr.	1.71 (1.50–1.92)
Heparan sulfate	tr.	0.41 (0.35–0.47)	0.47 (0.39–0.55)
Chondroitin sulfate	0.77 (0.75–0.80)	tr.	2.20 (2.11–2.30)

*These values represent the mean of total GAGs and the range (Nos. in parentheses) of duplicate determinations from the 48-h-point samples. Quantification was performed by scanning the autoradiograms with a soft laser beam densitometer and comparing the data to standard GAG curve run in parallel. The 32-h samples also showed a marked increase in hyaluronic acid and chondroitin sulfate, although the levels of hyaluronic acid in the SMC were below detection by this method (not shown). Using the values of Figure 2, the specific activity of the sulfated GAGs (as cpm ^{35}S/μg sulfated GAG) was determined. Values of 29,900 and 39,000 for the SMC and WiDr, respectively, and 31,000 for the co-culture were obtained. The predicted value for the co-culture was 34,450 (29,900+39,000/2), very close to the observed value. This indicates that the increase in sulfated GAGs was not due to a change in specific acitvity of the precursor pool of sulfate. Recovery of ^{35}S-labeled GAGs was 89% $\pm$ 9% (n = 15). For complementary data see Figures 2 and 3. tr. = trace amount.

subsequently cultured for an additional 24 h during which time they were labeled in SFM either continuously with [^{35}S]sulfate or for the last 4 h with [^{3}H]thymidine. Parallel duplicate samples were used to measure DNA content. The increase in [^{35}S]sulfate incorporation (Fig. 4A, closed circles) over the predicted values (Fig. 4A, dashed line) was evident at all the WiDr/SMC ratios tested, with the greatest augmentation occurring at 1:1 ratio. In contrast, the incorporation of [^{3}H]thymidine (Fig. 4B, closed circles) did not appreciably differ from the values predicted by calculating the [^{3}H]thymidine incorporated by each individual cell type (Fig. 4B, dashed line). Similarly, the amount of DNA in the co-cultures at various ratios did not appreciably change (not shown).

To determine whether co-culture affected the turnover of the newly synthesized proteoglycans, the fate of the ^{35}S-labeled macromolecules in the two cell types cultured either separately or at 1:1 combination was followed. The kinetics of degradation in WiDr and SMC cultured separately (Fig. 5A) were similar[1], with a half-life of about 6 h. Seemingly, the kinetics of degradation in co-cultures (Fig. 5B, closed circles) were indistinguishible from the predicted values (Fig. 5B, dashed line), as was the release of macromolecules in the medium and the generation of free sulfate (not shown).

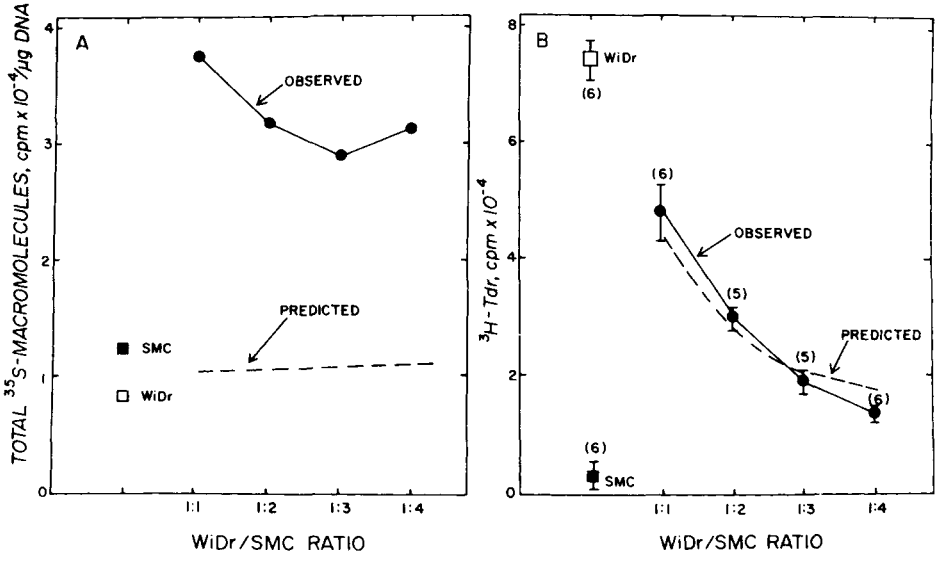

Fig. 4. Effects of various WiDr/SMC ratios on [^{35}S]sulfate (**A**) or [^{3}H]thymidine (**B**) incorporation. SMC (■), WiDr (□), or co-cultures (●) were grown for 4 days and subsequently cultured for an additional 24 h in SFM, during which time they were either labeled continuously with [^{35}S]sulfate or for the last 4 h with [^{3} H]thymidine. Parallel dishes were analyzed for DNA content. The values in A are the mean of duplicate samples, and those in B are the mean ± SD of n = 5–6 (numbers in parentheses).

[1]The kinetics of degradation of the proteoglycans are close to but not exactly first order. This derives in part from the fact that the turnover of proteoglycans reflects the summation of both secretion and intracellular degradation, two processes that may have different kinetics [21].

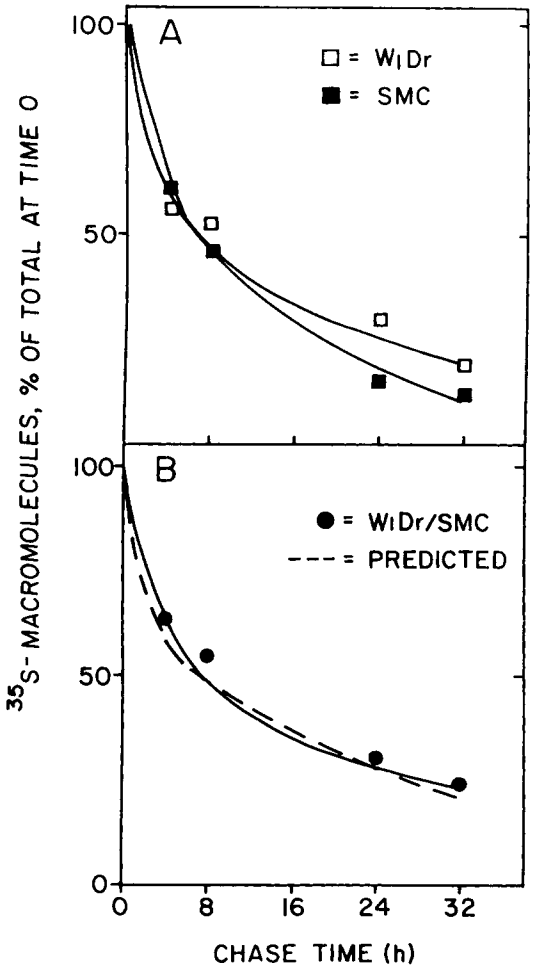

Fig. 5. Kinetics of degradation of ^{35}S-labeled macromolecules in WiDr and SMC cultured alone **(A)** or in a 1:1 combination **(B)**. The dashed line in B is derived from the values predicted from the cells cultured separately as in panel A. Cells were incubated with radiosulfate for 24 h, carefully washed in isotope-free medium supplemented with 1 mM SO_4, and chased for the designated time intervals. The values represent the average of the cell-associated radioactivity from duplicate dishes for each time point. The secretion of ^{35}S-labeled macromolecule as well as the generation of free $^{35}SO_4$ did not appreciably change (not shown).

Taken together, these results indicate that the observed increase in proteoglycan was due to a net increase in biosynthesis inasmuch as it was associated with neither a stimulation of cell proliferation nor with an inhibition of intracellular breakdown.

Isolation and Characterization of Proteoglycans

To investigate in more detail the nature of the ^{35}S-labeled macromolecules, the two cell types, either cultured alone or in various combinations, were incubated with radiosulfate for 48 h in SFM, and the macromolecular radioactivity in the media and cell layers was analyzed separately by DEAE-Sephacel chromatography. In both cell types over 95% of the ^{35}S activity bound to the column, indicating that most of the

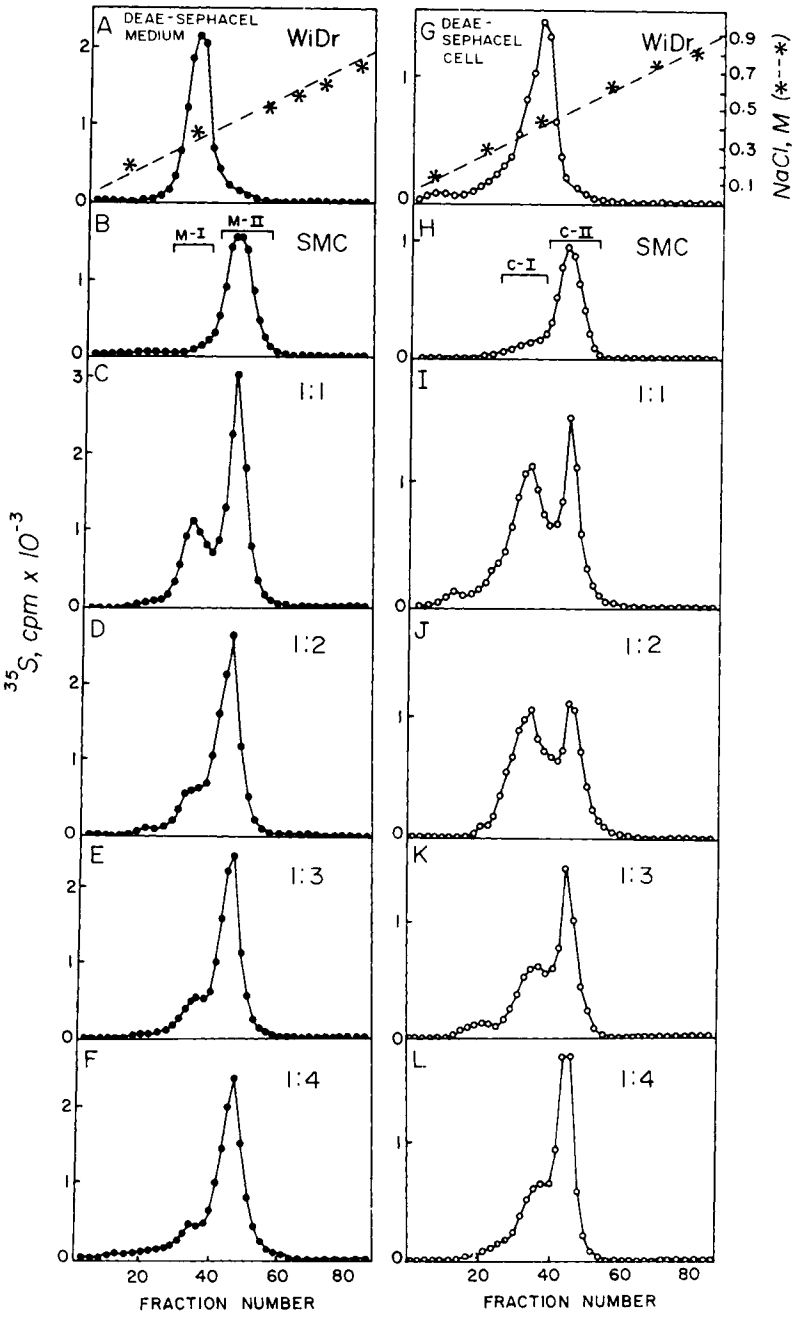

Fig. 6. DEAE-Sephacel elution profiles of Widr (**A,G**), SMC (**B,H**) and co-cultures at various WiDr/SMC ratios (**C–F, I–L**). The DEAE-Sephacel columns were washed with about 25 ml of 8 M urea buffer, 0.2% Triton X-100, 0.1 M NaCl, and then eluted with a linear 60-ml gradient between 0.1 and 0.8 M NaCl (*--*). Conductivity was measured and related to standard curve of various NaCl molarities in 8 M urea [20]. The peaks were pooled as indicated by the horizontal bars in B,H, dialyzed, and

^{35}S was incorporated into proteoglycans. In the WiDr there was a single peak eluting at about 0.4 M NaCl in both the medium (Fig. 6A) and cell layer (Fig. 6G). We have previously shown that this peak contains exclusively HS-PG [14]. In contrast, the SMC contained two peaks (Fig. 6B,H): i) one eluting at 0.4 M NaCl (pool I) which was primarily associated with the cell layer and contained HS-PG of different hydrodynamic size than the colon carcinoma cells (see below), and ii) another peak eluting at about 0.5 M NaCl (pool II). The relative amount of the more anionic compound increased progressively with the increase in the proportion of SMC so that at a 1:4 ratio it represented about 80% of the total proteoglycan (Fig. 6F,L). The various pools were isolated as indicated by the horizontal bars (Fig. 6B,H) and further analyzed by Sepharose CL-2B chromatography. In agreement with previous studies [14], the WiDr cells contained a major proteoglycan peak in the medium with $K_{av} = 0.50$ (Fig. 7A), and a slightly larger precursor associated with the cell layer with $K_{av} = 0.45$ (Fig. 7B) in addition to a smaller intracellular product with $K_{av} > 0.8$ (Fig. 7B). We have previously shown [21] that the medium and cell-associated proteoglycans contain heparan sulfate chains of $M_r = 30{,}000$, whereas the intracellular species contain primarily free chains of about one-third their parent's size. In contrast, the SMC pool I accounted for less than 10% of the total [^{35}S]sulfate incorporation in the medium fraction (Fig. 6B) and was primarily associated with the cell layer accounting for 25-30% of the total radioactivity (Fig. 6H). When analyzed

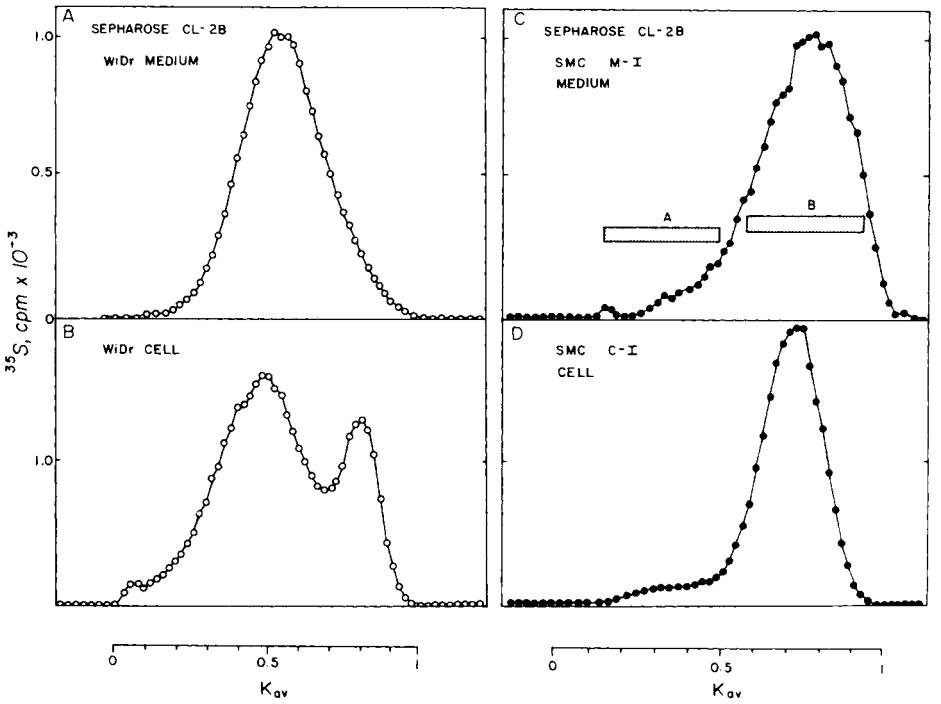

Fig. 7. Sepharose CL-2B elution profiles of proteoglycans from WiDr **(A,B)** and SMC **(C,D)** pool I (see Fig. 6A,B,G,H). The column was equilibrated and eluted with 4 M guanidine HCl, 0.5 M sodium acetate, pH 7.0, containing 0.2 % Triton X-100 and 25 mM EDTA. The two peaks, designated A and B, from the SMC were pooled as indicated by the horizontal bars (C), dialyzed, and lyophilized for further analysis.

on analytical Sepharose CL-2B, the SMC pool I contained primarily a smaller species with K_{av} = 0.70 in both the medium and cell layer (peak B, Fig. 7C and D, respectively) which represented about 75–80% of the total proteoglycan in this pool. The remaining material was composed of a larger product with K_{av} = 0.30 (Peak A, Fig. 7C,D).

The two SMC peaks were isolated and subjected to alkaline borohydride treatment before or after nitrous acid deamination. Sepharose CL-6B elution profiles of GAGs from C-IA and C-IB (Fig. 8A,B, filled circles, respectively) indicates that both species contained GAGs of similar size, with an estimated M_r = 35,000 [42], which were extensively degraded by nitrous acid (Fig. 8A,B, unfilled circles). Both samples contained a small radioactive peak near the total column volume (Fig. 8A,B) which likely represented ^{35}S generated by alkali treatment of heparan sulfate chains [43]. Nearly identical results were obtained with SMC M-IA and M-IB peaks, indicating that SMC pool I contained primarily HS-PG. The elution profiles of the co-culture pool I were essentially a summation of those shown in Figures 7 and 8, without the appearance of any new peak (not shown).

The SMC pool II contained two peaks with K_{av} = 0.30 (Peak A, Fig. 9A,B) and 0.65 (Peak B, Fig. 9A,B), the latter accounting for about 65% of the total ^{35}S activity recovered in this pool. The two proteoglycans from the medium contained similar GAG chains, M_r = 45,000 (Fig. 9C,D, filled circles), which were extensively degraded by chondroitinase ABC (Fig. 9C,D, unfilled circles). Chondroitinase AC II treatment digested >95% of the total ^{35}S activity in both Peaks A and B (not shown), indicating that <5% of the GAGs in this pool were in dermatan sulfate. The elution profiles of pool II proteoglycans from the co-culture gave identical results for the hydrodynamic size of both the proteoglycan and the GAGs, as well as for the susceptibility to chondroitinase digestion (not shown). Quantification of the two proteoglycan species showed a 300–400% increase of ^{35}S activity in CS-PG (Fig. 10, closed circles), while the ^{35}S activity in HS-PG (Fig. 10, open circles) did not appreciably change from the predicted values.

Taken together, the results indicate that the synthesis of CS-PG was selectively stimulated in co-cultures of WiDr and SMC. This increase was not accompanied by qualitative changes in either the size or type of GAG chains and accounted for the net increase in [^{35}S] sulfate incorporation. The accumulation of CS-PG and hyaluronic acid, as confirmed by GAG electrophoresis (Table I), was in agreement with the increase in extracellular matrix detected by the ultrastructural studies.

Analysis of the Protein Core of the Major Proteoglycan Synthesized by SMC

To determine the overall size of the protein core of the SMC, DEAE-Sephacel purified C-II and M-II pools, double labeled with [^{3}H]leucine and [^{35}S]sulfate, were digested with protease-free chondroitinase ABC and analyzed by 10% SDS-PAGE. In the control C-II sample, there were two major bands, one that did not penetrate the gel and one that migrated into the gel with an apparent molecular mass of 130 kDa (Fig. 11, lane 1). On a 8.5% SDS-PAGE the 115-kDa band migrated with an apparent mass of 130 kDa (not shown). The proportion of the material that did not penetrate the gel varied in intensity from experiment to experiment. Following chondroitinase ABC digestion (Fig. 11, lane 2) the 130-kDa product was converted into a 45-kDa band, indicating that this represented the protein core of the major CS-PG synthesized

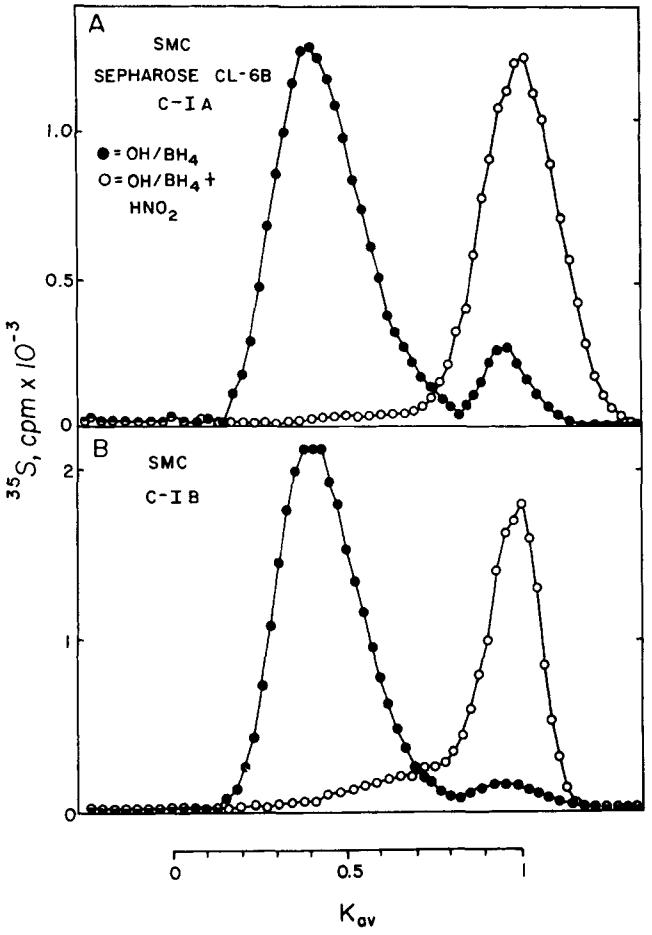

Fig. 8. Sepharose CL-6B elution profiles of alkaline borohydride-released GAGs from C-IA SMC before (●) or after (○) nitrous acid treatment at pH 1.5. The column was equilibrated and eluted with 0.5 M sodium acetate, pH 7.0, 25 mM EDTA. Similar elution profiles were obtained with the M-IA pool.

by SMC, i.e., the proteoglycan with a K_{av} of 0.65 on Sepharose CL-2B (cf. Fig. 9B). Similar results were obtained with M-II peak (not shown).

To determine whether the CS-PG was immunologically related to the PG-40 a gene product of human fibroblasts whose protein core has a molecular mass of 40 kDa [39], we reacted cell extracts of SMC labeled for 1 h with 100 μCi/ml [^{35}S]methionine with an affinity purified antiserum raised against the amino terminal peptide of PG-40 [39]. Following immunoprecipitation (Fig. 11, lane 3), two major bands were resolved by 8.5% SDS-PAGE, corresponding to the mature CS-PG and the precursor protein. The larger product migrated to a similar position of the [^{35}S]sulfate-labeled CS-PG (Cf. Fig. 11, lane 1) with an apparent mass of about 130 kDa, while the faster-migrating product was about 40 kDa, slightly smaller than the chondroitinase ABC-treated CS-PG (cf. Fig. 11, lane 2). This difference in electrophoretic mobility is likely due to the fact that chondroitinase ABC leaves attached to

the protein core the linkage oligosaccharide region plus one or two sulfated disaccharides [44]. No labeled material could be immunoprecipitated from [^{35}S]methionine-labeled WiDr samples, indicating that these epithelial cells do not express this gene product (see also below). The anti-PG-40 antiserum did not react with the material present on top of the gel (cf. Fig. 11, lane 1), suggesting that this material was not immunologically related to PG-40.

Taken together, the results indicate that the CS-PG of SMC could contain one or two chondroitin sulfate side chains of 45 kDa covalently linked to a 40-kDa protein core. The results further show that this CS-PG is immunologically and structurally related to the PG-40.

Cellular Source of Proteoglycan Synthesis

To determine the cellular source of proteoglycan synthesis, we reacted cultures of SMC, WiDr, or co-cultures with the anti-PG-40 antiserum [39] or with an affinity-purified antiserum raised against the HS-PG of EHS tumor [40], two markers specific for SMC and WiDr, respectively. The anti-PG-40 intensely stained the cytoplasm of SMC (Fig. 12A) and SMC pericellular areas (Fig. 12B arrowheads), but it did not react with the WiDr cells (Fig. 12A,B, asterisks). In contrast, with the anti-HS-PG

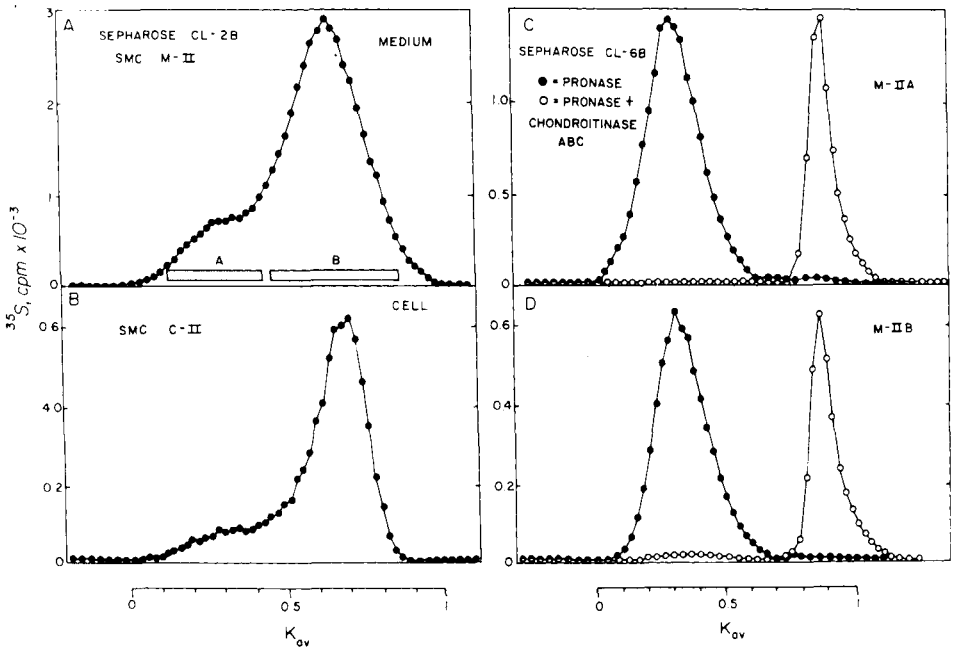

Fig. 9. Sepharose CL-2B **(A,B)** and CL-6B **(C,D)** elution profiles of proteoglycans and GAGs from pool II SMC, respectively. The two proteoglycan peaks, designated A,B, were pooled as indicated by the horizontal bars in A, and further subjected to Pronase treatment (C,D) before (●) or after (○) chondroitinase ABC digestion. The chromatographic conditions are the same as those in Figures 7 and 8, respectively. Similar profiles were obtained with C-IIA and C-IIB peaks. Less than 5% of the ^{35}S activity of the total pool II was resistant to chondroitinase AC II digestion (not shown), indicating that, at best, 5% was in dermatan sulfate. Similar results were obtained with alkaline borohydride-released GAG chains (not shown).

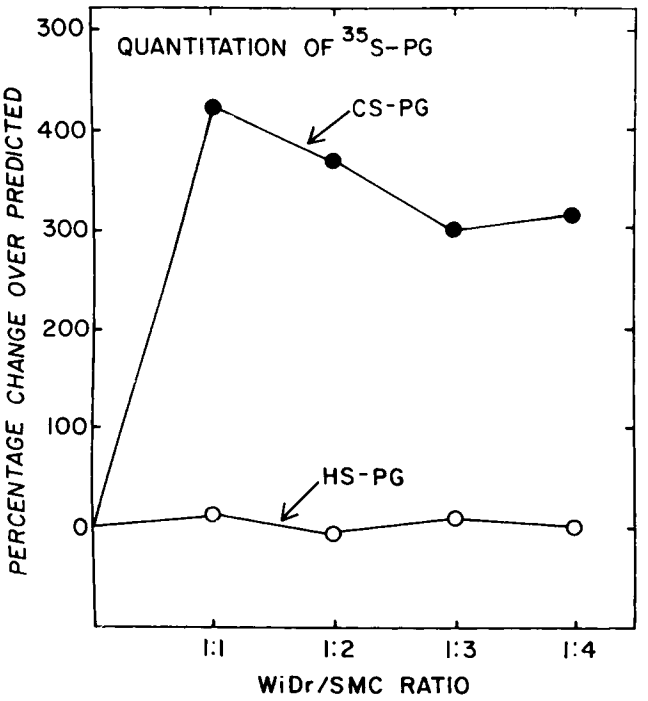

Fig. 10. Quantification of ^{35}S-labeled proteoglycans from co-cultures of WiDr and SMC at various ratios expressed as percentage change over the predicted values (the zero point on the ordinate).

antiserum the reverse situation occurred, i.e., the WiDr cells reacted intensely, while the SMC remained unstained (Fig. 12C). In addition, the anti-PG-40 antiserum recognized the tunica muscularis (the tissue of origin of SMC) and adventitial tissue of human colonic wall (Fig. 12E), as well as human dermis (Fig. 12G). Control sections incubated with normal rabbit serum for the co-cultures (Fig. 12D), colonic wall (Fig. 12F), and skin (Fig. 12H) were unreactive.

These results support the immunoprecipitation data described above and demonstrate that the SMC were indeed the source of CS-PG synthesis in the co-culture system.

Effects of Tumor-Conditioned Medium

To determine whether the stimulatory effects described above could be mediated by tumor cytokines, serum-free medium conditioned by WiDr was tested on cultured SMC. In these experiments, the total incorporation of [^{35}S]sulfate was stimulated by about 1.9-fold over the cells incubated in the absence of tumor-conditioned medium (not shown). Chondroitin sulfate was again the main component (>85%) of the ^{35}S-activity incorporated into the total macromolecules. Reciprocal experiments with medium conditioned by SMC revealed no stimulation of radiosulfate incorporation by the WiDr cells (not shown).

These results thus indicate that the stimulation of CS-PG can be partially reproduced by tumor cytokines, though to a lesser extent than the co-culture system (1.9- vs. 3-5-fold increase). This suggests that some of the interactions may require direct cell contact and/or a continuous production of the active metabolites.

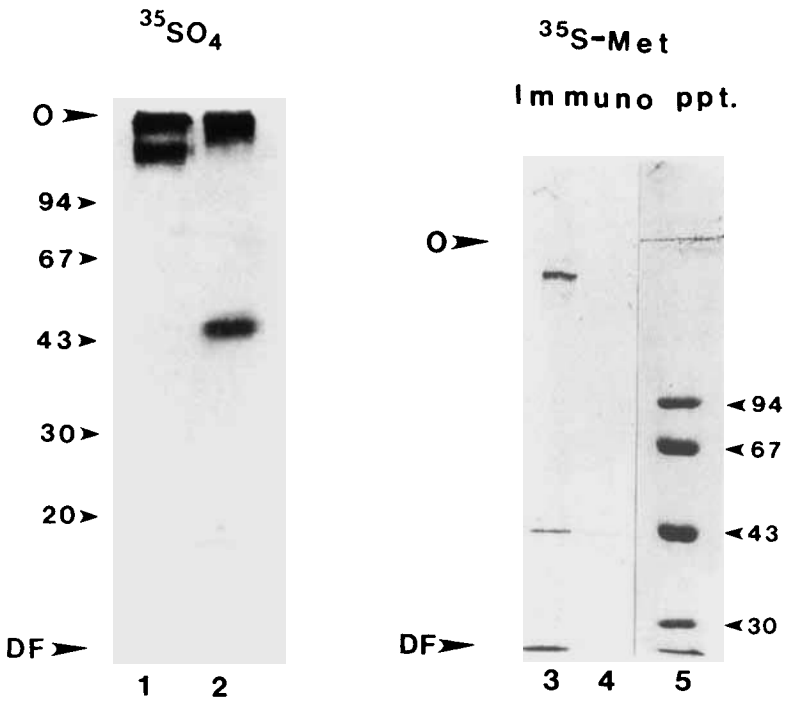

Fig. 11. Analysis of the protein core of the major CS-PG synthesized by SMC. **Lanes 1,2** Fluorograms of 10% SDS-PAGE of [^{3}H]leucine/[^{35}S]sulfate-labeled proteoglycan from the cell layer of SMC, before and after chondroitinase ABC digestion, respectively. The numbers on the left indicate the $M_r \times 10^3$ of standard proteins stained with Coomassie brilliant blue. **Lanes 3,4:** Fluorograms of 8.5% SDS-PAGE of immunoprecipitates and control, respectively, from SMC incubated for 1 h with 100 μCi/ml [^{35}S]methionine. **Lane 5:** Marker proteins stained with Coomassie brilliant blue. Both gels were run under reducing conditions. Notice that the larger product in lane 1, corresponding to 115–130 kDa, is converted to 45 kDa following chondroitinase ABC digestion (lane 2), and that the anti-PG-40 antiserum immunoprecipitates both the mature proteoglycan (the 130-kDa product) as well as a 40-kDa precursor (lane 3). 0, origin; DF, dye front.

DISCUSSION

The results demonstrate that co-culture of human colon smooth muscle and colon carcinoma cells leads to the formation of an extracellular matrix enriched in CS-PG and hyaluronic acid. Several lines of evidence indicate that these changes reflected a real increase in synthesis. First, quantification of total labeled and unlabeled GAGs showed a parallel increase in both hyaluronic acid and chondroitin sulfate, indicating that the increased [^{35}S]sulfate incorporation was not due to changes in specific activity of the precursor pool. Second, the metabolic incorporation of [^{3}H]thymidine as well as the total amount of DNA did not significantly change in the co-cultures, indicating that the changes described above were not due to stimulation of cell proliferation. Third, the kinetics of degradation of ^{35}S-labeled macromolecules in the co-cultures were indistinguishable from the predicted values, indicating that the accumulation of sulfated macromolecules was not due to an inhibition of intracellular breakdown.

The SMC synthesized primarily a small CS-PG representing 60–70% of the total ^{35}S-labeled proteoglycans, a larger CS-PG, probably similar to that synthesized

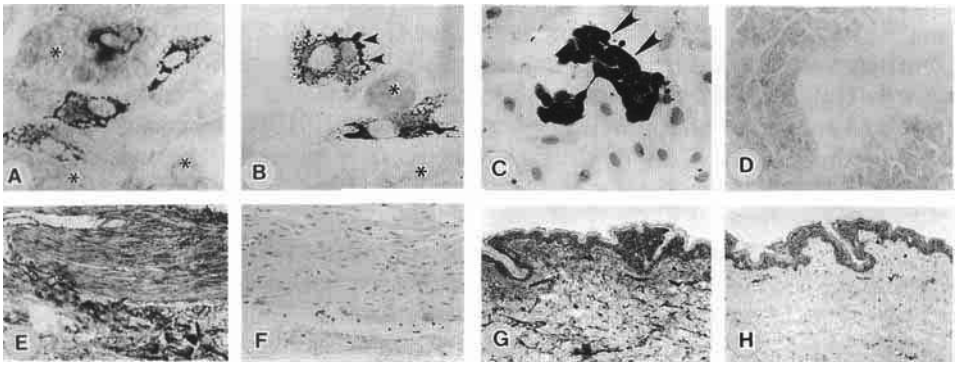

Fig. 12. Immunohistochemical localization of CS-PG and HS-PG in co-cultures of WiDr and SMC. The anti-CS-PG antiserum reacts intensely with the SMC cytoplasm **(A)** and focally with pockets of SMC pericellular matrix **(B,** arrowheads). In contrast, WiDr cells do not react with the anti-CS-PG antiserum (A,B, asterisks), but are stained with an anti-HS-PG antiserum **(C,** arrowheads). The anti-CS-PG antiserum reacts also intensely with the tunica muscularis (the tissue source of SMC) and adventitial connective tissue of human colonic wall **(E),** as well as with human dermis **(G).** Control sections incubated with normal rabbit serum for co-cultures **(D),** colonic wall **(F),** and skin **(H)** are unreactive. Samples in panels C,E–H were lightly counterstained with hematoxylin. A,B ×550; C,D ×400; E,F ×250; G,H ×130.

by other SMC [45,46], and only small amounts of HS-PG. In contrast, WiDr cells synthesized only HS-PG. When the SMC were co-cultured with WiDr, there was a marked (3–5-fold) stimulation of CS-PG synthesis, whereas HS-PG synthesis did not appreciably change. These results strongly indicate that the synthesis of CS-PG, a gene product expressed only by the SMC, was selectively stimulated by WiDr cells, and that this increase could account for the net increase in [^{35}S]sulfate incorporation by the co-cultures. Interestingly, the stroma around colon carcinoma is enriched in CS-PG [8] and colon carcinoma tissues maintained in organ culture synthesize a product nearly identical to the CS-PG synthesized by the SMC [9], indicating that the mesenchymal cells are the major producers of CS-PG. This concept is also supported by the following data: i) only the SMC-synthesized a CS-PG which could be precipitated by the anti-PG-40 antiserum; ii) only the SMC in co-cultures were specifically stained with the anti-PG-40, in contrast to the WiDr cells which reacted with an anti-HS-PG but not with the anti-PG-40 antiserum; and iii) medium conditioned by WiDr cells stimulated, though to a lesser degree than the co-culture, the synthesis of CS-PG in SMC, whereas medium conditioned by SMC had no effect on WiDr cells. The latter finding indicates that CS-PG stimulation can be partially reproduced by tumor cytokines as shown before [15], but also suggests that some of the interactions may require direct cell contact and/or a continuous production of active tumor metabolites.

Recent studies have confirmed the concept that tumor cells modulate proteoglycan metabolism in host mesenchymal cells. Knudson et al. [17] have demonstrated that co-cultures of human skin fibroblasts and various cancer cell lines derived from lung, pancreas, or skin synthesize threefold more hyaluronic acid than the sum of that produced by each cell type cultured alone. These investigators have reproduced similar results using tumor extracts [18] or medium conditioned by tumor cells [47]. Following the original observations by Merrilees and co-workers [48–50] that the production of GAGs can be affected in the normal state by cell-cell interactions, these

authors have shown that media conditioned by a variety of human tumor cells stimulate net synthesis of sulfated and unsulfated GAGs [19]. Of particular interest is that medium conditioned by HT-29 colon carcinoma cells, a cell line that synthesizes primarily heparan sulfate, is capable of markedly stimulating the synthesis of hyaluronic acid and chondroitin sulfate in human fibroblasts [19]. This is in strong support of our findings since Chen et al. [51] have recently provided chromosomal and isozymic evidence indicating that the WiDr cells used here are indeed derived from HT-29 cells.

The results of our study are closely linked to some of the concepts of tumor progression [52], and in particular to the aberrant expression of a gene product that may contribute to the capacity for tumor growth, local invasion, and metastasis [for reviews see 1–3, 53–56]. Indeed, altered GAG expression and metabolism can be associated with the metastatic process [57] and, as mentioned above, both hyaluronic acid and chondroitin sulfate have been implicated in cancer growth and infiltration. For instance, the synthesis of hyaluronic acid is enhanced in carcinoma cell variants with high metastatic potential [58] and its content is much higher around invasive rabbit V2 carcinoma [59]. It has been postulated that the selective deposition of this highly viscous product may favor tumor growth and infiltration [59]. Using the same V2 carcinoma cells, we have shown that these cells induce a pronounced increase in mesenteric mass, with a concomitant increase in hyaluronic acid and chondroitin sulfate synthesis [16]. The total amount of collagen also increases with time after implantation [60], as does the density of the collagen fibers and proteoglycan granules detected ultrastructurally [16].

The CS-PG that we have found in elevated amounts in human colon carcinoma tissue [8,9] or whose synthesis can be induced in colon fibroblasts [15] and SMC by the colon carcinoma cells, probably belongs to a class of ubiquitous CS-PGs that is present in a variety of mesenchymal tissues [39,61–67]. The protein core found in this proteoglycan class is about 40 kDa, thus called PG-40, and its sequence has been recently elucidated [39]. Furthermore, in a variety of human tumors including leiomyomas and fibromas, the CS-PG protein core has an apparent mass of 45–48 kDa following chondroitinase treatment, indicating that both smooth muscle- and fibroblast-derived neoplasms express similar gene products [67]. Our present results have shown that the chondroitinase ABC-treated CS-PG of SMC is also about 45 kDa and that the anti-PG-40 antiserum precipitates a [^{35}S]methionine-labeled precursor protein of about 40 kDa. Therefore, it is likely that this gene product is the same as that we found in elevated concentration in colon carcinoma tissue [8,9] and that variations in average size of this class of gene products reported previously [61–67] may reflect different degrees of post-translational modification. Indeed, hybridization of genomic DNA restriction fragments suggests that no more than two genes code for PG-40 and proteoglycans related to it [39]. In future studies we plan to use the newly developed cDNA probes specific for PG-40 [39] to determine whether the stimulation of CS-PG, both in vivo and in vivo, is associated with increased levels of protein core specific mRNA and whether these changes reflect alterations in transcription rates or RNA stability.

In conclusion, this study has been able to reproduce in vitro, at least in part, a tumor-associated matrix with a proteoglycan composition similar to that observed in vivo and provides further support to the notion that the formation of a proteoglycan-rich extracellular environment around tumors is regulated by specific host-neoplastic cell interactions.

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