Neoplastic Modulation of Extracellular Matrix: Proteoglycan Changes in the Rabbit Mesentery Induced by V2 Carcinoma Cells

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ABSTRACT

Previous studies have shown that the invasion of V2 carcinoma cells in the rabbit mesentery is associated with marked extracellular matrix synthesis leading eventually to an overall increase in mesenteric mass. The purpose of the present study was to investigate the structural and biochemical composition of the extracellular matrix in tumor-free parts of rabbit mesenteries at various stages after intraperitoneal implantation of V2 carcinoma cells. The overall thickness of the tumor-implanted mesenteries increased progressively and peaked at about Day 14, when it was about 8 times greater than the untreated or liver-implanted controls. This was mainly the result of an accumulation of extracellular matrix components. In particular, there was a marked increase in both collagen fibers and proteoglycan granules, as well as filaments, probably hyaluronic acid, as visualized by ruthenium hexamine trichloride. Stereological analysis showed a 6-fold increase in collagen fibers and a significant increase in the density and average diameter of proteoglycan granules. Biochemical analysis revealed a marked elevation in uronic acid content in the tumor-implanted mesenteries. Specifically, they contained 2.6 and 8.6 times the amount of hyaluronic acid and chondroitin sulfate, respectively, than did controls. Furthermore, the relative percentage of chondroitin sulfate was elevated markedly (26 versus 6% in controls). However, the content of heparan or dermatan sulfate did not vary significantly. Stereological analysis of the fibroblasts showed that their absolute number had doubled and that the cell volume of the individual fibroblast had increased markedly. This suggests that the fibroblasts were responsible for the excessive production of the extracellular matrix. These results support the concept that carcinoma cells can modulate their surrounding extracellular environment by stimulating the synthesis of connective tissue in the host mesenchymal cells.

INTRODUCTION

Current views (1–3) indicate that neoplastic cells can modulate their surrounding extracellular environment by at least three, often interrelated mechanisms: (a) abnormal production of extracellular matrix; (b) abnormal degradation of extracellular matrix; and (c) induction of matrix biosynthesis by host stromal cells, also known as desmoplasia. During tumor development one of these three basic mechanisms may become predominant or all of these may take place simultaneously in different locations of the same tumor (1). In particular, the process of excessive connective tissue formation around growing neoplasms is a well-recognized phenomenon, and it appears to be more accentuated in some invasive carcinomas of breast, prostate, pancreas, and large bowel (4, 5). Desmoplasia may be so accentuated that it may be solely responsible for the clinical presentation of the tumor as a “lump” (6). In our previous studies we have demonstrated that several human tumors exhibit aberrant contents of certain glycosaminoglycans (7) and that tissue extracts of human colon carcinoma (4) contain a 12-fold increase in chondroitin sulfate when compared with normal colon. Subsequent studies (8) have indicated that human colon carcinoma tissue in organ culture synthesizes high amounts of chondroitin sulfate proteoglycan and that the major source of the sulfated proteoglycans is the connective tissue surrounding the tumor cells (4). Therefore, our findings have raised the possibility that the neoplastic cells may induce proteoglycan production by host mesenchymal cells and that this results in an abnormal accumulation of sulfated proteoglycans which are generally present in only trace amounts in the normal state. This concept is supported by a recent study (6) demonstrating that the desmoplastic reaction occurring in certain human breast carcinomas manifests a 10-fold increase in type V collagen which is synthesized primarily by host connective tissue cells. In both this work and ours, the phenomenon observed is basically similar, namely an increased production of a specific matrix component by adjacent host stromal cells. To date, the factors that are involved in regulating the interaction between tumor and host mesenchymal cells are virtually unknown.

Here we have extended this line of research to an experimental animal model using implantation of V2 carcinoma cells into the peritoneal cavity of rabbits. It has been shown that, apart from tissue destruction (9, 10), invasion of V2 carcinoma cells into the mesentery is associated with an overall increase in mesenteric tissue in areas free of neoplastic involvement. This process includes: (a) multiplication of host connective tissue cells; and (b) enhanced production of fibrillar and non-fibrillar constituents of the extracellular matrix (11).

In the present study we have characterized the glycosaminoglycan content of the mesentery of normal, liver-implanted, and V2 carcinoma-implanted animals. Furthermore, we have investigated the ultrastructural changes in proteoglycans and collagen that occur in the extracellular matrix. The results indicate that tumor-free parts of the mesentery from the V2-implanted rabbits expressed a marked increase in hyaluronic acid and chondroitin sulfate content and that the number and size of the proteoglycan monomers observed ultrastructurally increased concomitantly. The results support the concept that tumor cells may modulate their surrounding environment by inducing a proteoglycan-rich extracellular matrix.
MATERIALS AND METHODS

Animals. Swiss silver rabbits, at the age of 5–7 months, were given injections intraperitoneally with 5 ml each of a suspension of V2 carcinoma cells, a tumor which is routinely propagated by intraperitoneal implantation in rabbits. Previous investigations (10) have shown that the neoplastic cells colonize primarily the omentum and, to a lesser extent, adhere to other organs in the abdominal cavity. Control animals were either untreated or injected intraperitoneally with rabbit liver homogenates. The V2 carcinoma has a predilection for the omentum, where it forms large nodules, but it is also adheres to the mesentery, where it generally forms smaller neoplastic nodules (10). For this study a total of 20 animals was used, and animals were sacrificed at Day 3–4 (early stage), Day 7–9 (middle stage), and Day 12–16 (late stage).

Morphological Studies. The control, liver-, and tumor-implanted animals were sacrificed by an intravenous overdose of sodium nembutal, and the mesenteric tissue was excised under reproducing conditions of stretching and held in this state throughout the subsequent stages of tissue processing (10). To preserve the extracellular matrix, 0.4% RHT (Johnson Matthey Chemicals, Royston, U. K.) was added to all the following fixatives and washing solutions (12). Prefixation was performed for 2 h at room temperature in 2% glutaraldehyde:1% formaldehyde freshly prepared from paraformaldehyde, 0.05 M sodium cacodylate buffer, pH 7.0. After three washes (0.05 M sodium cacodylate, 0.15 M NaCl, pH 7.4), the tissue was postfixed for 2 h at room temperature with 1% OsO4 in 0.1 M sodium cacodylate buffer, 0.1 M NaCl, pH 7.2. The specimens were washed three times in the same buffer, dehydrated through graded ethanol, and embedded in Epon.

Mesenteric areas free of blood vessels and, in the case of V2-implanted animals, without tumor nodules, were examined in vertical sections. For light microscopy, 0.7−μm sections were stained with 1% toluidine blue in 1% aqueous borax and photographed in a Zeiss Axiosimat. Thin sections were mounted on formvar-coated grids, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 10 CR electron microscope operated at 80 kV.

Quantitative Studies. The diameter of collagen fibrils was measured using profiles of cross-sectioned fibers. Twenty fibrils within fibers and 20 single fibrils were randomly selected per animal. Similarly, 20 proteoglycan granules associated with collagen fibrils within fibers and 20 proteoglycan granules which were not associated with collagen fibrils were randomly selected per animal. The diameter of fibrils and granules was measured on micrographs at a final magnification of ×72,000 using a ×8 magnifier with a 0.1-mm scale. The magnification was calibrated using a carbon grating replica with 21,600 lines/cm (Balzers Union, Balzers, Liechtenstein).

For stereological analysis, data were collected from light micrographs using standard techniques (13, 14). Ten mesenteries were analyzed: five from controls and five from V2 carcinoma-implanted animals in middle to late stages. Three random blocks from each animal provided three random sections. Three random light micrographs were taken per block, using an oil immersion objective. The final magnification was calibrated using an object micrometer operated at 80 kV.

For collagen fibers, the values of the Vv and the absolute amount per tissue column perpendicular to the mesenteric membrane was estimated by LC = Vv/ls, where Lc is the total profile length of a structural component (c) on a test line perpendicular to the mesenteric membrane. Ac is the total profile area of c and ls is the length of the test area as estimated by LT = Ac/ls, where Lc is the average total profile length of a structural component (c) on a test line perpendicular to the mesenteric membrane. Ac is the total profile area of c and ls is the length of the test area as described above. The average volume of individual fibrils was estimated by Vi = Vv/NIc.

The statistical significance of differences between control and tumor-implanted animals was estimated using Student’s t-test.

Isolation and Characterization of Glycosaminoglycans. Because of the small amount of sample (less than 200 mg average), it was impossible to study in detail the intact proteoglycans of normal and tumor-implanted mesenteries. Therefore, the analysis was focused on characterizing the glycosaminoglycan moieties. Mesentaries from control or liver-implanted and V2 carcinoma rabbits (free of tumor nodules) were excised and lyophilized. Glycosaminoglycans were isolated from each sample as described previously (4). In addition, a modification was used to each sample an aliquot of [35S]sulfate-labeled heparan sulfate proteoglycan (approximately 50,000 cpm/sample) was added (15). This [35S]labeled proteoglycan was used to calculate the percentage of recovery during all of the purification steps thereafter. The lyophilized samples were delipidized in ethanol (2:1, v/v) overnight and then in ether/ethanol (4:1, v/v) for 2 h. The supernatants were discarded, the samples were dried in an oven at 60°C for 1 h, and the dry defatted weight was determined. The samples were then subjected to extensive proteolysis with papain (250 units/ml; Sigma Chemical Company, St. Louis, MO) at 65°C for 22 h in 2 ml of phosphate-buffered saline, pH 7.0, containing 10 mM cysteine and 10 mM sodium EDTA. After 6 h of initial incubation, 50 units/ml of fresh enzyme was added to each sample. At the end of each incubation, equal volumes of a solution containing 1 M NaOH and 2 M NaBH4 were added, and each sample was incubated for an additional 6 h at 45°C. This step was used in order to cleave the residual O-linked carbohydrate chains from the protein cores under conditions that prevent the degradation of the sugar chains (16). The reactions were stopped by drop-wise addition of diluted glacial acetic acid. The samples were centrifuged at 12,000 × g for 1 h at 4°C to remove cellular debris, and the supernatants were dialyzed against double distilled water for 72 h at 4°C and lyophilized. In order to remove nucleic acid contaminants which interfere with the glycosaminoglycan analysis, the dry samples were subjected to a sequential enzymatic digestion with ribonuclease (45 units/ml) (Calbiochem-Boehringer, San Diego, CA) for 4 h at 37°C in 50 mM sodium acetate.

The abbreviation used is: RHT, ruthenium (III) hexamine trichloride.
buffer, pH 7.0, followed by deoxyribonuclease I (250 units/ml) for 4 h at 37°C in the presence of 5 mM MgCl₂. The solutions were mixed with equal volumes of 20% trichloroacetic acid to reach a final concentration of 10% and were left on ice for 4 h in order to precipitate the undigested nucleic acid and proteins. The solutions were then centrifuged at 15,000 \( \times g \) for 1 h, dialyzed at 4°C against double-distilled water, and lyophilized. Recovery of the \( ^{35} \text{S} \) sulfate-labeled heparan sulfate was greater than 70%. The lyophilized samples were resuspended in distilled water and subjected to cellulose acetate electrophoresis in 0.3 mM cadmium acetate buffer, pH 4.1, as described previously (17). The various glycosaminoglycans were identified by their co-migration with glycosaminoglycan standards and by their susceptibility to chondroitinase ABC (Proteus vulgaris; Miles Laboratory, Elkhart, IN) (18), Streptomyces hyaluronidase (Miles Laboratory), or nitrous acid (19) treatment. Standard glycosaminoglycans were used to test the specificity of the various enzymatic reactions, and they included hyaluronic acid from human umbilical cord, chondroitin sulfate from shark cartilage, heparan sulfate from pig intestine, and dermatan sulfate from skin (Miles Laboratory). The alcian blue-chondroitin sulfate from shark cartilage, heparan sulfate from pig intestine, and dermatan sulfate from skin (Miles Laboratory). The alcian blue-stained bands of the cellulose acetate strips were quantitated using a scanning densitometer (Helena Laboratory, MO) interfaced with an Hewlett Packard computer (4). Samples were measured with reference to standard curves with a range of sensitivity between 4 and 40 \( \mu \)g. Uronic acid content of each sample run in triplicate was determined by the carbazole method of Bitter and Muir (20) with \( \alpha \)-glycuronolactone as reference standard.

RESULTS

Morphological Studies of Mesentery from Control and Liver-implanted Animals. The whole embedded tissue, processed in a stretched state, was transparent and only lightly stained in shades of gray by the RHT treatment (Fig. 1a), and it measured about 5 \( \mu \)m in thickness.

The morphology of the normal rabbit mesentery has been described previously (10). Briefly, this transparent membrane (Fig. 1a) was composed of a delicate connective tissue stroma lined on both sides by a monolayer of mesothelial cells and a thin basement membrane. The connective tissue comprised different structures which were symmetrically stratified and included a network of elastic and a mesh of reticular fibers on both sides and a more central area containing primarily large collagen bundles with an average thickness of 2.0 ± 0.3 (SD) \( \mu \)m (Fig. 1, b and c and Fig. 4, a and b). Quantitative studies (Table 1) revealed that the average collagen fibrils within fibers had a diameter of 62 ± 4 nm, a value significantly larger than that of the single fibrils (48 ± 4 nm). Proteoglycans appeared as electron-dense granules 17 nm in average diameter (Table 1) when stained in the presence of RHT. This value was observed for granules both associated closely with collagen fibrils (Fig. 4, a and b) and dispersed throughout the extracellular matrix. This pattern of collagen-proteoglycan interaction was similar to that described in rat tail tendon (21) and may represent primarily dermatan sulfate (22). In addition, numerous RHT-positive filaments were observed throughout the extracellular matrix (Fig. 4a, double arrowheads) and appeared to connect portions of the non-fibrillar material and proteoglycan granules. These filaments, which probably represent hyaluronic acid (23), had an average diameter of about 4 nm.

The normal rabbit mesentery contained large areas free of proteoglycans or other visualizable structure (Fig. 1, b and c), even after processing in the presence of RHT. In addition to the connective tissue matrix components, the normal mesentery contained fibrocytes and scattered macrophages which were generally located near the surface (not shown).

After liver implantation, the macroscopic appearance and the thickness of the mesentery, as well as the diameter of the fibrillar constituents of the extracellular matrix (Fig. 1, d-f), were similar to the untreated controls. The overall density of the matrix granules was also indistinguishable from the controls.

Morphological Studies of Mesentery after Implantation of V2 Carcinoma Cells. Both the mesenteric thickness (Chart 1, open circles) measured after Epon embedding and the mesenteric weight (Chart 1, closed circles) increased significantly with time after tumor implantation (see also Figs. 2 and 3). At late stage the thickness was nearly 8-fold greater than in control or liver-implanted animals (compare Figs. 3, a and d with Fig. 1, a and d, respectively). This increase in volume was not a direct result of the formation of neoplastic nodules but was primarily due to an increase in the extracellular matrix. These qualitative

<table>
<thead>
<tr>
<th>Extracellular matrix component</th>
<th>Diameter of fibrils or granules (nm)</th>
<th>P-values (^p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen fibrils</td>
<td>Control ((n = 5))</td>
<td>V2-implanted ((n = 5))</td>
</tr>
<tr>
<td>Within fibers</td>
<td>62 ± 4 (^d)</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>Single fibrils</td>
<td>48 ± 4</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Proteoglycan granules</td>
<td>17 ± 3</td>
<td>24 ± 3 (^e)</td>
</tr>
<tr>
<td>Collagen-associated</td>
<td>17 ± 2</td>
<td>30 ± 2 (^e)</td>
</tr>
<tr>
<td>Single granules</td>
<td>17 ± 2</td>
<td>30 ± 2 (^e)</td>
</tr>
</tbody>
</table>

\(^a\) v2-carcinoma-implanted animals at middle to late stages.
\(^b\) Using Student’s t-test.
\(^c\) The average thickness of the collagen fibrils was 2.0 ± 0.3 and 3.2 ± 0.7 \( \mu \)m for control and V2-implanted animals, respectively (\(P < 0.005\)).
\(^d\) Mean ± SD.
\(^e\) The average diameter of a proteoglycan granule can be estimated assuming sphericity (4); \(V = \left( \frac{4}{3} \pi r^3 \right)\). This gives a value of 2.6 and 7.2-14.1 \( \times 10^4 \) \( \mu \)m for control and V2-implanted animals, respectively.

Chart 1. Increase in thickness (C) and wet weight (W) of tumor-free mesenteric tissue following intraperitoneal implantation of V2 carcinoma cells. The thickness was measured from perpendicular sections of Epon-embedded tissue processed in a controlled stretched-state. The values represent the mean (\(n = 3-6\)); bars, SD.
observations were confirmed by the quantitative studies summarized in Table 1 and 2. The average diameter of the proteoglycan granules increased significantly, and this change was more pronounced in the proteoglycans not associated with collagen fibers (Table 1; Fig. 4). Consequently, the average volume of a matrix granule was markedly increased in V2 carcinoma-implanted animals, since this value is greatly affected by minute changes in the radius (see Table 1, Footnote e). In contrast the diameter of individual collagen fibrils increased only slightly (5 nm on the average) for both fibrils within fibers and those occurring singly (Table 1). Their diameter, however, appeared to be greatly increased in the electron micrographs (Fig. 4) because of associated, electron-dense proteoglycan granules. The fine HRT-positive filaments remained of constant thickness (about 4 nm) throughout all of the tumor stages, whereas their density increased with tumor development (Fig. 4c). The above-mentioned changes were not a localized phenomenon but occurred uniformly throughout the mesentery free of tumor aggregates.

Stereological evaluation of collagen fibers and fibroblasts uncovered several differences (Table 2). The absolute amount of collagen fibers (Table 2, Lc) increased more than 6-fold. This was due to an increase in the number (compare Fig. 3, a and d with Fig. 1a) and average thickness (3.2 versus 2.0 μm) of collagen fibers. However, because the volume density of collagen fibers (Table 2, Vc) decreased by 37%, it is likely that extracellular matrix components other than collagen had been synthesized and accumulated in the V2-implanted mesentery. This was in agreement with the concurrent elevation of glycosaminoglycan content (see below) and the increase in the average size (Table 1) and density of proteoglycan granules (Fig. 3, b and e). Furthermore, the volume density and numerical density of fibroblasts (Table 2, Vf and Nf) decreased by 28 and 85%, respectively, whereas the total amount (Table 2, Lf) and number (Table 2, Nf) increased more than 6- and 2-fold, respectively, in the V2-implanted mesentery. Consequently, the average volume of fibroblasts (Table 2, Vf) increased by approximately 5-fold.

In summary, these results indicate that, following tumor implantation, the mesentery free of neoplastic aggregates contained elevated concentrations of collagen and proteoglycans. The larger RHT-positive granules in the matrix of V2-implanted mesenteries also suggest an increase in the size of single proteoglycan monomers (4). Furthermore, the increased density of the RHT-positive filamentous network interconnecting the proteoglycans suggests an increase in hyaluronic acid content. Finally, the increased number and cell volume of the fibroblasts suggests that these cells may be primarily responsible for the marked elevation of extracellular matrix constituents.

Analysis of Glycosaminoglycans. Because uronic acid is a specific marker for glycosaminoglycans (24), a quantitative analysis of its content provides an estimate of the glycosaminoglycan content in a tissue. Analysis of the mesenteries revealed a marked and progressive increase in total uronic acid in the animals implanted with V2 carcinoma cells (Table 3). The uronic acid increased as a function of time after tumor implantation, concurrently with the increase in total weight and thickness of the mesentery (Chart 1). At late stage the total uronic acid was 2.52 times that of the control animals and 2.85 times that of the liver-implanted animals. Interestingly, no significant differences were observed when the uronic acid was expressed on a wet weight basis. This probably reflected a higher water content induced by the increased amounts of the hydrophilic proteoglycan molecules and hyaluronic acid (see below). When the values were expressed on a mg of dry-defatted weight (Table 3), the neoplastic-free mesentery of the V2-implanted animals at late stage contained twice more than the uronic acid found in the control or liver-implanted samples, respectively.

The glycosaminoglycans were purified from each sample and identified using cellulose acetate electrophoresis (Fig. 5) and a combination of chemical and enzymatic treatments as described in "Materials and Methods." Quantitation of the various glycosaminoglycans was accomplished by scanning densitometry with reference to standard curves and is summarized in Chart 2. The major difference between the control (Chart 2, unfilled bars) and the V2-implanted animals at late stage (Chart 2, filled bars) was in the content of hyaluronic acid and chondroitin sulfate (P < 0.001), while heparan and dermatan sulfate were not changed significantly. In particular, the content of hyaluronic acid in the V2-implanted mesenteries was 2.6 and 8.6 times higher than that of controls. Furthermore, the relative percentage of chondroitin sulfate in V2-implanted mesenteries was 26% of the total glycosaminoglycan versus only 6% in the control. Similar patterns of

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 5)</th>
<th>V2-implanted (n = 5)</th>
<th>Δ (%)</th>
<th>P-valuesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblast, Vf (%)</td>
<td>7.4 ± 0.9c</td>
<td>5.3 ± 2.0</td>
<td>−28</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Collagen fiber, Vc (%)</td>
<td>25.9 ± 2.4</td>
<td>16.3 ± 2.9</td>
<td>−37</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Total length</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fibroblast, Lf (μm)</td>
<td>0.29 ± 0.03</td>
<td>1.92 ± 0.66</td>
<td>+662</td>
<td>&lt;0.0025</td>
</tr>
<tr>
<td>Collagen fiber, Lc (μm)</td>
<td>1.01 ± 0.14</td>
<td>6.25 ± 2.26</td>
<td>+619</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Total number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblast, Nf (10⁶/mm³)</td>
<td>6.7 ± 2.3</td>
<td>14.2 ± 7.4</td>
<td>+212</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Numerical density</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblast, Nf (10⁶/mm³)</td>
<td>189.0 ± 69</td>
<td>28.0 ± 21</td>
<td>−85</td>
<td>&lt;0.0025</td>
</tr>
<tr>
<td>Average volume</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fibroblast, Vf (μm³)</td>
<td>392.0 ± 93</td>
<td>1,893.0 ± 1,060</td>
<td>+483</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

a: V2-implanted animals at middle to late stage.  
b: Using Student’s t-test.  
c: Mean ± SD.  
d: These values were corrected for systemic errors due to finite section thickness as described in "Materials and Methods."  
e: Corresponding to the sum of all fibroblasts or collagen profiles contained in a thin column running vertically through the mesentery.  
f: These values represent the absolute number of fibroblasts per unit length (mm) of vertical section through the mesentery.  
g: These values derive from Vf = Vc/Nf.

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total uronic acid (μg)β</th>
<th>Uronic acid/dry defatted wt. (μg/mg)β</th>
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</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>61 ± 8β</td>
<td>0.50 ± 0.18</td>
</tr>
<tr>
<td>Liver-implanted (n = 3)</td>
<td>54 ± 3</td>
<td>0.45 ± 0.09</td>
</tr>
<tr>
<td>V2-implanted, early stage (n = 3)</td>
<td>56 ± 2</td>
<td>0.41 ± 0.10</td>
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<tr>
<td>V2-implanted, middle stage (n = 3)</td>
<td>80 ± 10</td>
<td>0.68 ± 0.12</td>
</tr>
<tr>
<td>V2-implanted, late stage (n = 4)</td>
<td>154 ± 18</td>
<td>0.67 ± 0.15</td>
</tr>
</tbody>
</table>

β: Uronic acid was calculated according to the method of Bitter and Muir (20) with D-glucuronolactone as reference standard.  

Mean ± SD.
NEOPLASTIC MODULATION OF EXTRACELLULAR MATRIX

Fig. 5. Representative cellulose acetate electrophoretograms of standard glycosaminoglycan mixture (Lane 1) and glycosaminoglycans isolated from control mesentery (Lane 2) or V2 carcinoma-implanted mesentery at middle (Lane 3) and late stage (Lane 4). Electrophoresis was performed in 0.3 M cadmium acetate buffer, pH 4.1, for 4 h at constant current of 1.2 mamp per strip. The mixture of glycosaminoglycan standard, 20 µg in total, include hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate (DS), and chondroitin sulfate (CS). The alcian blue-stained bands were also identified by their relative susceptibility to enzymatic and chemical treatments as detailed in "Materials and Methods." Quantitation of triplicate samples was done by scanning densitometry (see Chart 2).

In summary, the biochemical studies indicate that the mesenteries of V2 carcinoma-implanted animals contained an increased amount of hyaluronic acid and proteoglycans rich in chondroitin sulfate, both of which could contribute to the increase in weight and water retention. The increase in chondroitin sulfate may also provide an explanation for the quantitative changes observed in the density and size of the RHT-positive granules.

DISCUSSION

This study has demonstrated that the connective tissue cells of rabbit mesentery, following implantation of V2 carcinoma cells, actively synthesized and deposited a connective tissue matrix rich in hyaluronic acid and chondroitin sulfate. The neoplastically induced mesentery increased up to 8-fold in thickness and several times in weight, and this increase was not due to the proliferation of tumor cells and/or the formation of large neoepithelial aggregates. Indeed, only a few neoplastic cells were seen in the desmoplastic (hypertrophic) parts of the mesentery examined here. Previous studies using time-lapse cinematography (25) have shown that V2 carcinoma cells injected into the peritoneal cavity invade the mesentery and move diagonally between the host cells (fibrocytes and adipocytes) and the fibroblasts and non-fibroblasts, as constituents of the extracellular matrix, where they may eventually form neoplastic nodules. These tumor aggregates, however, become only a minor component of the mesenteric tissue and were excluded from the present investigation. On the other hand, stereological analysis revealed a 2-fold increase in the absolute number of fibroblasts and a 5-fold increase in their average cell volume. In addition, their cytoplasm contained large amounts of rough endoplasmic reticulum in tumor-implanted animals (11). This suggests that the fibroblasts were induced, directly or indirectly, by the V2 carcinoma cells to proliferate and synthesize extracellular matrix components. A similar induction of proliferation and activation has been proposed for the stromal fibroblasts of invasive human colon carcinomas (26). Interestingly, the activation of these mesenchymal cells is also accompanied by an increase in their cytoplasmic volume and in the amount of rough endoplasmic reticulum (26).

All of our findings indicate that the hypertrophy of the rabbit mesentery was due to an increase in number and thickness of collagen bundles together with a marked increase in proteoglycan granules and hyaluronic acid filaments which all contributed to the formation of an overall denser structure. It is noteworthy that the proteoglycans of V2-implanted mesentery appeared to be different from control in both their glycosaminoglycan composition (Chart 2) and average size (Table 1). In contrast, recent studies (11) using the same animal model used here have shown that there is no qualitative alteration in the composition of collagen, in the ratio of type I to type III collagen, or in their relative amounts on a dry weight basis. The fact that intraperitoneal implantation of liver homogenates did not cause any abnormal production of extracellular matrix suggests that the changes observed here were induced by a specific interaction between neoplastic and host mesenchymal cells. The latter cells might have also been stimulated to divide, since a significant proportion of fibroblasts had entered the S phase following tumor implantation (11), and their absolute number had doubled.

The relative contribution of neoplastic cells and host mesen-
The correlation between increased glycosaminoglycan and cell migration has been also observed in tumors. For instance, Toole et al. (44) have demonstrated that hyaluronic acid content in the stroma around invasive V2 carcinoma in the rabbit is 3–4 times greater than around the same tumor grown in the nude mouse, in which it is not invasive. Also, the relatively small amount of chondroitin sulfate is increased in the stroma surrounding the invasive tumor. These observations are in close agreement with the present study. Furthermore, our results indicate that similar increases in hyaluronic acid and chondroitin sulfate content can occur away from the tumor cells, suggesting that a direct neoplastic-stromal cell contact may not be a prerequisite for stimulation of matrix biosynthesis.

In conclusion, our findings indicate that tumor cells with a capacity to invade neighboring tissues have the potential to stimulate host connective tissue cells to produce a hydrated, proteoglycan-rich environment which may be important for tumor progression and metastasis. Therefore, the possibility that neoplastic cells may "condition" their own pericellular environment offers a new perspective in understanding the mechanisms involved in the complex host-tumor cell interactions. Future characterization of the molecular mechanisms involved in the regulation of the desmoplastic response may allow a better understanding of its biological function in vivo.

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REFERENCES


Fig. 1. Light and electron microscopic photomicrographs of control (a–c) and liver-implanted (d–f) mesenteries. No significant difference in the overall thickness of the mesentery and the density and diameter of collagen fibers between control (c) and liver-implanted mesenteries (f) is noted. The tissue was processed in a stretched state and fixed in the presence of ruthenium hexamine trichloride as described in the text. a and d, 0.7-μm Epon sections counterstained with toluidine blue, × 780; b and e, transmission electron micrographs, × 7,250; c and f, transmission electron micrographs, × 28,500.

Fig. 2. Light and electron microscopic photomicrographs of V2 carcinoma implanted mesenteries at early (a–c) and middle (d–f) stage. Notice the absence of tumor cell aggregates and the progressive increase in overall thickness of the mesentery together with an increase in density of the collagen fibers and proteoglycan granules. The magnification of each figure is identical to that of Fig. 1.
Fig. 4. Transmission electron micrographs of mesenteric tissue from control (a, b) and V2 carcinoma-implanted animals at late stage (c, d). Note the increase in the density of collagen fibers and proteoglycan granules (PG). The size of the proteoglycan associated with the collagen fibers (empty circles) appear also to be increased in the V2 carcinoma-implanted animals. The fine RHT-positive filaments (double small arrowheads) are clearly more visible in the V2-implanted mesentery (c), where they appear to interconnect proteoglycan granules. a-d, × 120,000.

Fig. 3. Light and electron microscopic photomicrographs of V2 carcinoma-implanted mesenteries at late stage (a–f). Notice the absence of neoplastic aggregates and the overall marked increase in thickness of the mesentery (a, d), which is nearly 8 times thicker than control or liver-implanted animals (compare Fig. 1, a and d). a–c and d–f represent cross-sections of mesenteries from two different animals, respectively, and display a marked increase in the density of the proteoglycan granules (*,e) and collagen fibers (f). Magnification of each figure is identical to those of Figs. 1 and 2.
Neoplastic Modulation of Extracellular Matrix: Proteoglycan Changes in the Rabbit Mesentery Induced by V2 Carcinoma Cells

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