

# Glycoprotein, elastin, and collagen secretion by rat smooth muscle cells

(differentiation/extracellular matrix/smooth muscle sublines)

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**ABSTRACT** Smooth muscle cells from rat heart secreted extracellular matrix components at high rates for many generations in culture. The matrix proteins remained anchored to the culture dish and were characterized after removal of cellular material with sodium dodecyl sulfate. Sequential enzyme digestion demonstrated the presence of at least three components, including glycoprotein(s), elastin, and collagen. Prolonged extraction of the matrix with detergent under reducing conditions solubilized a fucosylated glycoprotein having an apparent molecular weight of 250,000 and two other proteins with molecular weights of 72,000 and 45,000, respectively. Sublines derived from discrete colonies of smooth muscle cells synthesized all of the matrix components, and the proportion of collagen secreted by some sublines increased with time in culture. The biosynthesis of a mixed extracellular matrix and the relationships among the component proteins were therefore studied in one system producing milligram quantities of material.

The extracellular matrix proteins are of great importance to the functioning of an organism. Present culture models for their biosynthesis are not altogether satisfactory because most cells tend to lose their differentiated properties when they are removed from their normal environment (1)—for example, 3T3 and 3T6 cells, which are often used for studies of collagen biosynthesis, have largely lost their ability to synthesize this protein (2, 3). However, it has recently been shown that primary avian tendon cells synthesize physiological amounts of collagen under the correct culture conditions (1).

The current interest in the proteins comprising the extracellular matrix (4-7) involves not only collagen and elastin but also the high molecular weight glycoproteins which probably play a major role in the organization and properties of the matrix. The microfibrillar protein of the elastic fiber has an apparent molecular weight of 270,000 (8) and has been suggested to be involved in the organization of the fiber (7). Fibronectin (LETS protein) has also been shown to be a predominantly matrix protein (9), to bind to collagen (10), and possibly to act as a bridge in the binding of cells to collagen (11). Our understanding of the biosynthesis, processing, and interactions of these proteins has been retarded not only by the lack of culture systems that produce them rapidly in physiological amounts but also by the lack of quantitative data on the matrix components. There is little information in the literature as to the actual quantities of material secreted by cultured cells, and no studies have been reported on the biochemical analysis of a mixed matrix produced *in vitro*.

In this report we describe a culture system in which large amounts of an extracellular matrix are formed in a relatively short time. Smooth muscle cells cultured from rat heart form a multilayered structure containing milligram quantities of connective tissue proteins in a crosslinked, insoluble form within

2 weeks, even after many generations in culture. These proteins remain firmly anchored to the culture dish after removal of the cells with detergent, allowing their quantitation and analysis by enzymatic techniques. We therefore studied the production of all of the insoluble matrix proteins in one culture system and the relationships among the components.

## MATERIALS AND METHODS

**Heart Cell Cultures.** Twelve hearts obtained from 3-day-old rats were excised and cut into small pieces with a pair of scissors. These pieces were washed twice in calcium- and magnesium-free phosphate-buffered saline and then trypsinized at room temperature in freshly prepared 0.1% trypsin (1:250; Difco). The first harvest of cells, obtained after 10 min, was discarded. The next four successive 30-min harvests were collected and the cells were obtained by centrifugation. They were then cultured in Eagle's minimal essential medium containing 10% calf serum, 10% tryptose phosphate broth (Difco), penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were seeded into 75-cm<sup>2</sup> tissue culture flasks (Falcon) at 4-5 × 10<sup>6</sup> cells per flask and incubated in a CO<sub>2</sub> incubator (95% air/5% CO<sub>2</sub>) at 37°C for 90 min. The supernatant medium was then poured off, the attached cells were washed once, and fresh medium was added. The cultures were passaged shortly before confluence at a ratio of 1:4, and the secondary cultures were trypsinized and frozen in liquid nitrogen at approximately 1.5 × 10<sup>6</sup> cells per 2-ml vial in medium containing 10% dimethyl sulfoxide. Each vial was used to establish one 75-cm<sup>2</sup> flask, and the cells reached confluence 6 days after seeding.

**Growth Curve Analysis and Production of Matrix.** In all experiments in which the behavior of the cells was being studied or radioactive matrix was being prepared, cultures received daily additions of 50 µg of ascorbic acid (Merck, Darmstadt, Germany) per ml; this was added as a 100-fold concentrated stock solution. Cultures were initiated at 10<sup>5</sup> cells per 35-mm culture dish (Corning) and the medium was changed every 2 days thereafter. The cell number was determined in a Coulter Counter after dispersion of the cells with 0.25% Viokase containing 0.05% collagenase. Trypsin was not adequate for this task because the cells became enmeshed in the extracellular matrix which was only partially digested by trypsin.

When production of radioactively labeled matrix was required, isotopes were added to culture medium at a level of 1 µCi/ml at a stage when matrix started to appear (days 6-7 of culture). The isotopes used were the following: L-(3,4(n)-

Abbreviations: NaDodSO<sub>4</sub>, sodium dodecyl sulfate; BAPN, β-amino-propionitrile.

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[<sup>3</sup>H]proline, 30 Ci/mmol; L-[1-<sup>3</sup>H]fucose, 3 Ci/mmol; L-[2,3,4-<sup>3</sup>H]valine, 15 Ci/mmol; L-[<sup>35</sup>S]cystine, 40 Ci/mmol; L-[U-<sup>14</sup>C]valine, 225 mCi/mmol.

The amounts of matrix produced were determined in dishes in which the cellular components were dissolved with sodium dodecyl sulfate (NaDodSO<sub>4</sub>). After the medium was removed, the cultures were washed once with water and allowed to stand for 30 min with 1% NaDodSO<sub>4</sub>. The first wash was discarded, and the dishes then were treated for a further 2–6 hr with the detergent. The dishes were then washed with a stream of distilled water, rinsed four times with 70% ethanol, and allowed to dry. The amount of protein present on the dish was determined by the method of Lowry *et al.* (12) after overnight dissolution of the matrix in 1 ml of 2 M NaOH at 37°C in a humid environment. A standard curve was prepared for this purpose from a 1:1 mixture of rat tail collagen and bovine elastin (Sigma) dissolved in 2 M NaOH. Alternatively, the protein on the dish was stained directly with Coomassie brilliant blue R250 (Sigma).

**Isolation of Sublines.** Fourth-passage cells were plated into 60-mm dishes (1–3 × 10<sup>3</sup> cells per dish) and allowed to grow as isolated colonies for 12–14 days. The plating efficiency was approximately 3.5%, and 70% of the colonies present were actively secreting matrix. Discrete colonies, containing vigorously growing cells, were then ring-isolated, trypsinized, and cultured in 35-mm dishes. Two sublines were grown to confluence and frozen in liquid nitrogen for further studies.

**Enzymatic Hydrolysis of Labeled Matrix.** Dried, radioactively labeled matrices were subjected to proteolytic digestion by trypsin (Sigma type III, pretreated with 5 mg of bovine elastin per ml to adsorb contaminating elastase), elastase (Worthington, ESFF), and collagenase (Worthington, CLSPA). The enzymes were all used at concentrations of 10 μg/ml in 0.1 M Tris-HCl, pH 7.6/10 mM CaCl<sub>2</sub>. The progress of digestion was followed by removing 100-μl samples at various times and determining the released radioactivity in 5 ml of Instagel (Packard). All liquid was decanted at the end of the enzymatic digestions, and the dishes were washed and treated with 2 M NaOH as described above. This solution was neutralized and the radioactivity in 200-μl samples was determined as above.

**Electrophoresis.** The electrophoretic system described by Porzio and Pearson (13) was used to analyze solubilized matrix components. Gels were calibrated by using a preparation of myofibrillar proteins obtained from rat heart (13).

## RESULTS

**Growth of Cells and Production of Matrix.** The primary cultures were subjected to a selection procedure to eliminate cardiomyocytes which take longer than other cell types to attach to plastic (14). This was accomplished by washing the primary cultures 90 min after plating; subsequently, little or no beating activity was seen. Both polygonal and fibroblastic cells were present in the cultures. Electron microscopy showed that the majority of the adherent cells had the typical appearance of smooth muscle cells (Fig. 1). The cells grew as tightly packed multilayers, and considerable extracellular material including collagen fibrils and smooth muscle basal laminae was often apparent. The detergent-insoluble matrix had a three-dimensional network appearance (Fig. 2).

The growth rate of and production of insoluble extracellular matrix by fourth-passage cells are shown in Fig. 3. The cells grew with a doubling time of approximately 48 hr and formed multilayered structures containing 1.5 × 10<sup>6</sup> cells per 35-mm culture dish. No change in morphologic appearance consistent with fibroblast overgrowth was observed, and the cells maintained a polygonal shape throughout the experiment. Extra-

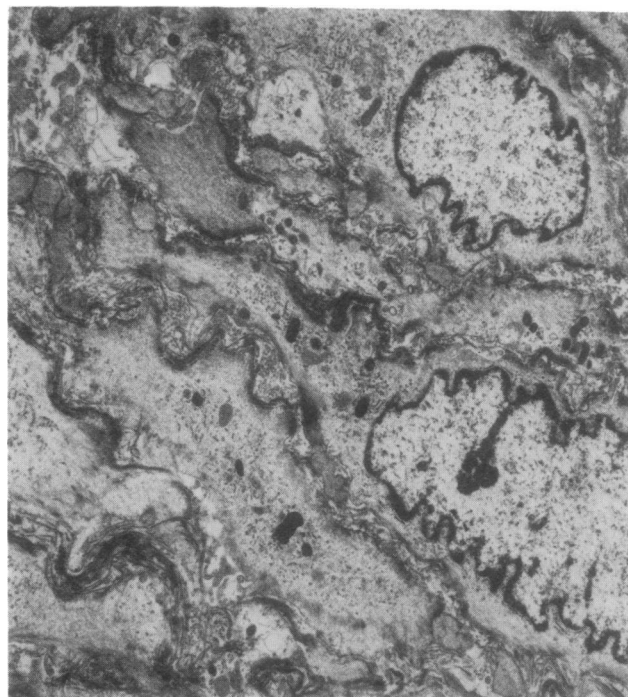


FIG. 1. Rat smooth muscle multilayer showing typical smooth muscle morphology. The section was cut at right angles to the plane of the culture dish. (×5000.)

cellular material began to appear 6–7 days after seeding and subsequently rapidly increased in quantity. Because as much as 33% of the total protein in the layer was matrix material, the cells could not be dissociated by trypsin alone, and a mixture of Viokase and collagenase was required to dissolve the structure and release the cells. In other experiments, up to 3 mg of matrix proteins was formed per 35-mm culture dish when cultures were maintained for 10 weeks. The deposition of insoluble matrix could be reversibly inhibited by the addition to the culture medium of the lathyrogen β-aminopropionitrile (BAPN), a compound that prevents the crosslinking of connective tissue proteins (15).

The composition of the insoluble matrix was probed by biosynthetic labeling with radioactive fucose, cystine, proline, or valine followed by sequential enzymatic digestion. Fucose and cystine were selected for their abilities to preferentially label the glycoprotein(s) components [these two compounds are

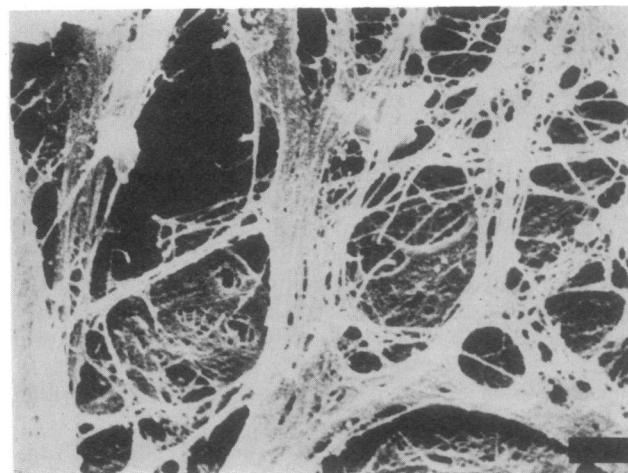


FIG. 2. Scanning electron micrograph of NaDodSO<sub>4</sub>-insoluble extracellular matrix produced by cultured smooth muscle cells. (×8500; bar = 1 μm.)

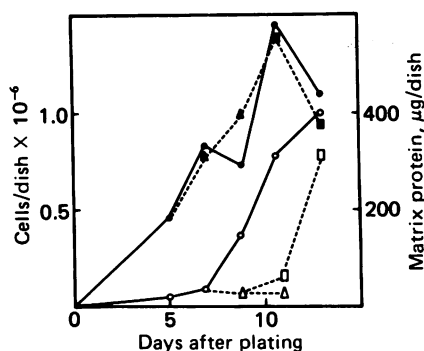


FIG. 3. Growth curves and production of NaDodSO<sub>4</sub>-insoluble matrix proteins by rat smooth muscle cells. Cultures were initiated in 35-mm dishes with 10<sup>5</sup> cells each; ascorbic acid (50 µg/ml) was added daily, and medium was changed every second day. Solid symbols, cell number; open symbols, amounts of NaDodSO<sub>4</sub>-insoluble material. ○, ●, Control; △, ▲, β-aminopropionitrile (BAPN) (50 µg/ml) added on day 5; □, ■, BAPN added on day 5 and removed on day 9.

present in glycoproteins but largely absent from elastin and collagen (8, 16)]. Proline was chosen as a label for all components, and valine is 10 times more prevalent in elastin than in collagen (7, 17). Because crosslinked elastin is not hydrolyzed by trypsin, but elastase digests proteins other than elastin (18), the sequence of enzyme treatment was always trypsin followed by elastase. Collagenase digestion was the final treatment used because collagen is insensitive to most proteases (19) and its digestion by this enzyme was retarded by the presence of the other matrix compounds.

Trypsin rapidly solubilized 93% of the fucose and 69% of the cysteine radioactivity from the matrix (Fig. 4*a* and *b*). In contrast, only 52% of the proline and 59% of the valine radioactivity were released from duplicate matrices by the same treatment. These results indicate that trypsin preferentially hydrolyses fucose-labeled glycoprotein components of the matrix. It is also likely that trypsin removes noncrosslinked precursor molecules which are known to be protease sensitive (20).

Elastase treatment released a further 32% of the proline-labeled and 35% of the valine-labeled matrix components (Fig. 4*c* and *d*) but only 5% of the fucose and 17% of the cysteine label. This suggests that the trypsin-insensitive material, which

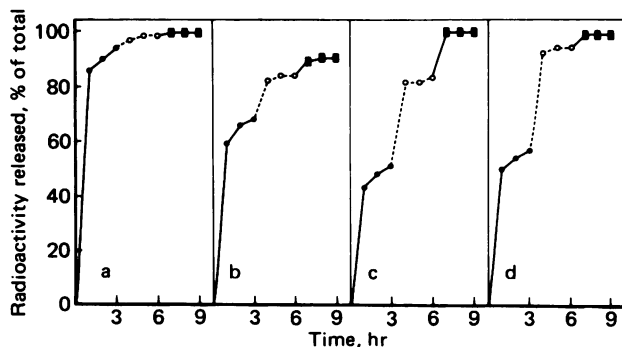


FIG. 4. Sequential release of radioactivity from NaDodSO<sub>4</sub>-insoluble matrix by trypsin (●—●), pancreatic elastase (○—○), and bacterial collagenase (■—■). Dishes containing labeled matrix were incubated in 0.1 M Tris-HCl, pH 7.6/10 mM CaCl<sub>2</sub> and the indicated enzyme (10 µg/ml) at 37°C. Samples were withdrawn at the indicated times for radioactivity determinations. The replacement of one enzyme for another after the 3-hr treatment time was carried out after rinsing with water. (a) Matrix labeled with [<sup>3</sup>H]fucose; (b) matrix labeled with [<sup>35</sup>S] cysteine; (c) matrix labeled with [<sup>3</sup>H]proline; (d) matrix labeled with [<sup>3</sup>H]valine.

Table 1. Solubility of extracellular matrix in various solvents

Solvent	% of total [ <sup>3</sup> H]proline solubilized	
	6-day cultures	13-day cultures
1% NaDodSO <sub>4</sub>	16	7
10 M urea	7	2
8 M urea + 1% NaDodSO <sub>4</sub> + 1% mercaptoethanol	28	9
5 M guanidinium chloride + 10 mM EDTA + 1% mercaptoethanol	25	11

[<sup>3</sup>H]Proline-labeled matrices were prepared by detergent treatment of 6-day or 13-day-old cultures of fifth-passage smooth muscle cells. The percentage of the total radioactivity present in the matrix that was solubilized by the indicated solvents in 5 days at 37°C was then determined.

was rapidly solubilized by elastase, represents crosslinked elastin. The fibrillar material remaining after trypsin and elastase treatments was solubilized by purified bacterial collagenase and, because it contained virtually none of the incorporated fucose, cysteine, or valine, it is therefore collagen. More recent experiments have shown that the presence of collagen and hydroxyproline in the matrix is absolutely dependent upon the addition of ascorbic acid to the growth medium. Furthermore, the percentage of collagen can be readily modulated by varying the ascorbic acid concentration (unpublished data). Amino acid analyses of the material solubilized by sequential trypsin, elastase, and collagenase treatments were in good agreement with published values for the microfibrillar protein, elastin, and collagen, respectively (unpublished data). These experiments therefore suggest that the matrix contained at least three components including glycoprotein(s), elastin, and collagen.

The matrix was highly insoluble in various denaturing solvents (Table 1). The solubility in all solvents decreased with increasing maturity of the cultures, an observation consistent with an increase in the degree of crosslinking with time. The material solubilized by the urea/NaDodSO<sub>4</sub> mixture from matrices produced after 6 days in culture contained three major polypeptide species when analyzed by polyacrylamide gel

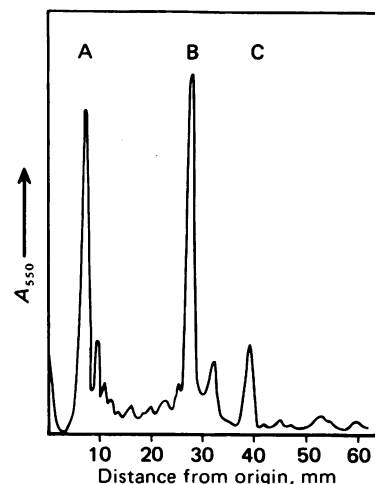


FIG. 5. Polyacrylamide gel analysis of material solubilized by 8 M urea/1% NaDodSO<sub>4</sub>/1% mercaptoethanol/0.5 M Tris-glycine buffer, pH 8.8 from matrix produced by cells after 6 days in culture. Gels stained with Coomassie blue were scanned on a Unicam SP 1700 apparatus at 550 nm.

Table 2. Analysis of matrices produced by smooth muscle sublines

Subline	Passage	% of total radioactivity solubilized by		
		Trypsin	Elastase	Collagenase
R22C1D	8	39	38	23
	20	44	16	37
R22C1F	8	19	28	51
	20	13	2	82

Sublines derived from mass cultures of rat smooth muscle cells were grown in the presence of [<sup>3</sup>H]proline and daily supplements of ascorbate. The matrices produced by the cells were then analyzed by sequential enzyme hydrolysis.

electrophoresis (Fig. 5). Band A, which was sensitive to trypsin digestion, had an apparent molecular weight of 250,000 and contained all of the incorporated [<sup>3</sup>H]fucose radioactivity when isolated from fucose-labeled matrices. Band B, molecular weight 72,000, contained high levels of [<sup>3</sup>H]proline and [<sup>14</sup>C]valine when isolated from cells cultured in the presence of these two isotopes (7). This was also found to be true for band C, molecular weight 45,000. A number of less-well-defined bands, which contained high levels of [<sup>3</sup>H]proline, were apparent between bands A and B, with molecular weights consistent with those reported for the  $\alpha$  and  $\beta$  chains of collagen (5).

Analysis of the matrices produced by two sublines isolated from the parental cultures was undertaken to determine whether individual cell types in the mass cultures secreted all components of the matrix or whether the constituent proteins were synthesized by different cell types in the mixed cultures. Two such sublines isolated from discrete colonies were found to secrete glycoprotein(s), elastin, and collagen, and the percentage of collagen synthesized increased with increasing passage level (Table 2). The results are unlikely to be due to overgrowth of one cell type by another because no morphologic changes were observed. Fibroblast contamination was also unlikely because it was difficult to isolate, from the parent cultures, colonies that grew with a fibroblastic appearance.

Not all of the sublines tested demonstrated increases in the collagen component with increasing passage level, but no diminution in the total amount of matrix synthesized by the cultures at high passage was observed. For example, one of the parental cell strains (R9) secreted 400  $\mu$ g of matrix per 35-mm dish at passage 4 and 930  $\mu$ g at passage 90.

## DISCUSSION

Any definition of differentiation must include not only the quality of gene expression but also the quantity. This has become particularly apparent with the realization that certain proteins once thought to be the products of specific cells (e.g., myosin and collagen) are synthesized by a wide variety of diverse cell types. It therefore follows that culture conditions should be such that differentiated cells can express their phenotype to an extent comparable to the *in vivo* situation (1).

The smooth muscle cells derived from rat heart synthesize and process large amounts of connective tissue proteins. Although we are unable to relate the quantities of protein produced to the situation in the intact animal, the composition of the matrix is similar to that reported for the aortic media (7). The matrix materials and cells actually formed a "tissue" in culture so that the structure was resistant to trypsin and could only be disaggregated by elastase and collagenase. From an experimental standpoint, it was also important that the matrix

proteins remained firmly attached to the culture dish after removal of the cellular material with detergent. Two other properties of the rat heart smooth muscle cell cultures commend them as a valuable experimental resource: these cells maintained their differentiated features for more than 90 passages, and sublines derived from individual colonies could be obtained with relative ease. These features have enabled us to obtain quantitative results and perform compositional analyses on the complete extracellular matrix elaborated by cultured smooth muscle cells. The rat system therefore has many advantages over other smooth muscle culture systems currently in use (21-24).

The glycoprotein(s) component of the matrix with a molecular weight of 250,000 was trypsin-sensitive and shares many of the properties of the microfibrillar protein (8) and fibronectin (9-11). This protein(s) probably plays a major role in the organization and structure of the matrix. The elastin component is highly crosslinked as shown by its resistance to denaturing solvents and also to trypsin. It is likely that the protein species of molecular weight 72,000, which was extractable from the matrix and contained a high ratio of valine to proline, is tropoelastin (7). The collagen in the matrix contained little cysteine or valine, was resistant to trypsin and elastase, and was highly susceptible to purified bacterial collagenase. Banded collagen fibrils have been seen in electron micrographs. The recent results of Mayne *et al.* (24) show that monkey smooth muscle cells synthesize types I and III collagen. It is also possible that the peptide with molecular weight 45,000 is identical to the CP45 species secreted by monkey cells (24).

Chen *et al.* (25) identified actin in the matrix prepared from chicken fibroblasts by treatment with a nonionic detergent, and it is therefore possible that actin is also present in the smooth muscle matrices. The extreme insolubility of the mature matrix in strongly denaturing solvents argues against any large-scale contamination by cellular proteins, particularly because no protein bands could be resolved by gel analysis of extracts of 12 to 14-day-old matrices. Experiments with [<sup>14</sup>C]thymidine (not shown) demonstrated that <19% of the cellular DNA remained associated with the matrix (i.e., <2  $\mu$ g of DNA per 400  $\mu$ g of matrix proteins). Although we have evidence that the cells produce sulfated proteoglycans, these molecules do not remain in the matrix preparation used here. The use of an ionic detergent such as NaDodSO<sub>4</sub> may therefore allow the preparation of a more insoluble matrix than that prepared with nonionic detergents (25, 26).

The interaction of the matrix proteins with each other and with cells is of fundamental importance to the properties of connective tissue. It is therefore significant that sublines of smooth muscle cells elaborated both the elastin and collagen components of the matrix and that the composition of the matrix changed in certain cultures with repeated subculturing. This latter observation is of particular significance in view of the fact that the extracellular material produced in atherosclerotic plaques is mainly collagen, whereas elastin is the major component of the aortic media (27). The changes in collagen/elastin ratio may mimic the pathological situation and it will be of interest to define conditions that alter the relative ratios of these proteins. It is also interesting that the cells secrete such large amounts of collagen at high passage in contrast to other cultured cells which rapidly lose their differentiated properties *in vitro* (1).

The availability of a matrix radioactively labeled in its specific components has made it possible for us not only to investigate the biosynthesis of all the insoluble proteins in one system but also to study the degradation of the matrix by hydrolytic enzymes produced by cultured cells. We have been able to

demonstrate the breakdown of the matrix by activated macrophages and malignant cells. These studies have a direct bearing on inflammation, tissue remodeling, and tumor invasion.

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