Effects of Vitamin A and Dexamethasone on Collagen Degradation in Mouse Mammary Adenocarcinoma

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ABSTRACT

Collagenases and other neutral proteases in tumors may facilitate tumor extension, invasion, and subsequent metastasis. We report the effects of vitamin A and dexamethasone, known inhibitors of collagenase production in vitro, on the collagen metabolism of mouse mammary adenocarcinoma and its capsule, borne by C3H/HeJ mice. The weight of the capsule was about 4% of the tumor, yet the total collagen content of the capsule was about 10-fold greater than that of the tumor tissue; tumor cells had no detectable collagen. With tumor growth, the collagenase and other neutral protease activities were increased in the tumor tissue; a negative correlation existed between collagenase activity and collagen content of the capsule. The protease activities of the tumor borne by vitamin A-treated hosts were about 50% lower than those of the controls; this coincided with a slight increase in the collagen content of the capsule. In contrast, the collagen content of the capsule borne by dexamethasone-treated hosts was 50% less than that of the controls; the protease activities were similar to the controls and occurred with tumor invasion and metastasis. Results suggest that the collagen metabolism of the capsule may be an indicator of proteolytic events within the tumor and the metastatic potential of the tumor that, in turn, suggests the possibility of preventing metastasis by inhibiting the production of collagenases and other neutral proteases, thereby localizing the tumor cells within the capsule. Vitamin A could be used for that purpose.

INTRODUCTION

Neoplastic invasion causes disruption of connective tissue barriers. It is considered likely that the collagenase (EC 3.4.24.7) that specifically degrades interstitial collagens (types I, II, and III collagens) may be important in this process (9) and that the collagenase that specifically degrades type IV collagen, the collagen of basement membrane, may promote metastasis (17). Collagenase activities have been found in many malignant tumors (1, 7, 12, 18, 23, 44) and in culture media of tissues and cells as an inactive form enzyme associated with a plasminogen activator and other proteases (38, 41, 42). The collagenases are found in the extracellular space surrounding tumor cells where degradation of connective tissue is quite evident or where extracellular matrix has been lost. This suggests that collagenase activity facilitates connective tissue breakdown associated with tumor invasiveness. The cellular origin of the collagenases in tumor tissues has yet to be identified conclusively (1, 12, 43, 44), but it is relevant that tumors are often infiltrated by macrophages, neutrophils, lymphocytes, and fibroblasts. Whatever the cellular origin, the production of collagenases appears to be controlled by a variety of factors.

Recently, it has been demonstrated (10) that antiinflammatory agents, such as medroxyprogesterone or DEX, known inhibitors of collagenase production in vitro (5, 6, 29), when locally applied to the tumor tissue blocked both collagenase production and neovascularization in vivo. In turn, tumor growth and invasion were prevented. Those results suggest that tumors elaborate or activate collagenases that facilitate vascularization of normal or peritumor tissues.

Inhibition by retinoic acid of collagenase production by rheumatoid synovial cells in culture (5) and the protection by VA against corneal ulceration vis a vis the production of collagenase have been reported (30). Those and other studies (22, 24, 26) suggest that VA may be involved in the mechanisms that control extracellular matrix degradation. That both inflammatory agents (retinoic acid and VA) and antiinflammatory steroids inhibit collagenase production or activation is surprising. Perhaps, each affects different aspects, e.g., production or activation.

More recent studies have shown that VA appears to be a potent promoter of differentiation of embryonal cell carcinomas (4, 31). The tumorigenicity of these cultures can be decreased effectively by exposure of the cells to retinoic acid (4, 31). However, neither the collagenase activities nor the collagen production of these tissues has been studied. In this paper, we report the effects of VA and of DEX on some aspects of collagen metabolism on C3HBA tumor and its capsule borne by C3H/HeJ mice, and how these relate to tumor invasion and metastasis.

MATERIALS AND METHODS

Materials. Reagents were obtained as follows: dansyl: amino acids and DEX from Sigma Chemical Co. (St. Louis, MO); VA from Nutral Biochemicals (Cleveland, OH); dansyl chloride from Pierce Chemical Co. (Rockford, IL); dansyl hydroxide and dansyl: amine from Seikagaku Kogyo Co. (Tokyo, Japan); HPLC-grade solvents from Fisher Scientific Co. (Pittsburgh, PA); 4'-6-diamino-2-phenylindoledihydrochloride from Boehringer Mannheim GmbH (West Germany); and Dubesco’s modified Eagle’s medium (39) from Grand Island Biological Co. (Grand Island, NY). The octadecyl-C-18 column was obtained from IBM Instrument, Inc. (Danbury, CT). Female C3H/HeJ mice at 5 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME). The starter inoculum C3HBA cells were provided by Dr. Eli Seifter, the Departments of Surgery and Biochemistry in this Institution. Other reagents were the same as described in previous publications (3, 35–37).

Inoculation and Administration of Reagents. Inocula were prepared

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3311
from 2-week-old tumors as follows: The tumor tissue described below was cut into small pieces and then sonicated in 50 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 10 mM NaN₃ (1 g tissue per 9 ml) at 4°C. Portions of the tissue were used for isolation of collagenase as follows. A portion of the skin and tail were excised and were studied as nontumor tissue, collagenous tissue such as stromal, capsule, or fascial tissue mainly capsule was homogenized. Fascia adjacent to the capsule and a portion of skin and tail were excised and were studied as nontumor tissues from tumor-bearing mice. Additionally, excised tissues corresponding to the above, from age-matched noninoculated controls, were prepared. A portion of each of these tissues was used for determination of hydroxyproline (Hypro) content and for RIA. The remaining tissues were cut into small pieces and then sonicated in 50 mM Tris-HCl, 5 mM CaCl₂, 0.2 mM NaCl (Tris:NaCl buffer) (pH 7.5) containing 0.1% Triton X-100 and 10 mM NaN₃ (1 g tissue per 9 ml) at 4°C. Portions of the sonicate were used for enzyme assays and for isolation of collagenases by an affinity column chromatography using Sepharose 4B conjugated with anti-granuloma collagenase IgG antibody as described elsewhere (37); the rest was used for other assays. For RIA, to assure that maximal enzyme protein was measured as described previously (37), the collagenase in the tumor was solubilized by urea as follows. The tumor tissue was homogenized and extracted twice with 4.5 mg/mL of tissue of 4 M (pH 7.75) and 20% Solvent B (methanol) with a flow rate 1 ml/min and measured by fluorescence detector. The yield of dansyl:Hypro or dansyl-[3H]Hypro from Hypro or purified [3H]Hypro, in the presence or absence of hydrolysates of BSA equal to 0.05 μmol of amino acids, was 90 ± 5% of the standard dansyl:Hypro. The dansylation was linear from 0.1 to 110 nmol of Hypro. The yield of dansyl:Hypro of guinea pig skin type I collagen was 0.082 μmol of Hypro per μmol of leucine. In this mobile phase, the expected amino acids that have retention time close to the Hydropro (30 min) are asparaginase, glutamic acid, 5-oxoproline, and serine. However, none of the first 3 amino acids is present in acid hydrolysates, while the retention time of serine (40 min) is longer than that of the Hydropro. The optimum dansylation of Hydropro was tested for pH, 6 to 12; temperatures, 4–70°C; reaction time, 5 to 200 min; and the molar ratio of amino acid to dansyl chloride, 1:1 to 1:11; and we found that, at pH 11, 50°C for 15 min reaction time with a 1:2 ratio gave the highest yield.

Enzyme Assays. Latent collagenase was activated proteolytically with trypsin using an enzyme:protein ratio of 1:100, at 25°C for 10 min. To assure maximal collagenase activity, this treatment was followed by addition of 6-fold molar excess of soybean trypsin inhibitor to further inhibit trypsin activity as described (35). Collagenase activity was measured by a reconstituted collagen fibril assay (35) using [14C]glycine-labeled guinea pig skin type I collagen substrate (3000 cpm/500 μg of substrate) in 50 mM Tris-HCl, 5 mM CaCl₂, 0.2 mM NaCl buffer, pH 7.5, containing 10 mM NaN₃ and 1 mM disopropyl fluorophosphate at 35°C. Enzyme activity was expressed as units: 1 unit of collagenase is the amount that degrades 1 μg of native collagen per min at 35°C. Other neutral protease activity was measured using soluble casein acetylated with [14C]acetic anhydride as substrate (17,500 cpm/500 μg of substrate) in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaN₃ at 35°C (36). Enzyme activity was expressed as units: 1 unit of protease is the amount that hydrolyses 1 μg of casein per min at 35°C.

RIA of Collagenases. The collagenase protein was measured by RIA using rabbit anti-collagenase IgG antibody raised against purified non-tumorous origin mouse granuloma collagenase. The competition curve for RIA was established by competitive binding of 125I-labeled and unlabeled antigen collagenase to the anti-collagenase IgG antibody as described previously (37). In brief, 125I-labeled antigen (41,000 cpm/100 ng) and various amounts of unlabeled antigen (0.1 to 500 ng) were reacted in the presence of anti-collagenase IgG (2.4 μg) that yields a bound/free ratio of approximately 1.0, for 40 h at 4°C; this was followed by reaction with second antibody (goat anti-rabbit IgG antiserum) for 2 h at 4°C. The immune complex formed was precipitated by centrifugation at 27,000 × g for 5 min at 4°C, and the 125I radioactivity in the precipitate was counted. The amount of 125I-labeled collagenase bound to antibody was 94 ± 2%. The recovery of 125I-labeled antigen in precipitates made with 10% trichloroacetic acid:0.25% BSA was 99 ± 1%, and the nonspecific binding of 125I-labeled antigen to normal rabbit serum was 6.4 ± 2%.
Each RIA included “total counts” tubes containing 125I-labeled collagenase alone, tubes in which normal rabbit serum replaced first antibody, and tubes in which RIA buffer replaced the test sample.

Morphological Examination. For the light microscopic examination, the excised tissues were immediately fixed in phosphate-buffered formalin. The thin slices of the tumor with capsule, the liver, and both lungs were embedded in paraffin, sectioned at 3-μm thickness, and stained with hematoxylin and eosin, Mallory’s trichrome, mucicarmin, periodic-acid Schiff with and without diastase digestion, and for reticulin. For ultrastructural studies, grossly viable areas of the tumor were fixed for 3 h in a solution of 3% glutaraldehyde:0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 1% phosphate-buffered Millonig’s osmium for 1 h. After rinsing and dehydration, the specimens were embedded in Epon (3), sectioned at 0.5- to 1.0-μm thickness, and stained with alkaline toluidine blue. The selected areas showing good tissue preservation were cut into thin sections and stained with uranyl acetate and lead citrate.

Other Methods. Amino acid content was determined colorimetrically (25) using leucine as a standard. Protein content was determined also colorimetrically (36) with BSA as a standard. DNA content was determined fluorometrically (16) using sonicated calf thymus DNA as a standard. Molecular weight was determined by gel filtration, SDS-polyacrylamide gel electrophoresis, and immunological cross-reactivity by double immunodiffusion analysis as described (3, 36). Significance of the data was tested by analysis of variance and the Yates corrected χ² test to determine sufficient differences between groups; P values <0.05 were considered significant.

RESULTS

Tumor Growth. The tumor growths at inoculation sites at 2, 4, 6, and 8 weeks after tumor cell inoculation were, respectively, about 1, 2, 4, and 5.5 g; the capsule weight was about 4 ±2% of the tumor. The established VA dose had no detectable animal morbidity; also, it did not decrease the size of the tumor as compared with the controls. Before experiments, we tested the dose of DEX (0.5 to 10 μg/ml) on hosts’ morbidity and examined changes in the weights of spleen, liver, and total body (used as parameters), since administration of DEX (10 μg/ml) to the age-matched, non-tumor-bearing normal controls decreased markedly and reversibly the spleen and body weights from those of the untreated normal controls. A dose of 7 μg of DEX per 3.5 ml per day per animal, estimated from water intake containing 5 μM DEX, caused no evident morbidity on non-tumor-bearing normal controls and had least effects on these parameters in the tumor-bearing hosts; DEX did decrease reversibly the size of tumor by 50%, as compared to that of the untreated controls. However, that decrease was accompanied by increased tumor invasion and metastases (lung, liver, spleen, and pancreas), as contrasted with VA-treated hosts and untreated controls (Table 1). This was statistically significant (P < 0.0001). However, the difference between the VA-treated and untreated controls on tumor metastases was not statistically significant (P < 0.3). The 2- to 3-fold increased dose of DEX decreased further the size of tumor and the other weight parameters; yet tumor invasion and metastases perhaps increased, and animals died within 1 to 3 weeks. One-half of the established dose of DEX had no effect on tumor growth, as compared with the untreated controls. Those results were the average of 4 independent experiments with 5 animals for each time point. It should be noted that the capsule of the DEX-treated hosts often fused with the fascial tissue; the properties of the capsule may have been changed by the DEX treatment, as contrasted with the VA-treated hosts or untreated controls.

Morphology of the Tumor. Light microscopic examination of the tumor revealed extensive areas of necrosis. Some had the appearance of typical coagulation necrosis, with cells displaying very pale cytoplasm and ghost nuclei. Other areas were infiltrated by recent hemorrhages. The well-preserved areas of the main tumor showed a well-developed collagenized capsule at the inoculation site, especially at the dermal level in the VA-treated and untreated controls (Fig. 1a, b, c, and d). The tumors in all 3 groups of mice showed similar histological patterns. The most common presented as solid fields of packed cuboidal or polygonal cells with inconspicuous vascular supply and collagen stroma (Fig. 1a, b). Intermingling with solid areas, tumor cells were arranged in gland formations or small acini surrounded by thin capillaries (Fig. 1c). In other areas, frequently connected with solid areas, the tumor grew as papillary formations. These had a delicate stromal core with vessels and a few collagen fibers, lined by a single layer of cuboidal cells (Fig. 1d). Cytologically, the tumor cells had polygonal or round shapes with large nuclei, prominent nucleoli, and slightly acidophilic vacuolated cytoplasm (Fig. 1c and d). Mucicarmin stain was negative. Besides extensive areas of necrotic tumor, there were isolated necrotic cells displaying strong eosinophilia, contracted cytoplasm, and picnotic nuclei. Mitotic figures were found in the 3 histological types (Fig. 1c) being more frequent in the solid-type tumors, ranging from 2 to 10 per high-power field with ×40 objective.

DEX-treated hosts showed tumors with a more aggressive pattern. Besides metastasizing to distant organs, the tumors invaded adjacent structures. The main pathways of spread were through lymphatic permeation and by direct extension, especially noticeable in the abdominal muscles and in the diaphragm (Fig. 2).

Tumor metastases tended to grow as solid masses. The tumor cells were clustered together, and there was no evidence of capsular encirclement and only scanty blood supply (Fig. 3a to c).

Our ultrastructural observations of samples of the transplanted tumor were consistent with previous reports (20). Most cells were very undifferentiated. They showed a polygonal shape, with large, bizarre nuclei and scanty cytoplasm. The plasma membranes were usually simple, straight, with rare projections towards neighboring cells. Rudimentary microvilli were frequently seen delineating small canaliculi. Desmosomes and hemidesmosomes were rarely found. Occasional cells, especially those arranged in an acinar fashion, were resting on a thin, uniform, electron-dense basal lamina.
Cytoplasmic organelles were sparsely distributed throughout a background of ribosomes mostly as free particles or arranged in small clusters. Rough endoplasmic reticulum was scanty, and only isolated, short ribosome-studded cisternae were seen. Some of the rough endoplasmic reticulum cisternae contained some finely granular electron-dense material suggestive of secretion material within their lumina. Smooth endoplasmic reticulum was absent, and the Golgi apparatuses were small, with 3 or 4 flattened cisternae. Mitochondria were small, short, with few cristae, and their matrices showed low electron density. Lysosomes were inconspicuous, and no peroxisomes were identified. Glycogen was absent, and cells undergoing degeneration showed large lipid vacuoles. Type A and type B viral particles, having characteristics previously described (2, 14), measuring between 75 and 100 nm, were seen on numerous occasions within the cytoplasm and in the intercellular spaces. Nuclei were usually large and with numerous indentations. The heterochromatin was uniformly and densely distributed. Nucleoli were frequently single and of large size with prominent associated chromatin.

The stroma was virtually absent except for very thin capillaries and rare bundles of collagen. Occasional fibroblasts were seen in tumor areas adjacent to the capsule.

**Collagen Content of the Tumor.** The collagen content of the capsule was about 10-fold greater than in the tumor tissue; tumor cells had no detectable collagen (Chart 1). The collagen content of the capsule and tumor tissue borne by DEX-treated hosts was about 50% less than that of the controls (Chart 1). In contrast, the collagen content of the capsule borne by VA-treated hosts was about 15% greater than that of the untreated controls; however, this was statistically not significant. The collagen content of the capsule of the controls appeared about equal to the fascial tissues adjacent to the capsule; the collagen content of the capsule of VA-treated hosts appeared about equal to non-tumor-bearing normal fascial tissues (Chart 1).

**Collagenase and Other Neutral Protease Activities.** Along with tumor growth, collagenase and other neutral protease activities were increased from 2 to 8 weeks (Chart 2). Enzyme activities of the tumor borne by VA-treated hosts were about 50% of the controls; DEX-treated hosts showed no change from that of the controls (Chart 2). Not only was collagenase activity decreased in the tumor of the VA-treated hosts, but so was the amount of enzyme protein (25%). Approximately one-half of the enzyme protein was present as trypsin activatable inactive form, so that the specific activity appeared to be decreased by 25%. The collagenase activity of the tumor borne by the DEX-treated hosts did not change from the controls and was present as an active form (Table 2).

**Properties of C3HBA-associated Collagenase.** The SDS-polyacrylamide gel electrophoretic patterns of the degradation products resulting from the action of collagenase on type I collagen revealed that the action of tumor-associated collagenase is similar to that of the known tissue collagenases (Fig. 4A). The collagenase was insensitive to diisopropyl fluorophosphate (1 mm) but was inhibited by 2-phenanthroline (1 mm), EDTA (10 mm), and to mouse serum (1:50, v/v). In double immunodiffusion analysis, the collagenase cross-reacted with anti-collagenase IgG raised against purified non-tumor-origin mouse liver granuloma collagenase (3, 35).

**Competition for **125I-labeled Collagenase Binding to Anti-
none of the interaction effects was significant. In other words, the relations of collagenase and time and of collagen and time were the same for all 3 groups. However, Newman-Keules *pathoc* tests revealed that the specific type of variation among the 3 groups differed for collagenase and collagen. For collagenase, there were significant differences between the VA-treated group (mean = 24.1) and both the DEX-treated group (mean = 43.4) and the untreated control group (mean = 44.8). Newman-Keules Q probabilities were P < 0.01 for both comparisons. The DEX and control groups did not differ significantly. For collagen, on the other hand, significant differences occurred between the DEX-treated group (mean = 18.8) and both the untreated control (mean = 37.1) and VA-treated (mean = 42.6) groups, with Q probabilities of P < 0.01 and P < 0.001 for the 2 comparisons, respectively. The control and VA-treated group did not differ significantly. A regression plot of the data of Table 3, obtained for each experimental group (VA-treated, DEX-treated, and untreated controls), was linear when the collagen content of the capsule was plotted against the amount of collagenase activity (correlation coefficient, 0.958, 0.983, and 0.951, respectively).

Results indicate that the correlation between the collagenase activity of the tumor and the collagen content of the capsule appears to be negative. The unchanged collagenase activity of the tumor borne by DEX-treated hosts coincided with a 50% decreased collagen content of the capsule as compared with that of the controls. This occurred with the distinctly more aggressive behavior of the tumor as revealed by invasion of neighboring structures (abdominal wall, diaphragm, omentum, pancreas) and metastases to distant organs.

**DISCUSSION**

C3HBA mouse mammary adenocarcinoma alters its growth and metastatic properties significantly in response to VA supplementation of the host C3H/HeJ mice (22, 28). If mice are inoculated with 1 to 5 x 10^4 cells, the tumor develops in 2 weeks; the tumors grow and then metastasize to liver and lung. Mice die from these metastases about 10 weeks after inoculation. If mice are fed chow supplemented with VA, tumor incidence is decreased. Interestingly, those tumors that do develop and become encapsulated are not highly invasive but grow to very large proportions; and animals survive more than twice as long as the controls. If tumors are removed from moribund animals and inoculated into normal mice, tumors develop and metastasize: VA does not alter the capacity for metastasis of cells per se (22, 28). Based on these and other studies (4, 5, 26, 27, 30, 31), we have postulated that VA inhibits collagenase production in the tumor and/or increases collagen deposition in the tumor capsule or both. That postulate is supported by the present studies.

Malignant tumors appear to contain subpopulations of tumor cells with widely differing metastatic potential (8, 21). The interactions among various tumor cell subpopulations or between tumor cells and host cells or their products may be important in regulating the expression of the metastatic phenotype and, in particular, in controlling the production of proteinase activities. This is in accord with the report that addition of medium obtained from the elicited macrophage cultures increased production of collagenase activity in Lewis lung carcinoma cultures (13). We also observed that the amount of collagenase protein and activity found in the medium of primary C3HBA tissue cultures was...
COLLAGEN METABOLISM IN MALIGNANT TUMOR

Table 2

Collagenase activity and protein

These values were the average of 2 independent experiments with 5 animals, in duplicate samples for each time point.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Time after inoculation (wk)</th>
<th>Collagenase activity (units/mg DNA)</th>
<th>Collagenase protein (μg/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor homogenate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>4</td>
<td>35 ± 1.9* (35 ± 1.3)</td>
<td>10.4 ± 0.3 (9.5 ± 0.3)*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>52 ± 2.2 (50 ± 1.8)</td>
<td>12.0 ± 0.3 (11.3 ± 0.3)</td>
</tr>
<tr>
<td>VA treated</td>
<td>4</td>
<td>23 ± 0.9 (12 ± 0.6)</td>
<td>7.5 ± 0.3 (6.2 ± 0.2)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>27 ± 1.6 (13 ± 0.9)</td>
<td>8.0 ± 0.2 (7.3 ± 0.2)</td>
</tr>
<tr>
<td>DEX treated</td>
<td>4</td>
<td>41 ± 1.3 (40 ± 0.9)</td>
<td>11.0 ± 0.3 (10.0 ± 0.3)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>46 ± 1.6 (45 ± 1.6)</td>
<td>12.2 ± 0.3 (10.8 ± 0.2)</td>
</tr>
</tbody>
</table>

*The collagenase in the homogenate of C3HBA tumor was measured.
*The collagenase protein in the 4 M urea extracts of the same homogenate as above was measured by RIA.
pector, mean ± SE.
*Activity obtained before trypsin digestion.
Protein measured in the supernatant in Footnote a.

![Chart 3. Competition for binding of 125I-collagenase to anti-collagenase IgG antibody by unlabeled antigen collagenase and collagenase in the urea extracts of the C3HBA tissue. Ordinate, percentage of control binding in presence of competing agent divided by cpm bound in the absence of competing agent. Abscissa, upper scale (μl) applicable to all competing agents and lower scale (ng) applicable only to the antigen collagenase. Competitive agents containing collagenases were obtained from the 4 week growth tumor homogenates by extraction with 4 M and 8 M urea and fractions obtained from the chromatography of the 4 M urea extraction on a Sephacryl S-200 column. Competing agents are as follows: open symbols indicate the collagenase in the 4 M urea extraction; solid symbols indicate 8 M urea extraction of the tumor homogenates; C, tumors obtained from the control hosts; O, tumors obtained from the VA-treated hosts; G, tumors obtained from DEX-treated hosts; O and V, fractions at 1.8 times the void volume; O, fractions at 3 times the void volume; O, unlabeled antigen collagenase.

Fig. 4. SDS-polyacrylamide gel electrophoresis. A, enzyme:collagen reaction mixture. The reaction mixture that contained 0.05% guinea pig skin type I collagen, 4 units of collagenase in 50 mM Tris-HCl:5 mM CaCl2:0.2 M NaCl buffer (pH 7.5), was reacted for 2 h at 25°C. The enzyme activity was terminated by addition of 10 mM EDTA. The mixture was then electrophoresed in gels of 0.1% SDS:10% polyacrylamide. Gels are: Lane 1, reaction mixture without enzyme; Lane 2, reaction mixture with the supernatant of the tumor homogenate; and Lane 3, reaction mixture with mouse granuloma collagenase [a known mammalian collagenase prepared by us (33)]. Arrows, bands that correspond, respectively, to β and α collagenase polypeptide chains; α and α', fragments of the α chains. B, collagenases. C3HBA-associated collagenases eluted from an anti-granuloma collagenase IgG conjugated Sepharose 4B column were electrophoresed in 0.1% SDS:10% polyacrylamide gels. The protein bands were detected by staining with silver. Gels represent: on the left, antigen collagenase; on the right, tumor-associated collagenases. Apparent molecular weight was determined by coelectrophoresis of known protein standards (phosphorylase b, M, 94,000; BSA, M, 68,000; ovalbumin, M, 43,000; chymotrypsinogen A, M, 25,000); buffer front.

greater when the cultures contained various cell populations and decreased when the cultures became less heterogeneous, suggesting that some cells or combinations of cells in the heterogeneous cell population appear to be responsible for the increased production of collagenase activity (data not shown). These observations suggest that the target cells have a potential to produce greater amounts of collagenase activity under certain conditions. Specific activity of the enzyme, on the other hand, can be influenced by the amounts of procollagenase activator and other neutral proteinases present during the process of activation or by the extent of glycosylation of the enzyme protein (19, 39). Procollagenase appears to have an autocatalytic property; thus, it autoactivates quantitatively without noticeable loss in molecular weight. Proenzyme was also activated by treatment with a variety of agents (34).

In many respects, the C3HBA-associated collagenase is similar to the enzyme purified from tissues of various species. Thus, the apparent molecular weight values of the inactive and active
Table 3
Collagenase activity of the tumor and collagen content of the capsule

<table>
<thead>
<tr>
<th>Samples</th>
<th>Time after inoculation (wk)</th>
<th>Collagenase activity of the tumor (units/mg DNA)</th>
<th>Collagen contents of the capsule (μmol Hypro/mmol leucine)</th>
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<tr>
<td>Untreated</td>
<td>2</td>
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<td>3</td>
<td>33.8 ± 1.9</td>
<td>38.5 ± 1.5</td>
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<td>38.2 ± 1.5</td>
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<td>44.2 ± 3.0</td>
<td>38.0 ± 1.3</td>
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<td>55.2 ± 3.1</td>
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<td>8</td>
<td>57.4 ± 3.2</td>
<td>34.5 ± 1.1</td>
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<tr>
<td>VA treated</td>
<td>2</td>
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<td>44.3 ± 2.1</td>
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<td>43.4 ± 1.5</td>
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<td>43.5 ± 1.5</td>
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<td>30.2 ± 2.4</td>
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<tr>
<td></td>
<td>8</td>
<td>32.2 ± 2.2</td>
<td>40.5 ± 1.8</td>
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<tr>
<td>DEX treated</td>
<td>2</td>
<td>34.2 ± 2.8</td>
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* Mean ± SE.

forms are in the range of literature values (M, 35,000 to 84,000) (11), and the specific activity (4,000 units/mg of protein) is also in the range of literature values (33 to 8,700 units/mg of protein) (18, 36, 44). The antibody raised against purified non-tumor origin M, 60,000 collagenase reacted with C3HBA-associated collagenases with molecular weights of 55,000 and 60,000, suggesting that immunologically they have structural similarities. Even though some of the several collagenases studied have immunological cross-reactivity, one has no a priori reason to believe all should have identical molecular weight as reported by us (37) and also by other workers (19, 39). However, a marked distinction between the 2 enzymes is their specific activity. If the 100-fold greater specific activity of the tumor-associated collagenase over that of the antigen granuloma collagenase is due to the presence of greater amounts of activator and other neutral proteinase in the tumor tissue, then the contribution of these proteinase activities may also play an important role in the collagen metabolism of the tissue. However, there is yet no consensus concerning the relationship between amounts of collagenase activity found in vivo and in vitro.

Overall, the results suggest that the invasiveness of the tumor may be related to the collagenase and other neutral proteinase activities. The increased collagenase activity correlated in some respect with the decreased collagen content of the capsule, and a 50% decreased collagen content of the capsule borne by the DEX-treated hosts coincided with the distinct invasion of the tumor and metastases, as contrasted with the VA-treated hosts and controls. Possibly, proteolysis at the site of the tumor cell invasion may be a prelude to the subsequent metastasis. In that regard, an agent that inhibits or limits the production of collagenases and other neutral proteinases, so that an adequate connective tissue barrier can form around the tumor cells, may in some degree prevent tumor cells from spreading. In that respect, VA may be acting as a positive agent to the hosts. The protection by VA may be more effectively demonstrated as the difference in the time sequence in tumor growth that occurs in early tumor growth between VA-treated hosts and untreated controls. Thus, the VA-treated hosts show much longer lag periods for the detectable tumor growth at the inoculation site (e.g., about 4 weeks, when 1 to 5 × 10⁶ cells are inoculated) as compared with that of the untreated controls (about 2 weeks). Since the current study was designed to demonstrate the metabolism of collagen in the tumor and its capsule, using fast-growing tumors, probably it could not allow observation of significant protective effects of VA as compared with untreated controls, because of the short duration of the experiment. DEX, on the other hand, may act as a negative agent to the hosts, by not only leaving collagenase and other neutral proteinase activities unchanged, but also by decreasing collagen content of the capsule. In any experiment of short duration, agents that enhance tumor spread are more easily detected than agents that inhibit tumor spread.

In part, in DEX-treated hosts, the decreased collagen content of the capsule that consists of major type I and minor type IV collagens may be due to the decreased synthesis of capsule collagen. Thus, treatment of neonatal chicks with DEX was reported to selectively decrease skin collagen synthesis in a time- and dose-dependent fashion, as evidenced by occurrence of decreased type I procollagen mRNAs, while other protein mRNAs remained relatively unaltered (32, 33). In that study, the maximum decrease (78%) in collagen synthesis occurred when the chick was given a single injection of 1.6 mg of DEX per kg of chick. Whether the unchanged collagenase activity of the tumor borne by DEX-treated hosts is due to certain genetic or epigenetic characteristics of the given cell strains, as reported for the effects of retinoic acid on the production of various proteinase activities in cultures (5, 27, 29), requires further investigation. One may note that the dose of DEX used did not affect significantly the collagen content of the skin and tail as compared with that of the untreated or 0.25% ethanol-treated controls (data not shown). That suggests that the effect of DEX is more specific in tissue actively growing or tissue in the remodeling stage as compared with tissue in the phase of stationary growth. However, it is not yet known if the tumor capsule is actively growing in the DEX-treated host, what the origin is of the stromal-like tissue of the tumor, and whether the metastases are related to the degradation of vascular basement membranes by relative increase in type IV collagenase activity.

Results suggest that collagen metabolism of the capsule may be an indicator of the proteolytic events within the tumor and of the metastatic potential of the tumor. They suggest the possible prevention of metastasis by inhibiting the production of collagenases and other neutral proteinases by VA, thereby localizing tumor cells within the capsule.

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REFERENCES

COLLAGEN METABOLISM IN MALIGNANT TUMOR

Fig. 1. Mouse with s.c. tumor implant. a, control mouse. The skin structures and the tumor itself are well preserved. The tumor is made of polyhedral cells with minimal stroma. The capsule is thin. H & E, x 320. b, vitamin A-treated mouse. It shows a thick, collagenized capsule and 2 venules. H & E, x 320. c, acinar arrangement of tumor cells with scanty stroma. A mitotic figure is shown in the upper part. H & E, x 800. d, papillary growth of the tumor. The tumor cells are mostly arranged in a single cell layer over a delicate fibrovascular stroma. Epon-embedded, 1-μm-thick section. Toludine blue, x 800.
Fig. 2. Mice treated with DEX. Three different degrees and modes of tumor infiltration of adjacent tissues are shown. In Fig. 2a, the expanding tumor has broken through the capsule and invaded the abdominal muscle layers. Some tumor cells have permeated between muscle fibers. Additionally, Fig. 2b shows nests of malignant cells in the interstitium of the diaphragm or within lymphatics. In other areas, groups of closely packed malignant cells have wedged through the capsule, invading the muscle as in Fig. 2c. The infiltration by tumor is associated with various degrees of degenerative changes of the muscle fibers. H & E, \( \times 155 \) (a) and \( \times 280 \) (b and c).
Fig. 3. Mouse treated with DEX. a, lung showing several metastases. The tumor cells are arranged in gland-like formation with no evidence of encapsulation. H & E, × 75. b, liver metastasis. The normal hepatic parenchyma (L), close to a portal space, is infiltrated by tumor cells (T). H & E, × 480. c, pancreatic metastasis. Acini of normal exocrine pancreas (P) are infiltrated by tumor cells (T). H & E, × 480.
Effects of Vitamin A and Dexamethasone on Collagen Degradation in Mouse Mammary Adenocarcinoma

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