Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 33-38

COLLAGENOLYTIC ACTIVITY OF RABBIT V_2-CARCINOMA GROWING AT MULTIPLE SITES*

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Received November 18, 1977

SUMMARY: Rabbit V_2-carcinoma was maintained by serial transfer of cell suspensions prepared from solid invasive tumors growing in the thigh muscles of rabbits. No collagenolytic enzyme, active or activatable, was found in extracts of 17 of 19 tumors removed from this site. In contrast, collagenolytic activity was detected in all extracts prepared from V_2-carcinoma growing in a subcutaneous site in all of seven rabbits. In five rabbits, tumor growth was initiated both subcutaneously and intramuscularly. Homogenates prepared from these tumors showed collagenolytic activity in both sites. No activity was extracted from tumors growing at two different intramuscular sites in the same animal. These results suggest that the site of tumor growth influences collagenolytic activity and the expression of this enzyme at an intramuscular site may be influenced by a second, subcutaneous, neoplasm.

INTRODUCTION: The potential role of collagenolytic enzymes in tumor invasion has led us to study this question in rabbits bearing a strain of V_2-carcinoma which has been serially transferred by intramuscular passage for many years. McCroskery et al. (1) prepared homogenates from a different strain of this neoplasm which was carried in the ascites form but transferred to an intramuscular (IM) site prior to use in their experiments. They found high levels of active collagenase in tumor extracts. Steven and Itzhaki (2) detected trypsin-activatable collagenase, but not spontaneously active collagenase, in extracts of IM V_2-carcinoma which also had been maintained in the ascites form.

We report here, our studies on the influence of the site of tumor growth on collagenolytic activity of an invasive rabbit carcinoma.

MATERIALS AND METHODS: Reagents and their sources included: Ultrapure ammonium sulfate (Schwarz-Mann, Orangeburg, NY); Tris, trypsin and mersalyl acid (Sigma

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* This is publication No. 741 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. This research is supported by NIH grants (CA19158) and (AM03564) and a grant from the Massachusetts Chapter, Arthritis Foundation.

0006-291X/78/0801-0033$01.00/0

Chemical Corp., St. Louis, MO); l-tosylamido-2-phenylethylchloromethyl ketone (TPCK)-trypsin and soybean trypsin inhibitor (Worthington Biochemical Corporation, Freehold, NJ); ^{14}C-labelled acetic anhydride (New England Nuclear Corp., Boston, MA).

Maintenance of V_2-Carcinoma in Rabbits

The V_2-carcinoma (3) was obtained from Dr. J. Folkman, Children's Hospital, Boston, Massachusetts. The tumor was maintained by serial intramuscular passage in rabbits. For transplantation, a rabbit bearing the tumor for 10-12 days was killed by injection of sodium pentobarbital and the tumor dissected from the surrounding muscle. The central tumor mass was cut into small slices in lactated Ringer's solution and pressed through a stainless steel grid (60 mesh). The resulting cell suspension was injected into each thigh muscle of a 2 kg New Zealand white male rabbit. 2.5 ml of cell suspension representing approximately 1 gm of tumor, was injected at each site. For subcutaneous initiation of tumor growth the same volume of cell suspension was injected subcutaneously in the scapular region.

Preparation of Tumor Extracts

Extracts were prepared from tumor initiated at intramuscular (IM) or subcutaneous (SC) sites 2-4 weeks earlier. Rabbits were killed and tumors excised as described above. The central tumor mass was sliced and homogenized at 4°C in 0.05M Tris-HCl (pH 7.4) - 1.0 M NaCl-5mM $CaCl_2$-0.02% sodium azide in a Brinkmann polytron (PT-350) homogenizer at high speed for two 30-second intervals. The homogenate was frozen in liquid N_2, thawed at room temperature and centrifuged at 29,000 x g for 30 minutes (1). The resulting pellet was re-extracted with buffer containing 0.05M Tris-HCl (pH 7.4), 0.2M NaCl-1mM $CaCl_2$ and 0.02% sodium azide, followed by centrifugation. The second supernatant was combined with the first and the combined supernatants were adjusted to 20% saturation with $(NH_4)_2SO_4$. The precipitate was saved for assay and the supernatant adjusted to 50% saturation with $(NH_4)_2SO_4$. The second precipitate and the supernatant were collected after centrifugation at 29,000 x g for 30 minutes and the precipitate was dissolved in .05M Tris-HCl (pH 7.4), 0.2M NaCl-1mM $CaCl_2$-0.02% sodium azide. Solutions were dialyzed overnight against the same buffer at 4°C and all fractions were tested for collagenolytic activity. In all instances, enzyme was found only in fractions precipitated by $(NH_4)_2SO_4$ between 20 and 50% saturation.

Assay for Collagenolytic Activity

Collagenolytic activity was measured by the collagen fibril lysis assay (5) using reconstituted ^{14}C-acetylated rat tail tendon collagen (6); 100 µl of 0.2% collagen solution having a specific activity of approximately 12,700 cpm/mg was mixed with 100-200 µl of the enzyme preparation and incubated at 37°C for 4 hours. Substrate plus buffer alone and substrate plus 0.01% trypsin were included as controls in each assay. Results were expressed as units of collagenase activity per gm of tumor tissue; 1 unit of collagenase represents the quantity of enzyme capable of lysing 1 µg of collagen/minute.

Trypsin Treatment of Tumor Extracts

The trypsin-activatable form of collagenase was detected after treatment of the tumor extract with TPCK-trypsin (90µg/ml) at 37°C for 5 minutes. Trypsin activity was inhibited by the addition of a fivefold excess of soybean trypsin inhibitor; the treated extract was assayed as described above. Non-enzymatic activation of tumor extracts was attempted by incubation of the enzyme preparation with 0.021 M mersalyl at 37°C for one hour (Harris, E.D., personal communication).

For identification of collagen degradation products, reaction mixtures (0.3 ml) containing 100 µg of acid soluble rat tail tendon collagen in 10 mM Tris-0.4M NaCl (pH 7.4), 200 µl of tumor extract and L-arginine (50mM) (added to inhibit fibril formation) were incubated at 35°C for 2 hours. The reaction products were subjected to polyacrylamide disc gel electrophoresis at pH 4.0 as described by Nagai et al. (7).

RESULTS AND DISCUSSION:

A. Effect of site of tumor growth on collagenolytic activity

Extracts were prepared from the intramuscular growths of 19 rabbits and collagenolytic activity was assayed before and after activation with trypsin. Trypsin-activatable enzyme was detected in only two of 19 extracts (Table I). In 17 inactive preparations, no enzyme was detected in any of the fractions. Several procedures known to dissociate enzyme-inhibitor complexes in other systems were tested for their ability to reveal collagenolytic activity in inactive IM tumor extracts. These procedures included dialysis of the initial homogenate against 3 M NaSCN (8); heating the pellet obtained by centrifugation of the tumor homogenate at 60°C for 4 minutes (9), extraction of the pellet with 0.1% Triton-100, or 5 M urea in the extraction buffer (10). No enzyme activity was detected in any instance. Treatment of the IM extracts with mersalyl also failed to elicit collagenolytic activity.

In order to explore the possible influence of the in vivo environment of the tumor, we examined the collagenolytic activity in extracts of tumors initiated at a subcutaneous site. Extracts were prepared from such tumors established singly in each of seven animals. Following activation with trypsin, collagenolytic activity was detected in all extracts (Table I). Collagenolytic activity was also detected in extracts treated with mersalyl. Thus, the site of tumor implantation may determine whether or not extractable collagenase is produced.

In an attempt to account for the difference in collagenolytic activity between V_2-carcinoma initiated at an intramuscular and a subcutaneous site, the tumors were examined histologically. No obvious difference in the appearance of the tumor or in surrounding cellular infiltrate was noted. Rabbits bearing growths at either intramuscular or subcutaneous sites regularly exhibited metastasis to regional lymph nodes.

TABLE I

COLLAGENOLYTIC ACTIVITY OF TUMOR INITIATED AT A SINGLE SITE: INTRAMUSCULAR OR SUBCUTANEOUS

No. of Animals	Site of tumor implantation	Tissue assayed for collagenase activity	µg collagen degraded/ min./gm tumor tissue
17	Muscle	IM tumor[a]	0
1	"	"	2.18
1	"	"	1.82
Total: 19			
7	Subcutaneous	SC tumor[b]	2.07 ± .14[c]

a = Tumor implanted in muscle

b = Tumor implanted subcutaneously

c = Mean value ± standard deviation

B. Effect of a subcutaneous tumor on collagenolytic activity of an intramuscular tumor

We tested the effect of a SC tumor on the collagenolytic activity of an IM tumor by initiating growth at both sites simultaneously. Extracts were prepared from both tumors and both extracts were found to have trypsin-activatable collagenolytic activity (Table II). This observation was confirmed with extracts prepared from the IM and SC tumors of five rabbits. Collagenolytic activity in IM and SC tumor extracts from the same animal was approximately equal on a weight basis although there was considerable variation among animals. Both IM and SC tumor extracts were shown by polyacrylamide disc gel electrophoresis to produce the same reaction products characteristic of the animal collagenases (5).

Our observation that the presence of an SC tumor leads to the appearance of collagenase activity in an IM tumor might be explained by the production of stimulator by the former. If such a substance was released into the circulation it could possibly elicit collagenase production by the intramuscular V_2-carcin-

TABLE II

COLLAGENOLYTIC ACTIVITY OF TUMORS INITIATED AT TWO SEPARATE SITES: INTRAMUSCULAR AND SUBCUTANEOUS

No. of Expts.	Site of tumor initiation[a]	Tissue assayed for collagenase activity[b]	µg collagen degraded/ min./gm tumor tissue
1	IM and SC	IM	1.0
	"	SC	1.0
2	"	IM	1.45
	"	SC	1.50
3	"	IM	0.19
	"	SC	0.15
4	"	IM	1.7
	"	SC	3.2
5	"	IM	6.47
	"	SC	6.09

a = Tumor implanted at both sites simultaneously

b = Tumor implanted intramuscularly and subcutaneously in the same animal were assayed separately for enzyme activity.

oma. It is not known whether the appearance of collagenolytic activity in the IM tumor extract requires the presence of tumor at a subcutaneous site or whether an inert mass or an inflammatory reaction at this site would lead to similar results. We are currently testing these possibilities.

We cannot as yet explain our inability to extract collagenase from the IM tumor (in the absence of SC tumor). Conceivably, enzyme might not be synthesized, or, alternatively, it might exist as an irreversible enzyme-inhibitor complex.

ACKNOWLEDGEMENT: We thank Dr. R. B. Colvin for reviewing the histologic sections and for his interpretation of the pathologic findings.

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