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Collagenolytic Activity in Malignant Melanoma: Physicochemical Studies[1]

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SUMMARY

Human malignant melanoma was shown to contain collagenolytic enzymes. With the use of radioactively labeled reconstituted collagen substrate, the enzyme activity of the tumor homogenate supernatant released radioactivity up to 24 times greater than normal skin and 100% more than benign melanotic tumors such as a junctional nevus and a cellular blue nevus. The known specific collagenase inhibitors significantly inhibited the enzyme activity, whereas soybean trypsin inhibitor did not. Kinetic studies demonstrated a linear increase of collagenolytic activity with respect to enzyme concentration and time of incubation. The optimum pH for enzyme action was 8.5. Disc electrophoresis of the reaction mixture showed collagen degradation products such as β^A, α^A, and α^B. Viscometric studies demonstrated that melanoma crude enzyme decreased the specific viscosity of salt-soluble guinea pig tropocollagen. The denaturation temperature of tropocollagen was decreased as a result of incubation with the melanoma homogenate. These results clearly indicate that the tumor contained active collagenase *in vivo*.

INTRODUCTION

Although the cause of neoplastic proliferation of the epidermal melanocytes is not known, the invasion of the melanocytes into the dermis, as it occurs in primary malignant melanoma of the skin, requires rupture of the basal lamina and dissolution of dermal collagen and ground substances. Since the basal lamina contains collagen (13), it is reasonable to presume that collagenase activity in the tumor facilitates the dissolution of this barrier. Histologically, it was found that collagen degeneration is quite evident in the vicinity of the invading tumor masses.[5] This study, as the 1st part of 2 reports, deals with biochemical and physicochemical demonstrations of collagenoltyic activity of primary malignant melanoma of the skin.

MATERIALS AND METHODS

The melanoma was retrieved from a disarticulated lower extremity. This tumor measured 11 x 8 x 3.5 cm and protruded sharply from the underlying skin. It originated from a nevus located on the plantar aspect of the right foot. For many years there was no change in the state of the lesion, but 2 years prior to definitive surgery it grew rapidly, became fragile, and bled easily. The patient was a 67-year-old Negro female who also had regional lymphatic and pulmonary metastases.

A junctional nevus measuring 2.5 x 2 cm was surgically excised under 1% Xylocain anesthesia from the forearm of a healthy 20-year-old white male. This nevus had been present for 8 to 10 years without any noticeable change in character.

A healthy 24-year-old Negro female was the source of the cellular blue nevus. It was located on the dorsum of the right foot and measured 1.5 x 1 cm. It was surgically excised under 1% Xylocain anesthesia. Surgically removed normal skin from the lower extremities was used in control studies. Human sera were drawn from 3 normal individuals for inhibitor studies. Histopathological diagnosis of each condition was established in hematoxylin and eosin-stained tissue sections.

Preparation of Tumor Homogenate. The excised specimen was immediately frozen and kept at $-20°$. The tumor was homogenized with a ground-glass homogenizer in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.001 M $CaCl_2$. Since the melanoma was very soft, it took only a few minutes to grind the tumor. The homogenization was done in an ice bath. As this melanoma was distinctly black and protruded above and over the surrounding uninvolved skin, there was little likelihood of including normal skin in the homogenate. Quantitative determination of tumor protein was done by the method of Lowry *et al.* (17). The homogenate was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was used in the following studies.

Preparation of Substrate. Uniformly labeled proline-^{14}C in doses of 25 μCi each or uniformly labeled glycine-^{14}C in 2 doses of 50 μCi each was injected i.p. into randomly bred white weanling guinea pigs. The animals were killed by decapitation 48 hr after the injection. The pelt was removed

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and s.c. tissue was scraped off mechanically. Neutral salt-soluble collagen was extracted and purified by the method of Gross (8). The final products were lyophilized and stored in a desiccator at −20°. The isotopes were obtained from Schwarz/Mann, Orangeburg, N. Y.

Preparation of Incubation Mixture. A highly viscous solution of reconstituted collagen was prepared by the method of Nagai *et al.* (18). Aliquots of 0.5 ml of this solution were pipetted into plastic centrifuge tubes and allowed to gel in a 37° water bath for at least 12 hr. Prior to admixture with the tumor homogenate, the reconstituted collagen substrate was disrupted with a steel needle in order to ensure good contact with the homogenate. A 0.2-ml aliquot of the supernatant of the tumor homogenate (crude enzyme solution) was added to each tube. All tubes were incubated at 37° for 18 hr with constant agitation. Homogenates of the junctional nevus, cellular blue nevus, and normal skin were similarly admixed with the substrate and incubated. After incubation, tubes were centrifuged at 59,000 × *g* at room temperature for 30 min to sediment undissolved collagen. A 0.5-ml aliquot of the supernatant was added to 10 ml of Insta-Gel (Packard Instrument Co., Downers Grove, Ill.), and the radioactivity was counted in a liquid scintillation spectrometer.

Kinetic Studies. Aliquots of 0.2 ml of the supernatant of the tumor homogenate were used for the studies of (*a*) enzyme concentration and collagenolytic activity and (*b*) incubation time and collagenolytic activity. For the study of pH-dependent collagenolytic activities, aliquots of 0.2 ml of the same crude enzyme solution were incubated at pH 5 and 5.5 in acetate buffer, at pH 6.0 and 6.5 in Tris-maleate buffer, at pH 7.0, 7.6, 8.0, 8.5, and 9.0 in Tris-HCl buffer, and at pH 9.5 in glycylglycine buffer. Salt-soluble, reconstituted guinea pig collagen which was labeled with glycine-^{14}C (see above) was used as substrate.

Caseinolytic Activity. Caseinolytic activity of the tumor homogenate was measured by the methods of Kunitz (14) and of Nagai *et al.* (16).

Inhibition Studies. Aliquots of 0.2 ml of supernatant of tumor homogenate were added to various reaction mixtures containing 0.5 ml of salt-extracted guinea pig collagen which had been labeled with either proline-^{14}C or glycine-^{14}C; Tris-HCl buffer, pH 7.6; and one of the following inhibitors, to make a total of 1.0 ml of each reaction mixture: (*a*) normal human whole serum at final dilutions of 1:10, 1:50, 1:250, and 1:500; (*b*) EDTA at a final concentration of 0.001 M; (*c*) 100 μg of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.); and (*d*) cysteine at a concentration of 0.01 M.

Disc Electrophoresis. Aliquots of several incubation mixtures were analyzed by disc electrophoresis through acrylamide gels with a Canalco apparatus. In addition, Fractions 2A and 2B of acid-soluble tropocollagen were prepared from calf skin by the method of Rubin *et al.* (19) and were used as substrate. The purity of these fractions was checked by amino acid analysis. The data were provided in our previous publication (10). The reaction mixture was first incubated for 18 hr at 27°, a temperature below that of tropocollagen denaturation. EDTA (0.001 M) was added to the reaction mixture to prevent further action of the enzymes, and then the reaction mixture was denatured at 45° for 10 min at pH 4.8. A 50-μl aliquot of the mixture was run at pH 4.5 at room temperature. The electrophoretic analysis was carried out as previously described by Dabbous *et al.* (2).

Viscometric Method. Viscosity was measured in Ostwald viscometers with a flow time for water of 80 to 95 sec. The method of Gallop *et al.* (7) was used with slight modifications. The temperature of the water bath was maintained at 27 ± 0.1°. The reaction mixture consisted of 5 mg of Fractions 2A or 2B of acid-soluble tropocollagen in 3 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.04 M $CaCl_2$ and 1.0 ml of supernatant of tumor homogenate. The viscosity was measured after various periods of incubation.

Optical Rotation and Melting Curves. A Zeiss polarimeter with a hydrogen light source (365 nm) was used to measure optical rotation. The concentration of tropocollagen used in these experiments was calculated from the optical rotation measurement obtained on denatured samples with −460° as the specific optical rotation of denatured gelatin. Melting curves were constructed by measuring the optical rotation of samples as the temperature was increased from 27 to 42°. Before each measurement, the samples were allowed to equilibrate at that temperature for 30 min. The enzyme action was terminated by lowering the pH to 3.6 to 3.8 and also by the addition of EDTA to a final concentration of 0.01 M. Samples, at pH 3.8, were kept in water-jacketed 1-dm polarimeter tubes during measurement.

RESULTS

Collagenolytic Activities on Salt-soluble, Isotope-labeled Guinea Pig Collagen

Release of Radioactivity. Collagenolytic activities of melanoma, junctional nevus, cellular blue nevus, and normal skin were tabulated in terms of cpm for solubilized collagen gel, percentage lysis of collagen gel, and percentage lysis of collagen gel per mg tumor protein (Table 1); the last

Table 1

Release of radioactivity and caseinolytic activity

Substrate[a]	Tissue protein (mg/0.2 ml)	cpm solubilized (above blank)	Collagen gel lysed (%)	% lysis/mg protein	Caseinolytic activity (μg trypsin equivalent/mg protein)
		Melanoma			
Glycine-^{14}C-labeled	4.3	812	12.6	2.9	9.9
Proline-^{14}C-labeled	4.3	600	14.9	3.5	9.9
		Junctional nevus			
Glycine-^{14}C-labeled	0.5	0	0.0	0.0	5.2
		Cellular blue nevus			
Glycine-^{14}C-labeled	0.52	0	0.0	0.0	9.6
		Normal skin			
Glycine-^{14}C-labeled	1.5	34	0.5	0.4	7.7
Proline-^{14}C-labeled	4.7	60	1.4	0.3	3.0

[a] Salt-soluble guinea pig collagen.

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value was the most convenient measure for a comparison of the activities of 4 kinds of specimens. Both glycine-^{14}C- and proline-^{14}C-labeled collagens were lysed significantly more by melanoma (2.9 and 3.5) than by the nonmalignant tumors and normal skin (0.4 and 0.3) (Table 1). No collagenolytic activity was detected in the junctional nevus or the cellular blue nevus (Table 1). Caseinolytic activity (trypsin-like activity) of melanoma was 9.9 μg trypsin equivalent per mg tumor protein, whereas those of the normal whole skin and dermis alone were 7.7 and 3.0 μg, respectively; the activity of the junctional nevus was 5.2 and the cellular blue nevus showed a value of 9.6 (Table 1). Thus, the caseinolytic activity of each specimen did not necessarily correlate with its collagenolytic activity and was not significantly higher in melanoma than in others (Table 1).

Enzyme Inhibitors. With glycine-^{14}C labeled substrate, it was demonstrated that hunan serum at a dilution of 1:10 inhibited the collagenolytic activity of melanoma to 72.0%, but the inhibitory effect rapidly dropped to 11.7% at the dilution of 1:500 (Table 2). EDTA at 0.001 M inhibited collagenolytic activity to 70%; however, the inhibitory effect did not increase when the concentration of EDTA was increased to 0.01 M. Cysteine (0.01 M) inhibited the collagenolytic activity only moderately (64.3%) (Table 2). Soybean trypsin inhibitor did not inhibit the activity more than 7.4%. This agreed with the finding that caseinolytic activity, *i.e.*, trypsin-like activity, was low in melanoma (Table 1) and indicated that trypsin was not responsible for the major part of the collagenolytic activity of the tumor. Since the cpm of the glycine-^{14}C-labeled substrate collagen in the incubation mixture was 6440, 566 cpm released by 50 μg trypsin is 8.7%. A similar range of collagenolysis by trypsin has been observed.[5]

Kinetic Studies. Collagenolytic activity increased linearly with the increase of crude enzyme concentration (Chart 1). The radioactivity released was linearly increased with incubation time. In each experiment, radioactivity released was significantly higher in experimental than in control specimens (substrate alone). Radioactivity released from both the 0-hr and 18-hr controls were not markedly different, indicating that substrate degradation due to prolonged incubation was relatively small. The amount of radioactivity released from the substrate alone between the 0-hr and 18-hr incubation times increased only 12 to 19%, whereas the substrate degradation by the crude enzyme increased linearly to greater than 140% (Chart 2). The optimum pH for the enzyme action was between 8 and 9, with a peak at 8.5 (Chart 3). The pH optimum remained the same at 12- and 18-hr incubation periods.

Disc Electrophoresis. The control tropocollagen yielded a characteristic pattern (Fig. 1). The fast-moving α chains (α_2 and α_1) were sequentially followed by the relatively slow-moving β chains and the slow-moving higher-molecular-weight components (Fig. 1, *left*). The pattern produced by the reaction products was different in that there appeared discrete sets of new bands. Simultaneously, the density of all the bands seen in the control (α_2, α_1, β, and higher-molecular-weight components) diminished or disappeared (Fig. 1, *right*), indicating that the new bands were produced by degradation of tropocollagen molecules. For example, fast-moving dense bands appeared in front of α_2, representing a set of α^A, while the original α_2 and α_1 became obscured or disappeared (Fig. 1). In the β chain region, a new set of bands moving faster than the original β components appeared (Fig. 1). This new band region seemed to represent β^A. Most of the high-molecular-weight components have disappeared. Low molecular-weight components appeared toward the buffer front and could represent molecules related to β^B (Fig. 1).

Viscosity Changes. The viscosity of acid-soluble calf skin tropocollagen was reduced as a result of incubation with supernatant of melanoma. At 27°, a temperature well below that of collagen denaturation, the specific viscosity of dilute tropocollagen solution at pH 7.6 was reduced in an approximately linear fashion by about 50% in 26 hr (Chart 4), whereas no significant viscosity change was observed in the control sample (Chart 4). This period of linear decrease was then followed by a slower rate of viscosity drop. After 48 hr of incubation (not shown in Chart 4), the loss of specific viscosity amounted to 75% of the value of the control collagen solution. With boiled melanoma superna-

Table 2

Inhibition studies

	Glycine-^{14}C, salt-soluble		Proline-^{14}C, salt-soluble	
	cpm	% inhibition	cpm	% inhibition
Melanoma complete	812		600	
Melanoma with serum (1:10)	227	72.0	163	72.9
Melanoma with serum (1:50)	588	27.6	478	20.3
Melanoma with serum (1:250)	642	20.9		
Melanoma with serum (1:500)	738	11.7	526	12.3
Melanoma with EDTA (0.001 M)	240	70.5	164	72.7
Melanoma with cysteine (0.01 M)	290	64.3	314	47.7
Melanoma with soybean trypsin inhibitor (100 μg)	752	7.4	600	00.0
Trypsin (50 μg)	566		346	
Trypsin with soybean inhibitor (100 μg)	77	86.4	50	85.5
Total radioactivity of incubation mixture	6440 cpm		4031 cpm	

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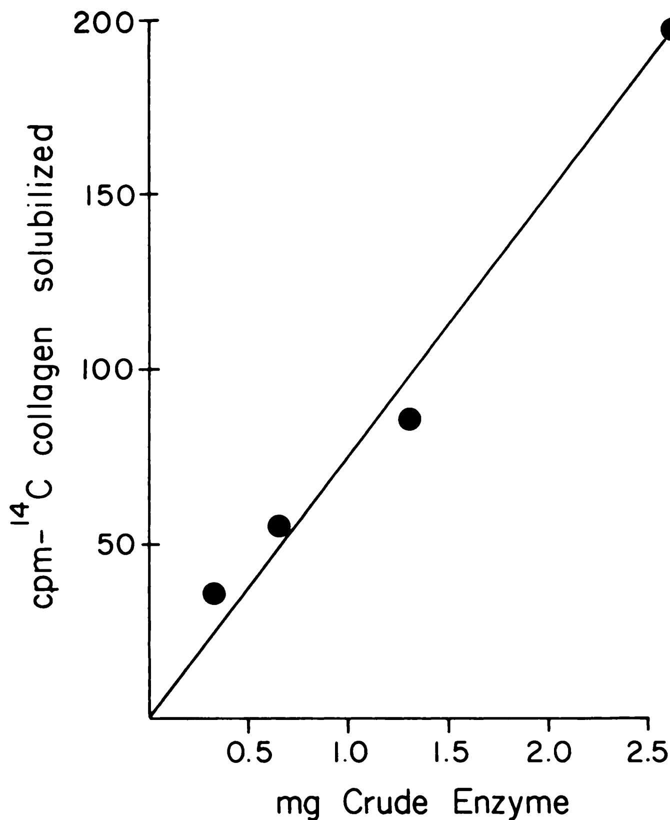

Chart 1. The degradation of salt-extracted, glycine-^{14}C-labeled guinea pig collagen substrate by melanoma collagenase as a function of crude enzyme concentration is measured by release of radioactivity after 18 hr of incubation (see text for details).

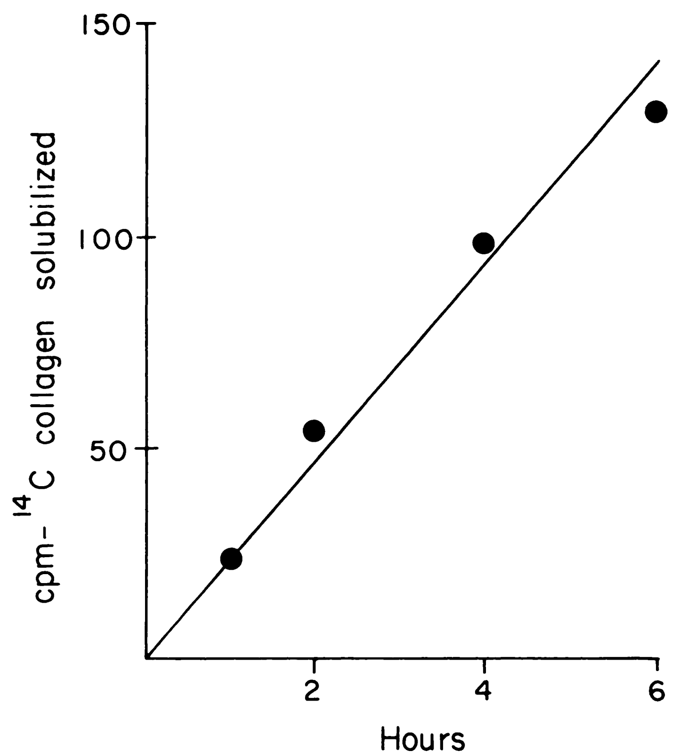

Chart 2. The degradation of collagen by melanoma crude collagenase as a function of time is measured by release of radioactivity from glycine-^{14}C-labeled, salt-soluble, guinea pig-reconstituted collagen (see text for details).

tant, no viscosity drop was observed. For this control experiment, the supernatant was previously heated in a boiling water bath for 5 min to inhibit enzyme activity.

Melting Curves. Denaturation of acid-extracted tropocollagen under mild conditions results in the formation of gelatin as a result of unwinding of the triple helix and the disorganization of the poly-L-proline type of structure. The process involved was previously reviewed by Von Hippel (21).

The denaturation temperature midpoint (*Tm*) of the substrate tropocollagen was about 40° (Chart 5). No detectable change in the negative optical rotation of acid-soluble calf skin tropocollagen solutions was observed as a result of incubation with the melanoma supernatant at 27° (Chart 5). This indicated that the triple-helical structure of the tropocollagen macromolecule remained intact during the treatment. When the reaction mixture was heated to 45° in a step-wise manner, it was noted that the denaturation temperature midpoint (*Tm*) of the same substrate was lowered to 31°. This decreased stability toward heat indicated that some of the stabilizing structures along the collagen macromolecule were lost as a result of cleavage of the tropocollagen helical polypeptide chains. The melting curve showed a plateau region at −650, approximately 78% denaturation (Chart 5). This biphasic characteristic of the

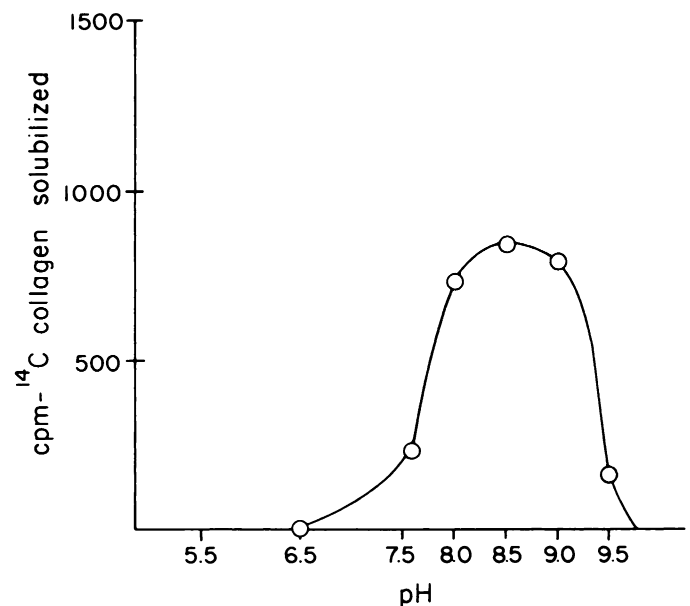

Chart 3. The degradation of collagen by melanoma crude collagenase as a function of pH dependence is measured by release of radioactivity from glycine-^{14}C-labeled, salt-soluble, reconstituted collagen after 18 hr of incubation (see text for details).

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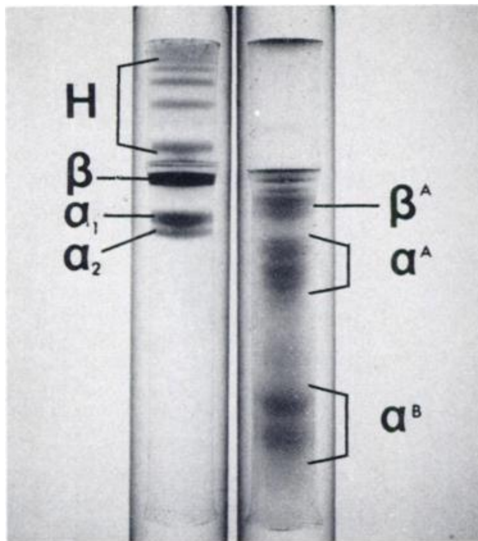

Fig. 1. Polyacrylamide disc electrophoretic patterns of control tropocollagen after denaturation (*left*) and melanoma-treated denatured tropocollagen after 24 hr of incubation at 27° (right). In the control (*left*), α (α_2,α_1), β, and γ, or heavier chain components (*H*) can be seen. In a melanoma-treated sample (*right*) those original components became lighter or obscure, and new bands such as α^B, α^A, and β^A representing degradation products of the corresponding original components appeared.

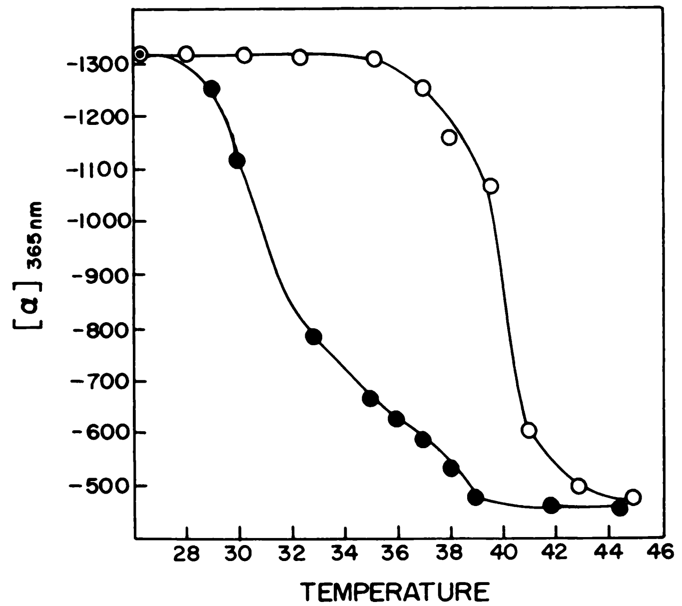

Chart 5. Melting curves of normal acid-soluble tropocollagen control (O) and melanoma crude enzyme-treated tropocollagen (●). Denaturation was carried out in 0.05% acetic acid (pH 3.8), and the temperature was raised in discrete steps to obtain the melting curves.

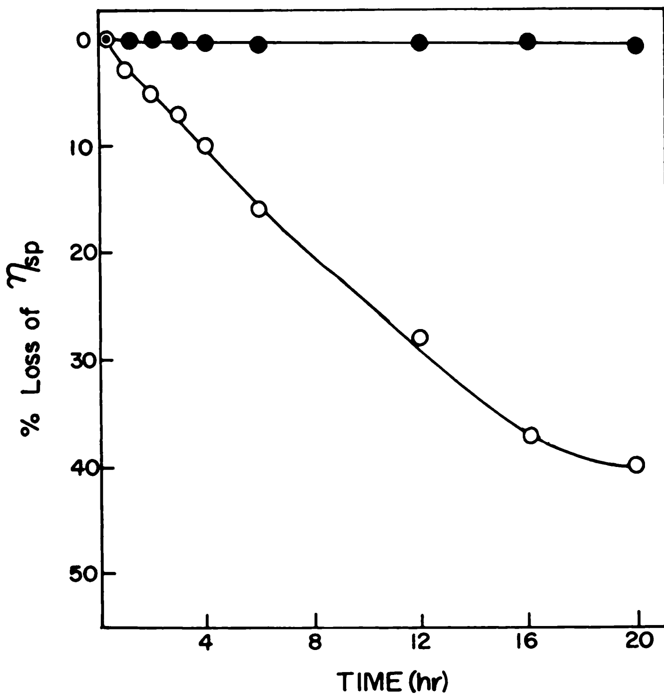

Chart 4. Viscometric determination of the collagenolytic activity of melanoma supernatant (1 ml/4 ml reaction mixture) at 27 ± 0.1°. The substrate collagen concentration was 0.1% in 0.05 M Tris-0.04 M $CaCl_2$ (pH 7.6). The fractional change in specific viscosity (η *spt*/η *spto*) at each measurement is plotted as a function of time; ●, substrate alone (control); O, substrate and melanoma homogenate (reaction mixture).

curve may suggest an admixture of predominantly cleaved products and an unaltered residual macromolecule, as previously reported in other tissue collagenases (3, 5, 11).

DISCUSSION

This investigation demonstrated the presence of collagenolytic activities in invasive malignant melanoma of the skin. Since the epidermis was not included in the homogenate, there is no possibility that we measured the epidermal and papillary dermis collagenases (5, 15). Human skin collagenase activity was shown to fall off markedly above pH 8.0 (5), whereas melanoma collagenase showed a peak activity at pH 8.5. Contamination by granulocytes is always possible in ulcerated tumors such as the specimen we used. Like melanoma collagenase, granulocyte collagenase is one of a few collagenases that are active *in vivo* and could be isolated directly from the granules. They are, however, not inhibited by 7% normal human serum (15), whereas melanoma collagenase was inhibited to 72% by 10% human serum. This suggests that granulocyte collagenases are not contributing significantly to the observed collagenolytic activity of our tumor. Thus, it seems that the source of the collagenolytic enzyme or enzymes demonstrated in this study is indeed the tumor cells *per se*. The absence of collagenolytic activity in the cellular blue nevus may suggest that macrophages such as melanophages, admixed in abundance in this tumor (16), do not contain active collagenolytic enzymes. As demonstrated in our control studies, melanocytes in the junctional nevus and cellular blue nevus did not contain a measurable amount of

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collagenases. The former is the most common source of malignant melanoma, and the latter can give rise to cancer. Production of tumor collagenase may coincide with the malignant changes of melanocytes.

Another exceptional collagenase that can be extracted directly in active form is that of basal cell epithelioma of the skin (22), a locally invasive but nonmetastasizing tumor. Compared with the collagenases of this tumor, collagenase of melanoma is not inhibited to the same extent by normal human serum; the basal cell epithelioma collagenase could be inhibited to 93% with 10% human serum, to 97% with 0.01 M EDTA, and to 75% by 0.01 M cysteine (22). A lesser degree of inhibition was also observed in the collagenase of squamous cell carcinoma of the skin (11); it was inhibited only to 75% by 10% human serum, to 71% by 0.01 M EDTA, and to 59% by 0.01 M cysteine. Dresden *et al.* (4), using a tissue culture method, noted 100% inhibition of basal cell epithelioma collagenase by 10% human serum, whereas collagenase of squamous cell carcinomas was inhibited only to 90% by the same concentration of the human serum. Further studies are in progress to see whether rapidly invasive tumors of the skin contain enzymes that are less sensitive to known inhibitors, particularly to serum collagenase inhibitor and, hence, are more malignant.

It is obvious that purification and characterization of enzymes from these tumors should be done before meaningful comparison can be made. Crude enzymes, as such, may contain a 2nd system of nonspecific proteases to degrade the collagen molecules initially cleaved by a specific collagenase. Such a 2nd system has been demonstrated in crude granulocyte collagenases (15). Specificity of melanoma collagenase could not be clearly demonstrated by disc electrophoresis because specific cleavage products such as α^A, α^B, and β^A were obscured by other degradative products. However, it will be demonstrated in a subsequent report that melanoma collagenase severs collagen molecules into 75 and 25% segments at the $\beta_2{}^2$ region in the segment-long spacing-type tropocollagen macromolecule.[5] This is strong evidence for the presence of a specific collagenase as found in other tissues (1, 5, 6, 9, 12, 22) and granulocytes (15), since an erosion of the molecule may occur by the action of other proteases (20) but not a complete cleavage across the molecule at this specific site.

REFERENCES

1. Bauer, E. A., Eisen, A. Z., and Jeffrey, J. J. Studies on Purified Rheumatoid Synovial Collagenase *in Vitro* and *in Vivo*. J. Clin. Invest., *50:* 2056-2064, 1971.
2. Dabbous, K. M., Seif, M., and Brinkley, E. The Action of Tetranitromethane on Acid-soluble Tropocollagen. Biochem. Biophys. Res., Commun., *48:* 1586-1591, 1972.
3. Danoff, R. B., McLennan, J. E., and Grillo, H. C. Preparation and Properties of Collagenases From Epithelium and Mesenchyme of Healing Mammalian Wounds. Biochim. Biophys. Acta, *227:* 639-653, 1971.
4. Dresden, M. H., Heilman, S. A., and Schmidt, J. P. Collagenolytic Enzymes in Human Neoplasms. Cancer Res., *32:* 993-996, 1972.
5. Eisen, A. Z., Jeffrey, J. J., and Gross, J. Human Skin Collagenase Isolation and Mechanism of Attack on the Collagen Molecule. Biochim. Biophys. Acta, *151:* 637-645, 1968.
6. Fullmer, H. M., and Lazarus, G. S. Collagenase in Bone of Man. J. Histochem. Cytochem., *17:* 793 798, 1969.
7. Gallop, P. M., Seifter, S., and Meilman, E. Studies on Collagen. I. The Partial Purification, Assay and Mode of Activation of Bacterial Collagenase. J. Biol. Chem., *227:* 891-906, 1957.
8. Gross, J. Studies on the Formation of Collagen. I. Properties and Fractionation of Neutral Salt Extracts of Normal Guinea Pig Connective Tissue. J. Exptl. Med., *107:* 247-263, 1958.
9. Gross, J., and Nagai, Y. Specific Degradation of the Collagen Molecule by Tadpole Collagenolytic Enzyme, Proc. Natl. Acad. Sci. U. S., *54:* 1197-1204, 1965.
10. Hashimoto, K., Yamanishi, Y., and Dabbous, M. K. Collagenolytic Activity of Basal Cell Epithelioma of the Skin. II. Electron Microscopic Observations *in Vivo* and *in Vitro*. Cancer Res., *32:* 2561-2567, 1972.
11. Hashimoto, K., Yamanishi, Y., Dabbous, M. K., and Maeyens, E. Collagenolytic Activity of Squamous Cell Carcinoma of the Skin. Demonstrations *in Vivo* and *in Vitro*. Cancer Res., in press.
12. Jeffrey, J. J., and Gross, J. Collagenase From Rat Uterus. Isolation and Partial Characterization. Biochemisty, *9:* 268-273, 1970.
13. Kefalides, N. A. Comparative Biochemistry of Mammalian Basement Membranes. *In:* E. A. Balazs (ed.), Chemistry and Molecular Biology of the Intercellular Matrix. Collagen, Basal Laminae, Elastin, Vol. 1, pp. 535-573. New York: Academic Press, Inc., New York, 1970.
14. Kunitz, M. Crystalline Soybean Trypson Inhibitor. J. Gen. Physiol., *30:* 291-310, 1947.
15. Lazarus, G. S., Daniels, J. R., Lian, J., and Burleigh, M. C. Role of Granulocyte Collagenase in Collagen Degradation. Am. J. Pathol., *68:* 565-578, 1972.
16. Lever, W. F. *In:* Histopathology of the Skin, Ed. 4, pp. 579-593. Philadelphia: J. B. Lippincott Co., 1967.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Rea-ent. J. Biol. Chem., *193:* 265-275, 1951.
18. Nagai, Y., Lapiere, C. M., and Gross, J. Tadpole Collagenase Preparation and Purification. Biochemistry, *5:* 3123-3130, 1966.
19. Rubin, A. L., Drake, M. R., Davidson, P. F., Pfahl, D., Speakman, P. T., and Schmitt, F. O. Effects of Pepsin Treatment on the Interaction Properties of Tropocollagen Macromolecules. Biochemistry, *4:* 181-190, 1965.
20. Seifer, S., and Haper, E. The Collagenases. *In:* P. D. Boyer (ed.), The Enzymes, Vol. 3, pp. 649-698. New York: Academic Press, Inc., 1971.
21. Von Hippel, P. H. *In:* G. N. Ramachandran (ed.), Treatise on Collagen, Vol. 1, p. 253-335, New York: Academic Press, Inc., 1957.
22. Yamanishi, Y., Dabbous, M. K., and Hashimoto, K. Collagenolytic Activity of Basal Cell Epithelioma of the Skin. I. Effect on Reconstituted Collagen and Kinetics. Cancer Res., *32:* 2551-2560, 1972.

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