6. The S-band radar facility of the Arecibo Observatory, Arecibo, Puerto Rico, had the following system characteristics at the time of these observations: operating frequency, 2.38 Ghz; antenna gain, 70 db; one-way antenna full beamwidth at half power, 2.7 arc min; average transmitter power, 400 kw (continuous wave); and receiving system noise temperature, 45°K when not observing Jupiter directly (Jupiter added about 30°K).
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12. The parameter f_0 (center-to-limb Doppler shift) is related to the satellite radius a, rotation period P, and radar wavelength λ by $f_0 = 4\pi a/\lambda P$ if the rotation axis is normal to the line of sight.
13. The Doppler frequency corresponding to the echoes from the subradar point is related to the orbital motion of the satellite and will be used in improving estimates of the orbital parameters of the Jovian system.
14. Before making this comparison, we corrected for the absorption effects of Venus' atmosphere and, more important, for the surface roughness of Callisto [see, for example, T. Hagfors, in *Radar Astronomy*, J. V. Evans and T. Hagfors, Eds. (McGraw-Hill, New York, 1968), pp. 187–218].
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Tumor Cell Collagenase and Its Inhibition by a Cartilage-Derived Protease Inhibitor

Abstract. *Human osteosarcoma and mammary carcinoma cells were cultured separately in a medium supplemented with fetal calf serum, until they were confluent. The medium was then replaced by serum-free medium supplemented with heparin. Both cell cultures secreted collagenase, and this activity was inhibited by a cartilage-derived protein of low molecular weight. Since cartilage is rarely invaded by neoplasms, the presence of this inhibitor may play an important role in the regulation of tumor invasion.*

The discovery of a collagenase present in normal tissues under physiological conditions (*1*) stimulated interest in this enzymatic activity in tumors (*2*). Neoplastic human tissues possess a collagenase which in most cases is similar, if not identical, to that isolated from normal human skin, and yield the specific peptide fragments TC^A and TC^B (*3*). Abramson *et al.* (*4*) extended these observations by correlating the clinical aggressiveness of epidermoid carcinomas of the head and neck with a high specific activity of tumor collagenase.

In the area of bone neoplasms, the most common primary malignant tumor is the osteosarcoma. This tumor erodes and replaces bone tissue, whose major organic component is collagen. Cartilage, another collagenous tissue adjacent to many bones, is rarely or less readily invaded by osteosarcomas. This phenomenon is also observed in bony metastases from breast carcinomas. We present data demonstrating that human osteosarcoma and mammary carcinoma cells secrete collagenase in culture, and that this activity can be inhibited by a cationic protein of low molecular weight isolated from bovine hyaline cartilage (*5*).

For the present experiments we used human osteosarcoma cells (TE-85), a well-defined cell line of McAllister *et al.* (*6*), and human breast carcinoma cells (ALAB, lung metastasis), a cell line described by Reed and Gey (*7*).

Tissue culture medium, RPMI-1640 (Gibco), was supplemented with 10 percent fetal calf serum (FCS) (Reheis Chemical) which had been heat inactivated for 50 minutes at 56°C; 50 μg of gentamycin (Schering) per milliliter; and 5 μg of amphotericin-B (Squibb) per milliliter.

The bovine cartilage protease inhibitor was prepared as recently described (*5*). In brief, the cationic low molecular weight protein is obtained by ultrafiltration of an extract of bovine scapula cartilage followed by affinity chromatography on insoluble trypsin.

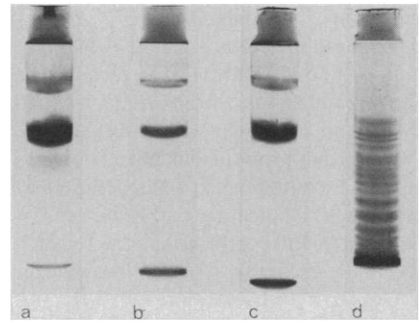

Fig. 1. Polyacrylamide gel electrophoresis. The effect of human mammary carcinoma collagenase on guinea pig collagen and its inhibition by a cartilage-derived protease inhibitor. (a) Collagen control. (b) Collagen plus trypsin. (c) Collagen plus mammary carcinoma collagenase and cartilage inhibitor. (d) Collagen plus mammary carcinoma collagenase.

Tumor cells (initial seeding 4×10^4 cells per milliliter) were cultured in Falcon tissue culture flasks (75 cm^2 growth area) each containing 15 ml of FCS-supplemented medium (37°C, in a humidified atmosphere of air and 5 percent CO_2). After the cells reached confluence (usually 5 days for the TE-85 cells and 4 days for the ALAB cells) the cell layers were extensively rinsed with FCS-free medium, according to the method of Werb and Burleigh (*8*). The flasks were then divided into two groups; both groups were cultured in FCS-free medium, but to one group sodium heparin (50 unit/ml; Sigma) was added. The cells were cultured at 37°C as described above, and the media were changed at 2-day intervals. The heparin-free cultures were maintained for up to 6 days, while the cells cultured with heparin were maintained for 14 days. The media were decanted, cleared from cellular debris by centrifugation, and those from each experiment were pooled. The media were adjusted so that they contained 50 m*M* tris and 5 m*M* $CaCl_2$, by means of 1*M* tris-HCl buffer, *p*H 7.6, and solid $CaCl_2$. They were then dialyzed against 100 m*M* tris-HCl buffer, *p*H 7.6, containing 5 m*M* $CaCl_2$ as described (*8*). Samples were then concentrated (50 : 1) with an Amicon PM-10 membrane and assayed for collagenase activity.

Collagenase and its inhibition by the cartilage inhibitor was measured by means of an assay for the release of ^{14}C-labeled glycine peptides from 100-μl portions of a solution of undenatured guinea pig skin collagen, as described for the inhibition of human collagenase with different inhibitors (*5*). Two hundred microliters of the concentrated, processed medium were used either directly or after prior incubation for 30 minutes at 22°C with 10 μg of the cartilage inhibitor. The reaction mixture was then incubated with the collagen at 37°C for 4½ hours. The reaction was terminated and the radioactivity measured in an automatic liquid scintillation counter (*5*).

The reaction products resulting from collagenolytic activity were separated by polyacrylamide gel electrophoresis according to the method of Nagai *et al.* (*9*).

In FCS-free media, neither of the tumor cell cultures could be maintained for more than 6 days because of severe necrosis and cell degeneration. When these media were examined for enzyme activity, only a negligible amount of collagenase could be found.

Table 1. Determination of the inhibition of collagenase from human osteosarcoma cell media with bovine cartilage inhibitor. Assays were carried out at 37°C for 4½ hours. Protein was determined according to the Warburg and Christian method (A_{280}/A_{260}). The total number of counts per minute per assay was 1700.

Reaction mixture	[^{14}C]glycine (count/min)	Inhibition* (%)
Buffer (0.05*M* tris, 0.005*M* $CaCl_2$, *p*H 7.4)	439	
Buffer + 0.02 percent trypsin	513	
Buffer + 150 μg of bacterial enzyme	1660	
Buffer + 752 μg of osteosarcoma cell medium	1463	
Buffer + 752 μg of osteosarcoma cell medium + 10 μg of cartilage inhibitor	805	69.3

*Expressed as $[1 - (E + I) - T/E = T] \times 100$, where the symbols E, I, and T are the enzyme, inhibitor, and trypsin, respectively, and the value of each is expressed in terms of counts per minute.

Table 2. Determination of the inhibition of collagenase from human mammary tumor cell media with bovine cartilage inhibitor. Assays were carried out at 37°C for 4½ hours. Protein was determined according to the Warburg and Christian method (A_{280}/A_{260}). The total number of counts per minute per assay was 1330.

Reaction mixture	[^{14}C]glycine (count/min)	Inhibition* (%)
Buffer (0.05*M* tris, 0.005*M* $CaCl_2$, *p*H 7.4)	351	
Buffer + 0.02 percent trypsin	398	
Buffer + 428 μg of mammary tumor cell media	578	
Buffer + 428 μg of mammary tumor cell media + 10 μg of cartilage inhibitor	484	52.3

*Expressed as $[1 - (E + I) - T/E - T] \times 100$, where the symbols E, I, and T are the enzyme, inhibitor, and trypsin, respectively, and the value of each is expressed in terms of counts per minute.

When the tumor cells were cultured in the presence of heparin, however, the cells remained viable throughout the culture period of 2 weeks.

After the heparin-supplemented media were processed, a significant amount of collagenolytic activity was found, as shown in Tables 1 and 2. The activity of the osteosarcoma cell culture medium was inhibited by 69 percent, and that of the mammary carcinoma cell medium by 52 percent, as a result of the prior incubation with cartilage-derived inhibitor.

We demonstrated the inhibition of collagenolytic activity in mammary carcinoma tissue culture media by observing the reaction products of the assay on polyacrylamide gel electrophoresis (Fig. 1). The mammary carcinoma collagenase produces a large spectrum of reaction products of the collagen during incubation, and proteolysis is prevented by prior incubation with the cartilage inhibitor. These multiple bands are most probably caused by the presence of other proteases within the media which degrade the reaction products after the tumor collagenase cleaves the collagen.

Primary or metastatic bone tumors easily erode the bone tissue but rarely invade cartilage. Bone and cartilage contain genetically distinct types of collagen, but both types of collagen are susceptible to collagenase cleavage although cartilage collagen is the more resistant of the two (*10*).

Kaufman *et al.* (*11*) demonstrated that when heparin was added to organ cultures of mouse calvaria, there was a marked increase in the amount of bone resorbed in a system containing suboptimal concentrations of parathyroid hormone. The release of peptides containing hydroxyproline into solution paralleled bone resorption, indicating that destruction of collagen occurred. Sakamoto *et al.* (*12*) showed that heparin (50 unit/ml) increased the production or the release of the collagenase from cultures of mouse bone in vitro. Therefore, we added heparin to the cultures of isolated sarcoma and carcinoma cells and found that heparin not only stimulated the release of collagenolytic activity from these cells but also maintained their viability throughout the culture period of 14 days.

It is known that both osteosarcoma and mammary carcinoma cells respond in vitro to treatment with estrogen (*13*). Parathyroid hormone has been shown to affect transplantable osteogenic sarcomas (*14*), indicating that bone resorption or erosion can be enhanced by such physiologic agents. To our knowledge, however, no observation has been reported that heparin can enhance collagenase release from tumor cells.

The finding that the protein isolated from cartilage inhibits the collagenolytic activity released by tumor cells into FCS-free media may be of physiological importance. Cartilage is one of the few tissues within the body that is rarely invaded by neoplasms. We reported previously that cartilage is resistant to vascular invasion (*15*), and that cartilage extracted by low concentrations of guanidine hydrochloride can be invaded by blood vessels (*16*). We therefore suggest that the naturally occurring resistance of cartilage to both normal invasion (vascular invasion) and pathologic invasion (tumor invasion) may be due to a collagenase inhibitor which seems to be an integral component of the cartilage matrix.

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