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ELASTIN DEGRADATION BY PROTEASES FROM CULTURED HUMAN BREAST CANCER CELLS

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SUMMARY: Potent elastinolytic activity was elaborated into the media of several cultured human breast cancer cell lines. Cultured stromal cells from normal and tumor-bearing human breast tissue contained no such activity. This elastase-like activity was resolved into three major peaks of activity by DEAE-cellulose column chromatography. Two of the three activities were separated from the collagenolytic activity also produced by these cells. Inhibition profiles of the elastinolytic activities indicated that two were serine proteases and one was a metalloprotease. Each of these three proteases were in an inactive form and required trypsin pretreatment for activity. The desmoplastic reaction to human breast cancer contains both collagen and elastin. The present findings indicate that the breast tumor cells themselves may modulate the turnover of these stromal proteins.

INTRODUCTION:

Elastin is a connective tissue component in a variety of tissues and organs. It is present in skin, yellow tendons, and in blood vessels (1). Elastin is also a prominent component of breast carcinoma (2,3). The elastic tissue is associated with the desmoplastic or stromal reaction to the tumor. In certain invasive carcinomas, desmoplasia is so marked that it alone is responsible for the tumor "lump". During the metastatic process, elastinolytic proteases may be required for cells to penetrate the surrounding stroma. Hormonal modulation causes remission in some cases of human breast cancer. During this remission, it is the stromal reaction which recedes as the total tumor mass regresses. Here also, elastinolytic proteases must be invoked. Previous studies have shown that human cancer cells possess the capacity to degrade the collagenous component of their surrounding stroma (4, 5). Therefore we examined cultured breast tumor cells for an elastase-like activity.

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We describe herein elastinolytic activity elaborated by cultured human breast cancer cells. This activity has been partially purified and characterized as being associated with at least three separate proteinases. Thus, in the desmoplastic reaction to breast cancer, it appears the breast tumor cells themselves modulate the turnover of stromal components.

MATERIALS AND METHODS:

Cell Cultures: The human breast cell lines used in these experiments were ZR75-1, ZR75-30, and ZR75-31A, kindly provided by Ms. Linda Engel, NIH and MCF-7 from Dr. Marvin Rich, Michigan Cancer Research Foundation. The RMF* and PTF cells are primary human breast fibroblast cultures generously provided by Drs. W. Jones and R. Hallowes of the Imperial Cancer Research Laboratory, London. The NBF fibroblasts and HeLa cells were obtained from the UCSF Cell Culture Facility. All cells were cultured in RPMI 1640 with 4% FCS. After 24 hrs, the cells were washed three times with HBSS. The cells were then cultured with either serum free medium or medium supplemented with 4% FCS as indicated. The cells were grown at 37°C in an atmosphere of 95% air and 5% CO_2. The medium was harvested and changed every 48 hrs.

Enzymatic Assays: Elastin was radiolabeled using ^3H-$NaBH_4$ according to Banda and Werb (6). For each assay, 175 µl of the conditioned media was trypsin activated. Activation was effected by using 12.5 µl of 0.025% trypsin for 5 min and terminated by the addition of 12.5 µl of 0.125% soybean trypsin inhibitor. The 200 µl sample was then added to 100 µl of a 2 mg/ml suspension of ^3H-elastin. The reaction was performed at 37°C for 24 hrs. A unit of elastinolytic activity is defined as 1 µg of elastin degraded per hour. Inhibition studies were performed by incubating the activated samples with inhibitor for 10 min at 37°C. The substrate was then added and incubation continued as described.

Collagenolytic activity was assayed by the method of Johnson-Wint (7). For the collagenase assay, trypsin activation was required and performed as described for the elastinolytic sample. A unit of collagenolytic activity is defined as 1 µg of collagen degraded per min.

DEAE Cellulose Chromatography: DE-52 (Whatman) was packed into a 1 x 25 cm column and equilibrated with 50mM Tris-HCl containing 5mM $CaCl_2$ and 0.02% NaN_3, pH 8.3, at 4°C. A 40 ml sample conditioned by confluent cell layer was used for partial purification. The sample was dialyzed against the running buffer overnight and applied with a flow rate of 60 ml/hr. The bound proteins were eluted with a linear gradient of from 0.0 to 0.15 M NaCl in the same buffer, and 5 ml fractions were collected. The total gradient was 800 ml. This was followed by a subsequent wash of 1 M NaCl.

RESULTS:

Several lines of human breast cancer cell lines were grown and the conditioned media examined for elastinolytic activities (Table 1). Elastinolytic activities were found in the media of all the human breast tumor cell lines examined. Very low activity was found in the media of fibroblasts. Fibroblasts

*Abbreviations used are: RMF, fibroblasts derived from a reduction mammoplasty; PTF, fibroblasts derived from a surgical mastectomy specimen from a peritumor area; NBF, fibroblasts obtained from newborn foreskin; FCS, fetal calf serum; HBSS, Hank's basic salt solution; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; and NEM, N-ethyl malleimide.

TABLE I

Elastin Degradation by Human Cells in Culture

Cell Type	Elastin Degradation (cell culture supplemented with 4% FCS) [cpm]	Elastin Degradation (cells cultured without FCS) [cpm]
ZR75-31A	696	8,760
ZR75-30	796	4,278
ZR75-1	486	4,982
MCF 7	283	2,169
NBF	201	879
PTF	121	381
RMF	107	206
HeLa	206	728

Survey of elastinolytic activities in various human cell cultures. Cells were plated at a density of 5×10^5 cells/well in FCS supplemented culture medium. After 24 hrs, serum free or 4% FCS supplemented medium was used. The counts represent the elastin degraded by 100 µl of 48 hr conditioned medium, obtained between days 2 and 4. The mean of experiments in triplicate from three separate cultures are presented here. The specific activity of the elastin was 140-195,000 cpm/mg.

from a variety of sources were examined. The PTF and RMF cells, fibroblasts obtained from malignant and normal breast tissues, respectively, had low activity as did foreskin fibroblasts. HeLa cells, another human malignant epithelial cell line, was examined and also found to have low activity. The ZR75-31A cells produced the highest activity and was selected for further study. These activities could be detected only when cells were grown in the absence of FCS.

The period of culture when maximal elastinolytic activity occurs was determined. Cells were cultured in the presence and absence of 4% FCS. Elastinolytic activity was highest between day 0 and 2 under serum free conditions (Figure 1). Activity decreased during subsequent culture periods. We have observed that cells also have vacuolization and granularity of their cytoplasm by day 6 of growth in serum free conditions. In contrast, cells cultured in presence of 4% FCS had little elastinolytic activity during any period.

Elastinolytic enyzmes fall into all classes of proteases, cysteine, serine, and metalloproteases (8). To characterize the present activities further, partial purification was undertaken. Dialyzed culture medium was applied to a column of DEAE-cellulose and the proteins eluted with a shallow gradient of buffered NaCl. Each fraction was trypsin activated and assayed for elastino-

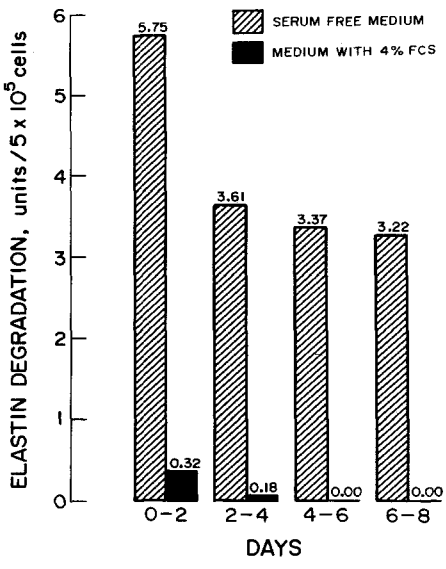

Fig. 1. Timecourse of elastinolytic protease production by ZR75-31A cells. Medium was allowed to be conditioned for 48 hr period. On the days indicated, the medium was harvested and elastinolytic activity was examined, as described in Methods. The mean of experiments in triplicate from three separate cultures are presented here.

lytic activity. Three activities were separated (Fig. 2a) and designated proteases I, II, and III. Since these cells also produce collagen-degrading activity (5) collagenolytic assays were performed (Fig. 2b). Most of the collagenolytic activity was found in the column void volume and in the eluate following application of the high-salt wash. It cannot be concluded from these observations whether protease III also had collagenolytic effect. These experiments suggest that breast tumor elastinolytic activity was the result of three separate enzymes, at least two of which had no associated collagenolytic activity.

The three proteases were compared by inhibition studies (Table II). A number of inhibitors specific for cysteine, serine, and metalloproteases were used. Protease I and III activities were inhibited by the serine protease inhibitor PMSF. Partial inhibition by cysteine protease inhibitors was observed, whereas an enhancement of activities was observed in the presence of the reducing agent DTT. This suggests that there may be thiol-sensitive activity in this fraction. Protease II activity decreased in the presence of metalloprotease inhibitors. Since it is well known that serum contains various protease

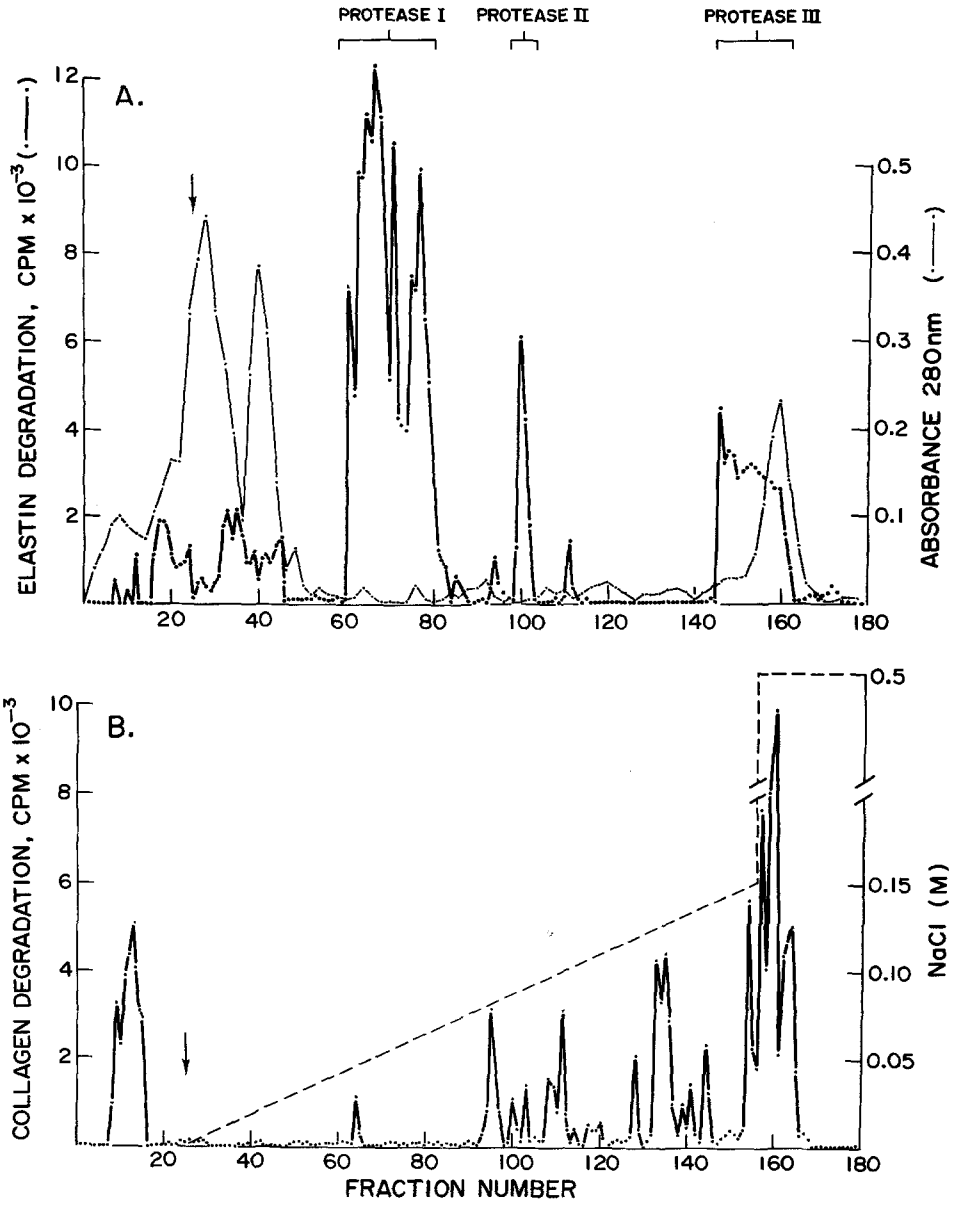

Fig. 2. DEAE cellulose chromatography of crude ZR75-31A culture medium. 40 ml of crude culture medium conditioned by confluent cell layer was dialyzed against the elution buffer and was then applied to the column. The gradient was begun at fraction 25 and 1 M NaCl was applied at fraction 155. In A, the absorbance at 280 nm is represented by the thin line and elastinolytic activity by the heavy line. In the lower panel, the collagenolytic activity is shown. The elastinolytic fractions which were pooled and used in the inhibition studies are indicated by brackets.

inhibitors, FCS was also tested. FCS was an effective inhibitor for all three proteases.

From these experiments we can conclude that FCS inhibits activity and also suppresses the capacity of these cells to produce elastinolytic proteases. Even

TABLE II

Inhibition Profile of the Elastase-like Enzymes from the Human Breast Tumor Cell Culture Medium

Preincubation Concentration	Protease I [% of control]	Protease II [% of control]	Protease III [% of control]
4.0% Fetal Calf Serum	0	0	0
0.4% Fetal Calf Serum	0	0	0
0.4% FCS supplemented RPMI conditioned by ZR75-31A cells for 7 days	0	0	0
10mM EDTA	103	47	102
10mM EGTA	99	37	98
10mM 1,10 phenanthroline	101	20	104
10mM DTT	131	68	110
10mM PMSF	17	88	36
10mM Benzamidine	9	76	27
10mM NEM	79	98	89
10mM Iodoacetate	63	103	93

Inhibition profile of elastinolytic-like proteases separated by DEAE chromatography. Three pooled fractions of elastinolytic protease activity was obtained from the DEAE chromatography as shown in Figure 2A. The pooled fractions were trypsin activated and allowed to incubate with the inhibitor for 15 min. The concentration of the inhibitor at this stage is the preincubation concentration shown above. PBS was added to the control. To 100 µl sample of the enzyme-inhibitor mixture, 100 µl of the substrate was added. The data depicted represents the mean of triplicate assay.

low levels of serum effectively suppress elaboration of these enzymes. The relationship to the physiological situation *in situ* is unknown.

DISCUSSION:

Elastin is one of the major fibrous proteins found in the stromal reactions to breast cancer. We wished to establish whether human breast tumor cells synthesize proteases which can degrade elastin. At present, there are three well characterized mammalian elastases. In the mammalian pancreas, there are two types of elastases (10). The best characterized is the porcine pancreatic elastase I, which is a serine proteinase secreted in a zymogen form by the pancreatic β-cells. The second class of mammalian elastase is the neutral proteinase found in neutrophil granules (11). A third mammlian elastase is a metalloproteinase secreted by inflammatory macrophages (6).

In the present communication, we present evidence that human breast tumor cells in culture elaborate into the media elastinolytic-like enzymes. There are

at least three activities, two of which are serine proteases and one a metallo-protease. Each exists in an inactive form and requires trypsin pretreatment for activity. These enzymes may be involved in the modulation and turnover of the elastin-containing desmoplastic reaction found in most human breast cancers.

An elastinolytic activity has been demonstrated previously by Hornebeck and Robert in extracts of human breast carcinomas (12). As shown in the present studies it is the malignant epithelial cells themselves and not the surrounding stromal cells which elaborate this activity. This observation corroborates the work of Gullino and coworkers (13, 14) that it is the neoplastic cell population that regulates the over-all amount of scleroprotein produced in the neoplastic mass.

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