Isolation of a Protease from the Cell-Free Medium of in vitro Cultured Mammary Carcinoma

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Summary. Trypsin-like protease with wide spectrum of enzymatic activities have been isolated from cell-free medium from in vitro cultured human mammary carcinoma cells, and from peripheral blood lymphocytes of patients with mammary carcinoma cultured in presence of cell-membrane carcinoma-associated glycoprotein.

Several studies have suggested that plasma membranes of cancer and inflammatory cells exhibit abnormal 'permeability' preferentially releasing lysosomal enzymes. Cathepsin D has been found to be raised in homogenates of various organs of patients with malignant disease[1-3]. When cultured in vitro, many tumors release biologically active mediators. HeLa cells. severe tumorigenic hamster cell lines[4] and cells transformed by DNA and RNA tumor viruses, and several human tumor cell lines[5] release an inhibitor of macrophage migration. A possible role for this inhibitor is to modify the surface properties of tumor cells, and to alter cell-mediated immune responses directed against the neoplastic cells[6,7]. The present studies report the release of a protease with wide spectrum of activities from human mammary carcinoma into the medium.

Human mammary tumor cells were derived from ductaf malignant breast carcinomas which were confirmed by histological examination. The cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 20% heat inactivated fetal calf serum, 2 m*M*-glutamine, 100 U penicillin/ml and 100 µg streptomycin/ml of medium. In this medium, in 24 h the cells adhered to the glass, and growth attained a plateau within 93 to 120 h. At concentrations of 5–2 µg/ml, insulin accelerated the growth and delayed plateau formation to longer than 260 h. Similar effects were obtained with either pancreatic or soybean trypsin inhibitors. At the period of the plateau, the cell-free medium was carefully withdrawn and fresh medium was added to the cells. The cell-free medium was passed through a series of Amicon Diaflo ultrafiltration membranes (UN 2, PM 10, PM 30, XM 50, XM 50, XM 100 and XM 300) a procedure that excluded differentially the various compounds according to their molecular weight, and concentrated the samples 10- to 12-fold. Fractions obtained through the membrane XM 50 inhibited migration of peritoneal macrophages as assessed by the capillary tube technique reported earlier[8]. Aliquots of this fraction were dialyzed against distilled water and then lyophilized. The dry powder was reconstituted in 2 ml of 0.15 *M* NaCl-0.01 *M Tris* HCl buffer, pH 8.4 and applied onto a 1.6×100 cm Sephadex G-100 column. The column was precalibrated with molecular weight markers, bovine serum albumin (MW 67,000), salt-free chymotrypsinogen (MW 25,000) and lysoeyme (MW 17,000). Fractions which eluted from the column with the chymotrypsinogen marker inhibited macrophage migration. When examined on polyacrylamide gel electrophoresis, this fraction showed 1 single band. This fraction hydrolyzed benzoyl-1-arginine ethyl ester, tosyl-1-arginine methyl ester, lysine methyl ester, acetyglycyl-1-lysine methyl ester, z-gly-prol-gly-gly-prol-ala-OH, BZ-prol-leu-gly-gly-

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Table I. Action of cell-free medium from cultures of the mammary carcinoma or of peripheral blood lymphocytes of normal or cancer patients with and without the carcinoma associated glycoprotein (CAA)

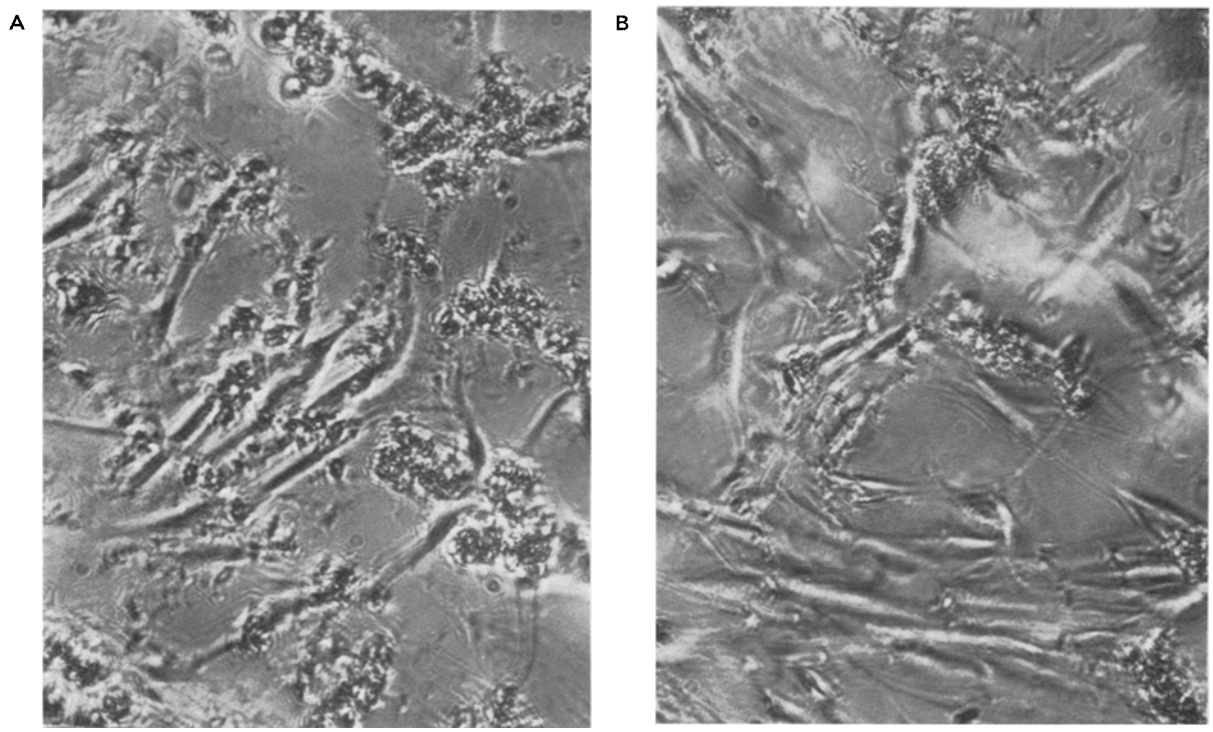
Substrate	Mammary carcinoma	Peripheral blood lymphocytes				Trypsin
		Normal Donors		Cancer patients[a]		
		With CAA	Without CAA	With CAA	Without CAA	
N-Benzoyl-1-arginine ethyl ester (BAEE)	625 ± 24	14 ± 2	—	425 ± 72	27 ± 5	3067 ± 60
N-Tosyl-1-arginine methyl ester (TAME)	413 ± 21	8 ± 2	—	532 ± 63	41 ± 7	1.25×10^5 ± 354
N-Acetyl-1-tyrosine ethyl ester (ATEE)	25 ± 8	—	—	—	—	8 ± 2
Lysine methyl ester (LME)	645 ± 7	27 ± 3	—	976 ± 81	56 ± 9	2153 ± 21
Hemoglobin	323 ± 14	9 ± 2	—	394 ± 44	—	3663 ± 43
Human Fibrinogen[a]	75 ± 6	—	—	98 ± 19	—	1265 ± 23
Human Plasminogen[b]	1609 ± 41	—	—	1765 ± 93	52 ± 8	6473 ± 68
z-gly-prol-gly-gly-prol-ala-OH[c]	264 ± 12	—	—	1538 ± 84	41 ± 6	23 ± 8
pz-Prol-leu-gly-prol-gly-arg-OH	864 ± 32	—	—	2165 ± 108	76 ± 8	178 ± 34

The above results are expressed as units per mg protein in the cell-free medium. 1 unit of tosyl-1-arginine methyl ester, or N-benzol-1-arginine ethyl ester is equivalent to 1 µ*M* ester liberated per mg protein.
[a] The results are expressed in µg of peptides solubilized from 500 mg of fibrinogen per mg protein. [b] The results are expressed in units of N-tosyl-1-arginine methyl ester per mg of protein. [c] The results are expressed in µg of the peptide split per min per mg of protein.

Table II. Effect of enzyme inhibitor on the migration inhibition agent(s) in cell-free medium enzyme from in vitro cultures

In vitro treatment of migration inhibition agent(s)	Migration inhibition activity (%)		
	Lymphocytes from		Mammary carcinoma
	Normal donors	Cancer patients	
Untreated	2.4 ± 8.3	87 ± 10.9	61 ± 9.1
Pepstatin (20 μg/mil)	2.1 ± 10.7	46 ± 9.0	49 ± 7.8
Human-α-anti-trypsin (0.5 mg/ml)	3.8 ± 9.6	21 ± 8.7	59 ± 11.6
Soybean trypsin inhibitor (0.5 mg/ml)	1.8 ± 7.6	69 ± 12.4	31 ± 9.3
Pancreatic trypsin inhibitor (0.5 mg/ml)	1.5 ± 6.4	73 ± 10.6	52 ± 8.1
p-Aminobenzamidine (2×10^{-4} *M*)	2.0 ± 11.2	43 ± 10.3	39 ± 8.1
Ovomucoid (0.5 mg/ml)	1.2 ± 8.7	88 ± 12.7	68 ± 10.5
Enzyme substrates			
TLCMK (5×10^{-4} *M*)	1.7 ± 10.1	80 ± 11.9	58 ± 11.3
N-Tosyl-1-Arginine methyl ester (2×10^{-3} *M*)	2.3 ± 12.7	86 ± 14.6	60 ± 12.2
N-Benzoyl-1-Arginine Amide (2×10^{-4} *M*)	2.7 ± 11.9	81 ± 12.7	58 ± 10.6
N-Acetyl-1-Tyrosine ethyl ester (1×10^{-4} *M*)	1.6 ± 9.3	81 ± 13.1	71 ± 12.1
N-Benzoyl-1-Arginine ethyl ester (2×10^{-3} *M*)	1.9 ± 14.3	91 ± 14.6	58 ± 9.7
TPECMK (1×10^{-4} *M*)	2.7 ± 11.9	81 ± 12.7	58 ± 10.6
N-Acetyl-1-Tyrosine ethyl ester (2×10^{-2} *M*)	2.7 ± 15.5	89 ± 11.7	65 ± 9.3
Z-gly-prol-gly-gly-prol-ala-OH	2.2 ± 3.6	16.9 ± 8	23 ± 9.1
PZ-prol-leu-gly-gly-prol-D-arg-OH [a]	3.8 ± 9.4	24.7 ± 8	35.6 ± 11.6
Inhibitors of the serine active center			
Diisopropylfluorophosphate (5×10^{-3} *M*)	0.4 ± 9.7	2.5 ± 4.2	12 ± 8.2
Epsilon-Amnio Caproic Acid (5×10^{-2} *M*)	0.5 ± 8.9	10.7 ± 9.7	23 ± 4.6

TLCMK, N-tosyl-l-lysylchloromethyl ketone; TPECMK, N-tosyl-l-phenyl ethyl-chloromethyl ketone. [a] The data for the splitting of the peptide is reported in Table 1.



Effect of the enzyme from cell-free medium on in vitro growth of the mammary carcinoma. Mammary carcinoma cells were allowed to grow in Eagle's essential medium alone (A), and in the medium containing 10 μg of the cell-free medium enzyme (B). Growth taken 120 h after seeding.

prol-D-arg-OH, hemoglobin, casein and catalyzed the conversion of human plasminogen to plasmin (Table I).

Mammary carcinoma cells were allowed to grow in the Eagle's essential medium alone (Figure A), and in the medium, to which was added 10 µg of the purified protease per ml (Figure B), and growth was examined at 48 h intervals. Clusters of round cells predominate in Figure A, while fusiform cells predominate in Figure B.

An antigenic glycoprotein was prepared and purified from the cell surface of the carcinoma cultured cells described earlier[9]. When added to cultured peripheral blood lymphocytes of patients with mammary carcinoma the glycoprotein released a protease which also inhibited migration of guinea-pig peritoneal macrophages, while it had no effect on peripheral blood lymphocytes of normal donors. The data summarized in Table I indicate similarities between the protease activities of the 2 cell-free media. In the experiments presented in Table II, the lymphocytes were first incubated at 37 °C for 120 °C in Eagle's essential medium containing 10 µg of the carcinoma associated glycoprotein, washed 3 times with the fresh medium, and then cultured for 120 h. The cell-free media were harvested and the protease was separated. The series of protease inhibitors listed in Table II blocked the migration inhibition of peritoneal macrophages caused by the 2 proteases. At concentrations of 5 to 25 µg/ml, insulin inhibited the proteolytic activity of the proteases in the 2 types of cell-free media.

Discussion. There is accumulating evidence for cell-mediated immune reactions against antigens associated with breast cancer. These reactions are revealed by reactivity of lymphocytes from breast cancer patients against tissue culture cells derived from breast cancer in the microcytotoxicity assay[10,11], inhibition of the migration of autologous leukocytes with crude extracts of breast cancers, and stimulation of autologous lymphocytes of breast cancer patients with tumor cell-membrane extracts[9,12–17]. Combined with the data presented, these observations are in strong agreement with the release of biologically active substances during stimulation of sensitized lymphoid cells either with non-specific mitogens, or with the specific sensitizing antigen. The results reported identify the macrophage migration inhibition factor with a wide spectrum of enzymatic activities, which explain several observations: The destructive properties of tumors, the decreased mutual adhesiveness of cells and a variety of local and systemic pathological reactions in cancer and inflammatory diseases.

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Hemagglutination of Neuraminidase-Treated Human Erythrocytes by *Leishmania enriettii* Infected Guinea-Pig Sera

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Summary. Sera from guinea-pigs infected with the protozoan parasite *Leishmania enriettii* showed higher hemagglutination (HA) titres for neuraminidase treated human erythrocytes than those of normal guinea-pig sera. This HA activity was associated mostly with the 19S fraction of the immune serum and could be absorbed out with an antigenic fraction of the parasite membrane. Antigenic determinants involved in this HA reaction consisted of, at least, β-D-galactosyl or lactosyl residues.

Treatment of human erythrocytes and lymphocytes with *Vibrio cholera* neuraminidase (VCN) removes cell surface sialic acids and exposes hidden antigenic determinants (Thomsen or T-antigens) which have been shown to react with normal human and various animal sera as well as with plant lectins[2–9]. Although the earlier workers[2–4] used the term Thomsen or T-agglutinins to refer to these activities in normal sera, more recently these are shown to be due to the presence of naturally occurring antibodies of IgM class[6,7]. The mechanism for the production of such antibodies and their functional significance still remains to be discovered, though several possibilities exist[10,11]. In the present communication, direct experimental evidence has been presented to show that these antibodies can be produced due to an immune response against a discrete set of antigenic determinants present on the surface of a microorganism. The system described here is the infection of guinea-pig with the protozoan parasite *Leishmania enriettii* and agglutination of VCN-treated human erythrocytes by normal (NGPS) and immune (IGPS) sera.

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