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ELASTASE ACTIVITIES OF HUMAN BLADDER CANCER CELL LINES DERIVED FROM HIGH GRADE INVASIVE TUMOURS

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Elastase activities in intact human bladder cancer cell lines, established from three patients, were measured using a fluorogenic substrate highly specific for elastase, under conditions of physiological pH and ionic strength. This method allowed separation of cell-associated from secreted enzyme activity. As secreted elastase accounted for only 8% of the total, we concluded that the elastases were present at the cell surface. Inhibition studies using extracts of cell-surface elastases showed them to be serine proteinases which were also inhibited by α_1-antitrypsin. Partially purified fractions showing the highest specific activity towards the fluorogenic substrate hydrolysed insoluble elastin thus confirming the presence of elastases. This is the first time that elastase activity has been demonstrated in human bladder cancer cells and may represent a mechanism involved in tumour invasion.

1.INTRODUCTION

Proteinases produced by cancer cells may facilitate cancer cell invasion of normal tissue [1]. Two neutral proteinases which have been implicated in the penetration of normal tissue by cancer cells are collagenase [2] and plasminogen activator [3]. Another which has recently attracted interest in tumour cell studies is elastase. Elastase is the only enzyme which can hydrolyse insoluble elastin, a component of elastic tissues which is generally resistant to proteolysis [4]. In addition, elastase can hydrolyse several other tissue components including connective tissue macromolecules, plasma proteinase inhibitors, and immunoglobulins [4]. Therefore the production of elastase by tumour cells could increase their ability to invade normal tissues. Several studies have shown that tumour cells secreted either inactive [5] or active [6] elastases into culture medium or, when grown directly in contact with the matrix, degraded components of an extracellular matrix which included elastin [7].

Abbreviations: HH, Hanks balanced salt solution buffered with 25mM Hepes; APA-AMC, AcAlanylProlylAlanine-7-amino-4-methylcoumarin; PPE, porcine pancreatic elastase; HLE, human leukocyte elastase; PMSF, phenylmethone sulphonyl fluoride; SBTI, soya bean trypsin inhibitor; Aust., Australia.

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Using a substrate which is highly specific for elastase [8], we have demonstrated the presence of active elastase in intact human bladder cancer cells. Production of this proteinase may be involved with cancer cell reimplantation observed in superficial bladder cancer [9].

2.MATERIALS AND METHODS

2.1 Cell culture

B10 [10] and 13A are clones derived from continuous cell lines UCRU-BL-17 [11] and UCRU-BL-13 [12], originally established as xenografts in nude mice from primary human bladder transitional cell carcinomas. UCRU-BL-28 (BL28) is a continuous cell line derived from biopsy material obtained from a patient with a highly invasive transitional cell carcinoma of the bladder (unpublished). All cell lines, developed at the Urological Cancer Research Unit, grow as tumours in nude mice when injected subcutaneously. Cell lines were cultured in RPMI 1640 tissue culture medium (Flow, Aust.) minus antibiotics, with 0.21% $NaHCO_3$, 4mM L-glutamine (Flow), and 10% foetal calf serum (CSL, Aust.) heated to 56°C for 30 min, in tissue culture flasks (Corning, Aust.) at 37°C in a humidified incubator in 7.5% CO_2, 5% O_2 and 87.5% N_2.

2.2. Fluorimetric assay

Cells ($2.5x10^5$/well) in 24 well tissue culture plates were washed twice in Hanks balanced salt solution buffered with 25mM Hepes, pH7.4(HH). HH buffer (200ul) was added to 4-6 replicate wells; 20ul of the elastase substrate [8] AcAlanylAlanyl-ProlylAlanine-7-amino-4-methylcoumarin (APA-AMC), was added to begin the reaction (final concentration 350uM) and the plates incubated in a moist atmosphere at 37°C for 40 min. Solution was removed from the wells, placed on ice then centrifuged (10,000g, 10min, 4°C) and 150ul of the supernatant assayed in a microcuvette for released product, 7-amino-4-methylcoumarin (AMC) in a Hitachi 4000 fluorimeter at wavelengths of 380nm (excitation) and 448nm (emission). To determine the amount of elastase secreted by the cells during the assay period, at 40 min, solution was removed from wells in which cells had been incubated in buffer only, centrifuged and 200ul of supernatant assayed with substrate in fresh wells for a further 40 min. Porcine pancreatic elastase (PPE) E0258 from Sigma, U.S.A., was used as a standard. Results were not corrected for the presumed partial inactivity of this commercial enzyme.

2.3. Extraction and partial purification of cell-surface elastase

Subconfluent cells in 75cm^2 flasks were washed twice in a solution of NaCl (132mM), KCl (5mM), and Hepes (20mM). Cell-surface elastase was extracted by rocking (3-5min) the flasks containing 2.5ml of the same solution with butanol (1-4%) included. After centrifugation (10 min, 1200g) the supernatant containing cell-surface proteins was concentrated 100 fold in Centricon 10 microconcentrators (Amicon, Aust.) at 4°C. The concentrate was added to the drained bed of a Blue Sepharose (Pharmacia, Aust.) column (1.6cm x 25cm) equilibrated with a buffer of NaCl (0.6M), Brij 35 (0.1%) and sodium borate (25mM, pH 8.3) following dilution 1:3 in buffer. The column was washed with the same buffer at a flow rate of 30ml/h and fractions (1 ml) were collected and tested for elastase activity using the fluorimetric assay. Protein determination was carried out using fluorescamine labelling of protein [13] with bovine serum albumin Fraction V as standard at wavelengths of 380nm (excitation) and 486nm (emission). Fractions showing the highest specific activity were concentrated 10 fold in fresh microconcentrators and used for elastin hydrolysis.

2.4. Hydrolysis of insoluble elastin

Aliquots (30-50ul) of fractions of partially purified elastase collected from the Blue Sepharose column were added to 50ul of elastin (10mg/ml, bovine nuchae ligamentum elastin, ES60, pass 400 mesh, Elastin Products, Md., U.S.A.) in a buffer containing 0.6M NaCl, 10mM $CaCl_2$, 25mM sodium borate, pH 8.3 (total volume

400ul) in siliconized, screw-topped Sarsted microtubes. Samples were incubated at 37°C for 24h with constant rotation then centrifuged (10min, 10,000g, 4°C) to pellet residual elastin. Supernatants (250ul) were carefully removed to tubes containing 500ul sodium borate (pH8.5) and 250ul fluorescamine (660ug/ml) was added while vortexing. Peptides released by elastolysis were measured as detailed (2.3). To determine the amount of fluorescence due to background protein, partially purified elastase was added to elastin which had been incubated for 24h in buffer containing an amount of detergent equivalent to that in the test sample. This value was used as the 0 time point and deducted from the total fluorescence measured at 24h. Results are expressed as equivalent to the fluorescence obtained from the peptide Leucyl leucine (Serva,Aust.) used as a reference standard.

2.5. Assays with inhibitors

Human leukocyte elastase (HLE) with an activity of 160mU/mg towards SucAlaAlaVal-paranitroanilide, was generously provided by Dr. M. Smith, Raymond Purves Laboratory, Royal North Shore Hospital, St. Leonards, N.S.W., Australia. Inhibitors, Trasylol (aprotinin), Bayer, Aust; α_1-antitrypsin and soya bean trypsin inhibitor (SBTI), Sigma, U.S.A.; phenylmethone sulphonyl fluoride (PMSF), Calbiochem., Aust., and HLE, PPE or cell-extracted enzyme were preincubated at 37°C for 10 min in HH (160ul). Aliquots (140ul) were removed to a microcuvette containing 10ul of 3.5mM APA-AMC and the rate of AMC release monitored for 3min and compared with that of enzyme solutions preincubated in HH buffer only.

3.RESULTS

3.1. Cell-associated and secreted elastase

After an assay time of 40min, bladder cancer cells released up to 3.1uM AMC (Table 1). This was equivalent to that released by 962pg PPE. Of the total activity, only 8% could be attributed to secreted activity which suggested that the majority of measured enzyme was cell-associated. After >6h in the presence of substrate, cells remained intact and viable as determined by exclusion of trypan blue (0.2%) and the ability to grow in culture which suggested that the cell-associated activity was located at the cell surface. Cells viewed in a fluorescent microscope exhibited fluorescent product at the cell periphery which could be removed by washing leaving no evidence of internal fluorescence (results not shown). This was further evidence to suggest that elastase activity was located at the cell surface. Elastase could be readily extracted from the cell surface with 1%(cell lines BL28,13A) or 4%(cell line B10) butanol, leaving 95%(BL28,13A) or 75%(B10) of cells excluding trypan blue.

Table 1 Cell-associated and secreted elastase activity of intact cells measured with APA-AMC

	nM AMC released at 40 min	
Cell line	Total*	secreted (% of total)
B10	3,140	8
13A	1,783	8
BL28	600	7

*Values represent the average of two experiments.

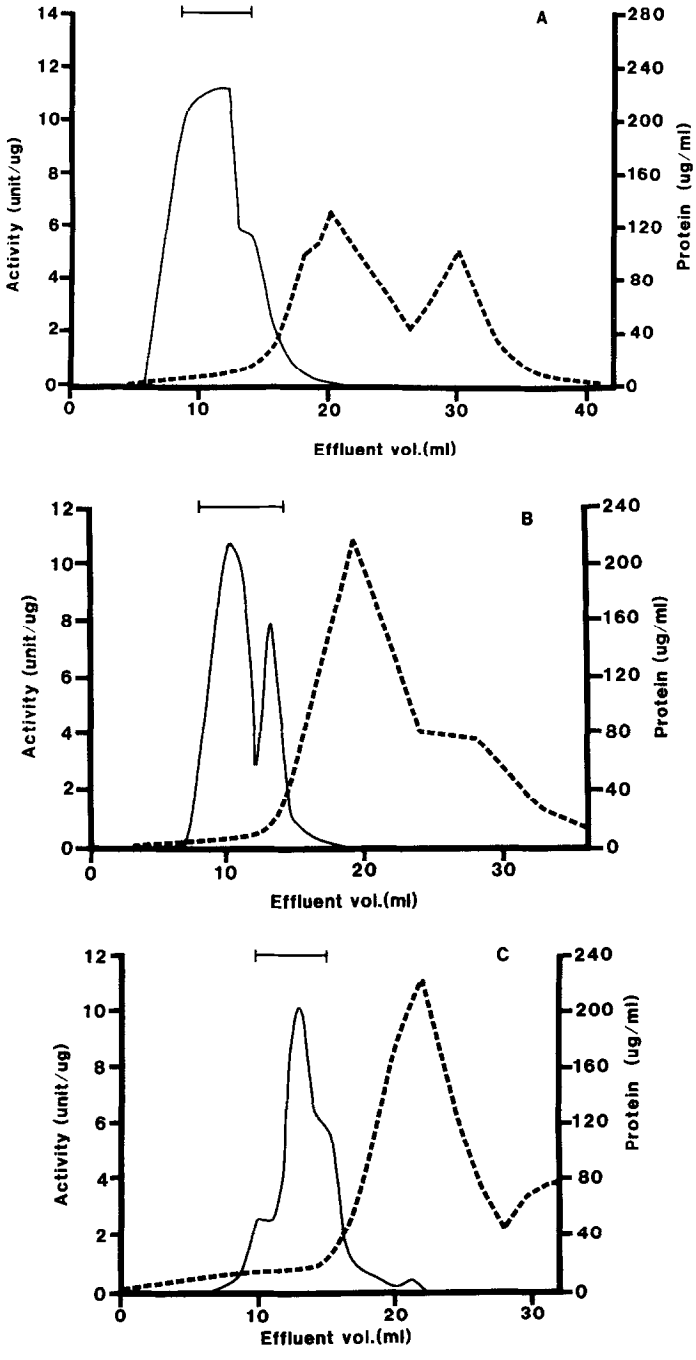

Figure 1. Profiles of elastase activity from the Blue sepharose column. (A) B10; (B)13A; (C) BL28.

—— Elastase activity as measured using APA-AMC as a substrate.

----- Protein concentration as absorption at 280nm.

⊢⊣ Fractions used for elastin hydrolysis.

3.2. Partial purification of cell-surface elastase

Figure 1 shows the profiles of elastase activity purified from three separate cell lines by passage through Blue Sepharose. Elastase activity eluted immediately following the

Table 2 Partial purification of cell-extracted elastase

Cell Line	Step	protein (ug)	Total Activity Units(a)	Specific Activity (b)	Yield %	Enrich-ment (fold)
A)B10	crude	7,000	3,941	0.563	100	-
	concentrate	1,584	1,057	0.667	27	-
	Blue Seph.(c)	35	785	22.44	20	40
B)13A	crude	11,270	947	0.084	100	-
	concentrate	2,718	1,082	0.398	114	5
	Blue Seph.(c)	78	197	2.51(d)	21	23
C)BL28	crude	9,608	2,071	0.216	100	-
	concentrate	1,981	2,298	1.16	111	5
	Blue Seph.(c)	46	776	16.88	37	70

(a) One unit of activity is equal to that of 1ng PPE/min as measured in the fluorimetric assay with APA-AMC as substrate.
(b) Specific activity is expressed as unit/ug protein.
(c) This step refers to the fractions showing the highest specific activity from the Blue Sepharose column after further concentration. For (A) and (B), 26-67% of added protein passed through the membrane during concentration increasing the specific activity. This did not occur with (C).
(d) In all cases specific activity was determined immediately prior to elastin hydrolysis. However, 13A was frozen at -70°C before assay and it was estimated that about 50% of the total units of activity was lost during freezing and thawing. This could account for the lower apparent specific activity compared with individual fractions collected from the Blue Sepharose column.

void volume for all cell extracts showing that elastase was excluded from Blue Sepharose under these conditions. This gave very good separation of elastase activity from other proteins. An enrichment in specific activity (23-70 fold) was achieved (Table 2). Fractions from the Blue Sepharose column which showed the highest specific activity towards APA-AMC, hydrolysed insoluble elastin thus confirming the presence of elastase (Table 3). Elastase from 13A was the most efficient of the three bladder cancer cell elastases in hydrolysing insoluble elastin but still less so than PPE which has been found to be the elastase species that most readily solubilizes bovine elastin [4].

3.3. Inhibition of cell-surface extracted elastase

Table 4 shows that none of the cell-surface extracts of elastase was inhibited by EDTA but all were significantly inhibited (85-96%) by 1mM PMSF indicating that these

Table 3 Elastin hydrolysis by partially purified cell-surface elastase extracts from the Blue Sepharose column

Sample	pmol leucine equivalents released/ng* elastase at 24h
PPE	318
B10	44
13A	178
BL28	54

*Activity was determined using APA-AMC as substrate and PPE as a reference standard.

Table 4 Effect of proteinase inhibitors on elastase activities

Inhibitor	Concentration	Inhibition (%)				
		HLE	PPE	B10	13A	BL28
Trasylol	88ug/ml	76	0	0	0	0
EDTA	10mM	0	0	0	0	0
PMSF	1mM	97	90	96	90	85
SBTI	100ug/ml	98	0	0	0	0
α_1-antitrypsin	100ug/ml	96	98	75	63	72

Inhibition is expressed as the percentage inhibition compared with enzyme preincubated in the absence of inhibitor. PMSF was dissolved in propan-2-ol before dilution in HH buffer. An equivalent amount of propan-2-ol was included in the control samples. All other compounds were in aqueous solution.

bladder cancer elastases are serine proteinases. They were also inhibited (63-75%) by α_1-antitrypsin inhibitor (100ug/ml). As was the case for PPE, Trasylol and SBTI had no effect on any of the three bladder cancer cell elastase activities.

4.DISCUSSION

As early as 1968, Gilfillan [14] reported that tumorigenic human, hamster and mouse cells were able to phagocytose and degrade elastin when cultured over 72h in the presence of elastin. More recently Kao and Stern [5] observed that human breast carcinoma cell lines secreted pro-elastases into serum-free conditioned medium and this activity was inhibited in proportion to the amount of serum present. They deduced that serum elastase inhibitors caused this inhibition. Mouse mammary adenocarcinoma cloned cells and cells from primary culture secreted active elastase into serum-free medium which also was inhibited (90%) by a 1/50 dilution of mouse serum [6]. By contrast, elastase measured in intact human bladder cancer cells represents active enzyme synthesized in the presence of serum and therefore reflects enzyme levels which were not inactivated by serum inhibitors. Although still an artificial system, this is more likely to represent the levels of enzyme which are active in vivo, than those studies in which cells are cultured in serum-free medium before examination of cell-associated or secreted enzymes.

Evidence to suggest a cell-surface location of elastase comes from fractionation of porcine aortic smooth muscle cells [15], fractionation of monocytes which had been labelled with a synthetic elastase inhibitor [16] and, from studies of extracellular matrix degradation by human fibrosarcoma cells [7]. That elastase produced by human bladder cancer cells was also located at the cell-surface, was inferred from the low (8%) amount of the total activity which could be attributed to enzyme secreted by the cells during assay, the fact that fluorescent product could be removed by washing, and that elastase activity could be extracted into solution with butanol leaving the majority of cells excluding trypan blue.

The location of proteinases at the cell surface raises several physiologically important implications:

(1) Production of enzymes at the cell surface may provide a higher concentration of enzyme than if the same amount were dispersed in a fluid phase and would thus increase the effective level of proteinase activity within a localized area.

(2) Immobilization of proteinases at the cell surface may help to confine proteolytic activity to one location thus preventing widespread tissue degradation.

(3) The ability of inhibitors to bind to the active site of enzymes may be influenced by the stereo-conformation of immobilized enzyme molecules. Large inhibitors may bind less easily to molecules bound to the cell surface than to enzyme molecules which are free in solution. Considering cell and tissue abuttment, it is likely that studies of proteinases present at the cell surface rather than those secreted into conditioned medium, may be of more relevance in investigating the potential role of proteinases in tissue degradation and tumour invasion. Although cells from normal bladder epithelium could be grown in primary culture, attempts to passage these cells were unsuccessful, therefore a comparison of elastase levels between normal and cancer cells under similar conditions was not possible. However, all three cell lines were derived from tumours which were highly invasive in the patient. Theoretically, the presence of this broad spectrum proteinase is potentially advantageous to cancer cells in their invasion of normal tissues.

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