

Cathepsin B-like Enzymes

SUBCELLULAR DISTRIBUTION AND PROPERTIES IN NEOPLASTIC AND CONTROL CELLS FROM HUMAN ECTOCERVIX*

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Distribution of cathepsin B-like activity was determined in fractions of control and neoplastic epithelial cells from human ectocervix. Cells were isolated from specimens obtained at surgery and cultivated in chemically defined media. Neoplastic cells with a moderate rate of proliferation (NCE-1) were separated from those in the same primary tumor with higher replication rates (NCE-2). Both tumor and control cells were harvested in the logarithmic phase of growth and were disrupted and fractionated in buffered isotonic sucrose. In each cell line, activities of acid phosphatase and β -glucuronidase were concentrated in mitochondria-lysosome (ML) fractions. Cathepsin activity was also concentrated in ML fractions of control but not in those of neoplastic cells, with activity largely in crude nuclear (CN) fractions of NCE-2. Plasma membranes (PM) with densities of 1.13–1.16 g/ml were purified from CN fractions. The molar ratio of cholesterol to phospholipid was 0.91 in PM of controls and 0.88 to 0.89 in PM of cancer cells. Activities of plasmalemma marker enzymes in PM were enriched by 30 to 40 times homogenate levels and specific binding sites for wheat germ agglutinin were enriched by 34- to 44-fold. In neoplastic, but not control, cells, cathepsin activity was also enriched in PM to as high as 14 times homogenate levels in NCE-2. This activity was reduced no more than 6% by extraction of membranes with hypotonic or high salt buffers. Concentration of proteinase was also found in Triton-purified nuclei. Enzyme in fractions of tumor and control cells cleaved the endopeptidase substrate, benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy- β -naphthylamide, at a pH optimum of 6.2 at 37 °C, with a K_m of 0.038 mM in ML of controls and 0.056 to 0.058 mM in PM and purified nuclei of NCE-2. Activities were inhibited strongly by iodoacetic acid, ZnSO₄, leupeptin, antipain, and tosyl-L-lysine chloromethyl ketone, whereas pepstatin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, EDTA, and CaCl₂ were less effective. Cathepsin B-like enzyme in ML fractions of control and cancer cells was unstable at pH 8.0 and sensitive to heat denaturation at 65 °C. In contrast, activity in PM and nuclei of NCE-2 cells showed considerable stability at pH 8.0 and at 65 °C. Thus, heat- and alkaline-stable cathepsin B-like activity occurs in

plasma membranes and nuclei of neoplastic cervical cells.

The studies of Carrel and Ebeling in 1928 (1) offered the first indication that malignant tumors exhibit increased activity of acid proteinase at or near the external cell surface. The occurrence of elevated acid proteinase activity in the extracellular milieu of neoplastic cells has since been well established (2–6). Such tumor-associated activity appears to be largely attributable to a thiol proteinase with properties similar to that of cathepsin B, as characterized from other sources (6–8).

Several lines of evidence indicate that neoplastic cells *per se* are the major source of the thiol proteinase activity found extracellularly. Recent studies have shown that serum activity of a cathepsin B-like enzyme is markedly elevated in patients with a wide variety of cancers and falls precipitously upon successful excision or chemical treatment of the malignancy (9, 10). Moreover, malignant cells obtained at surgery and cultivated *in vitro* continue to release thiol proteinase into their extracellular media at levels which correlate well with serum levels of enzyme activity that prevailed *in vivo* before excision of the tumors (4). Other investigations have shown that the rate of secretion of cathepsin B-like activity *in vitro* by cells undergoing neoplastic transformation (11, 12) and by malignant cells (4, 5, 13) exceeds that by their untransformed counterparts by as much as 50-fold. Such secretion of thiol proteinase by neoplastic cells has also been found to exhibit a positive correlation with the rate of cell growth in soft gels (4).

Biochemical data on the subcellular distribution of cathepsin B-like activity in homogenates of tumor cells are not presently available. Investigations of several normal tissues have shown that cathepsin B occurs predominantly in mitochondria-lysosome fractions obtained by relatively mild homogenization methods (7, 14). Using cytochemical methods, others have also localized the enzyme in lysosomes of normal pancreatic β -cells observed by electron microscopy (15) and in cytoplasmic granules of normal fibroblasts viewed by fluorescence and bright field microscopy (16). In contrast, 1 report provides evidence for a substantially different distribution of cathepsin B in neoplastic cells. Using fluorescein-labeled antisera directed against a partially purified preparation of cathepsin B, Sylvén *et al.* (17) found that antigen with immunological similarity to the lysosomal proteinase was present largely at or near the surfaces of malignant cells. Corresponding untransformed cells exhibited reactivity with the specific antisera only in cytoplasmic granules. Nevertheless, it is important to note that numerous independent investigations

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have indicated that other acid hydrolases, which are enriched in crude lysosomal fractions of normal tissues, occur predominantly in cytosol fractions of cancerous tissues (*cf.* 3). Similar results have also come from studies of cervical carcinomas and control cervical material (18, 19). However, the apparent predominance of lysosomal enzymes in the cytosol fraction of tumor cells might well have resulted from inadvertent extraction of the hydrolases by homogenization procedures (*e.g.* blender homogenization (19)) which elicit extensive damage to lysosomes and other cell structures (3, 20).

The present work seeks to determine the distribution of cathepsin B-like and other selected enzyme activities in cell fractions obtained after controlled homogenization of control and neoplastic cervical epithelial cells. As recommended by deDube (20) and others (21), we have adopted an analytical approach to subcellular fractionation. This report provides a balance sheet wherein are given the values of several marker enzyme activities, biochemical constituents, and specific ligand binding, as these are related to the levels of corresponding components in the homogenate. The properties of cathepsin B-like activities in the resultant subcellular fractions of control and neoplastic cervical cells are compared to those of cathepsin B isolated from other sources.

EXPERIMENTAL PROCEDURES

Materials—All solutions for cell fractionation and subsequent analyses were prepared with water ultrapurified to a specific resistance exceeding 18 megaohms-cm, using a Millipore Milli-RO reverse osmosis system in series with a Milli-Q water purification system (Millipore Corp.). Collagenase, type I, was purchased from Worthington. Minimum essential medium with D-valine and horse serum were from Grand Island Biological. Estradiol-17 β , cortisol, and gentamicin were obtained from Schering, while transferrin, phenylmethane sulfonyl fluoride, tosyl-L-lysine chloromethyl ketone, soybean trypsin inhibitor, iodoacetate, calf thymus DNA, yeast RNA, and fetuin were from Sigma. Highly purified albumin (Pentex) was purchased from Miles. Cytidine 5'-monophosphate-[4,5,6,7,8,9-¹⁴C]sialic acid (158 Ci/mol) and [³H]wheat germ agglutinin (4.37 Ci/mmol) were from New England Nuclear, while unlabeled wheat germ agglutinin and N-acetyl-D-glucosamine were from Calbiochem-Behring. Ultrapure sucrose of density gradient grade was purchased from Schwarz/Mann. Nylon mesh of 35- μ m pore size was from Tobler, Ernst, and Traber, Inc., Elmsford, NY. Leupeptin, antipain, and pepstatin were from the Peptide Institute, Inc., Osaka, Japan. Z¹-Ala-Arg-Arg-4-MeO β NA and Z-Gly-Gly-Arg-4-MeO β NA were obtained from Enzyme Systems Products, Indianapolis, IN. Bz-Arg- β NA and Bz-Arg-amide were purchased from Sigma. The sources of other reagents and materials used here have been stated earlier (10, 12, 22, 23).

Cell Cultures—Cervical epithelial cells were prepared from tissue specimens obtained from 6 patients at the UCLA Medical Center. Three control patients (40 \pm 5 years) with noncancerous ectocervix underwent hysterectomies for endometriosis, benign myoma of the uterus, or pelvic inflammatory disease. The remaining patients (56 \pm 4 years) were diagnosed as having squamous carcinoma of the cervix and donated tissue samples taken as incisional biopsies. Portions of all cervical excisions were submitted for histologic examination, and the remaining tissue was used in these studies. The 3 epidermoid carcinomas were classified as stages IB (n = 1) or IIB (n = 2) according to criteria proposed by the cancer committee of the International Federation of Gynecology and Obstetrics (24). This study was conducted with approval of the institutional committee for protection of human subjects; informed consent of the latter was obtained in all cases.

Tissue specimens obtained aseptically at surgery were maintained on ice and transported to the laboratory within 30–60 min. Cervical epithelial cells were isolated from underlying tissues by use of the collagenase procedure described previously (4, 22). Isolated cells were initially cultivated at a density of 1.5×10^4 cells/cm² in minimum essential medium with D-valine substituted for L-valine (4, 25, 26). Media were supplemented with 5% horse serum, 2×10^{-9} M estradiol-17 β , 2×10^{-9} M cortisol, 1×10^{-8} M insulin, 0.5 μ g of transferrin/ml, and 50 μ g of gentamicin/ml. Thereafter, media were progressively depleted of serum and estradiol, and the latter were replaced by 0.1% (w/v) albumin and 1 mM sodium pyruvate (4). Cultures were maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C. After 10 days, neoplastic cervical epithelial cells with a moderate rate of proliferation *in vitro* (NCE-1, cell doubling time of 30 h) were separated from a variant subline with a higher replication rate (NCE-2, cell doubling time of 20 h) by an intercellular aggregation method described in detail previously (4, 12). In these experiments, the number of NCE-2 cells initially isolated averaged $23 \pm 4\%$ of the tumor mass, with the remainder NCE-1 cells. Both lines of carcinoma cells and control cervical epithelial cells (CCE, cell doubling time of 111 h) were then cultivated for 3 weeks in media with serum and estradiol, followed by 1 week in chemically defined media lacking the latter supplements (4). Thereafter, cells were harvested in the logarithmic phase of growth. All cells retained an epithelial appearance (4), and at least 95% of cells sampled in each experiment excluded vital dyes such as nigrosin or trypan blue (final concentration, 0.05% in phosphate-buffered Ringer solution) after 10 min incubation at 37 °C (12).

Cell Fractionation—Plasma membrane and other subcellular fractions were prepared at 4 °C from the isolated cervical epithelial cells essentially by the method of Pietras and Szego (23). Cells were disrupted in 9 volumes of 0.25 M sucrose containing 0.1 mM calcium acetate and 5 mM Tris-HCl, pH 7.4, using a ball-shaped Teflon pestle in a Dounce homogenizer (approximately 0.15 mm clearance). The number of homogenizer strokes (forward and return) was critical for reproducible sedimentation of plasma membranes and other cellular organelles. The extent of cell rupture was ascertained each 8 strokes using a phase-contrast microscope for the first 24 strokes and each 2 strokes thereafter (27). Homogenization was stopped when about 98% of the cervical cells were disrupted (28). Apart from the few intact cells, predominantly free nuclei and apparent plasma membrane ghosts were visible along with cytoplasmic material under the phase microscope. About 28 strokes were used for disruption of neoplastic cells and 32 strokes for control cell preparations. The homogenate was then diluted with an equal volume of medium and filtered 2–3 times through 2 layers of 35- μ m nylon mesh to remove all unbroken cells. The filtrate (*e.g.* whole homogenate) was then centrifuged at 4,100 rpm for 20 min in a Sorvall SS-34 rotor to sediment the crude nuclear fraction. This fraction was washed 3 times with an equal volume of 0.25 M sucrose containing 5 mM Tris-HCl, pH 7.4, and finally suspended in 18 ml of 52% (w/w, 1.24 g/ml) sucrose in 5 mM Tris-HCl, pH 7.4. The following 2 discontinuous sucrose density layers were introduced above the latter suspension: from bottom to top, 18 ml of 37% (1.16 g/ml) and 2 ml of 31% (1.13 g/ml) sucrose in 5 mM Tris-HCl, pH 7.4. Gradients were centrifuged in a Spinco SW-27 rotor

at 22,400 rpm for 16 h ($\int_0^t \omega^2 \cdot dt = 3.2 \times 10^{11}$ rad²/s) at 4 °C using a

Beckman model L5-75 ultracentrifuge. After centrifugation, fractions at 1.13–1.16 g/ml (plasma membrane) and at 1.16–1.24 g/ml (particulate material) were each collected, diluted with 0.25 M sucrose in 5 mM Tris-HCl, pH 7.4, and washed twice by centrifugation at 13,700 rpm for 20 min in a Sorvall SS-34 rotor. The semipurified nuclear sediment was likewise diluted with medium and washed twice by centrifugation at 4,100 rpm in a Sorvall SS-34 rotor. All fractions were then suspended in 0.25 M sucrose containing 5 mM Tris-HCl, pH 7.4, to about 1 mg of protein/ml and stored at 4 °C. Analyses for ligand binding and enzyme activities were conducted within 24 h.

The combined supernatants and washes of the crude nuclear pellet (CN) were centrifuged twice at 9,300 rpm for 15 min in a Sorvall SS-34 rotor, yielding a mitochondrial-lysosomal sediment (ML). The postmitochondrial supernatants were combined and centrifuged at 40,000 rpm for 1 h in the type 40 rotor of a Spinco model L ultracentrifuge, yielding a microsome-rich pellet (Ms) and a supernatant (S). All fractions were diluted as appropriate with 0.25 M sucrose in 5 mM Tris-HCl, pH 7.4, to about 1 mg of protein/ml and stored at 4 °C.

Purification of Nuclei—The semipurified nuclear sediment obtained after sucrose density gradient centrifugation of the crude

¹ The abbreviations used are: Z, N^o-benzyloxycarbonyl-; -4-MeO β NA, -4-methoxy- β -naphthylamide; Bz-, benzoyl-; - β NA, - β -naphthylamide; Tos-, p-toluenesulfonyl-, -CH₂Cl, -chloromethyl ketone; CCE, control cervical epithelial cells; NCE-1, neoplastic cervical epithelial cells with moderate rate of replication *in vitro*; NCE-2, neoplastic cervical epithelial cells with high rate of replication *in vitro*; CN, crude nuclei; ML, mitochondria-lysosome; Ms, microsome-rich; S, 105,000 \times g supernatant; PM, plasma membrane; P, particulate; N, semipurified nuclei; UPN, ultrapurified nuclei.

nuclear fraction was purified further at 4 °C. Semipurified nuclei were first exposed to 0.01% (v/v) Triton X-100 in 0.25 M sucrose with 5 mM Tris-HCl, pH 7.4, followed by immediate centrifugation for 10 min at 4,100 rpm in a Sorval SS-34 rotor, Sorvall RC2-B centrifuge. Subsequent examination of treated nuclei by dark field ultraviolet fluorescence microscopy after staining with acridine orange (29) revealed that the resultant preparations were essentially devoid of extraneous cytoplasmic organelles. These preparations will be referred to as ultrapurified nuclei (UPN).

Lectin Binding—Binding of [³H]wheat germ agglutinin (4.37 Ci/mmol) to cervical cells was carried out by methods similar to those of Chang *et al.* (30). About 10⁸ isolated cells were incubated at 22 °C with 0.2 µCi of labeled lectin (39 ng/ml) for 3 min in Ringer solution with 1 mM sodium pyruvate (31). The free radioactivity in the medium was removed by sedimenting the cells at 4 °C for 5 min at 1000 rpm in a Sorvall SS-34 rotor, Sorvall RC2-B superspeed centrifuge, followed by 2 successive washes with ice-cold homogenization medium. Plasma membrane and other cellular fractions were then prepared as described above. Over 98% of [³H]wheat germ agglutinin binding to cervical cells was blocked in the presence of a 100-fold molar excess of unlabeled wheat germ agglutinin or by prior incubation of the lectin with 0.1 M N-acetyl-D-glucosamine.

Enzyme Analyses—All enzyme analyses were carried out at 37 °C and in the presence of 0.1% (v/v) Triton X-100 to overcome the problem of latency of enzyme activity (20, 32). Activity of 5'-nucleotidase (EC 3.1.3.5) was determined by the method of Touster *et al.* (33). Analyses for alkaline phosphatase (EC 3.1.3.1) were done as described in Pietras (12). Activity of ouabain-sensitive (Na⁺, K⁺-activated) adenosinetriphosphatase (EC 3.6.1.3) was determined as described earlier (31). Measurement of sialyltransferase (EC 2.4.99.1) activity was done by the method of Bernacki and Kim (34).

The assay of cathepsin B (EC 3.4.22.1) activity was conducted with procedures described by Szego *et al.* (7). In brief, determinations in triplicate were conducted in 40 mM sodium citrate-sodium phosphate, pH 6.2, in the presence of 5 mM dithiothreitol and 0.2 mM Z-Ala-Arg-Arg-4-MeOBNA. Fluorescence of the reaction product was measured at the emission peak of 420 nm on an Aminco-Bowman spectrofluorometer with an activation beam of 292 nm. A series of concentrations of the fluorescent product, 4-methoxy- β -naphthylamine, was analyzed concomitantly, as standard.

Acid phosphatase (EC 3.1.3.2) activity was analyzed as described by Szego *et al.* (35). β -Glucuronidase (EC 3.2.1.31) activity was determined by the method of Fishman as modified by Musa *et al.* (36). Succinate dehydrogenase (EC 1.3.99.1) activity was measured according to Pennington (37) and that of glucose 6-phosphatase (EC 3.1.3.9) by the method of Hübscher and West (38). For each enzyme activity, linearity of the reaction with time as well as sample protein was verified experimentally.

Chemical Analyses of Plasma Membranes—Plasma membranes collected at sucrose gradient interfaces were diluted with 5 mM Tris-HCl, pH 7.4, and pelleted by centrifugation. To remove adsorbed or occluded material (39, 40), pellets containing about 1.5 mg of protein were resuspended in 10 ml of 0.15 M NaCl and 5 mM Tris-HCl, pH 7.4, and incubated for 1 h at 4 °C, with repeated resuspensions at 10-min intervals with a Vortex stirrer. The membranes were then pelleted by centrifugation and resuspended in highly purified water at an approximate protein concentration of 1.5 mg/ml. Aliquots of this suspension were analyzed chemically for protein, cholesterol, phospholipid, sialic acid, DNA, and RNA. Protein was analyzed by the method of Lowry *et al.* (41) with bovine serum albumin as standard. Lipids were extracted from washed plasma membranes with chloroform by the method of Bligh and Dyer (42) as modified by Touster *et al.* (33); aliquots were analyzed for total cholesterol by the procedure of McDougal and Farmer (43) and for total organic phosphate by the method of Galliard *et al.* (44). Total phospholipid content was calculated by assuming 770 as the average molecular weight of the phospholipids (27). N-Acetylneuraminic acid was determined by the procedure of Warren (45) after hydrolysis of membranes in 0.1 M H₂SO₄ at 80 °C for 1 h. DNA and RNA were extracted according to Maggio *et al.* (46). DNA was analyzed by the diphenylamine method of Burton (47) with calf thymus DNA as standard. RNA was determined by the orcinol method described by Schneider (48) using yeast RNA as standard.

Data Analyses—Errors are expressed as standard errors of the mean. The *t* test was applied to assess the differences between the means of paired or independent data, as appropriate. The determination of *K_m* and *V_{max}* values for cathepsin B-like enzyme in subfrac-

tions of cervical epithelial cells was performed by the method of Lineweaver and Burk (49).

RESULTS

Distribution of Protein, Enzymes, and Specific Wheat Germ Agglutinin Binding Sites in Major Fractions of Cervical Cells

The distribution of cellular constituents was investigated in homogenates prepared by the mild procedures outlined above from control and neoplastic cell lines. The proportions and relative specific activities of protein, enzymes, and lectin binding in the major subfractions of the several cell groups are shown in Table I. In all cases, wheat germ agglutinin binding is concentrated predominantly in crude nuclear fractions and to a lesser extent in microsome-rich fractions. Cervical plasma membrane-marker enzymes, (Na⁺, K⁺-activated) adenosinetriphosphatase, 5'-nucleotidase, and alkaline phosphatase (27, 50, 51) are distributed similarly. The crude nuclear fractions also contain more than 96% of the DNA present in each homogenate (not shown). Glucose 6-phosphatase occurs largely in the microsome-rich fractions. Activities of succinate dehydrogenase, acid phosphatase, and β -glucuronidase are concentrated in mitochondrial-lysosomal fractions. The latter fraction also contains about 70% of cathepsin B-like activity in control cells (Table I). In contrast, substantially less cathepsin activity is recovered in the mitochondria-lysosome pellet of neoplastic cells, whether with moderate (*p* < 0.05) or high (*p* < 0.001) rates of proliferation. Especially evident in those tumor cells with high proliferative activity (*i.e.* NCE-2, Table I) is the prominent association of cathepsin B-like activity with crude nuclear fractions. Moreover, using the present method of cell disruption, little activity of cathepsin B-like or other sedimentable hydrolases was noted in the cytosol fractions.

Characterization of Plasma Membrane and Associated Cellular Fractions

The crude nuclear sediment is generally contaminated with plasmalemmal as well as microsomal membrane components released during cell disruption (21, 32). Further fractionation of the nuclear pellet was achieved by isopycnic centrifugation in a discontinuous sucrose density gradient. The resultant bands at 1.13–1.16 g/ml (plasma membrane), 1.16–1.24 g/ml (particulate), and the semipurified nuclear sediment were collected, washed at the centrifuge, and analyzed for lectin binding and enzyme activities (Table II). For additional analyses, ultrapurified nuclei were prepared by treatment of the nuclear sediment with Triton X-100 as described.

Among control and carcinoma cell lines, fractions at 1.16–1.24 g/ml apparently represent a mixture of particulate material with minimal enrichment of either plasma membrane-marker enzymes or binding sites for wheat germ agglutinin (*i.e.* particulate, Table II). The greatest enrichment of these plasmalemmal markers occurs in those fractions recovered at 1.13–1.16 g/ml (*i.e.* plasma membranes, Table II). In all cell groups, the relative specific activities of (Na⁺, K⁺-activated) ATPase, 5'-nucleotidase, and alkaline phosphatase in the latter fraction range from 30–40 times the homogenate, while specific lectin binding ranges from 34–44 times the homogenate. By interpolation from concentrations in the mitochondria-lysosome fractions (Table I), fractions at 1.13–1.16 g/ml (plasma membrane) exhibit a maximum relative specific activity of 0.003 for succinate dehydrogenase, indicating a mitochondrial contamination of 0.3%. Similar calculations for glucose 6-phosphatase activities suggest that the low density membrane fractions are composed of microsomal material to the extent of 0.9–1.8%. Based on determinations of β -glucuronidase activities, contamination with lysosomal components

TABLE I

Distribution of protein, wheat germ agglutinin binding, and enzymes in major subfractions of control and neoplastic cervical epithelial cells

Determinations were conducted on subfractions of control cervical cells (CCE), and neoplastic cells with either moderate (NCE-1) or high (NCE-2) rates of proliferation by methods given in the text. Data on subcellular fractions are from 2 or 3 independent experiments and represent the average percent distribution in each of the indicated fractions of the total homogenate activity. Corresponding values in parentheses represent the ratio of the specific activity of enzyme or lectin binding in the isolated fraction to that in the homogenate.

Determination	Cell group	Homogenate	Subcellular fraction					Recovery	
			Crude nuclear	Mitochondria-Lysosome		Microsome-rich	Supernatant		
		units	% homogenate (relative specific activity)					%	
Protein ^a	CCE	22.6±1.2	34.8±0.9	23.6±0.2		9.8±0.1		28.5±0.3	96.7±0.7
	NCE-1	24.6±0.8	34.2±3.1	19.5±1.8		13.9±1.9		31.9±2.2	99.6±0.8
	NCE-2	25.6±1.0	34.9±1.9	20.6±2.7		13.4±2.2		29.5±2.2	98.4±0.7
Wheat germ agglutinin binding ^b	CCE	12.4	52.5 (1.51)	13.3 (0.56)		27.2 (2.78)		2.1 (0.07)	95.1
	NCE-1	11.5	50.9 (1.49)	12.4 (0.64)		36.2 (2.60)		2.1 (0.07)	101.6
	NCE-2	11.1	52.9 (1.52)	11.3 (0.55)		33.5 (2.50)		0.6 (0.02)	98.3
Na ⁺ , K ⁺ -ATPase ^c	CCE	21.2±2.8	52.8±0.2 (1.52)	13.1±1.2 (0.56)		28.6±0.2 (2.93)		1.1±0.3 (0.04)	95.6±0.9
	NCE-1	22.6±1.5	51.7±2.0 (1.51)	15.1±2.1 (0.78)		31.0±4.2 (2.22)		1.5±1.0 (0.05)	99.2±3.1
	NCE-2	28.0±0.6	51.1±1.6 (1.47)	14.3±1.6 (0.71)		29.9±3.7 (2.27)		1.4±0.6 (0.05)	96.7±3.7
5'-Nucleotidase ^c	CCE	52.8±3.2	48.3±1.1 (1.38)	10.0±1.1 (0.42)		38.0±2.7 (3.89)		1.9±0.3 (0.07)	98.2±0.8
	NCE-1	53.7±4.4	46.7±2.9 (1.37)	7.2±1.7 (0.39)		41.8±6.5 (2.98)		1.6±0.8 (0.05)	97.3±3.2
	NCE-2	49.6±0.8	48.1±0.9 (1.40)	7.9±1.6 (0.38)		37.7±4.0 (2.89)		2.0±1.6 (0.07)	95.7±1.7
Alkaline phosphatase ^c	CCE	32.8	51.7 (1.56)	15.2 (0.66)		23.6 (2.39)		3.5 (0.12)	94.0
	NCE-1	34.4	42.1 (1.34)	17.3 (0.90)		33.6 (2.12)		0.8 (0.02)	93.8
	NCE-2	38.3	44.0 (1.31)	15.8 (0.86)		34.1 (2.17)		0.4 (0.01)	94.3
Cathepsin B ^c	CCE	15.3±0.9	15.9±0.9 (0.46)	69.7±1.0 (2.96)		6.4±0.1 (0.65)		1.9±0.1 (0.07)	93.8±0.8
	NCE-1	12.8±0.9	36.2±4.6 (1.05)	51.9±8.5 (2.66)		6.1±3.7 (0.58)		2.4±1.3 (0.07)	98.3±0.5
	NCE-2	11.0±0.6	48.9±6.4 (1.39)	33.3±9.9 (1.69)		11.8±3.1 (0.92)		4.0±2.2 (0.10)	98.0±1.8
Acid phosphatase ^c	CCE	16.2±1.6	14.3±0.8 (0.41)	72.5±2.1 (3.08)		6.8±1.4 (0.70)		1.5±0.0 (0.05)	95.2±0.9
	NCE-1	17.8±0.8	22.2±3.2 (0.70)	58.3±4.6 (3.33)		14.7±0.3 (0.93)		3.7±1.5 (0.11)	98.9±1.2
	NCE-2	20.2±1.2	24.4±2.5 (0.57)	57.5±8.8 (2.51)		8.6±3.2 (0.50)		3.5±0.9 (0.10)	93.9±2.8
β-Glucuronidase ^c	CCE	10.6±0.4	17.1±4.4 (0.49)	77.5±3.5 (3.30)		2.3±0.8 (0.24)		1.2±0.7 (0.04)	98.1±2.6
	NCE-1	12.2	15.2 (0.38)	69.2 (3.93)		12.0 (0.09)		2.1 (0.05)	98.5
	NCE-2	15.8±0.3	26.6±6.7 (0.73)	67.8±5.3 (2.96)		0.9±0.1 (0.08)		1.7±1.4 (0.07)	97.0±2.4
Succinate dehydrogenase ^c	CCE	14.1	7.5 (0.23)	85.9 (3.72)		0.5 (0.05)		0.6 (0.02)	94.5
	NCE-1	15.8	4.1 (0.14)	97.1 (4.53)		0.2 (0.01)		0.0 (0.00)	101.4
	NCE-2	15.3	7.7 (0.24)	88.4 (4.52)		0.2 (0.01)		0.0 (0.00)	96.3
Glucose-6-phosphatase ^c	CCE	0.7±0.2	13.3±1.6 (0.38)	3.8±1.3 (0.16)		75.7±4.1 (7.77)		0.1±0.0 (0.14)	96.8±2.2
	NCE-1	1.0±0.1	11.8±4.2 (0.33)	4.6±2.3 (0.26)		73.3±5.0 (5.38)		6.0±1.7 (0.19)	95.7±2.1
	NCE-2	1.0±0.2	16.9±8.4 (0.47)	5.9±4.8 (0.34)		70.1±5.6 (5.41)		4.7±3.6 (0.15)	97.7±1.4

^amg/10⁸ cells.

^b10⁻⁴ x (dpm/mg protein).

^cnmol/min/mg protein.

appears to range from 1.5–2.7%. To evaluate the potential contamination of plasma membrane subfractions of control and NCE-2 cells with elements of the Golgi complex, activity of sialyltransferase was determined (cf. Ref. 32). Enzyme activity averaged 0.011 and 0.014 nmol of sialic acid transferred/min/mg of protein in homogenates of control and NCE-2 cells, respectively ($n = 2$, not shown). Only 0.9% of activity in the homogenates was found in membrane fractions at 1.13–1.16 g/ml, with relative specific activity of 0.82 in control and 0.86 in NCE-2 preparations.

The enzymic analyses confirm a predominantly plasma membrane origin of cellular subfractions at 1.13–1.16 g/ml and are, in turn, consistent with additional evidence on the chemical composition of the several fractions. The latter results of analyses for cholesterol, phospholipid, sialic acid, DNA, and RNA are presented in Table III. Membranes of control cells showed somewhat higher cholesterol/protein ratios and lower phospholipid/protein ratios than those of neoplastic cells. The molar ratios of total cholesterol to phospholipid were 0.91, 0.89, and 0.88 for membranes of CCE, NCE-1, and NCE-2 cells, respectively. The values for sialic acid, which may be bound to either protein or lipid, were highest in CCE cell plasma membranes and lowest in NCE-2 cell membranes. DNA was not detectable in the several membrane preparations. RNA contents of all membrane fractions were low, indicating little ribosomal contamination of the plasma membranes.

Association of Cathepsin B-like Activity with Plasma Membranes and Ultrapurified Nuclei—Plasma membrane fractions of control cells exhibit a marginal enrichment of acid phosphatase (cf. Ref. 53) but no concentration of cathepsin B-like activity (Table II). In contrast, plasma membranes of neoplastic cells with low and high rates of proliferation show substantial cathepsin activity enriched to 3.5 and 14.5 times that of the respective homogenates. About 14% of total cellular cathepsin B-like activity is recovered in plasma membranes of cells with high replication rates (NCE-2, Table II).

Since entrapment of soluble enzyme inside plasmalemmal vesicles or adsorption to plasma membranes could account for a substantial portion of the cathepsin activity detected in cancer cell membranes, control experiments were instituted to investigate these potential artifacts. The acid proteinase activity of the latter plasma membranes was reduced by no more than 6% ($p > 0.30$) by extraction of membranes with hypotonic buffer (i.e. 5 mM Tris-HCl, pH 7.4) or physiologic saline (i.e. 0.15 M sodium acetate, not shown).

It is equally important to note that a small enrichment of cathepsin B-like activity occurs in ultrapurified nuclei of neoplastic, but not control, cells (Table II). In contrast, these ultrapurified nuclei exhibit essentially no specific binding sites for wheat germ agglutinin and little activity of the other predominantly extranuclear enzymes shown in Table II.

Substrate Specificity and pH Dependence of Cathepsin B-like Activity—The pH dependence of cathepsin B-like activity

TABLE II

Distribution of protein, wheat germ agglutinin binding, and enzymes in plasma membrane and associated subcellular fractions of control and neoplastic cervical epithelial cells

Determinations were conducted on subfractions of the crude nuclear pellet from control cervical cells (CCE) and neoplastic cells with either moderate (NCE-1) or high (NCE-2) rates of proliferation by methods described in the text. Data are from 2 or 3 independent experiments and represent the average percent distribution in each fraction of total homogenate activity (see Table I). Corresponding values in parentheses represent the ratio of the specific activity of enzyme or lectin binding in the isolated fraction to that in the homogenate (Table I).

Determination	Cell group	Subfraction of crude nuclear pellet			
		Plasma Membrane	Particulate	Semipurified nuclear	Ultrapurified nuclear
		% homogenate (relative specific activity)			
Protein ^a	CCE	1.1±0.1	6.2±1.2	24.7±0.2	15.9±0.6
	NCE-1	0.6±0.3	8.0±1.1	24.7±2.5	16.8±2.0
	NCE-2	1.0±0.3	7.0±1.6	24.6±2.6	15.9±2.6
Wheat germ agglutinin binding ^b	CCE	37.7 (34.27)	13.1 (2.11)	1.0 (0.04)	0.4 (0.03)
	NCE-1	26.5 (44.17)	23.0 (2.88)	0.8 (0.03)	0.3 (0.02)
	NCE-2	35.6 (35.60)	15.4 (2.20)	1.5 (0.06)	0.5 (0.03)
Na ⁺ , K ⁺ -ATPase ^c	CCE	34.2±3.9 (30.14)	14.8±2.2 (2.78)	3.8±1.8 (0.15)	1.4±0.6 (0.09)
	NCE-1	19.1±0.8 (33.22)	25.0±4.5 (3.07)	5.2±1.3 (0.22)	2.5±0.9 (0.15)
	NCE-2	29.8±6.7 (30.93)	14.0±4.0 (2.41)	3.9±1.1 (0.16)	1.8±0.6 (0.12)
5'-Nucleotidase ^c	CCE	38.5±0.4 (34.89)	8.8±1.1 (1.46)	1.8±0.1 (0.08)	0.5±0.1 (0.03)
	NCE-1	22.2±2.4 (38.35)	16.2±2.7 (2.10)	5.2±1.5 (0.21)	0.7±0.2 (0.05)
	NCE-2	36.3±4.8 (40.64)	5.7±2.1 (0.94)	4.7±3.3 (0.18)	2.4±2.0 (0.12)
Alkaline phosphatase ^c	CCE	26.9 (30.20)	15.2 (3.91)	9.4 (0.38)	2.1 (0.12)
	NCE-1	17.7 (33.27)	19.1 (2.66)	5.0 (0.22)	1.6 (0.08)
	NCE-2	22.4 (32.69)	14.5 (2.81)	4.9 (0.19)	1.3 (0.07)
Cathepsin B ^c	CCE	0.2±0.1 (0.15)	10.2±0.2 (1.81)	5.4±0.7 (0.22)	1.4±0.4 (0.09)
	NCE-1	1.9±0.4 (3.53)	7.3±3.4 (0.85)	25.8±2.0 (1.05)	19.3±3.3 (1.18)
	NCE-2	13.7±2.6 (14.51)	2.4±1.7 (0.28)	32.9±2.1 (1.36)	29.3±1.1 (1.94)
Acid phosphatase ^c	CCE	0.8±0.2 (0.73)	4.5±0.6 (0.75)	8.8±1.5 (0.36)	3.9±0.7 (0.24)
	NCE-1	0.1±0.1 (0.23)	11.6±1.7 (1.62)	6.8±0.9 (0.30)	2.5±0.1 (0.14)
	NCE-2	0.3±0.2 (0.30)	12.5±0.9 (2.01)	10.0±1.1 (0.32)	3.5±0.0 (0.16)
β-Glucuronidase ^c	CCE	0.1±0.1 (0.08)	11.9±2.2 (1.92)	3.0±2.7 (0.12)	1.3±1.2 (0.09)
	NCE-1	0.1 (0.06)	12.3 (0.64)	2.4 (0.28)	1.1 (0.18)
	NCE-2	0.1±0.0 (0.08)	15.1±3.4 (1.75)	7.5±3.6 (0.29)	2.3±1.2 (0.15)
Succinate dehydrogenase ^c	CCE	0.0 (0.01)	5.9 (1.51)	1.6 (0.06)	0.4 (0.02)
	NCE-1	0.0 (0.00)	3.5 (0.53)	0.2 (0.01)	0.1 (0.01)
	NCE-2	0.0 (0.00)	6.6 (1.14)	0.1 (0.01)	0.0 (0.00)
Glucose-6-phosphatase ^c	CCE	0.1±0.0 (0.07)	9.9±1.9 (1.63)	3.7±0.2 (0.15)	1.7±0.3 (0.11)
	NCE-1	0.1±0.0 (0.09)	8.5±2.1 (1.03)	1.9±1.6 (0.07)	0.6±0.4 (0.04)
	NCE-2	0.1±0.0 (0.10)	12.3±7.0 (1.52)	2.8±1.6 (0.11)	0.4±0.4 (0.03)

^amg/10⁸ cells.

^b10⁻⁴ × (dpm/mg protein).

^cnmol/min/mg protein.

in plasma membranes of control and neoplastic cervical cells was analyzed using the substrate, Z-Ala-Arg-Arg-4-MeOβNA, as shown in Fig. 1. Enzyme activity in cancer cell membranes is clearly optimal at about pH 6.2. At this pH, activity in equivalent amounts of normal cell membranes is 14–53 times less than that of neoplastic cell membranes. Among all membrane preparations, a second minor peak of enzyme activity occurred at pH 8.0. However, unlike the activity measured at pH 6.2 (see below), activity at pH 8.0 was inhibited strongly by 0.1 mM phenylmethane sulfonyl fluoride. Moreover, the latter, apparent serine proteinase showed little variation in specific activity in control as compared to neoplastic cell membranes.

The substrate specificity of cathepsin B-like enzyme in membranes of NCE-2 cells was determined with the various synthetic substrates indicated in Table IV. A comparison is also made with the substrate properties of enzyme in ultra-purified nuclei from NCE-2 cells and in mitochondria-lysosome fractions from control cells. As determined for enzyme

in plasmalemma, cathepsin B-like enzyme in the latter fractions was found to cleave Z-Ala-Arg-Arg-4-MeOβNA optimally at about pH 6.2 (not shown). At this pH, the latter substrate was hydrolyzed at the highest rate by enzyme in each of the subcellular fractions (Table IV). The rates of hydrolysis of Z-Ala-Arg-Arg-4-MeOβNA ranged from 25- to 32-fold higher than those determined with Bz-Arg-βNA, the traditional arylamide substrate for cathepsin B (54). Such preferential cleavage of substrate containing 2 adjacent arginine residues is characteristic of cathepsin B activity from other sources (6, 7, 55, 56). However, as reported by others (6, 7, 55, 56), the action of cathepsin B-like enzyme in the present investigation was not enhanced simply by increasing the peptide chain length of the substrate. This is shown by the substantially lower rate of hydrolysis of Z-Gly-Gly-Arg-4-MeOβNA, a substrate which is hydrolyzed by trypsin about 220 times more rapidly than the Bz-Arg-amide, which is widely used for the determination of cathepsin B2 (*i.e.* carboxypeptidase B (55, 57)).

TABLE III

Chemical analyses of plasma membrane subfractions of control and neoplastic cervical epithelial cells

The plasma membrane subfractions (fraction 2) of control (CCE) and neoplastic cells with low (NCE-1) and high (NCE-2) rates of proliferation *in vitro* were prepared and analyzed as described in the text. The results represent the average of analyses of 2 separate membrane preparations that agreed to within 5%.

Determination	Plasma membrane fraction		
	CCE	NCE-1	NCE-2
		$\mu\text{g}/\text{mg protein}$	
Cholesterol	187.0	183.6	184.0
Phospholipid	408.5	411.0	416.4
Sialic acid	7.8	7.1	6.7
DNA	ND ^a	ND	ND
RNA	5.6	6.1	5.3

^a Not detectable.

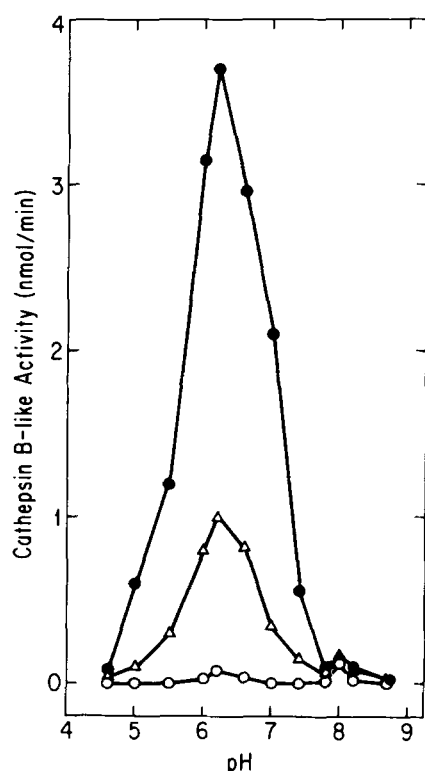


FIG. 1. Analysis of the pH dependence of cathepsin B-like activity in plasma membrane fractions of cervical epithelial cells. Membranes were purified by procedures given in the text from control cells (○), neoplastic cells with a low rate of proliferation (NCE-1, △), and neoplastic cells with a high replicative rate (NCE-2, ●). Duplicate determinations of cathepsin B-like activity were conducted at each pH point, using the synthetic substrate, *N*^α-benzyloxycarbonyl - alanyl - arginyl - arginyl - 4 - methoxy - β - naphthylamide. Plasma membrane samples contained 0.025 mg of protein.

The kinetic parameters for hydrolysis of various concentrations of Z-Ala-Arg-Arg-4-MeOβNA by cathepsin B-like enzyme in subcellular fractions of cervical cells were determined from data presented in Fig. 2. Substrate concentrations were limited to a range of 0.025–0.2 mM since apparent substrate inhibition was encountered at higher levels (55). For each preparation, the measured maximal velocity appears to closely approximate the theoretical maximum velocity (Fig. 2). With hydrolysis of substrate at pH 6.2 and 37 °C, K_m was 0.038 mM for enzyme in mitochondria-lysosome fractions of control cells. As reported for cathepsin B-like activity of breast cancers (6), values of K_m for enzyme in tumor cell fractions were somewhat

TABLE IV

Substrate specificity of cathepsin B-like activities in subcellular fractions of control and neoplastic cervical epithelial cells

The source of enzyme activity was either the mitochondria-lysosome fraction of control cells (CCE), plasma membranes of neoplastic cells (NCE-2), or ultrapurified nuclei of neoplastic cells (NCE-2). Except where otherwise noted, incubations were conducted in the presence of 0.2 mM substrate and 5 mM dithiothreitol at pH 6.2 for 30 min, as described in the text. Efficiency of Bz-Arg-amide was analyzed by application of the fluorescence method of Taylor *et al.* (79) and that of Bz-Arg-βNA by the fluorescence method of McDonald *et al.* (80). Fluorescent products resulting from hydrolysis of Z-Gly-Gly-Arg-4-MeOβNA or Z-Ala-Arg-Arg-4-MeOβNA were analyzed as described in the text. Enzyme activities are reported relative to that of Z-Ala-Arg-Arg-4-MeOβNA which is expressed as 100%. Values represent the averages of triplicate analyses in 2 independent preparations that agreed to within 6%.

Substrate	Relative specific activity		
	Mitochondria-lysosome (CCE)	Plasma membrane (NCE-2)	Ultrapurified nuclei (NCE-2)
	%		
Z-Ala-Arg-Arg-4-MeOβNA	100.0	100.0	100.0
Bz-Arg-βNA	3.1	3.4	4.0
Z-Gly-Gly-Arg-4-MeOβNA	1.1	1.6	0.8
Bz-Arg-amide	0.8	0.2	0.3

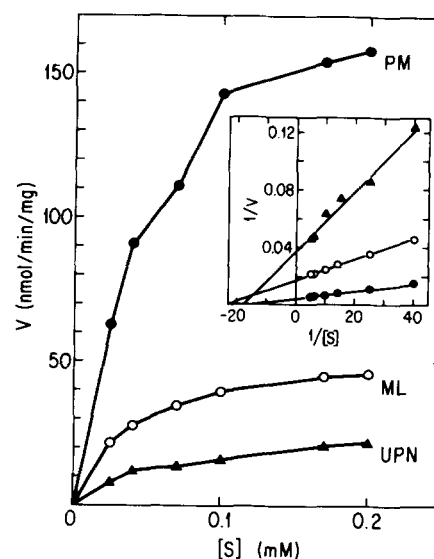


FIG. 2. Influence of concentration of Z-Ala-Arg-Arg-4-MeOβNA on reaction velocity. Sources of enzyme activity include the mitochondria-lysosome fraction of control cells (ML, ○), plasma membranes of NCE-2 cells (PM, ●), and ultrapurified nuclei of NCE-2 cells (UPN, ▲). Rates of hydrolysis of the substrate at concentrations ranging from 0.025–0.2 mM were determined in the presence of samples containing 0.05 mg of protein in each of 2 independent sets of experiments and otherwise as described in the text. Kinetic parameters were determined by the method of Lineweaver and Burk (49); a double reciprocal plot is shown as an inset. Values of V_{max} (nanomoles/min/mg of protein) are 54.5 in ML fractions of control cells and 212.3 and 26.2 in PM and UPN of neoplastic cells, respectively.

higher than that found in control cell fractions. The latter values averaged 0.058 mM and 0.056 mM for proteinase in plasma membranes and nuclei, respectively, of NCE-2 cells. These values correspond well with estimates of K_m for hydrolysis of Z-Ala-Arg-Arg-4-MeOβNA by cathepsin B of preputial gland (7) but are lower than that reported for enzyme from other sources (6, 56, 58).

Inhibition of Cathepsin B-like Activity—Thiol proteinase activity in mitochondria-lysosome fractions of control cervical

cells, as well as that in plasma membranes and ultrapurified nuclei of neoplastic cells (NCE-2), was strongly antagonized by agents shown to inhibit cathepsin B from other sources (7, 14, 54, 56, 58, 59). The latter inhibitors include ZnSO_4 , iodoacetic acid, leupeptin, antipain, and Tos-Lys- CH_2Cl (Table V). There was little or no inhibition of cathepsin B-like activity by pepstatin, an inhibitor of cathepsin D and of pepsin (60), nor by phenylmethanesulfonyl fluoride or soybean trypsin inhibitor, antagonists of serine proteinases (61). At a concentration of 1.0 mM, but not at 0.1 mM, CaCl_2 elicited a slight reduction of cathepsin B-like activity in the several subcellular fractions (Table V). A small inhibition of enzyme activity was also found in the presence of 2.0 mM EDTA.

Stability of Cathepsin B-like Activity—The effect of pH on the stability of cathepsin B-like enzymes in the several sub-

cellular fractions indicated in Table VI was investigated by preincubating samples for 6 h at 4 °C in buffers at pH 7.4 or pH 8.0 followed by assay under standard conditions. Enzyme in mitochondria-lysosome fractions of control cells (Table VI), as well as that in corresponding fractions of NCE-2 cells (not shown), was partially inactivated by prior incubation at pH 8.0, but not at pH 7.4. In contrast, enzyme in both plasma membranes and ultrapurified nuclei of NCE-2 cells was relatively stable to preincubation at pH 8.0 as compared to nonpreincubated controls (Table VI).

There was a manifest difference in the thermal stability of cathepsin B-like activity in the several cervical cell fractions (Table VI). The bulk of enzyme activities in mitochondria-lysosome fractions of control cells is destroyed by heating to 65 °C for 5 min, while that of plasma membranes and ultrapurified nuclei from NCE-2 cells is relatively resistant to the heat treatment. In contrast, cathepsin B-like activity in mitochondria-lysosome fractions of NCE-2 cells is substantially less resistant to heat denaturation at 65 °C for 5 min, showing a loss of 84% of thiol proteinase activity (not shown).

DISCUSSION

This communication provides new evidence on the occurrence of cathepsin B-like activity in plasma membranes and ultrapurified nuclei of neoplastic cervical epithelial cells. Although about 70% of cathepsin B-like activity occurs in the mitochondria-lysosome fraction of control cell homogenates, only 33–52% of such activity is found in this fraction of corresponding neoplastic cells. Especially among the cancer cells with a high rate of proliferation *in vitro*, a significant proportion (*i.e.* 13.7%) of total cellular cathepsin B-like activity is associated instead with plasma membrane subfractions sedimenting at a density of 1.13–1.16 g/ml. As in earlier studies on plasma membranes of HeLa cells (27, 50–52), the latter membrane fractions show marked enrichment of 5'-nucleotidase, alkaline phosphatase, and (Na^+ , K^+ -activated) ATPase to 31–41 times homogenate levels. Our plasma membrane preparations also concentrate specific binding sites for wheat germ agglutinin (30, 31) but exhibit little activity of acid phosphatase, β -glucuronidase, succinate dehydrogenase, or glucose 6-phosphatase (*cf.* Ref. 27). Plasma membranes of control and NCE-2 cells also show little enrichment of sialyltransferase activity which is concentrated in Golgi fractions (*cf.* Ref. 32). The high molar ratio of cholesterol to phospholipid found in plasma membranes of both control and neoplastic cells provides further evidence of their purity (27, 51).

The enrichment, to about 15 times the homogenate, of cathepsin B-like activity in plasma membranes of neoplastic cells has not heretofore been reported. However, our findings do correspond with previous observations on the immunohistochemical localization of cathepsin B at or near the surfaces of tumor cells (17). To investigate potential artifacts due to conditions of homogenization and processing which might give rise to the present results, we have attempted to extract the thiol proteinase activity of plasma membranes with hypotonic buffer or with media of high ionic strength known to promote the solubilization of proteins loosely associated with membrane preparations (23, 31, 32, 39). However, these treatments elicited a loss of no more than 6% of plasmalemma-associated cathepsin B-like activity. Such results indicate that the proteinase is not loosely bound to tumor cell membranes or entrapped within membrane vesicles. Further investigation is required to distinguish whether such cathepsin B-like activity of cervical carcinoma cells is an integral membrane protein, as may be the proteinase activities in erythrocyte membranes (62–64), or whether cathepsin B-like enzyme is firmly but transiently bound to membranes, possibly to specific receptor

TABLE V

Effects of potential inhibitors of cathepsin B-like activity in subcellular fractions of control and neoplastic cervical cells

Mitochondria-lysosome fractions of control cells and plasma membrane (fraction 2) or ultrapurified nuclear fractions of neoplastic cells (NCE-2) were prepared as described in the text. Activity in each sample (50 μg of protein) was determined \pm the various inhibitors indicated in the table, as described under "Experimental Procedures." Values represent the averages of triplicate analyses in 2 separate experiments that agreed to within 5%.

Inhibitor	Cathepsin B-like activity			
	Concentration	Mitochondria-lysosome (CCE)	Plasma membrane (NCE-2)	Ultrapurified nuclei (NCE-2)
	mM	% inhibition		
None	0.0	0	0	0
CaCl_2	0.1	0	0	0
	1.0	9	12	13
ZnSO_4	0.1	100	97	93
ZnSO_4 + EDTA	0.1 + 2.0	12	14	13
EDTA	2.0	10	12	11
Iodoacetic acid	0.1	100	100	100
Leupeptin	0.001	98	100	92
Antipain	0.001	88	91	85
Pepstatin	0.001	0	1	0
Tos-Lys- CH_2Cl	0.01	92	94	90
Phenylmethane sulfonyl fluoride	0.1	9	8	10
Soybean trypsin inhibitor	100 ($\mu\text{g}/\text{ml}$)	5	3	4

TABLE VI

Effect of pH and temperature on the stability of cathepsin B-like activity in subcellular fractions of control and neoplastic cervical cells

Mitochondria-lysosome fractions of control cells (CCE) and plasma membrane (fraction 2) or ultrapurified nuclear fractions of neoplastic cells (NCE-2) were prepared as described in the text. Samples were incubated in the absence of dithiothreitol either in the buffer of indicated pH for 6 h at 4 °C or at the indicated temperature for 5 min at pH 7.4. Aliquots of the latter were then assayed under standard conditions at pH 6.2 and 37 °C. Results are the averages of analyses in 2 separate experiments that agreed to within 5%.

Preincubation pH or temperature	Activity at pH 6.2 at 37 °C		
	Mitochondria-lysosome (CCE)	Plasma membrane (NCE-2)	Ultrapurified nuclei (NCE-2)
	% control		
No preincubation	100	100	100
pH 7.4	102	100	104
pH 8.0	74	97	100
22 °C	100	100	99
65 °C	4	95	96

components, in the course of its flux through the membrane compartment (*cf.* Refs. 65 and 66).

The present investigation provides additional evidence for enrichment of cathepsin B-like activity in ultrapurified nuclei of neoplastic, but not control cervical cells. Earlier studies had shown an increase in the level of cathepsin activities in partially purified nuclei of malignant tissues (67) and in ultrapurified nuclei of mitogen-stimulated tissues (7). However, there are conflicting views as to whether proteolytic enzymes are normal constituents of nuclear subfractions or "contaminants" of cytoplasmic origin (68, 69). Since our ultrapurified nuclei show minimal contamination with putative extranuclear enzymes, it is unlikely that the selective concentration of cathepsin B-like activity in tumor cell nuclei is attributable to gross cytoplasmic contamination. Nevertheless, it is not clear from the present data whether the differences in nuclear cathepsin activity between control and carcinoma cells reflect changes subsequent to or concomitant with neoplastic transformation.

The thiol proteinase activities of both control and neoplastic ectocervical cells exhibit many properties similar to those of cathepsin B as characterized in liver (14, 59, 70), spleen (54, 55, 71, 72), preputial gland (7), lung (56), parathyroid gland (58), brain (73), and breast (6). These properties include optimal activity at about pH 6.2, a preference for substrate with 2 adjacent arginine residues, and strong inhibition by ZnSO_4 , iodoacetic acid, leupeptin, antipain, and Tos-Lys- CH_2Cl , but not by pepstatin, phenylmethanesulfonyl fluoride, or soybean trypsin inhibitor. Cathepsin B-like activities of human ectocervix are also partially diminished in the presence of 2.0 mM EDTA and in media containing 1.0 mM, but not 0.1 mM, CaCl_2 . EDTA alone was initially reported to elicit partial activation of cathepsin B from bovine spleen (54). However, McDonald and Ellis (55) later found that activity of the enzyme could be enhanced by EDTA only if added in the presence of a sulfhydryl activator. Others have reported that activity of cathepsin B from preputial gland (7) and from liver (70) exhibits a requirement for micromolar concentrations of Ca^{2+} , but is inhibited by millimolar concentrations of the divalent cation. Our data are consistent with the latter observations. The apparent requirement for EDTA in determinations of the activity of cathepsin B from other sources (*cf.* Refs. 14, 54, and 55) could be attributable to the presence of trace contaminants such as Zn^{2+} , Cu^{2+} , or Hg^{2+} which are potent enzyme inhibitors at concentrations as low as 0.01 mM (54). Alternatively, it may represent a significant organ-specific difference in the properties of cathepsin B-like enzymes.

The stability of cervical cathepsin B-like enzyme under conditions of alkaline pH or heat denaturation appears to be a unique property of the extralysosomal enzyme in carcinoma cells. Unlike the latter, thiol proteinase activity localized in the mitochondria-lysosome fractions of both control and neoplastic cervical cells is partially inactivated by prior incubation at pH 8.0 and destroyed by heating to 65 °C, properties shared by cathepsin B from other organs (7, 8, 58, 59). However, a cathepsin B-like enzyme found in the extracellular media from explants of human mammary adenocarcinoma is reported to be stable to inactivation at pH 8.0 (6). Thiol proteinase secreted by cervical carcinoma cells was also found to retain 96% of its initial activity after treatment at 65 °C for 5 min (4). Hence, the cathepsin B-like enzyme associated with plasma membranes and nuclei of cervical cancer cells exhibits properties similar to those of thiol proteinase secreted by tumor cells from breast and ectocervix. Preliminary characterization of the extracellular enzyme associated with breast tumors indicates that it has a somewhat larger molecular size and a more acidic isoenzyme pattern than cathepsin B from

liver (6). Thus, as proposed earlier (4), it appears worthwhile to investigate the possibility that neoplastic cells produce an altered form or isoenzyme of cathepsin B which is able to escape the usual cellular recognition systems for the intracellular transport and insertion of the enzyme in lysosomes (65, 66). Alternatively, the lysosomal and extralysosomal enzymes may be 2 different activities which fortuitously share similar catalytic properties. Purification of these cathepsin B-like enzymes and subsequent production of specific antisera will be helpful in answering such questions.

In accord with findings in independent investigations (*cf.* Ref. 74), the present data indicate that variant subpopulations of malignant cells (*i.e.* NCE-2) occur in primary tumors of human ectocervix. The subcellular distribution of cathepsin B-like enzyme in NCE-2 cells is not only more aberrant than that of control cells, but also varies significantly from that of paired NCE-1 cells which were initially constituents of the same epidermoid tumor. It remains to be determined whether this pronounced biochemical difference or perhaps other subtle variations in lipid or sialic acid contents of plasma membranes from the 2 neoplastic cell populations may predispose NCE-2 cells for selective invasion of and proliferation in other host tissues. Cleavage by tumor thiol proteinase of several potential substrates in the cell interior or within the microenvironment of the cell surface could play a significant role in the expression of malignancy. For example, cathepsin B-like enzyme from other sources is known to participate in the degradation of collagen (75) and proteoglycans (76), 2 major constituents of the extracellular matrix. The occurrence of such activity at the surface membrane of neoplastic cells could promote their release from the primary tumor and facilitate their dissemination to distant sites (77, 78). The reported hydrolysis of specific histones by cathepsin B from brain (73) and preputial gland (7) suggests that availability of the proteinase in cell nuclei could result in alteration of chromatin function. We hope that questions raised by these and independent investigations (4-7, 9-13) will promote more definitive studies on the nature and functions of cathepsin B-like activities in neoplastic cells.

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