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CHARACTERIZATION OF CYSTEINE PROTEASE AND CYSTEINE PROTEASE INHIBITOR ACTIVITIES IN MCF-7, AND MCF-7/Adr^{R} HUMAN BREAST CANCER CELLS

by

Paul Blakely Scaddan

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Abstract

The expression of the lysosomal cysteine proteases, cathepsins B, H, and L, and cysteine protease inhibitors in intracellular (cells) and extracellular (media) fractions prepared from the MCF-7 human breast cancer cell line, its adriamycin resistant variant, MCF-7/Adr^R, and their somatic cell hybrid, M:A, was examined using synthetic substrates. Although these three cell populations had similar growth parameters, MCF-7/Adr^R demonstrated statistically significant 10-25 fold and 10-15 fold higher levels of intracellular cathepsins B and L activities respectively, than those found in the MCF-7 parent, or the M:A hybrid. Moreover, the increased levels of cathepsins B and L observed in MCF-7/Adr^R were not observed in either *gpt* or *ras/gpt* transfected MCF-7 cells. Cathepsin H activities were similar in all MCF-7 populations examined. The pattern of expression of acid/pepsin activatable cathepsin B in extracellular fractions of MCF-7, MCF-7/Adr^R, and M:A was similar to that observed in intracellular fractions. Analysis of free and total cysteine protease inhibitor levels in MCF-7 and MCF-7/ Adr^R cells using FPLC suggested MCF-7 cells possessed almost twice the total inhibitor level of MCF-7/Adr^R, but retained better than half of this activity in a protease bound (ie., inactive) form. These results support the conclusion that alterations in the levels of total cysteine protease inhibitors, may contribute to increased levels of cathepsin B and L activity in MCF-7/Adr^R cells.

for mom and dad

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Abs	absorbance
Adr^R	adriamycin resistant
AHH	aryl hydrocarbon hydroxylase
arg-arg	Nα-CBZ-argininyl-argininyl 4-methoxy-ß-napthylamide
auto-nano-water	autoclaved nanopure water
BSA	bovine serum albumin
Cat. B	cathepsin B
CBZ	carboxybenzyl
CPI	cysteine protease inhibitor
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
EU	enzyme unit
facs	fluorescence activated cell sorting
FPLC	fast pressure liquid chromatography
gpt	guanine phosphotransferase
KIU	kallikrein inactivating unit

K_{av}	separation coefficient, or proportion of stationary gel volume available for diffusion of a given solute
K_{cat}	enzymatic rate constant
kDa	kilodalton
K_m	Michaelis-Menten constant
MCF	Michigan Cancer Foundation
MEM	minimal essential medium
MDR	multidrug resistance
min	minute
M.W.	molecular weight
n.d.	not detected
PMSF	phenylmethylsulfonylflouride
ras	rat sarcoma
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
UV	ultraviolet
Z-arg-arg-Mec	Nα-CBZ-argininyl-argininyl 7-amido-4 methyl-coumarin.HCl
Z-arg-Mec	N-α-CBZ-L-argininyl-7-amido-4-methylcoumarin. HCl
Z-phe-arg-Mec	N-CBZ-L-phenylalanyl-L-argininyl-7-amido-4-methylcoumarin.HCl

Introduction

Normal homeostasis within animals involves a delicate and complex balance between cell division and cell death. In a young animal, cell multiplication exceeds cell death so that growth may occur. In adults, some cells terminally differentiate then die, and show little or no replacement. The growth of animal cells is a carefully regulated process that responds intrinsically to the specific needs of the body (Darnell *et al.*, 1990). Occasionally, however, the mechanisms involved in regulating homeostasis break down. A cell may begin to grow and divide, although the body has no need for further cells of its type. Descendants of this transformed cell may inherit their parents lack of for regulation, eventually producing a clonal population of cells able to grow and expand indefinitely. This mass of "immortal cells" is called a tumor.

There are two basic types of tumors manifested within an organism, benign and malignant. Benign tumors exist as an encapsulated, non-invasive and non-metastasizing (i.e. non-malignant) mass of cells that generally are not considered life threatening (Clark, 1991). Despite the appearance of proliferative excesses associated with "odd" histological cell patterns, these tumors usually appear well differentiated (Buckley, 1992). In contrast, malignant tumors develop abnormal histological cell patterns and also exhibit a loss of pattern, the most malignant tumors being devoid of any pattern (Buckley, 1992). These histological observations are consistent with evidence that malignant cells exhibit: deficiencies in differentiation, variability in phenotypes, rates of cell proliferation in excess of rates of cell death, and, failure to remain in their

natural positions, resulting in invasion of normal tissue and metastasis to distant sites in the body (Tryggvason *et al.*, 1987). Of all the malignant phenotypes, metastasis, the uncontrolled spread of malignant cells throughout the body, is the primary cause of death from cancer (Liotta, 1992).

1.1 Metastasis: Molecular Mechanism

Descriptively, the process of metastasis is well established. Cells dissociate from the primary tumor, enter the circulatory system, travel to distant sites, and initiate a secondary tumor. However, little is known concerning the molecular mechanism of the metastatic cascade since the inherent complexity of carcinogenesis and of host-tumor interactions have confounded the interpretation of results (Kerbel *et al.*, 1988; Stetler-Stevenson, 1990; Liotta *et al.*, 1991). Despite this restriction, research has provided some insight into the mechanism of metastasis. For example, Liotta and colleagues observed that expression of the metastasis phenotype required activation of a set of effector genes different from those for transformation to immortality (Liotta *et al.*, 1991). Such studies suggest that different malignant phenotypes are under different genetic controls. The diversity in genetic controls, in turn, suggests variability in expression of each malignant phenotype. Thus individual cells from the same tumor may possess different metastatic potential and might therefore be expected to demonstrate different efficiencies for initiating secondary tumors (Fidler and Hart, 1982; Liotta *et al.*, 1991).

Research comparing proteolytic activity in normal and cancer cells has also provided insight into the mechanism of metastasis. In 1925, A. Fischer reported that

malignant tumor tissue, but not normal tissue, released "factors" capable of degrading extracellular proteins (reviewed in Recklies *et al.*, 1980). Research in whole animal, tissue, and cell culture systems over the last 20 years has confirmed this result and has established that levels of protease activities are increased in malignantly transformed cells (Jones and DeClerk, 1980; Hocman, 1992). It has been suggested that this increase in activity is a prerequisite for the expression of the metastasis phenotypes (Liotta, 1990; Hocman, 1992).

The link between tumor-associated proteases and metastasis is a logical one for various reasons. First, there are several points during the metastatic cascade that would be facilitated by the action of proteases; for example, entry into and escape from the circulatory system, and penetration into normal tissue (Liotta, 1992, Hocman, 1992). Second, in normal cells, proteases are involved in degradation of the extracellular matrix, a complex component necessary to maintain the structural and functional integrity of each cell within a tissue. The extracellular matrix consists of two major structural components, the basement membrane and interstitial connective tissue. These differ with respect to composition, location, and function (Tryggvason *et al.*, 1987). Basement membranes are thin, continuous, extracellular structures present throughout the body. They separate organ cells, epithelia, and endothelia from the interstitial connective tissue. The interstitial connective tissue, on the other hand, is a complex matrix composed of cells located in a meshwork of collagen and elastic fibers, proteoglycans, glycoproteins, and hyaluronic acid substance. It is present in several different forms, including bone, tendons, cartilage, ligaments, fasciae, and stroma, and plays a mechanical and supportive role. Degradation is highly regulated and involves

synthesis, activation, and inhibition of pro-enzymes in the extracellular environment (Starkey, 1990). A breakdown in this regulation could result in an increase in the level of active protease. This, in turn, would facilitate metastasis of a cancer cell.

While there is increasing evidence that proteases are involved in metastasis, neither the identities of the proteases involved, nor the molecular and cellular basis for their increased activities is understood. Since some of the extracellular components, for example, collagen and elastin, are not efficiently degraded at physiological pH by general proteases, it might be anticipated that degradation would require a battery of hydrolytic enzymes (e.g., proteases) operating concomitantly (Recklies *et al.*, 1980). More recent evidence suggests that extracellular proteases may indirectly stimulate cell multiplication through the secretion and induction of other proteases via a multi-step, multi-path cascade of proteolytic events (reviewed in Scher, 1987). Regardless of the experimental details, the evidence thus far suggests that the phenotypic expression of metastasis (and invasion) most likely involves a number of proteases working together in a manner similar to the proteolytic cascades involved in biological processes such as blood coagulation (Liotta *et al.*, 1991). Further evidence suggests that the acid proteases, or cathepsins, play an important role in metastasis.

1.2 Proteases: The Lysosomal Cathepsins

Lysosomal exopeptidases (peptidases) and endopeptidases (proteinases) are responsible for the metabolism and function of numerous proteins (Neurath, 1989). The endopeptidases, some of which are also exopeptidases, can be grouped into four major families based on their catalytic site, optimum pH, cation requirements, and

susceptibility to inhibitors: cysteine proteases, serine proteases, aspartic proteases, and metalloproteases (Table 1). While all of these proteases have been implicated in various biological and pathological processes, the lysosomal acidic proteases, termed cathepsins, together with plasminogen activators and collagenases, have been most frequently associated with invasion and metastasis (Dresden *et al.*, 1972; Jones *et al.*, 1975; Rochefort *et al.*, 1992; Sloane *et al.*, 1992).

The cathepsins, for example cathepsin D, an aspartic protease, cathepsin G, a serine protease, and cathepsins B,H, and L, cysteine proteases, are present in most mammalian cells (Neurath, 1989). They are involved in normal turnover of cellular components including the degradation of hemoglobin, collagen, cartilage proteoglycans, immunoglobulin G, bacterial cell wall proteins (reviewed in Leonessa *et al.*, 1992), as well as components taken up from the extracellular environment (reviewed in Sloane *et al.*, 1990a). Cathepsins are synthesized on membrane-bound ribosomes as biologically inactive precursors (Figure 1), and are transferred co-translationally into the endoplasmic reticulum and later into the golgi apparata (Bohley and Seglen, 1992). After post-translational proteolytic processing and modification of carbohydrate moieties, they are transported to the lysosomes. Transport is mediated by a receptor that recognizes mannose-6-phosphate residues present on the precursor cathepsins. This process involves signal peptide cleavage, propeptide cleavage, and in some cases, carboxy-terminal processing (Nishimura *et al.*, 1990; Mach *et al.*, 1992).

In normal cells, proteolytic maturation to the active enzyme occurs as an early event within the lysosomes (Nishimura and Kato, 1987). Only a small amount of the proenzyme is secreted by default into the extracellular fluid. However, the secretion of

TABLE 1

Major Classes of Proteases

CLASS	EC NUMBER	EXAMPLES	pH RANGE for activity
Serine	3.4.21.-	trypsin	7-9
		chymotrypsin	
		plasmin	
		plasminogen activator	
		cathepsin G	
Cysteine or thiol	3.4.22.-	cathepsin B	3-8
		cathepsin H	
		cathepsin L	
Aspartic or acid	3.4.23.-	pepsin	2-7
		cathepsin D	
Metallo-	3.4.24.-	collagenases	7-9
		gelatinase	neutral
		stromolysin	neutral

FIGURE 1:

Intracellular Sorting of Cathepsin B

Cathepsin B is initially synthesized on polysomes of the rough endoplasmic reticulum (RER) as a 39-44 kDa pro-form that is co-translationally N-glycosylated (1). This pro-form then follows a secretory route into the golgi (2), where the majority of the protease acquires a phosphomannosyl residue (P). This residue serves as a high affinity ligand for binding to mannose-6-phosphate receptors (—◄) in the golgi (3). The protease/receptor complex exits the golgi via a clatharin (▬) coated vesicle (4) and is delivered to a pre-lysosomal acidified compartment (5) (early or late endosomes) where dissociation occurs. It is in these pre-lysosomal compartments that processing by limited proteolysis is believed to take place, prior to dissociation. Released protease is packaged into lysosomes (6), while the dissociated mannose-6-phosphate receptor recycles to the golgi (7), or moves to the plasma membrane (8) where it serves to internalize extracellular protease (9). The protease is thus targeted for the lysosome, and is separated from proteins which are destined for immediate secretion (10).

from Dahms *et al.*, 1989
modified by J. Kappos

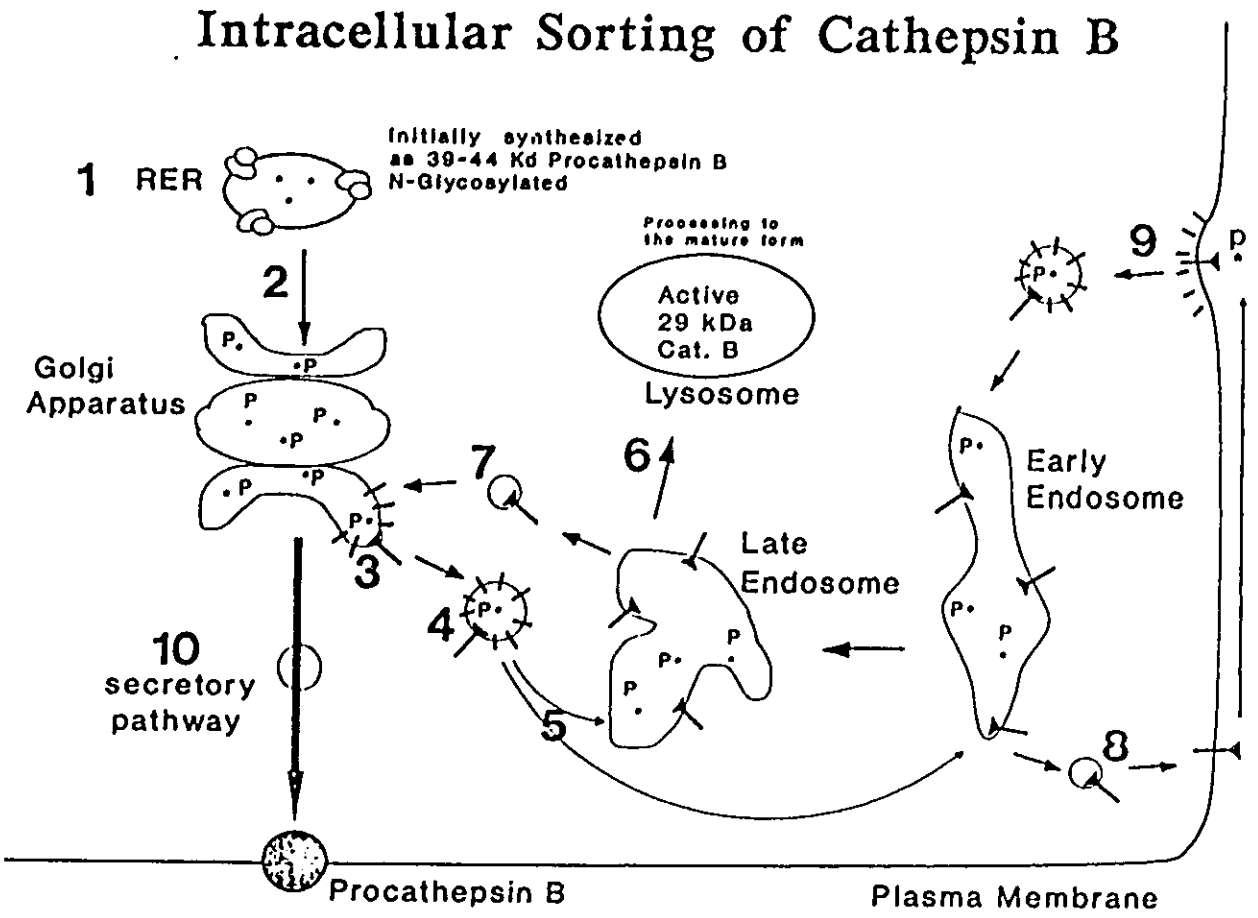

Fig. 1

active and activatable latent forms of cathepsin B and L has been shown to be elevated in response to malignant dedifferentiation (Recklies *et al.*, 1980; Mort *et al.*, 1981; Recklies *et al.*, 1982). It has been suggested that this elevation reflects, at least in part, an alteration in the normal intracellular trafficking of the lysosomal enzymes resulting in increased secretion of lysosomal cathepsins (Sloane *et al.*, 1990a). Other studies supported and expanded on this interpretation. Maciewicz and colleagues (1989), for example, reported high levels of pro-cathepsin B and L in all cell lines established from colorectal carcinoma. However, only clonally-derived, malignant cell lines were able to process them to the mature active forms. Based on these results, it was hypothesized that metastatic potential was better related to levels of active enzyme rather than levels of synthesized precursor (Lah *et al.*, 1992a). The data support this hypothesis. Elevated levels of cathepsin B activity have been reported in experimentally induced tumors (Sloane *et al.*, 1982; Rozhin *et al.*, 1987) as well as in lung (Higashiyama *et al.*, 1991), gastric, colorectal (reviewed in Higashiyama *et al.*, 1993) and breast cancers (Krepela *et al.*, 1989; Lah *et al.*, 1992a). In each study, tumors with higher grades of metastatic potential also demonstrated higher levels of active cathepsin B (reviewed in Sloane *et al.*, 1987; Sloane, 1990). Evidence for a similar correlation between metastatic potential and other cancer-associated enzymes such as cathepsin D (Sloane *et al.*, 1982; Rochefort *et al.*, 1992; Johnson *et al.*, 1993), plasminogen activator (Schmitt *et al.*, 1992), and collagenase (Recklies *et al.*, 1980) is not convincing at present.

1.3 Cysteine Proteases and their Endogenous Inhibitors

It has been suggested for some time that the correlation between cysteine protease activity (e.g., cathepsin B) and metastatic potential appears to be a qualitative one rather than a quantitative one (Rozhin *et al.*, 1990). This may reflect the fact that alterations in activity in malignant tumors can occur in several ways: 1) changes in transcription and translation (i.e., synthesis) of the enzyme (Moin *et al.*, 1989), 2) changes in processing and subcellular localization of the enzyme (reviewed in Sloane *et al.*, 1987), and 3) changes in its regulation by endogenous inhibitors (Lah *et al.*, 1989b). Of these, the third is receiving increased research attention.

Cathepsins, as cysteine proteases, react readily with, and are inhibited by, many thiol-blocking reagents such as peptidyl-diazo methanes or peptidyl-diazomethyl ketones (Polgár, 1989). *In vivo*, however, these proteases are inhibited by the cystatin superfamily of endogenous inhibitors generally known as cysteine protease inhibitors (CPIs). Despite their current popularity, research concerning these protein inhibitors is relatively recent. They consist of three evolution-related families: the stefins, the cystatins, and the kininogens (Anastasi *et al.*, 1983; Müller-Esterl *et al.*, 1985). Stefins and cystatins are low molecular weight inhibitors (11 kDa-15 kDa) relative to the kininogens (56 kDa-160 kDa). Stefins are structurally the simplest inhibitor of the three and contain no disulfide bridges. Cystatins are similar to stefins but possess two disulfide bridges. Gene triplication has generated the kininogen heavy chain which contains three cystatin-like domains (reviewed by Polgár, 1989). The cystatin superfamily of inhibitors all share a sequence of homology, the homology box, around the proposed active site (Sali and Turk, 1987). Of the three families, the low

molecular weight inhibitors appear to be particularly important in the regulation of cathepsins. Members of the cystatin family are found both intracellularly and extracellularly. They are heat, acid, and alkaline resistant (Green *et al.*, 1984; Sloane *et al.*, 1990a) and bind in a one-to-one stoichiometric fashion to the target enzymes with varying affinities (Chambers *et al.*, 1992). The high affinity of the cystatins for cathepsins has led to the hypothesis that their major role *in vivo* is to protect normal cells against increased and unscheduled (i.e., unregulated) protease activity (Chambers *et al.*, 1992). It follows that a breakdown in this inhibitor-mediated regulation could lead to the increased protease activities observed in metastatic cancer cells.

Although a great deal of scientific literature describes increased levels of protease activity, the role of endogeneous protease inhibitors in this increase and its relationship to metastasis has virtually not been investigated until recently. Itoh and colleagues (1987) found that the ascitic fluid and plasma of Sarcoma 180 tumor-bearing mice exhibited a potent inhibitory effect on the enzyme activity of papain (a plant cysteine protease), not observed in plasma from normal mice. Rozhin and coworkers (1990) demonstrated that the enhanced cellular and plasma membrane-associated activity of cathepsin B in isolated B16a murine melanoma cell subpopulations with different lung colonization potentials, did not reflect an increase in expression of the cathepsin B gene. Based on their results, they proposed that this increase reflected an alteration in the regulation of the cysteine proteases by their endogenous inhibitors. While some research is consistent with this explanation, other research is not. At present, there are reports indicating that malignant tumors contain higher, the same, and lower levels of cysteine protease inhibitors than those observed in normal tissues (Lah *et al.*, 1989b;

Rozhin *et al.*, 1990; Chambers *et al.*, 1992). However, this variation may reflect, at least in part, limitations in measuring both protease and protease inhibitor levels in a sample.

1.4 Determination of Endogenous Protease Inhibitor Levels

Levels of protease are determined in two general ways: 1) activity against a natural or synthetic substrate in a reaction mix (i.e., plate assays) or in solid-phase (i.e., gel electrophoresis)(reviewed in Sarath *et al.*, 1989), and 2) immunoassay (Recklies and Mort, 1982). The former measures only active enzyme and is limited by the sensitivity of the assay, the specificity of the substrate, and by interference from components originating from nonmalignant cells in the case of tumors, or the culture environment (e.g., serum) in the case of cell cultures (Dufresne *et al.*, 1993b). Some of the problems have been minimized by using more sensitive fluorometric substrates, synthetic inhibitors to non-relevant proteases, and stringent assay conditions, for example, pH (reviewed in Polgár, 1989). Immunoassay, while more sensitive, does not discriminate between latent, active, or inhibitor-inactive proteases (Recklies and Mort, 1982; Gabrijelic *et al.*, 1992; Higashiyama *et al.*, 1993).

Levels of inhibitor are also measured in two ways: 1) assay of percent inhibition against a standard, exogenous protease, for example, the plant cysteine protease papain, or purified cathepsin B- in a reaction mix, or more recently, embedded in a gel (Rozhin *et al.*, 1990; Ioannidis *et al.*, 1993), and 2) immunoassay. These methods present limitations similar to those for assay of protease levels. Under physiological conditions, for example, the inhibitor can exist in two forms which reflect protease

regulation (detailed in the Discussion) at the simplest level, bound (i.e., inhibitor-inactivated protease), and free. Assay of inhibitor levels under these conditions reflects free rather than total inhibitor. Moreover, since inhibitor levels are routinely determined by measuring percent inhibition against a known, exogenous protease, endogenous protease present in the sample could confound interpretation of results. One approach to this problem utilizes the heat and/or alkaline sensitivity of the lysosomal cysteine proteases and the resistance of their endogenous inhibitors (reviewed in Sloane, 1990). In a representative assay to measure CPIs, for example, cell or tissue homogenates are treated at 100°C for 5 minutes prior to addition of the exogenous protease: ie., papain or purified cathepsin B (Rozhin *et al.*, 1990; Chambers *et al.*, 1992; Sloane *et al.*, 1992). Theoretically, this treatment eliminates endogenous protease activity and dissociates any heat-resistant CPI from the protease:inhibitor complex. The significance of this approach, however, is based on several assumptions, for example: 1) that the elimination of protease activity and dissociation of inhibitor are complete; 2) that the experimental conditions do not vary over time or between experimental systems; and 3) that free dissociated inhibitors do not complex with their free and/or inactivated proteases. At present, these assumptions have not been established. Thus the significance of determinations of inhibitor-to-protease levels in both normal and malignant cell/tissue systems remains questionable as does their relevance to the molecular mechanisms underlying metastasis. The experiments described in this thesis were undertaken to address this problem.

1.5 MCF-7 Breast Cancer Cell Line

Much of what we know concerning the relationship between regulation of protease activity and the malignant phenotype of metastasis has been obtained from analysis of rodent and human malignant tumors (Sloane *et al.*, 1986; Sloane *et al.*, 1987; Moin *et al.*, 1989). Despite the importance of whole animal systems, biochemical and genetic analyses of the molecular and cellular mechanism underlying metastasis are limited by the inherent complexity and heterogeneity of normal and malignant tumors. It is well established, for example, that tumors consist of normal, initiated (i.e. transition), transformed, and malignantly transformed cells with different metastatic potential (Miller *et al.*, 1989). These different types of cells would certainly exhibit different phenotypes which, if not identified, could affect expression and measurement of the malignant phenotypes of interest. The use of cell culture has minimized this problem by permitting the isolation, establishment, and characterization of homogeneous subpopulations of mortal and immortal cells (reviewed in Freshney, 1990) from primary explants (Figure 2). Cell lines established from breast cancers are particularly good for analyses of malignant phenotypes since breast cancers usually metastasize early in the development of the tumor. One of the most interesting, versatile, and well studied breast cancer cell lines is MCF-7 (Vickers *et al.*, 1989; Scaddan and Dufresne, 1992; Scaddan and Dufresne, 1993a; Dufresne *et al.*, 1993a). This cell line was derived from the pleural effusion of a 69 year old Caucasian woman (Soule *et al.*, 1973). It has been studied extensively with respect to numerous malignant phenotypes including: growth factors (Aakvaag *et al.*, 1990), expression of cytochrome P450IA1 (Vickers *et al.*, 1989; Dufresne *et al.*, 1993a), transfection of v-ras^{H} DNA (Kasid *et*

FIGURE 2:

Evolution of a Cell Line

The vertical axis represents total cell growth (assuming no reduction in cell number at each passage); the horizontal axis represents time (weeks) in culture for a hypothetical cell culture. Although a continuous cell line is depicted as arising at 12½ weeks, it could with different cultures arise at any time, likewise senescence may arise at any time.

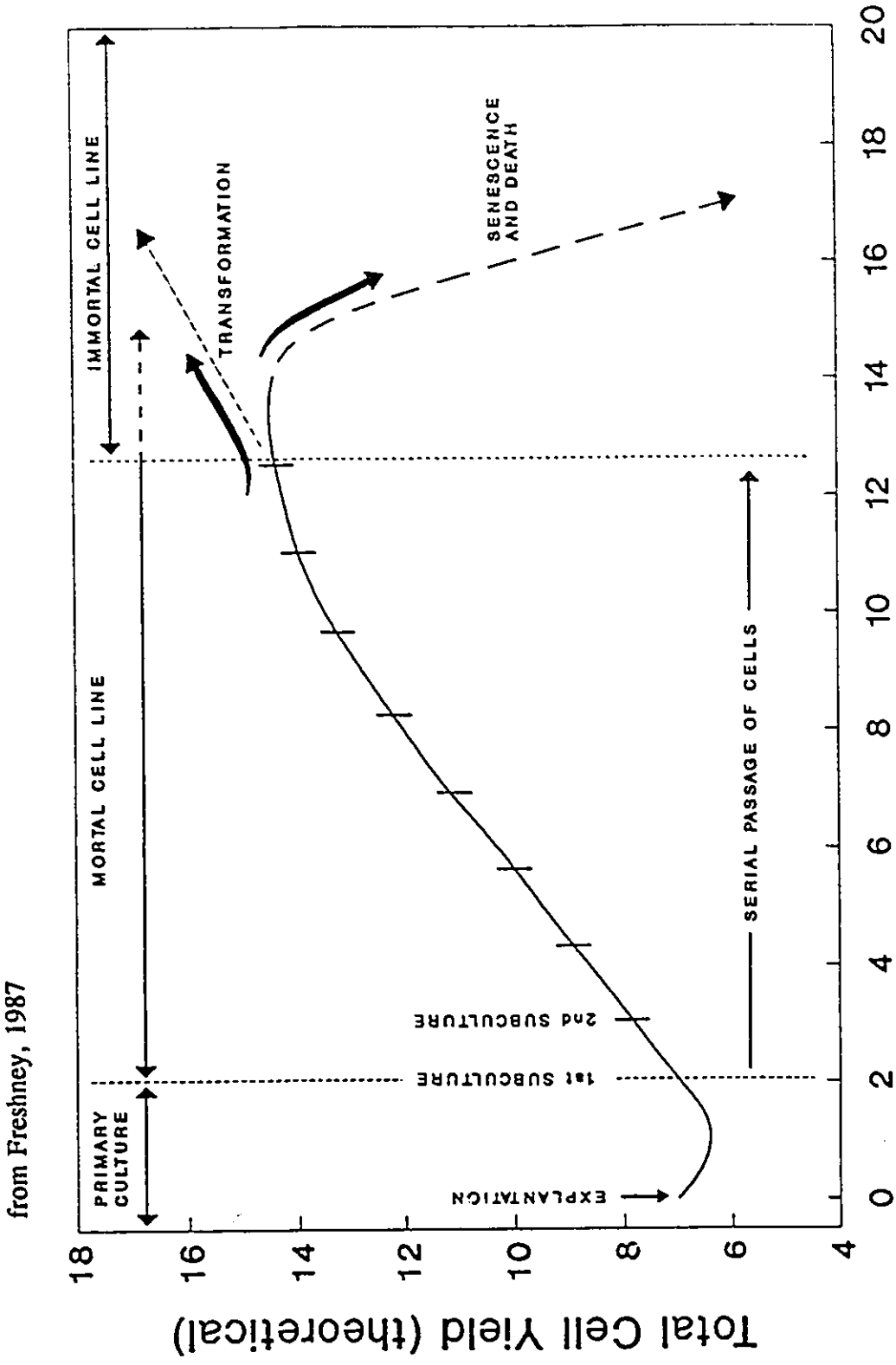
Total Cell Yield (theoretical)
Weeks in Culture
IMMORTAL CELL LINE
MORTAL CELL LINE
PRIMARY CULTURE
TRANSFORMATION
SENESCENCE AND DEATH
EXPLANTATION
1st SUBCULTURE
2nd SUBCULTURE
SERIAL PASSAGE OF CELLS
from Freshney, 1987

Fig. 2

al., 1985; Worland *et al.*, 1989; Gelmann *et al.*, 1992), vimentin expression (Thompson *et al.*, 1992), overexpression of P-glycoprotein and glutathione-S-transferase (Chen *et al.*, 1990; Whelan *et al.*, 1992), and estrogen receptor content (Vickers *et al.*, 1989; Thompson *et al.*, 1992). A great deal has been learned about the relationships between different malignant phenotypes and their practical application. An elevated level of estrogen receptor (ER), for example, is well correlated with prognosis (i.e. survival time) in both pre- and postmenopausal women, where a positive ER status predicts a longer disease free survival (Thompson *et al.*, 1992). Tumors which are most likely to respond to hormonal manipulation, via anti-estrogens, can also be identified by the presence of estrogen receptors. Identification based on these properties, however, is not absolute. Research has demonstrated that breast cancer cells undergo an almost inevitable progression towards a more malignant phenotype characterized by invasive/metastatic foci that ultimately become resistant to chemotherapeutic drugs and endocrine manipulation (Brünner *et al.*, 1993). It is the development of a metastatic, hormone-independent, and drug-resistant phenotype that appears to be responsible for the high percentage of treatment failures among breast cancer patients (Brünner *et al.*, 1993). The ultimate acquisition of the highly metastatic phenotype may be a fundamental biological property of many human breast cancer cells, one linked to alterations in the regulation of proteases (Leonessa *et al.*, 1992).

Although the molecular events that enable breast cancer cells to acquire a progressed phenotype remain unclear (Brünner *et al.*, 1993), it is clear that the MCF-7 breast cancer cell represents an early malignant breast cancer phenotype (Leonessa *et*

al., 1992). MCF-7 cells require physiological levels of estrogen for maximal growth *in vitro* and for tumor formation in nude mice (reviewed in Gelmann *et al.*, 1992). Because of its hormone-dependent properties, MCF-7 has served as a useful model for the study of signals which activate the malignant phenotype in breast cancer (Aakvaag *et al.*, 1990).

Early neoplastic populations like MCF-7 tend to be non-invasive and, by implication, non-metastatic (Leonessa *et al.*, 1992). The application of selective pressures, for example, immunological, nutrient deprivation, and therapeutic intervention, could over a period of time, induce the progression of these cells towards an invasive, metastatic, hormone- and cytotoxic drug-resistant tumor (Clarke *et al.*, 1986). Such is the case with the adriamycin (doxorubicin) resistant variant of MCF-7, MCF-7/Adr^R. This cell line exhibits multidrug-resistance by virtue of its overexpression of P-glycoprotein (Fairchild *et al.*, 1987), and altered expression of drug metabolizing enzymes, including glutathione-S-transferase (Batist *et al.*, 1986), and aryl hydrocarbon hydroxylase (AHH) (Vickers *et al.*, 1989). The MCF-7/Adr^R cell line also expresses significantly lower levels of estrogen receptor than those expressed in its MCF-7 parent cell line and is capable of estrogen-independent growth in nude mice (Vickers *et al.*, 1989). Lastly, the MCF-7/Adr^R cell line is more metastatic and invasive than MCF-7 as demonstrated by Thompson's Boyden chemoinvasion chamber studies (Thompson *et al.*, 1992). Taken together, these results make it clear that the MCF-7 and MCF-7/Adr^R cell lines represent an excellent model system to examine the mechanism of metastasis. First, the alterations in phenotypes accompanying resistance of MCF-7 to adriamycin (i.e. MCF-7 to MCF-7/Adr^R)

mimic the alterations in phenotypes accompanying progression of breast cancer cells to a more malignant state. Second, It has been reported that decreased ER levels are accompanied by increased cathepsin B and L activities, and decreased cysteine protease inhibitor levels (Lah *et al.*, 1992b).

1.6 Research Description and Objectives

The following work describes an analysis of the expression of three cysteine protease activities, cathepsin B, cathepsin H, and cathepsin L, and cysteine protease inhibitors in the human breast cancer cell line, MCF-7, and its adriamycin-resistant variant, MCF-7/Adr^R using Fast Performance Liquid Chromatography (FPLC).

The specific objectives of the study were:

- to examine and compare the growth parameters of MCF-7 and its adriamycin -resistant variant, MCF-7/Adr^R;
- to examine and compare intracellular levels of cathepsin B, H, and L activities in unfractionated samples of: a) MCF-7, b) MCF-7 transfected with *gpt*, c) MCF-7 transfected with *ras/gpt*, d) MCF-7/Adr^R, and e) MA, a somatic cell hybrid between MCF-7 and MCF-7/Adr^R, using two types of substrates, colorimetric and fluorometric;
- to examine and compare intracellular and extracellular levels of cathepsin B in MCF-7, MCF-7/Adr^R, and their somatic cell hybrid, MA; and
- to examine intracellular levels of cathepsins B, H, and L, and levels of free and heat-dissociated CPI after fractionation on a Superose 12 HR 10/30 FPLC column.

Materials and Methods

2.1 Materials

Fast garnet CBC salt, L-cysteine, bovine serum albumin (fraction V), p-hydroxy-mercuribenzoic acid, Nα-CBZ-argininyl-argininyl 4-methoxy-ß-napthylamide, Nα-CBZ-argininyl-argininyl 7-amido-4-methylcoumarin.HCl, E-64, leupeptin, pepstatin, 7-amino 4-methylcoumarin, pepsin, sodium selenite, and low molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, Missouri). N-α-CBZ-L-argininyl-7-amido-4-methlycoumarin.HCl, and N-CBZ-L-phenylalanyl-L-argininyl-7-amido-4 methylcoumarin.HCl were purchased from Bachem California (Torrance, California). Purified pork liver cathepsin B and Trayslol (aprotinin), were generous gifts from Dr. A. Warner (University of Windsor) and Dr. G. Stojanovic (Arizona State University) respectively. PMSF was purchased from Boehriinger Mannheim Canada (Laval, Quebec). Superose 12 HR 10/30 FPLC column was purchased from Pharmacia (Uppsala, Sweden). The protein assay kit was purchased from Bio-Rad Laboratories (Mississauga, Ontario). Medium (alpha MEM), calf and fetal calf sera, gentamicin sulfate, trypsin EDTA and tissue culture plasticware were purchased from Gibco Laboratories (Burlington, Ontario). All other chemical were purchased from Sigma Chemical Co. (St. Louis, Missouri), or Fisher Scientific Co. (Fair Lawn, New Jersey), and were of reagent grade or better.

2.2 Cell Cultures

MCF-7, MCF-7/Adr^R, MCF-7/*ras gpt*, and MCF-7/*gpt* human immortal breast cancer cell lines were originally obtained from Dr. Kenneth Cowan (National Institute of Health, Bethesda, Maryland, 20205). The somatic cell hybrid between MCF-7 and MCF-7/AdrR, MA, was also examined. This hybrid was formed by exposing mixed suspensions of these cells to decreasing concentrations of polyethylene glycol 6000 (Dufresne and Dosescu, 1985). Selection of putative hybrids was based on the dominance of the adriamycin-resistant phenotype, and true hybrids were identified by means of facs analysis of DNA (Dufresne *et al.*, 1993a). Cells were routinely maintained in alpha-MEM medium supplemented with 5% heat inactivated (56°C) serum (6 parts calf serum to 4 parts fetal calf serum, v/v) and 50 μg/ml gentamicin sulphate. Cell transfers were carried out using 0.25% trypsin-EDTA after washing with warm citrate saline solution (15 mM trisodium citrate, 134 mM potassium chloride, pH 7.8). All cell cultures were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

The assay of extracellular proteolytic activity required the use of serum free medium for 24 hours prior to the collection of cells and media. The serum free medium used was a modified version of Darlington's (Darlington, 1987) developed by Adeli and Sinkevitch (Adeli and Sinkevitch, 1990). It consisted of 3 parts alpha MEM and one part Waymouth's MB752/1 supplemented with: 2 mM L-glutamine, 30 nM sodium selenite, 1.0 mg/L i-inositol, 8.0 mg/L thymidine, 0.05 mg/L $CuSO_4.5H_2O$, 0.016 mg/L $MnSO_4.H_2O$, 0.03 mg/L $ZnSO_4.7H_2O$, 0.024 mg/L $Mo_7O_{24}.4H_2O$, 0.022 mg/L $CoCl_2.6H_2O$ and antibiotic-antimycotic solution consisting of penicillin,

streptomycin, amphotericin B and fungizone. This serum-free medium was adapted for use in protease analysis in human cells (Dufresne *et al.*, 1993b).

2.3 Cell Storage

Long term storage of cells is a routine cell culture procedure used to prevent loss of cells in culture due to factors such as contamination and incubator malfunction, and to minimize changes in genotypes and phenotypes which may occur with prolonged maintenance in culture. Storage was routinely carried out in liquid nitrogen and in an Ultra Low Revco, Revco Inc. (West Columbia, S.C., 29169) at -80°C. Trypsinized cells were diluted in regular alpha medium supplemented with 5% serum and centrifuged at 1000 rpm for 5 minutes to remove trypsin. Cells were then resuspended in freezing solution (7 parts alpha medium, 2 parts fetal calf sera, 1 part DMSO, v/v) and seeded into Nunc cryotubes at $1x10^6$ cells per vial. Vials were placed in a -20°C freezer for 2 hours before transfer to liquid nitrogen or the Revco freezer. One to two weeks after freezing, cell samples were checked for contamination and viability. Storage under these conditions provided better than 60% of the expected efficiency of plating over a 12 month period.

2.4 Cell Maintenance

Cells were routinely maintained in 25 cm^2 culture flasks, and were transferred to larger 75 cm^2 culture flasks to provide sufficient numbers for plating (e.g., "seeding"). Cells were seeded into 100 cm^2 culture dishes at a concentration of $2x10^5$ cells per dish and grown to near confluency. Cells were then collected in ice cold phosphate buffered saline solution (PBS) (136.9 mM sodium chloride, 2.7 mM potassium

chloride, 8 mM anhydrous sodium phosphate, 1.5 mM anhydrous potassium phosphate, pH 6.0) into 17 x 100 mm polypropylene tubes with a rubber policeman. The suspension was pelleted at 1000 x g for 5 minutes in a Damon/IEC HN-S Centrifuge (Needham Heights, Mass, 02194) and washed three times in cold PBS. Cell pellets were stored at -20°C until time of enzyme assay.

2.5 Growth Parameters

Cells were seeded into 100 mm culture dishes at a concentration of $2x10^5$ cells per dish, and incubated at 37°C. Each day thereafter (for 6-7 days), two cell counts were performed with a hemacytometer at each of two time points (i.e., morning and agternoon). At the completion of the experiment, cell number was plotted against time to form a growth curve where saturation density (the maximum number of cells that grow per cm^2 of surface area) and population doubling time (the length of time it requires a population of cells to double in number during exponential growth) were determined.

2.6 Enzyme Assays

Six hundred μl of cold homogenization buffer (135 mM potassium chloride, 0.1 mM EDTA, 0.1% tween 80, 15mM potassium phosphate, pH 6.0) was added to the thawed cell pellet. The pellet was then homogenized with a Brinkmann Polytron (Brinkmann Instruments, Oakville, Ontario) on setting 4 for 2 x 10 second pulses. The homogenate was clarified at 4,000 x g in a Beckman Microfuge E (Beckman Instruments Inc., Palo Alto, California) for 5 minutes to remove cellular debris. A protein assay was performed on the clarified homogenate (see Methods section 2.9).

Enzyme assays using colorimetric (ß-napthylamide) and/or fluorometric (4-methylcoumarin) substrates were conducted on both unfractionated and FPLC-fractionated cell homogenates (intracellular fractions), as well as on serum-free cell medium collected from cells (extracellular fractions). In the case of unfractionated homogenates, the protein concentration of each sample was adjusted down to 0.16 mg/ml, a concentration at which substrate was not limiting (Figure 4).

A. Unfractionated Homogenate

Cathepsin B was assayed in unfractionated homogenates using the colorimetric substrate, Nα-CBZ-arg-arg-4-methoxy ß-napthylamide (arg-arg), according to a modified procedure of Barrett (Barrett, 1972). In a volume of 300 μl, the pre-reaction mix contained 200 μl of sample homogenate and 100 μl of cysteine/incubation buffer (1.33 mM Na_2EDTA, 88.2 mM anhydrous potassium phosphate, 13.7 mM sodium phosphate, pH 6.0, with 0.32 mg/ml L-cysteine) and was allowed to preincubate at room temperature for 10 minutes. Twenty μl of arg-arg substrate was then added to the reaction mix and 50 μl of this was immediately transferred to a reaction stop tube (on ice) containing 50 μl of coupling reagent (3.57 g p-hydroxy-mercuribenzoic acid in 120 ml 0.5 M sodium hydroxide, with 50 mM Na_2EDTA in 1 liter, pH 6.0). The reaction mix was then transferred to a 37°C water bath. At 15 and 30 minute time intervals after transfer, 50 μl of reaction mix was removed and added to a stopper tube (on ice) containing 50 μl of coupling reagent. Upon completion of the last transfer, 50 μl of fast-garnet-Brij solution (2.5 mg fast garnet salt in 4.8 ml auto-nano-water with 200 μl 4% Brij 35 in water) was added to each reaction stop tube. The tubes were allowed to sit for at least 10 minutes at room temperature then 1 ml of butanol was

added to each. Tubes were vortexed vigorously, then centrifuged in a Precision Vari-Hi-Speed Centricone (Precision Scientific Co., Chicago, Illinois) at 1000 x g for 5 minutes. The liberated 2-napthylamine contained in the top butanol layer was then measured at 520 nm with a Beckman DU-64 Spectrophotometer equipped with a Quant II Quad Soft Pack Module. One unit of proteolytic activity (EU) was defined as that amount of enzyme which gives rise to an absorbance of 0.016 in one minute. Specific Activity was expressed as enzyme units (EU) per milligram of total protein.

Cathepsins B, H, and L activities were measured in unfractionated samples using fluorometric, 4-methylcoumaryl-amide substrates according to a modified procedure of Barrett. (Barrett, 1980; Barrett and Kirschke, 1981). In all cases, cell samples and synthetic substrates were kept on ice prior to addition to reaction mix. A 750 μl pre-reaction mix consisted of 500 μl of homogenate sample, diluted in 0.1% Brij 35 solution in water, which was preincubated for 2 minutes at 37°C with 250 μl activation buffer (**cathepsin B**: 352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM disodium EDTA, and 8 mM DTT, pH 6.0; **cathepsin H**: 200 mM KH_2PO_4, 200 mM Na_2HPO_4, 4 mM disodium EDTA, and 40 mM DTT, pH 6.8; **cathepsin L**: 340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, and 8 mM DTT, pH 5.5). The assays were started with the introduction of 250 μl of 0.02 mM substrate solution (**cathepsin B**: Nα-CBZ-arginine-arginine 7-amido-4-methyl-coumarin.HCl, or Z-Arg-Arg-Mec; **cathepsin H**: N-α-CBZ-L-arginine-7-amido-4-methylcoumarin.HCl, or Z-Arg-Mec; **cathepsin L**: N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methyl-coumarin.HCl, or Z-Phe-Arg-Mec). All substrate solutions were originally prepared as 1 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at 4°C. Stock

solutions were diluted to 0.02 mM working strength solutions with auto-nano-water (ie. 200 μl stock into 10 ml auto-nano-water). After 10 minutes incubation at 37°C, the reactions were terminated with the addition of 1 ml stopping reagent (100 mM sodium monochloroacetate, 30 mM sodium acetate, and 70 mM acetic acid, pH 4.3). [NOTE: The reaction blank was as described above except that 1 ml of stopping reagent was added before the activation buffer.] Since the specific activities for cathepsins B and L are of similar order (as are their K_{cat}/K_m values) and both proteases recognize the Z-Phe-Arg-Mec substrate, a correction was made for cathepsin L activity. Cathepsin B activity was determined with Z-Arg-Arg-Mec under conditions specific for cathepsin L (i.e., pH 5.5). These values were subtracted from the values obtained with the Z-Phe-Arg-Mec substrate to provide a corrected activity for cathepsin L (Nishimura *et al.*, 1988).

The fluorescence of free aminomethylcoumarin in each reaction tube was then measured at room temperature at an excitation wavelength of 370 nm and an emission wavelength of 460 nm with a Turner Model 430 Spectrofluorometer (G.K. Turner Associates, Palo Alto, California). Fluoresence readings were standardized with 0.5 μM 7-amino-4-methylcoumarin standard (prepared from 1 mM stock in DMSO diluted to 0.5 μM with a 1:1 mixture of activator buffer and stopping reagent) set at 1000. The spectrofluorometer was adjusted so that 1000 arbitrary units corresponded to the release of 1 nmol of product. One milliunit of proteolytic activity was defined as the quantity of enzyme releasing 1 nmol of aminomethylcoumarin per minute. For the 10 minute assay, a reading of 1000 therefore corresponds to 0.1 milliunits of activity in the tube.

B. FPLC-Fractionated Homogenate

Clarified homogenates were fractionated with a Superose 12 HR 10/30 FPLC column (Pharmacia, Uppsala, Sweden). One to two mg/ml of homogenate was loaded in 500 μl homogenization buffer (135 mM potassium chloride, 0.1 mM EDTA, 0.1% tween 80, 15mM potassium phosphate, pH 6.0) and eluted in column buffer (25 mM potassium chloride, 0.1 mM EDTA, 10% glycerol, 15 mM potassium phosphate, pH 6.0) at a flow rate of 1.0 ml/min with a Beckman Gradient Liquid Chromatograph. Eluates were detected with a Beckman Analytical Optical Unit at a UV wavelength of 280 nm. Sensitivity was generally between 0.08 and 0.32 absorbance units full scale (AUFS). Elution profiles were recorded on a Kipp and Zonen Chart Recorder at a chart speed of 1.0 cm/min. Fractions of 250 μl (for colorimetric analysis) and 500 μl fractions (for fluorometric analsis) were collected with a Pharmacia Frac-100 Fraction Collector and stored on ice until assay.

For colorimetric assays, a pre-reaction mix consisted of: 25 μl of fractionated homogenate which was preincubated with 50 μl of 3X cysteine/incubation buffer (1.33 mM Na_2EDTA, 88.2 mM anhydrous potassium phosphate, 13.7 mM sodium phosphate, pH 6.0, with 0.96 mg/ml L-cysteine) and 75 ul of incubation buffer (1.33 mM Na_2EDTA, 88.2 mM anhydrous potassium phosphate, 13.7 mM sodium phosphate, pH 6.0) for 10 minutes. The reaction was started with the addition of 10 μl arg-arg and allowed to proceed for 15 minutes following the initial removal of 50 μl reaction mix, which was added to 50 μl of coupling reagent (3.57 g p-hydroxymercuribenzoic acid in 120 ml sodium hydroxide, with 50 mM Na_2EDTA in 1 liter, pH 6.0). The reaction was terminated with the addition of 50 μl of reaction mix

to 50 μl coupling reagent. Zero and reaction tubes were measured colorimetrically as described above, following the addition of fast-garnet-Brij solution. Protease activity was expressed in enzyme units (EU).

Fluorometric assays of fractionated homogenates for cathepsins B, H and L activity involved diluting 50 μl of fraction with 450 μl 0.1% Brij solution in auto-nano-water. Diluted fractions were then preincubated for 5 minutes at 37°C with activation buffer (see fluorometric assay for whole homogenates for buffer composition). The reactions were started with the addition of 250 μl of 0.02 mM substrate (see fluorometric assay for whole homogenates). After 10 minutes incubation at 37°C, the reactions were terminated by the addition of 1 ml stopping reagent (see fluorometric assay for whole homogenates). The reaction blank was prepared as described above using a representative fraction. As well, the fluorescence was measured as described above.

C. Extracellular Medium

Levels of activatable cathepsin B were determined using a modified method of Mort (Mort et al, 1981). After 24 hours of growth in serum free medium, cells were collected and their homogenates assayed as described for whole homogenates. The serum free medium is collected into 50 ml conical tubes (for multiple samples) and clarified with a Damon IEC Clinical Centrifuge (Needham Heights, Mass, 02194) at 1000 x g for 5 minutes. Samples were stored at -20°C until time of assay. For the assay, 150 μl of extracellular medium was activated with 30 μl of 0.5 mg/ml pepsin in an acid buffer (0.8 M anhydrous sodium acetate, pH 3.8) at 40°C for 1 hour. The pH was returned to near 6.0 with 60 μl of 0.2 M potassium phosphate buffer (pH 9.0).

The reaction was initiated with the addition of 20 μl arg-arg and terminated at 0, 1, 2 and 3 hour time points with 50 μl cold coupling reagent and after the addition of fast-garnet-Brij solution, the reaction was measured colorimetrically, as described previously.

2.7 Inhibitor Assays

A. Exogenous (commercial) Inhibitor

The commercial inhibitor assay was performed exactly as the whole homogenate assay, excepting the following: the reaction mix consisted of 190 μl homogenate, 10 μl commercial inhibitor, 100 μl cysteine/incubation buffer, and 20 μl arg-arg.

B. Preparation of Commercial Inhibitors (Beynon and Salveson, 1989)

L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) inhibits cysteine proteases, has a molecular weight of 367.4, and an effective concentration of 1-10 μM. For 10X effective stock, 2.28 mg E-64 was dissolved in 2 ml auto-nano-water. At this concentration, this was stable for up to two months at -20°C. This was diluted by one tenth just prior to use. Leupeptin inhibits trypsin-like serine and some cysteine proteases, has a molecular weight of 426.6, and an effective concentration of 10-100 μM. For a 10X effective stock, 8.53 mg leupeptin was dissolved in 2 ml auto-nano-water. This was stable for one month at -20°C. This was diluted by one tenth just prior to use. Phenylmethylsulphonyl flouride (PMSF) inhibits serine proteases, has a molecular weight of 174.2, and an effective concentration of 0.1-1 mM. A 10X effective stock was prepared by dissolving 5.6 mg PMSF in 2 ml 100% methanol. This was stable for nine months at 4°C. This was diluted by one tenth just prior to

use. Ethylenediamine tetraacetic acid (EDTA) inhibits metallo-proteases, has a molecular weight of 372.24, and an effective concentration of 1-10 mM. A 10X effective stock was prepared by adding 1.6 ml EDTA (0.5 M) to 3.4 ml auto-nano-water. This was diluted by one tenth just prior to use. Aprotinin (trayslol) inhibits serine proteases, has a molecular weight of 6500, and an effective concentration of 100 KIU (kallikrein inactivating unit). This is diluted from 10,000 KIU/ml manufacturers stock solution just prior to use. Pepstatin inhibits aspartic proteases, and has an effective concentration of 1 μM. A 10X effective stock was prepared by dissolving 2 mg pepstatin in 2 ml 100% methanol. This was diluted by one tenth just prior to use.

C. Endogenous Inhibitor

The cathepsin B inhibitor assay was performed exactly as the fractionated homogenate enzyme assay, except for the following: the reaction mix consisted of 50 μl 3X cysteine/incubation buffer, 50 μl of column fraction, 50 μl of pork liver cathepsin B, and 10 μl arg-arg. Control vessels contained 50 μl 3X cysteine/incubation buffer, 50 μl incubation buffer, 50 μl pork liver cathepsin B, and 10 μl arg-arg. Control levels of proteolytic activity for pork liver cathepsin B were around 0.1 delta absorbance units at 520 nm. The pork liver cathepsin B was a generous gift from Dr. Alden H. Warner of the University of Windsor. Inhibitory activity was defined as the percentage drop in proteolytic activity in experimental vessels compared to the control levels of pork liver cathepsin B activity.

Endogenous cathepsin B inhibitor assays using fluorometric substrates involved diluting 100 μl of fraction with 100 μl 0.1% Brij solution, and preincubating with 50

μl pork liver cathepsin B and 250 μl cathepsin B activation buffer for 5 minutes at 37°C. The reaction was started with the addition of 250 μl of 0.02 mM Z-arg-arg Mec. After 10 minutes incubation at 37°C, the reaction was terminated with the addition of 1 ml stopping reagent. The enzyme control reaction mix consisted of 50 μl cathepsin B and 200 μl 0.1% Brij solution preincubated with 250 μl cathepsin B activation buffer. Both control and inhibitor samples were assayed fluorometrically as described previously. Total protease inhibitor activity was determined by the method of Green *et al* (1984). This involved heat treatment of a constant amount of homogenate protein at 80°C for 5 minutes, followed by centrifugation at 4,000 x g to remove debris. Fractions from FPLC were assayed for inhibitor activity as described previously for non-heat treated fractionated homogenates.

2.8 Selectivity Curve Using FPLC

Molecular weight standards were run on a Superose 12 HR 10/30 FPLC and their elution volumes recorded. Utilizing Blue Dextran to elicit the void volume (V_o), as well as bovine albumin (M.W. 66.2 kDa), ovalbumin (M.W. 42.7 kDa), chymotrypsin (M.W. 25.0 kDa) and cytochrome C (M.W. 13.0 kDa), a selectivity curve for the approximation of molecular weight was produced. Separation coefficients (K_{av}) were calculated using the following formula:

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

where V_e = the elution volume, V_o = the void volume, and V_t = the total volume.

2.9 Protein Determination

The standard protein microassay described by Bradford in 1976 and prepared by

Bio-Rad was followed. Bovine stock albumin, fraction V, was prepared by dissolving 29 mg BSA in 10 ml of autoclaved nano-pure water (auto-nano-water). 50 μl of this stock solution was then added to 1400 μl of auto-nano-water to give a concentration of 100 μg/ml BSA. 1 ml of this solution was diluted with 4 ml auto-nano-water to give a final stock solution concentration of 20 μg/ml. Protein standards and samples were prepared as follows:

protein amount	vol. stock	vol. water	vol. dye
2 μg	100 μl	700 μl	200 μl
3 μg	150 μl	650 μl	200 μl
4 μg	200 μl	600 μl	200 μl
5 μg	250 μl	550 μl	200 μl
8 μg	400 μl	400 μl	200 μl
12 μg	600 μl	200 μl	200 μl
blank	0 μl	800 μl	200 μl
sample	30 μl	770 μl	200 μl

Standards and samples were analyzed with a visible light wavelength of 595 nm using a Beckman DU-64 Spectrophotometer equipped with a Quant II Quad Soft Pac Module. Standard curves were deduced from the change in O.D. absorbance values.

2.10 Statistical Analysis

Statistical analysis was carried out using the SYSTAT for Windows 5.1 statistical program (SYSTAT Inc., Evanston, Illinois, 1992) with the generous assistance of Dr. I.M. Weis and Mr. Aaron Fisk, both of the University of Windsor.

Results

3.1 Growth Characteristics of MCF-7 and MCF-7/Adr^R

Before the two breast cancer cell lines, MCF-7 and MCF-7/Adr^R, could be compared for expression of cathepsin activities, it was important to establish that both cell lines behaved similarly in culture. To achieve this, general patterns of growth were observed and the growth parameters (i.e., saturation density, and population doubling time) were determined for each cell line (described in Methods). Both cell lines exhibited similar, classic growth curves (Freshney, 1990) consisting of a lag phase, a period of exponential growth, and a plateau. [NOTE: Growth curves are presented in Results section 3.4 and are therefore not reproduced here.] This is evident from the results in Table 2 for the two growth parameters calculated from the growth curve. Population doubling times, calculated during the exponential phase of cell growth, were similar in both cell lines. Saturation densities, the number of cells per cm^2 of surface area at the plateau phase of growth, were also similar for both cell lines. Previously, our laboratory had noted that both cell lines demonstrated similar efficiencies of plating, a parameter which defines the number of cells giving rise to colonies (Dufresne *et al.*, 1993a). Three general properties concerning the biology of MCF-7 and its adriamycin-resistant variant were judged to be important to the subsequent analysis. First, both cell lines are highly anchorage dependent and the proportion of cells liberated into the medium, even at saturation, is negligible. Second, both cell lines demonstrated stable growth characteristics and aneuploid chromosome

TABLE 2

Growth Parameters for the Human Breast Cancer Cell Lines MCF-7 and MCF-7/Adr^R

CELL LINE	POPULATION DOUBLING[a] TIME (hours)	SATURATION DENSITY[b] (cells/cm^2)
MCF-7	16	5.1×10^4
MCF-7/Adr^R	17	5.9×10^4

[a] Population Doubling Time represents the time (in hours) it takes for a population of exponentially growing cells to double in number.
[b] Saturation Density represents the maximum number of cells that will occupy a cm^2 of available growing space.

numbers over time in culture and between experiments. Third, cells from the two cell lines were similar in size and contained similar amounts of protein and DNA (per cell) (Dufresne *et al.*, 1993a).

3.2 Substrate Specificity

There is controversy with respect to the specificity of the synthetic substrate Nα-CBZ-arg-arg-4-methoxy-ß-napthylamide (arg-arg). Early studies indicated that arg-arg was specific for cathepsin B alone with no cross-reactivity for two other cysteine proteases, cathepsins H and L (Barrett and Kirschke, 1981). More recent work questions the absoluteness of this specificity and has resulted in a more cautious interpretation of specificity by some researchers. Sloane's group, for example, has modified Barrett's interpretation in such a way that acknowledges the preference of arg-arg for cathepsin B but does not exclude cathepsins H or L (Rohzin *et al.*, 1990). Despite the controversy, it is generally agreed that pH plays an important role in the definition of specificity. Each of the different cathepsins in the cysteine protease family is reported to have a different pH optimum. In the studies described in this thesis, assay conditions for each cathepsin were stringently controlled to favor that cathepsin. However, until the conditions for specificity are established, all interpretations are open to question.

While there is obvious debate about the absolute specificity of arg-arg for cathepsin B, it was possible to test if this substrate was specific for the cysteine family of proteases. In brief, homogenates from MCF-7 and MCF-7/Adr^R were assayed under conditions specific for cathepsin B, in the absence and presence of different

commercially available inhibitors. The results of this analysis are presented in Table 3. In both cell lines, E-64 (L-3-trans-epoxysuccinyl-leucyl amide-(4-guanidino)-butane: 1-10μM) and leupeptin (10-100μM), known inhibitors of cysteine proteases, eliminated protease activity using arg-arg. In contrast, inhibitors of the serine-, aspartic-, or metallo-proteases had no effect on levels of activity.

3.3 Cathepsin Activity in Unfractionated Cell Homogenates

As discussed in the introduction (section 1.5), the MCF-7 cell line has become the experimental system of choice for many researchers examining malignant phenotypes (Kasid *et al.*, 1985; Worland *et al.*, 1989; Vickers *et al.*, 1989; Chen *et al.*, 1990; Whelen *et al.*, 1992; Thompson *et al.*, 1992; Gelmann *et al.*, 1992; Scaddan and Dufresne, 1992). During the course of research in these different laboratories, the original MCF-7 cell line genotype and phenotype have been altered by mutation (e.g., resistance to chemotherapeutic agents) and by transfection of particular genes (e.g. oncogenes). Such manipulations can produce multiple changes at the level of phenotype (Vickers *et al.*, 1989). While many of the changes in phenotypes appear to be the result of a specific type of manipulation, it is possible that others will be observed in all variants of MCF-7, regardless of the manipulation. This possibility has obvious implications concerning the relationship between genotype and phenotype. For this reason, the expression of cysteine protease activity was first examined in unfractionated homogenates prepared from MCF-7 cells and three different variants of the MCF-7 cell line: a *gpt*-transfected variant, a *ras/gpt*-transfected variant, and the adriamycin-resistant variant used throughout this study. The assays were conducted with arg-arg

TABLE 3

Effect of Commercially Available Inhibitors on Cathepsin B Activity of MCF-7 and MCF-7/Adr^R

INHIBITOR	TARGET PROTEASE	EFFECTIVE[a] CONC.	INHIBITION[b] MCF-7	INHIBITION[b] MCF-7/Adr^R
Control	---	---	–	–
E-64	cysteine	10 uM	+	+
Trayslol	serine	100 KIU	–	–
PMSF	all serine	50 uM	–	–
Leupeptin	trypsin like serine some cysteine	50 uM	+	+
EDTA	metallo	5 mM	–	–
Pepstatin	aspartic	1 uM	–	–

[a] according to *Beynon and Salvesen, 1989.*
PMSF = phenylmethanesulphonyl flouride
EDTA = ethylenediamine tetraacetic acid
KIU = Kallikrein Inactivating Unit

[b] determined as complete elimination of intracellular enzyme activity, as described in materials and methods.

under conditions optimal for cathepsin B using homogenates with similar concentrations of protein. Levels of activity were measured 15 and 30 minutes after initiation of the reaction. The results of this study, presented in Figure 3, indicate that levels of activity measured in the *gpt*-transfected and the *ras* oncogene-transfected variants were similar to those measured in the original MCF-7 cell line. Levels of activity in the adriamycin-resistant variant, however, were significantly 20 to 25 fold greater than its MCF-7 parent ($p=0.003$). In all cell lines examined, the specific activities at 15 and 30 minutes were similar suggesting that the reaction was linear over the time.

The results obtained with arg-arg suggested that the increased cysteine protease activity phenotype was specific to MCF-7/Adr^R. However, the colorimetric substrates are not as sensitive as other substrates for measuring protease activity (reviewed in Polgár, 1989). To examine the possibility that the low levels of activity measured in MCF-7 and its two transfected variants reflected limited sensitivity, a fluorometric assay for cysteine proteases was adopted using methylcoumarylamide substrates. While these substrates can be prohibitively expensive to use, they are safer (napthylamide is a suspected carcinogen) and more sensitive than colorimetric substrates (Barrett and Kirschke, 1981). Their sensitivity reflects the liberation of 7-amino-4-methylcoumarin which can be measured using a fluorometer. The linear relationship between fluorescence and increasing concentrations of an aminomethylcoumarin standard is presented in Figure 4.

Three methylcoumarin substrates were used to re-examine levels of cysteine proteases in the different MCF-7 cell variants: Nα-CBZ-arginine-arginine 7-amido-4-methylcoumarin .HCl; Nα-CBZ-L-arginine-7-amido-4-methylcoumarin.HCl; and N-

FIGURE 3:

Intracellular Cathepsin B Activity in MCF-7 Variant

Unfractionated Homogenates Using Colorimetric Substrate

MCF-7 variants are represented by protein adjusted, unfractionated homogenates that were assayed with the colorimetric substrate arg-arg under conditions optimal for cathepsin B. Fifteen and 30 minute endpoint reaction times were performed to examine and confirm reaction linearity and the activity was measured as described in Materials and Methods, section 2.6A. Protease activity is expressed as Enzyme Units/mg total protein, where one enzyme unit (EU) is defined as that amount of protease which gives rise to an absorbance unit of 0.016 in one minute at a wavelength of 520 nm.

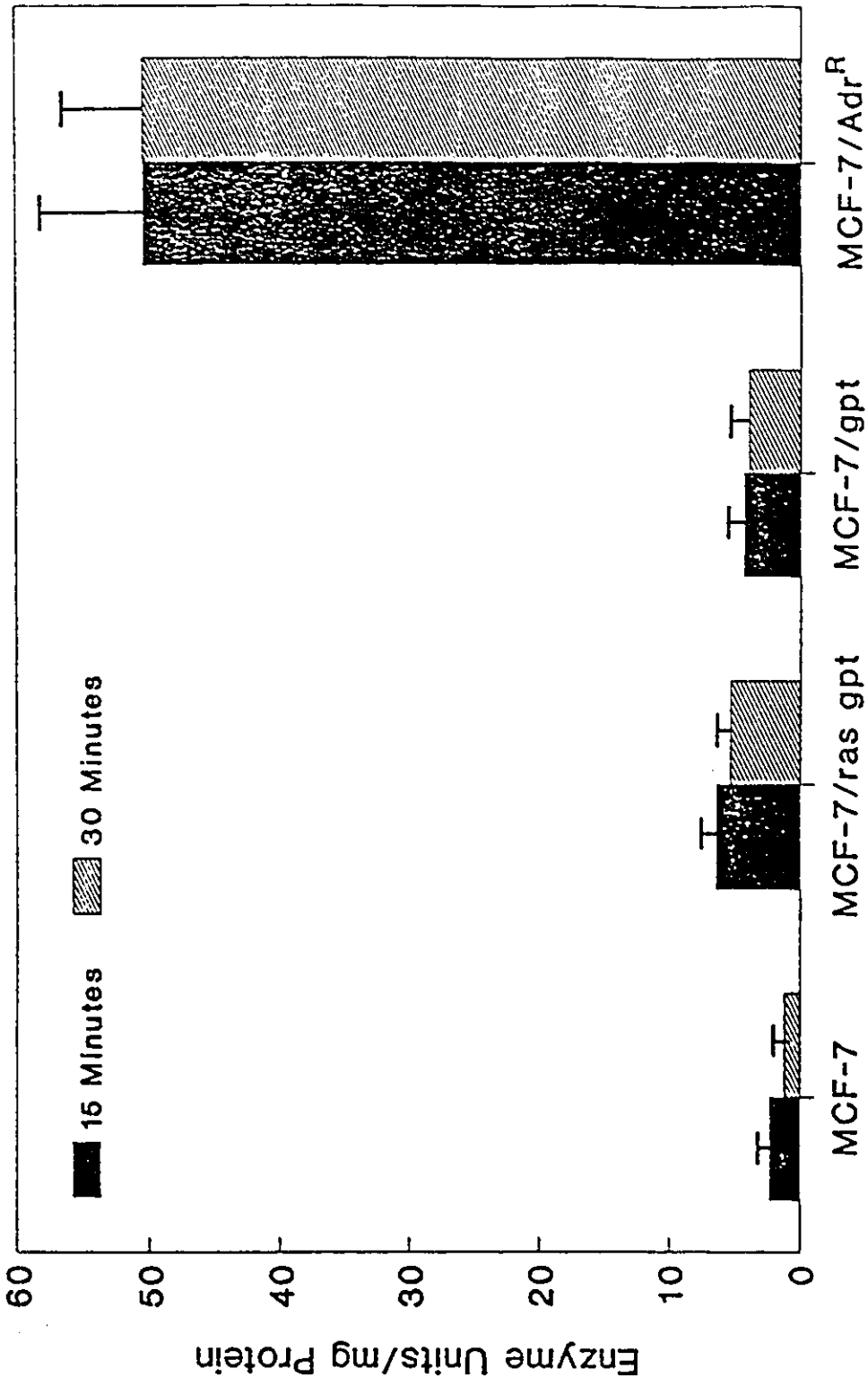
Enzyme Units/mg Protein
60
50
40
30
20
10
0
15 Minutes
30 Minutes
MCF-7
MCF-7/ras gpt
MCF-7/gpt
MCF-7/Adr^R
CELL VARIANT

Fig. 3

FIGURE 4:

7-Amino 4-Methylcoumarin Standard Curve

7-amino 4-methylcoumarin standards of 0.1-0.5 μM concentration were prepared from a 1.0 mM stock solution in DMSO by diluting with a 1:1 solution of activation buffer and stopping reagent. Fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm as described in Materials and Methods, section 2.6A.

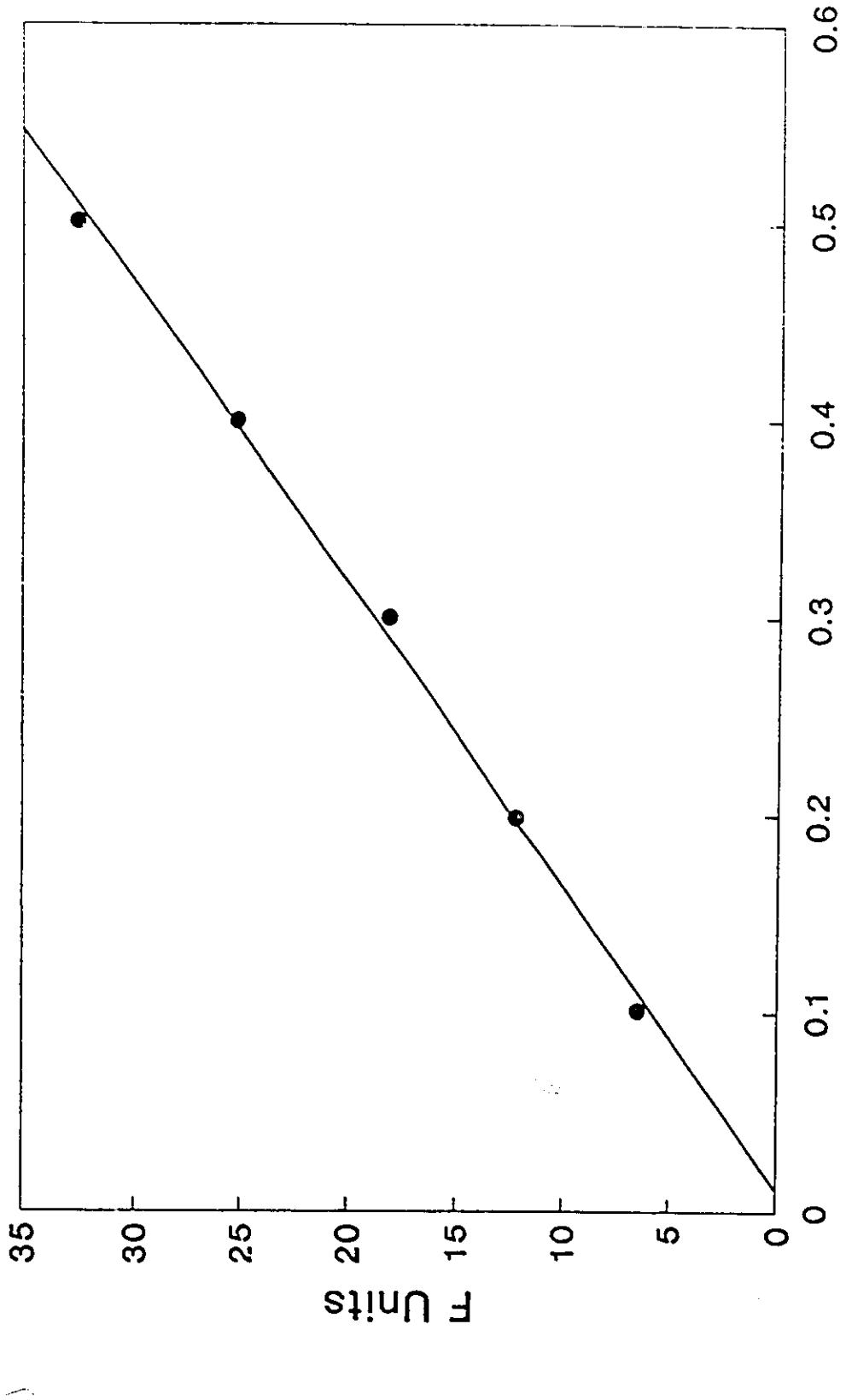

Fig. 4

CBZ-phenylalanyl-L-arginine-7-amido-4-methylcoumarin.HCl (Table 4). Historically, these substrates have been used to measure lysosomal cathepsins B, H, and L, respectively. However, as described in Methods (section 2.6), a correction for cross-reactivity between cathepsin B and cathepsin L is routinely introduced (Nishimura *et al.*, 1988). The corrected values for levels of cathepsins B, H, and L activities in unfractionated homogenates of different MCF-7-derived cell lines is presented in Figure 5. At the very least, the overall pattern of expression of total cysteine proteases (cathepsins B, H and L) among cell lines using fluorometric substrates is similar to that obtained with the colorimetric substrate, arg-arg (compare Figures 3 and 5). However, differences were observed in the expression of individual cathepsins (Figure 5). Levels of cathepsin B activity were reproducibly similar in MCF-7 and its two transfected variants, while levels in the adriamycin-resistant variant were 10-15 fold greater. A similar pattern of expression was observed in these four cell lines for cathepsin L activity. Levels of cathepsin H, however, were similar in all cell line homogenates. Moreover, in sharp contrast to cathepsins B and L, levels of cathepsin H activity did not increase in the adriamycin-resistant variant. Using Spearman rank correlation analysis (Zar, 1984), the descending order of expression of cathepsin activities in the adriamycin-resistant variant -cathepsin L first, cathepsin B second, and cathepsin H third- over a series of experiments was significant ($p=0.010$). Irrespective of this order, an increase in cysteine protease activity was observed only in the adriamycin-resistant variant of MCF-7. This is in agreement with the previous data using the colorimetric substrate, arg-arg.

TABLE 4

A Comparison of the Properties of Lysosomal Cysteine Proteases[a]
Cathepsins B, H, and L

	Cathepsin B	Cathepsin H	Cathepsin L
Recomended test substrate (pH optimum)	Z-Arg-Arg-NMec (pH 6.0)	Arg-NMec (pH 6.8)	Z-Phe-Arg-NMec (pH 5.5)
Aminopeptidase activity	No	Yes	No
Endopeptidase activity	Moderate	Variable	High
Peptidylpeptidase activity	Yes	No	No
Aldolase inactivation	Yes	No	Yes
Leupeptin sensitivity	High	Low	High
Z-Phe-Phe-CHN_2 sensitivity	Low	Low	High
pI	4.5-5.5	6.0-7.1	5.5-6.1
Concanavalin A - Sepharose	Not bound	Bound	Bound

[a] from *Barrett and Kirsche, 1981*

FIGURE 5:

Intracellular Cathepsin B, H, and L Activity in MCF-7 Variant Unfractionated Homogenates Using Fluorometric Substrates.

MCF-7 variant, protein adjusted, unfractionated homogenates were assayed with the fluorometric substrates Z-arg-arg-Mec, Z-arg-Mec, and Z-phe-arg-Mec under conditions optimal for cathepsins B, H, and L respectively. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm as described in Materials and Methods, section 2.6B. Protease activity is expressed as milliUnits/mg total protein, where one milliunit of proteolytic activity is defined as the quantity of protease that liberates 1 nmol of aminomethylcoumarin per minute.

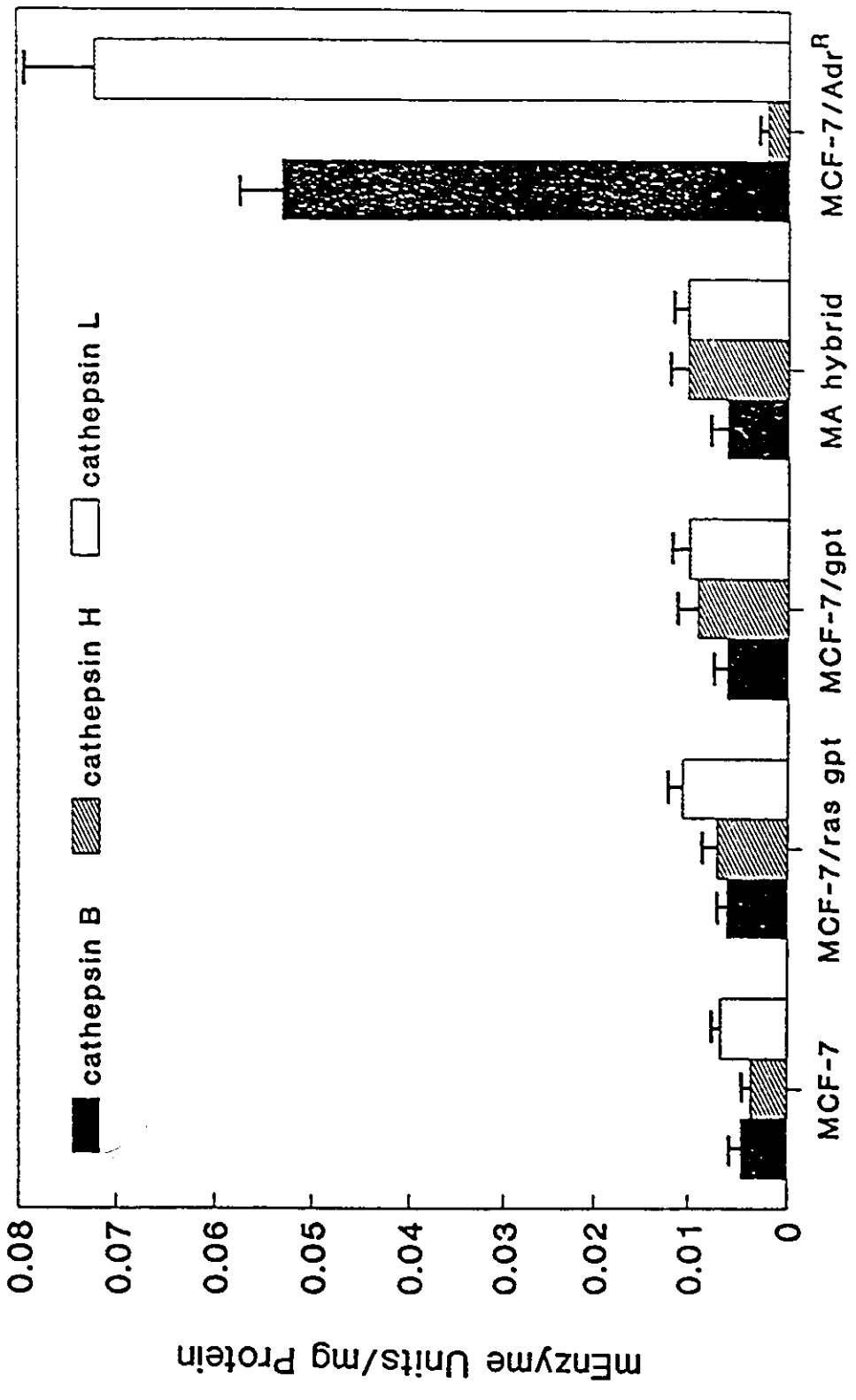
mEnzyme Units/mg Protein
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
cathepsin B
cathepsin H
cathepsin L
MCF-7
MCF-7/ras gpt
MCF-7/gpt
MA hybrid
MCF-7/Adr^R
CELL VARIANT

Fig. 5

Figure 5 presents data that has not yet been discussed, that for M:A, a somatic cell hybrid between MCF-7 and its adriamycin-resistant variant. Since questions of dominance of expression are routinely examined in cell hybrids between parents with different phenotypes (Dufresne and Dosescu, 1985; Manjunath and Dufresne, 1989), M:A provided a convenient opportunity to gain some insight concerning the mechanism underlying increased cysteine protease activity in the adriamycin-resistant variant of MCF-7. Since levels of cysteine proteases (i.e., cathepsins B, H, and L) in the hybrid cells were similar to those of the MCF-7 parent it appeared that the expression of increased cysteine protease activity observed in adriamycin-resistant cells was suppressed in the hybrid.

3.4 Levels of Cysteine Protease Activity in Cells and Media

It has been reported that the acquisition of the metastatic phenotype is accompanied by increases in both intracellular and extracellular levels of cysteine proteases such as cathepsin B (Introduction section 1.2). Early studies suggested that increases in extracellular levels reflected a multi-step process involving increased secretion of both active, mature forms and activatable proforms of cathepsins B and L, followed by subsequent activation of latent proforms via autoactivation at acidic pH and/or protease action (Recklies *et al.*, 1980; Mort, *et al.*, 1981; Recklies *et al.*, 1982). To examine this relationship, levels of cysteine protease were examined in unfractionated homogenates and medium collected from MCF-7 cells and their adriamycin-resistant variant. Because of the similar patterns of expression of cathepsin B activity observed with colorimetric and fluorometric substrates, the assays were conducted under

conditions optimal for cathepsin B using arg-arg as the substrate (described in Methods, section 2.6). In brief, cells were seeded and grown in culture medium supplemented with serum. At 24 hour intervals for each of seven days after initial seeding, the medium was removed from cells and replaced with serum-free, defined medium (Dufresne *et al.*, 1993b). Cells were then incubated at 37°C for 24 hours after which cell counts were determined and cells and their medium were collected. Fractions prepared from cells and medium were termed intracellular and extracellular fractions respectively. Latent pro-enzyme (e.g., pro-cathepsin B) in the extracellular fractions was activated according to established procedures and involved pre-incubation of conditioned serum free medium with the aspartic protease, pepsin, at pH 3.8 (described in Methods section 2.6). The results of these analysis for MCF-7 and MCF-7/Adr^R are presented in Figures 6A and 6B respectively. To facilitate comparison, the protease activity data are also summarized in Table 5. Within each cell line, levels of intracellular activity at each point of growth were reproducibly greater than levels of activated extracellular activity. Between cell lines, the MCF-7/Adr^R cell line demonstrated greater levels of intracellular cathepsin B activity at each point of growth than the MCF-7 parent (compare Figures 6A and 6B, and corresponding data in Table 5 for clarification). This result is consistent with the previous results (i.e., Figures 3 and 5). The pattern of expression of acid/pepsin-activated activity in extracellular fractions of MCF-7 and MCF-7/Adr^R cells is somewhat different in that higher levels of activity were apparent in the adriamycin-resistant variant late in cell growth (i.e., 144 and 168 hours). Prior to this, levels of protease (e.g., cathepsin B) in both cell lines were comparable. A decrease in activity in both intracellular and extracellular

FIGURE 6:

Intra- and Extracellular Levels of Cathepsin B Activity in MCF-7, MCF-7/Adr^R, and M:A Hybrid.

Panels A, B, and C represent intra- and extracellular levels of cathepsin B activity, coupled with growth curve analysis over a 7 day period. 2 x 10^5 cells were seeded in 100mm culture dishes for (A) MCF-7, (B) MCF-7/Adr^R, and (C) the M:A hybrid. Presented are cell numbers for each day of growth (-●-), following one day in serum-free medium. Cathepsin B activities of intracellular (solid bars) and extracellular (open bars) fractions of MCF-7, MCF-7/Adr^R, and the M:A hybrid, were determined using the synthetic substrate, arg-arg (extracellular cathepsin B activity was determined after acid/pepsin activation, as described in Materials and Methods, section 2.6C). Protease activity is expressed as Enzyme Units/mg total protein, where one enzyme unit (EU) is defined as the quantity of protease that gives rise to an absorbance unit of 0.016 in one minute at a wavelength of 520 nm.

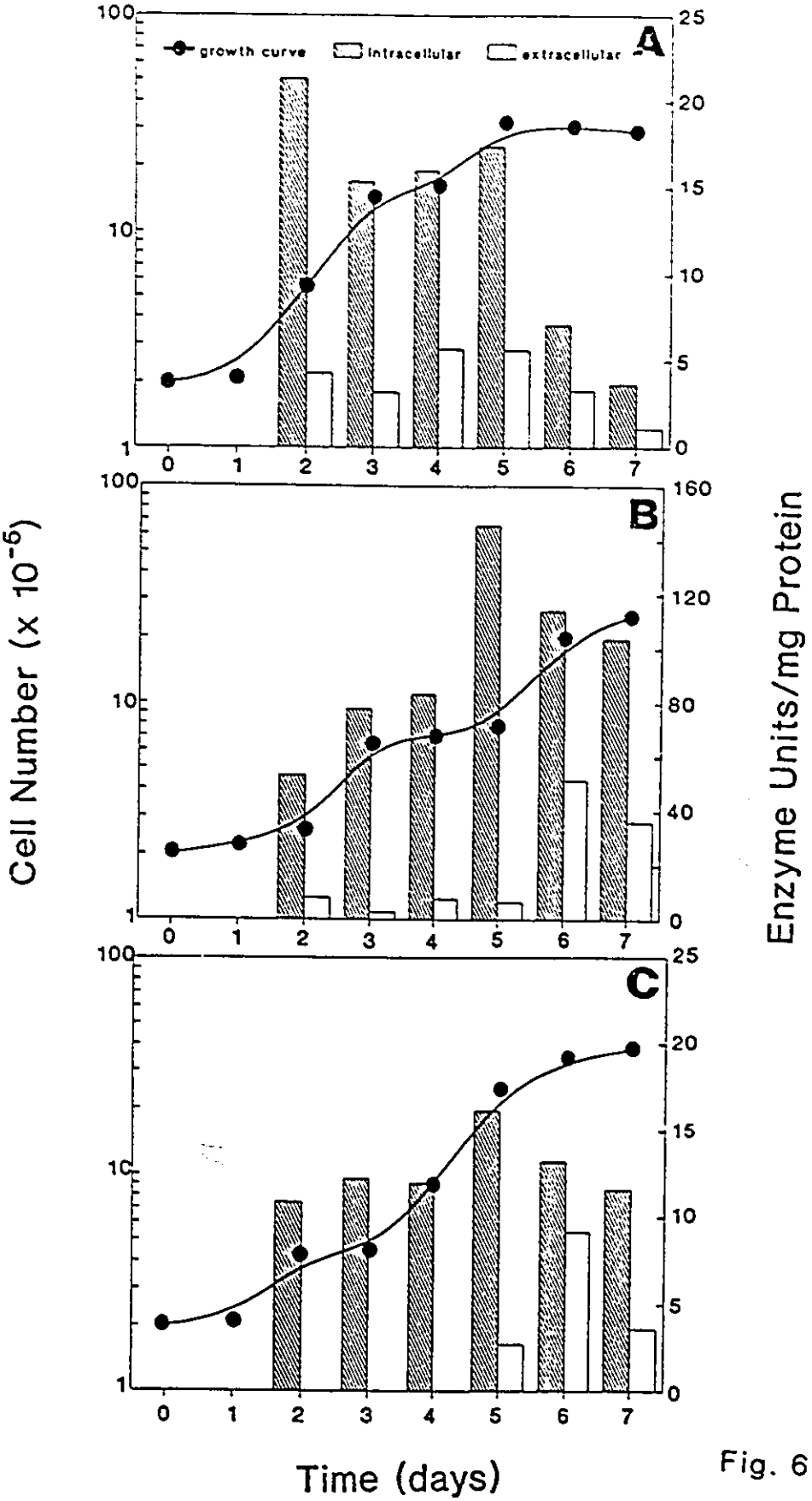
growth curve
intracellular
extracellular
A
B
C
Cell Number (x 10⁻⁶)
Enzyme Units/mg Protein
Time (days)

Fig. 6

TABLE 5

Summary of Intracellular and Extracellular Levels of Cathepsin B Activity in MCF-7, MCF-7/Adr^R, and M:A Hybrids

	Cathepsin B Specific Activity (Enzyme Units/mg Protein)[a]					
DAY	MCF-7		MCF-7/Adr^R		M:A Hybrid	
	Intra[b]	Extra[c]	Intra	Extra	Intra	Extra
0	---[d]	---	---	---	---	---
1	---	---	---	---	---	---
2	21.32	4.27	53.07	8.01	10.87	n.d.[e]
3	15.38	3.21	77.97	3.67	12.23	n.d.
4	16.03	5.66	83.54	7.63	11.97	n.d.
5	17.41	5.57	145.53	6.41	16.13	2.67
6	7.07	3.31	114.12	51.28	13.22	3.56
7	3.67	1.11	103.70	36.06	11.59	2.07

[a] Specific Activity is expressed in Enzyme Units per milligram of sample protein; each value represents the mean, where n=3. Standard deviations were less than 10%.

[b,c] Intra and Extra represent the intracellular and extracellular level, respectively, of cathepsin B activity after 1 day in serum free medium, for each day of growth.

[d] — represents points in the growth of the cells where cathepsin B activity cannot be determined, since cells will not adhear to a growing surface if they are plated into serum free medium.

[e] n.d. not detected.

fractions was observed by the last time point of the experiment (Table 5, day 7). The biological significance of this decrease is not known at present although it may reflect a decreased translation resulting from cell cycle inhibition in culture, or a post-metastatic decrease in protease activities of tumors *in vivo* (Veksler *et al.*, 1987). Regardless of the explanation, it is unlikely that these results reflect cell death since transfer to serum-free medium had no effect on cell growth or on cell viability (results not shown). This is consistent with previous observations indicating that MCF-7 cells showed the same growth characteristics in both the presence and the absence of fetal bovine serum (De Launoit *et al.*, 1991). A time course experiment to compare levels of activity in intracellular and extracellular fractions in the M:A somatic cell hybrid was also performed. For the purpose of comparison, these results were also included in Figure 6 (i.e., Figure 6C) and in Table 5 (third column). Once again, levels of activity in intracellular and acid/pepsin treated extracellular fractions of the hybrid were similar to those for MCF-7 at each time point of growth. The failure to detect any activity for day 2, 3, and 4 extracellular fractions may reflect a limited sensitivity. Regardless of these differences, increases in levels of acid/pepsin-activatable protease activity were eliminated by preincubating the extracellular fractions with either the cysteine proteinase inhibitor E-64 or the aspartyl proteinase inhibitor pepstatin (results not shown). These results are consistent with those suggesting an increased secretion of a latent, high molecular weight form of cathepsin B from malignant tissues in comparison with non-malignant tissue (Mort and Recklies, 1986).

3.5 FPLC Fractionation of MCF-7 and MCF-7/Adr^R Cells

The data obtained in unfractionated cell homogenates demonstrated that levels of cysteine protease activity in MCF-7/Adr^R, were greater than those measured in MCF-7. This is most evident in intracellular fractions. There are several mechanisms which could explain this increase (discussed in Introduction) including an alteration in the regulation of proteases by their endogenous protease inhibitors. Assay of inhibitor levels in unfractionated homogenates, however, can be problematic. Homogenates contain bound (i.e., complexed with target protease) and free forms of the inhibitor, as well as endogenous proteases that may interfere with measurements of inhibition against a standard, exogenous protease. Some investigators have approached this problem by using heat treatment, or alkaline pH, to dissociate inhibitor from th protease:inhibitor complex. These treatments presumably destroy the biological activity of proteases but not that of their endogenous inhibitors (Sloane *et al.*, 1992). While these treatments are useful, their effectiveness depends on complete dissociation and assumes that inactivated protease cannot reassociate with free inhibitor. The need to consider this assumption was bypassed in this study by separating inhibitors and proteases in fractions by FPLC on a Superose 12 HR 10/30 FPLC column. This column permits the efficient separation of molecules with molecular masses between 10 kDa and 300 kDa, a range that includes various proteases and protease inhibitors. A selectivity curve was prepared by calculating separation coefficients, K_{av}, (detailed in Methods, section 2.8) for different molecular weight standards (Figure 7). This curve was in excellent agreement with the separation curve provided by the manufacturer and demonstrated reproducible separation of both exogenous pork liver cathepsin B and

FIGURE 7:

Selectivity Curve Using a Superose 12 HR 10/30 FPLC

Molecular weight standards (bovine albumin, 66.2 kDa; ovalbumin, 42.7 kDa; chymotrypsin, 25.0 kDa; and cytochrome C, 13.0 kDa) were analyzed with FPLC and their elution volumes recorded. Void volume was determined with Blue Dextran and K_{av} values determined for each molecular weight using the formula outline in Materials and Methods, section 2.8. K_{av} values were determined from the elution volumes of cathepsins B, H, and L activity (B, H, and L, respectively) using fluorometric substrates, and cathepsin B inhibitory activity regions (I), in an effort to estimate their molecular weights.

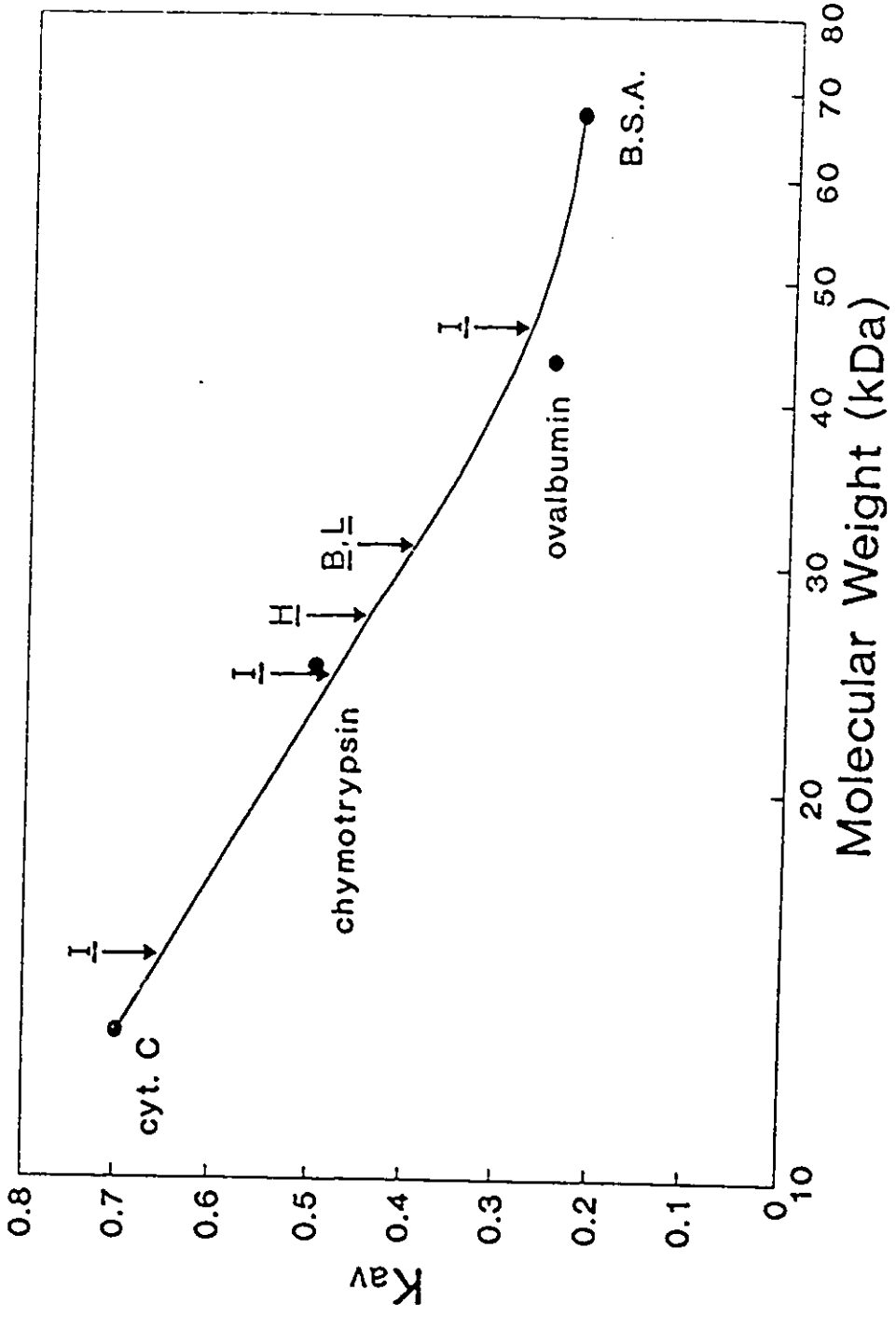
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
Kav
Molecular Weight (kDa)
cyt. C
chymotrypsin
ovalbumin
B.S.A.
I
I
H
B.L
I

Fig. 7

commercial egg white cystatin preparations without loss of their biological activity. For reference, this figure also includes the apparent molecular weight positions at which endogenous inhibitor, I (i.e., % inhibition of pork liver cathepsin B), and endogenous protease (i.e., cathepsins B, H and L) activity were detected from breast cancer cells. These patterns were identical for all three FPLC columns used during the course of this thesis research.

To determine if the column would separate cysteine proteases (e.g. cathepsin B) and their endogenous inhibitors in the human breast cancer cell system, cell homogenates were prepared from growing cultures of MCF-7 and MCF-7/Adr^R cells and similar amounts of protein from each, in homogenization buffer (pH 6.0), were injected separately onto a Superose 12 HR 10/30 FPLC column at room temperature. Fractions were eluted at room temperature with phosphate column buffer (pH 6.0) at a flow rate of 1.0 ml/min. Absorbance at 280 nm was monitored with a Beckman Analytical Optical Unit and recorded on a Kipp and Zonen chart recorder. Five hundred μl fractions were collected, stored on ice then assayed for cathepsin B activity using the colorimetric substrate, arg-arg, and for inhibitor activity using purified pork liver cathepsin B (detailed in Methods, section 2.7). The elution profile, protease (E) and inhibitor (I) assay profiles for MCF-7 and MCF-7/Adr^R are presented in Figure 8A and Figure 8B respectively. Distinct peaks of protease and inhibitor activity were observed at similar positions for both cell lines. Moreover, although an equal amount of protein was added, levels of protease activity in fractions from the adriamycin-resistant variant of MCF-7 were reproducibly greater than those measured in fractions from the original MCF-7 as evidenced from the different enzyme units scale.

FIGURE 8:

Colorimetric Analysis of Cathepsin B and Cathepsin B Inhibitor Activities in Fractionated Homogenates of MCF-7 and MCF-7/Adr^R.

Panels A and B represent FPLC tracings for fractionated homogenates of MCF-7 and MCF-7/Adr^R respectively. Homogenates for each cell line were clarified and protein adjusted before 500 μL of each sample were applied separately to a Superose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min over 30 minutes. Following the collection of 250 μL fractions, cathepsin B (E) and cathepsin B inhibitor (I) activities were detected. Protease activity is expressed as Enzyme Units, where one enzyme unit (EU) is defined as that quantity of protease that gives rise to an absorbance unit of 0.016 in one minute at a wavelength of 520 nm. Inhibitor activity is determined as percent inhibition of control pork liver cathepsin B activity, as described in Materials and Methods, section 2.7C.

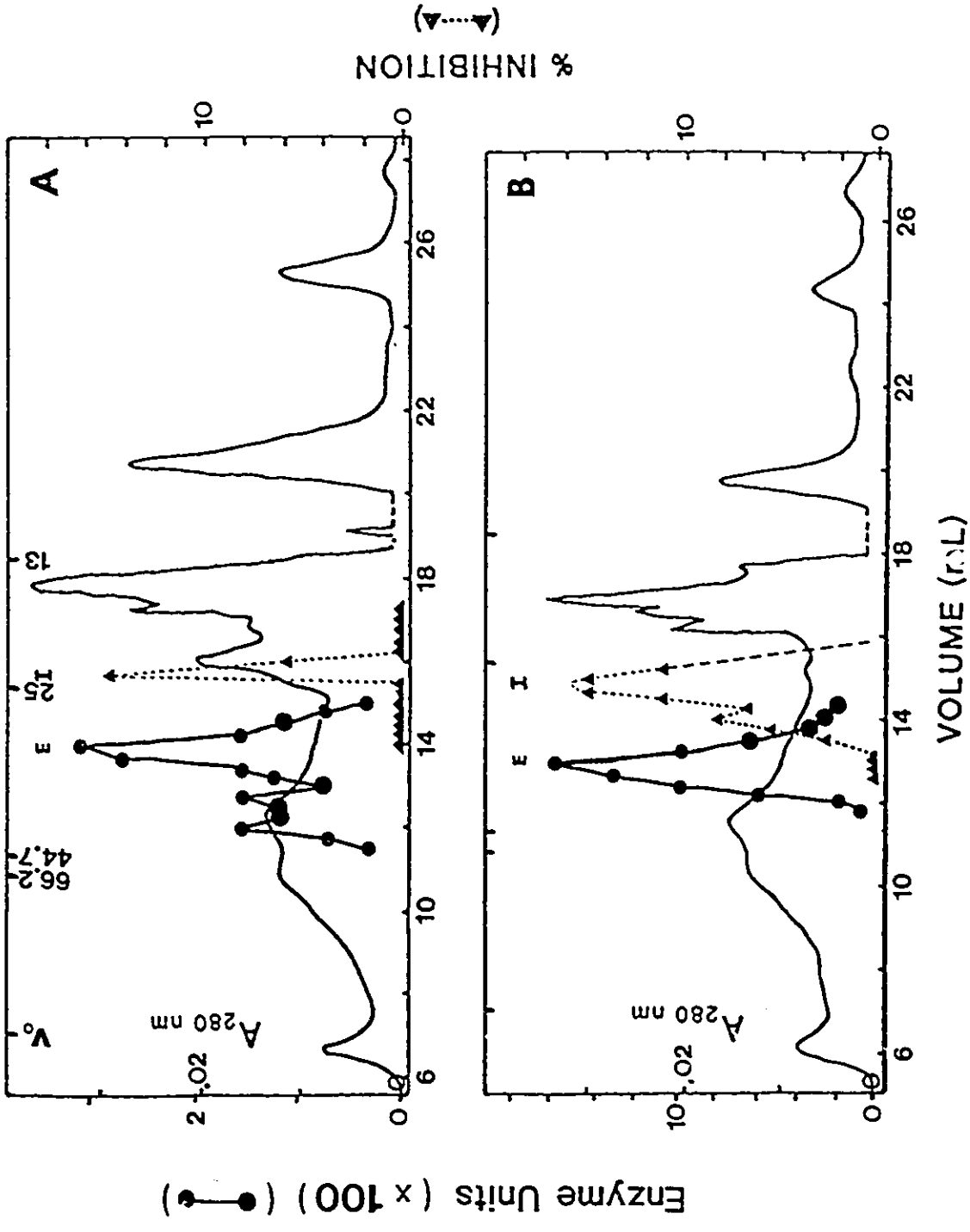
% INHIBITION
(▲---▲)
A
B
$A_{280\ nm}$
.02
V$_o$
66.2
44.7
E
I
13
12.5
VOLUME (mL)
Enzyme Units (x 100) (●—●)

Fig. 8

Having established that protease and inhibitor could be separated in both cell lines, the experiment was repeated using the more sensitive fluorometric assay and the 4-methylcoumarylamide substrates for cathepsins B, H, and L (detailed in Methods). Levels of inhibitor were again determined using purified pork cathepsin B and expressed as percent inhibition. Representative profiles for MCF-7 and MCF-7/Adr^R are presented in Figure 9A and Figure 9B respectively. The activity of the cathepsins in fractions collected from FPLC paralleled the order of activity observed in whole homogenates for both MCF-7 and MCF-7/Adr^R, with cathepsin L (corrected) expressing the greatest level of activity, followed by cathepsin B, then cathepsin H. Peak average levels -the calculated average of enzyme activity at each enzyme peak- for cathepsin B and cathepsin L activities were greater in MCF-7/Adr^R by 3.6 and 3.4 fold respectively than levels in MCF-7 fractions. Cathepsin H activity was similar in both cell lines. Based on the standard FPLC separation curves (Figure 7) cathepsin B and cathepsin L peak activities from MCF-7 and MCF-7/Adr^R fractions both eluted at a molecular weight position corresponding to 31.0 kDa. Cathepsin H activity in both cell lines eluted slightly later at a molecular weight position corresponding to 27.5 kDa.

Cathepsin B inhibitory peaks for both cell lines eluted after endogenous cathepsin B activity over a 5 ml elution volume from 14.5 to 19.5 mls. Two distinct inhibitory peaks at molecular weight positions corresponding to 25.0 kDa and 15.0 kDa were observed within this elution range. The 25.0 kDa peak for MCF-7 and MCF-7/Adr^R samples yielded peak average levels of inhibition -the calculated average % inhibition at each inhibitor peak- of 8.5% and 7.5% respectively, while the 15.0 kDa peak

FIGURE 9:

Fluorometric Analysis of Cathepsin B, H and L, and Cathepsin B Inhibitor Activities in Fractionated Homogenates of MCF-7 and MCF-7/Adr^R.

Panels A and B represent FPLC tracings for fractionated homogenates of MCF-7 and MCF-7/Adr^R respectively. Homogenates for each cell line were clarified and protein adjusted before 500 μL of each sample was applied separately to a Superose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min over 30 minutes. Following the collection of 500 μL fractions, cathepsin B, H, and L activities (B, H, and L respectively) and cathepsin B inhibitor activities were detected. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm as described in Materials and Methods, section 2.6B. Protease activity is expressed as mUnits, where one milliunit is defined as the quantity of protease that liberates 1 nmol of aminomethylcoumarin per minute. Inhibitor activity was determined as percent inhibition of control pork liver cathepsin B activity.

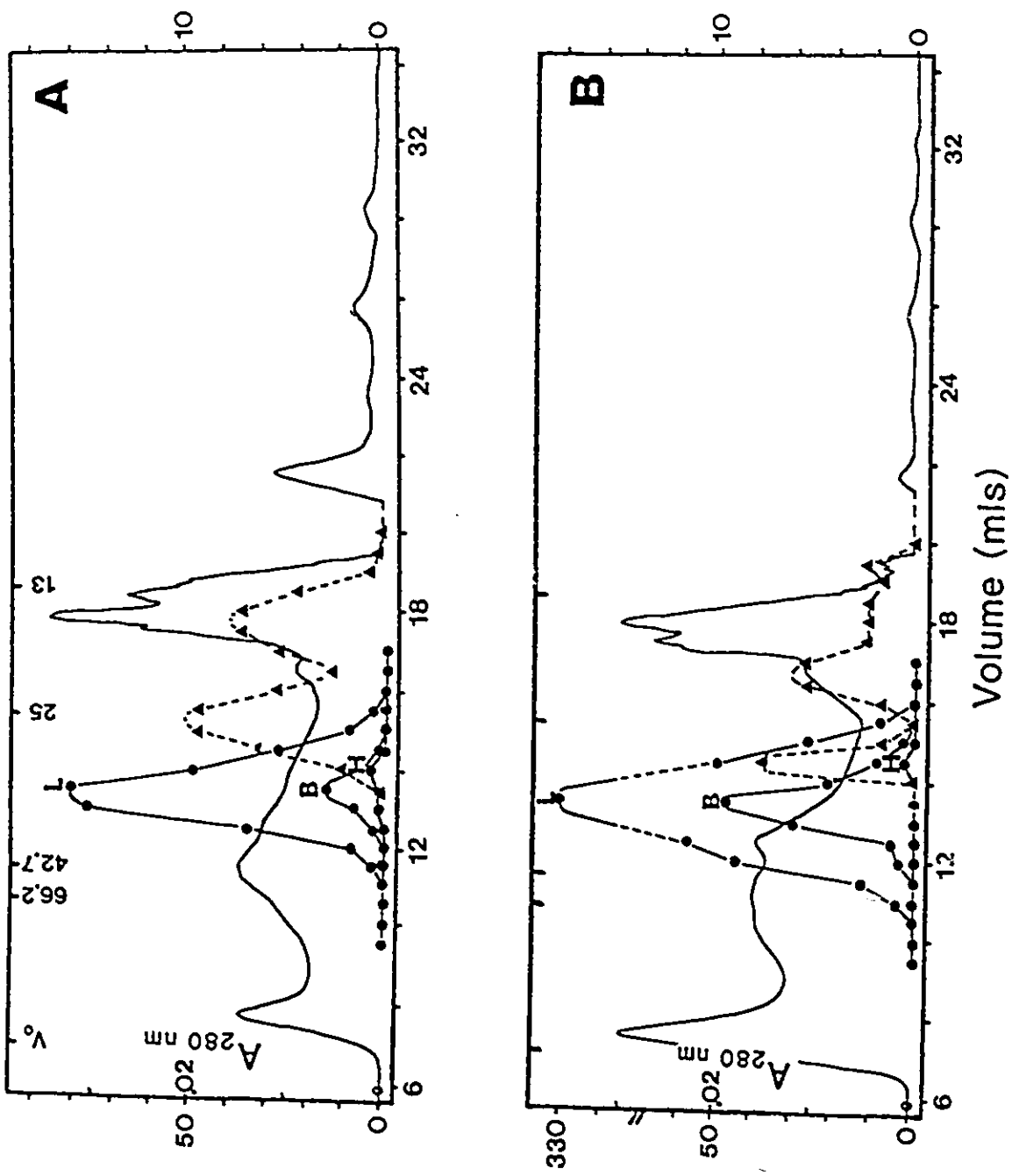
A
B
A280 nm
Vo
66.2
42.7
25
13
L
B
H
Units (x10⁵) (•—•)
% Inhibition (▲---▲)
Volume (mls)

Fig. 9

yielded peak average levels of inhibition of 6.8% and 5.5% inhibition respectively. The total peak average levels of inhibition (the sum of all peak average % inhibitions), for MCF-7 was 15.3%, while that for MCF-7/Adr^R was 13.0%. The relative values for average peak levels of cathepsin activities and of inhibition remained the same for both cell lines between experiments and with increased time in culture.

3.6 Heat Treatment: Total Endogenous Cathepsin B Inhibitor

The results obtained using FPLC column chromatography repeatedly demonstrated that levels of cysteine protease in the adriamycin-resistant variant were higher than those measured in MCF-7 when assayed under conditions optimal for cathepsin B. The results further suggested that this increase was not the obvious result of different levels of inhibitor of cathepsin B. Peak average levels of inhibition were similar in both cell lines. It was recognized, however, that this interpretation could be misleading since levels reflected the amount of free inhibitor available to interact with the extraneous cathepsin B. Inhibitor that is bound to endogenous protease would not be detected. The stability of cysteine protease inhibitors to heat has been used by many researchers to provide a more representative estimate of the total level of heat stable inhibitors (i.e., stefins, cystatins, and kininogens) present in each cell line. In NIH 3T3 cells, for example, incubation of homogenates at 100°C for 5 minutes effectively dissociated inhibitors from the cathepsin B:inhibitor complex. Moreover, while this treatment had no effect on inhibitor reactivity, it eliminated detectable, endogenous cathepsin B activity (Chambers *et al.*, 1992). The potential of this approach in the MCF-7 human breast cancer cell system was first examined by treating homogenates of MCF-7 and

MCF-7/Adr^R cells at different temperatures prior to colorimetric (arg-arg) assay of protease activity under conditions optimal for cathepsin B (Lah *et al.*, 1992). The results of a typical experiment are presented in Figure 10. For each temperature at which activity was detected, levels in MCF-7/Adr^R were signifigantly greater than those observed in MCF-7 ($p=0.000$). Levels of activity in both cell lines decreased at greater than physiological temperatures. At 70°C no activity was detected in either cell line.

While heat treatment of MCF-7 and MCF-7/Adr^R unfractionated homogenates (e.g., 80°C, 5 min) effectively eliminated cathepsin B activity, attempts to measure levels of inhibitor were unreliable and for the most part unsuccessful despite the manipulation of controls (e.g., addition of bovine serum albumin) suggested in the literature (Chambers *et al.*, 1992). In view of this result, an approach combining heat treatment of homogenates with subsequent fractionation by FPLC chromatography was attempted. In brief, homogenates prepared from each cell line were normalized for protein content and treated at 80°C for 5 minutes. After clarification by microcentrifugation, the heat-treated samples were injected onto the FPLC column. The column was eluted with column buffer at pH 6.0 and fractions were collected, then fluorimetrically assayed as described for non-heated FPLC fractions. The results of these analysis for MCF-7 and MCF-7/Adr^R are summarized in Figures 11A and 11B respectively. Heat treatment appeared to alter the absorbance profiles of regions eluting at molecular weights greater than 23.0 kDa in both the MCF-7 and MCF-7/ Adr^R samples (compare figures 9A and 9B, to 11A and 11B, respectively). Heat treatment also eliminated detectable cathepsin activity in both cell line extracts but did

FIGURE 10:

Heat Sensitivity of Cathepsin B Activity in MCF-7 and MCF-7/Adr^R Human Breast Cancer Cells

Clarified, protein adjusted, unfractionated homogenates of MCF-7 and MCF-7/Adr^R cells were treated for 5 minutes at 80°C. Samples were again clarified for an additional 5 minutes and subsequently assayed colorimetrically for their levels of cathepsin B activity. Protease activity is expressed as Enzyme Units/mg total protein, where one enzyme unit (EU) is defined as that amount of protease which gives rise to an absorbance unit of 0.016 in one minute at a wavelength of 520 nm.

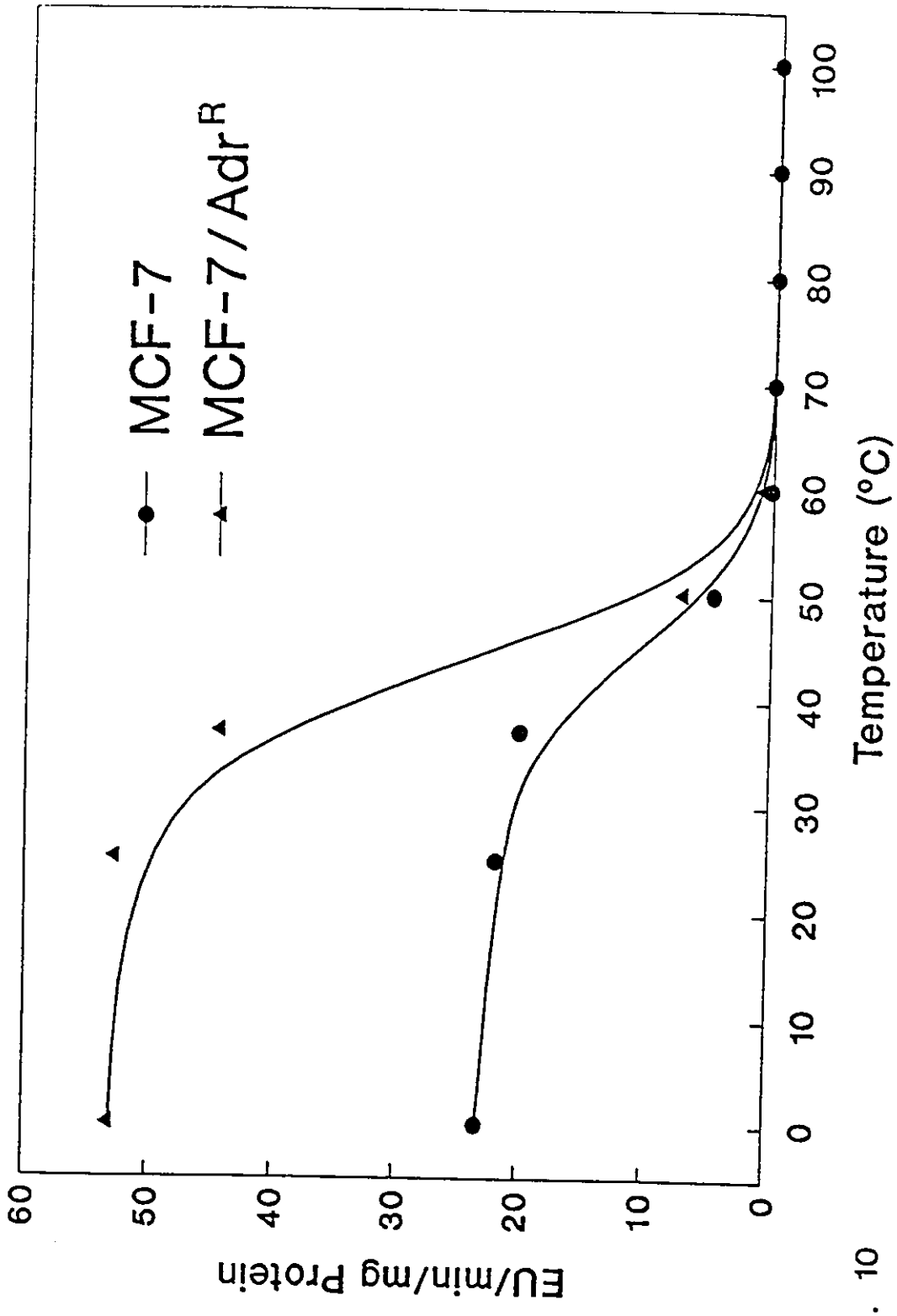
MCF-7
MCF-7/Adr^R
EU/min/mg Protein
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100

Fig. 10

FIGURE 11:

Fluorometric Analysis of Cathepsin B, H and L, and Cathepsin B Inhibitor Activities in Heat Treated, Fractionated Homogenates of MCF-7 and MCF-7/Adr^R.

Panels A and B represent FPLC tracings for heat treated fractionated homogenates of MCF-7 and MCF-7/Adr^R respectively. Clarified, protein adjusted, unfractionated homogenates of MCF-7 and MCF-7/Adr^R cells were treated for 5 minutes at 80°C. Samples were again clarified for an additional 5 minutes before 500μL of each sample was applied separately to a Superose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min over 30 minutes. Following the collection of 500 μL fractions, levels of cathepsin B, H, and L activity, and cathepsin B inhibitor activity were examined fluorometrically. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm, as described in Materials and Methods, section 2.6B. Protease activity is expressed as mUnits, where one milliunit is defined as the quantity of protease that liberates 1 nmol of aminomethylcoumarin per minute. Inhibitor activity was determined as percent inhibition of control pork liver cathepsin B activity.

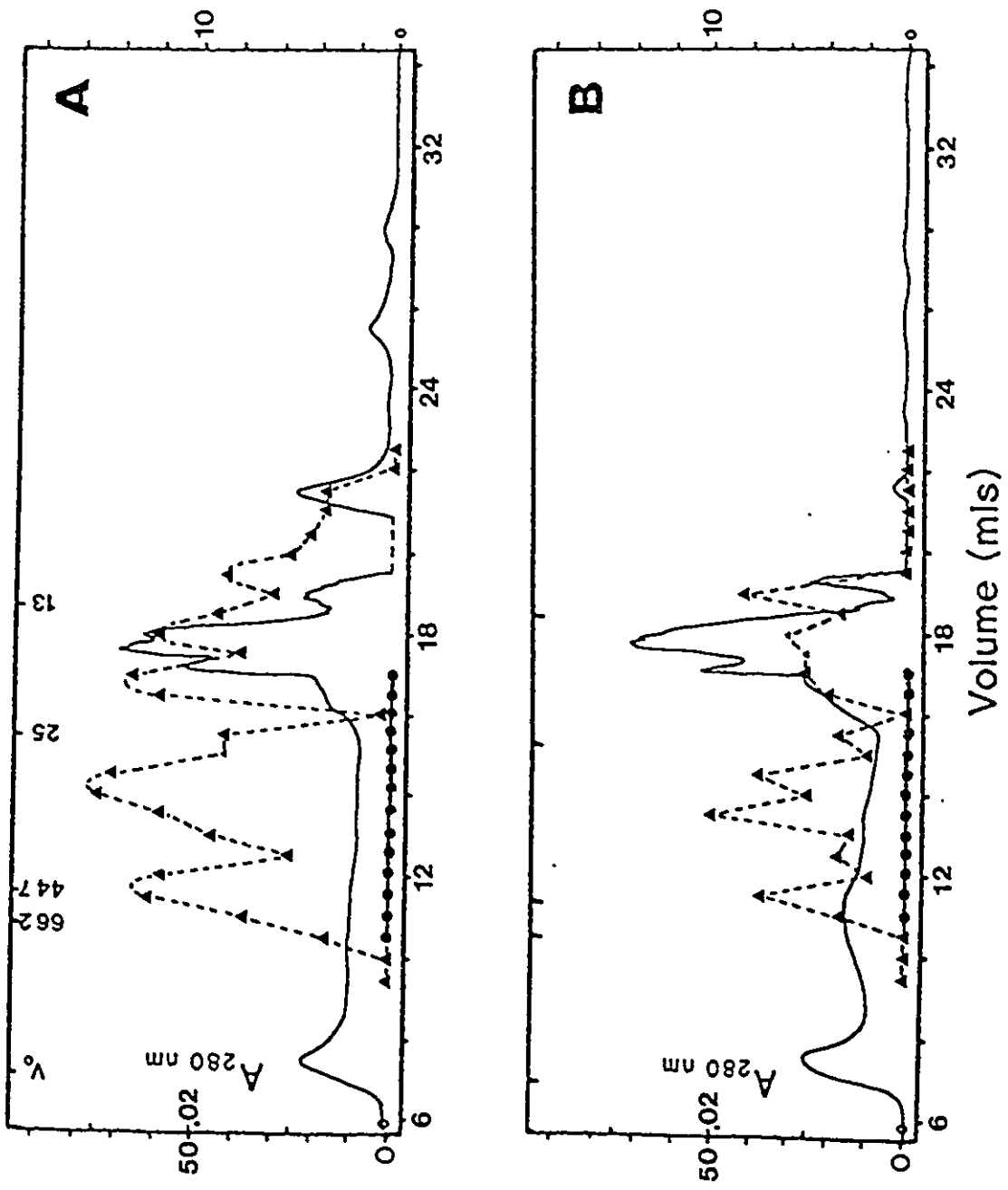
A
B
A280 nm
.02
50
0
6
12
18
24
32
10
0
% Inhibition (▲---▲)
mEnzyme Units (x10⁵) (●—●)
Volume (mls)
662
447
25
13

Fig. 11

not eliminate inhibition of cathepsin B activity. Inhibition was detected between elution volumes of 10.0 mls and 22.0 mls for the MCF-7 sample (Figure 11A) and between 10.5 mls and 19.5 mls for the MCF-7/Adr^R sample (Figure 11B). Within these elution volumes, three distinct regions of inhibition were observed in both MCF-7 and MCF-7/Adr^R heat-treated, fractionated samples. The first inhibitory region eluted at a position corresponding to an average, apparent molecular weight of 45.0 kDa. The peak average level of inhibition (%) for this region was 2.5 fold greater in MCF-7 than in MCF-7/Adr^R. The second inhibitory region eluted at a position corresponding to an average, apparent molecular weight of 28.5 kDa. At this region, the peak average level of inhibition (%) was 1.8 fold greater in MCF-7 than in MCF-7/Adr^R. The third inhibitory region was reproducibly broader and eluted at positions corresponding to apparent molecular weights between 11 kDa and 19 kDa. At this region, the peak average level of inhibition (%) was 1.8 fold greater in MCF-7 than in MCF-7/Adr^R. The positions of the 28.5 kDa and 11-19 kDa inhibitory regions corresponded, in an approximate manner, to regions obtained after FPLC chromatography of non-heated homogenates prepared from MCF-7 and MCF-7/Adr^R (compare Figures 9 and 11). The data for non-heated and heat-treated homogenates of MCF-7 and MCF-7/Adr^R after FPLC chromatography are summarized in Table 6. While the absolute values for levels of inhibition varied from experiment to experiment, the relative ratios between levels in the cell lines remained constant. In general, although heat treatment increased levels of inhibition in both MCF-7 and MCF-7/Adr^R, compared to non-heated samples, levels of increase (e.g., 1.3 fold) in the adriamycin-resistant variant of MCF-7 were lower than those measured in MCF-7 (e.g., 2.2 fold). At the same time,

TABLE 6

Summary of Protease and Protease Inhibitor Activity
in Non-Heated and Heated MCF-7 and MCF-7/Adr^R Fractionated Homogenates

Treatment	Cell Line	Peak avg. Cathepsin B[a] Activity (mUnits x10^5)	Peak avg. % Inhibitory Activity[b] 45 kDa	24-28.5 kDa	11-19 kDa	Total %[c] Inhibition
NON-HEATED	MCF-7	9.1	n.d.[d]	8.5	6.8	15.3
	MCF-7/Adr^R	33.3	n.d.	7.5	5.5	13.0
HEATED	MCF-7	n.d.	10.6	13.6	9.9	34.1
	MCF-7/Adr^R	n.d.	4.2	7.7	5.5	17.4

[a] **Peak avg. Cathepsin B Activity** represents the averaged protease activity of peak fractions for the cathepsin B activity peak; measured in milliUnits. Results have been multiplied by 10^5.

[b] **Peak avg. % Inhibitory Activity** represents the averaged % inhibitory activity of peak fractions for each inhibitory peak; measured in percentage decrease of control pork liver cathepsin B activity.

[c] **Total % Inhibition** represents the sum of the peak average % inhibitions for each treated sample.

[d] **n.d.** not detected.

assuming that heat-treatment and FPLC fractionation permit a more realistic estimate of total inhibitor, fold differences in protease activity between the two cell lines after FPLC chromatography of non-heated homogenates were always greater (i.e., 2-5 fold) than fold differences in levels of inhibition after FPLC chromatography of heated homogenates. Thus, while the adriamycin-resistant variant of MCF-7 demonstrated higher levels of protease activity and lower levels of inhibition than those in the MCF-7 parent, the differences were not complementary.

Discussion

Malignant transformation of a somatic cell is a multi-step process that begins with the transformation of a normal cell to a cell that reproduces and differentiates abnormally. Through cell division, this transformed cell gives rise to a population of similarly transformed cells termed a clone. Cells in this clone can demonstrate two general patterns of growth: they can remain encapsulated and not spread, in which case the tumor is usually benign, or they can detach and spread to other parts of the body, in which case the tumor is usually malignant. Some cancers are more malignant than others. The degree of malignancy depends in part on the rate at which the cells reproduce, but a more critical property appears to be how readily cells metastasize from the primary cancer (Schirrmacher, 1985; Hill, 1987; Thompson *et al.*, 1992).

According to Thompson (1992), "Metastasis is a multifactorial process by which tumor cells escape from the primary tumor, disseminate through blood and lymph vessels, evade host immune defense, and home to specific target organs where they extravasate and re-colonize". Cancers that tend to metastasize quickly (i.e., when the number of cancer cells is still small) are generally more malignant. There are several reasons for this including that secondary cancers often form in tissues such as brain, lung, liver, or bones and are more difficult to detect and usually more difficult to treat. Moreover, a secondary cancer may also metastasize and initiate tertiary cancers in still other sites.

The correlation between malignancy and metastasis has resulted in considerable international research into the molecular mechanisms by which cells detach from the primary tumor. Although the research is relatively recent, there is some consensus that decreased cell-to-cell adhesion and increased cell motility -phenotypes readily observed in cancer cells- are important to metastatic potential. These phenotypes have several molecular explanations. For example, they may be a result of changes that occur in the surface of the cell membrane when a normal founder cell is first converted to a cancer cell. Alternatively, they may also be the result of changes in the ability to degrade and invade through basement membrane (i.e., the extracellular matrix) by proteolytic enzymes (reviewed in: Recklies *et al.*, 1980; Nicolson, 1991; Liotta, 1992).

A variety of degradative enzymes, including heparanases, collagenases, and lysosomal metallo-, serine-, aspartic-, and cysteine proteases, have been reported to contribute, directly and indirectly, to metastasis (Sloane, 1990; Liotta *et al.*, 1991; Chambers, 1992; Chambers *et al.*, 1992; Sloane *et al.*, 1992). Of these, alterations in the activities of lysosomal cysteine proteases (e.g., cathepsins B and L) appear to be particularly significant (reviewed in Recklies *et al.*, 1986 and Tryggvason *et al.*, 1987; Sloane, 1990; Chambers *et al.*, 1992).

Several hypotheses have been suggested to explain the molecular basis of increased lysosomal cysteine protease activity in cancer cells. Not surprisingly, these hypotheses reflect the complexity of protease regulation in normal cells and include: 1) enhanced transcription of protease genes as a result of oncogene-mediated amplification, up-regulation, or increased efficiency of the transcription molecular machinery (Nishimura and Kato, 1987; Moin *et al.*, 1989); 2) changes in subcellular processing and

localization (Mort and Recklies, 1986); and 3) changes in regulation by endogenous cysteine protease inhibitors (CPIs) (Lah *et al.*, 1989b; Chambers *et al.*, 1992; Lah *et al.*, 1992b). While these hypotheses are neither mutually exclusive nor exhaustive, the research described in this thesis focused on the latter, specifically, the regulation of cathepsin B by its endogenous inhibitors in various human MCF-7 breast cells in culture. The rationale for using the MCF-7 breast cancer cells has been discussed in detail in the Introduction of this thesis. In short, it provides a highly controlled, well characterized, relevant, isosytem to study tumor progression in a human cancer that is characterized by early metastasis. The MCF-7 wild type cell line is characteristic of "early" breast tumor progression in that it is: a) fully hormone dependant (i.e., estrogen receptor (ER) positive), b) sensitive to chemotherapeutic drugs, and c) poorly metastatic. MCF-7 cells rarely form tumors without the influence of hormones and require physiological levels of estrogen for maximal growth in vitro and for tumor formation in nude mice (Aakvaag *et al.*, 1990; Gelmann *et al.*, 1992). In contrast, MCF-7/Adr^R, an adriamycin resistant variant derived from MCF-7 is characteristic of "late" breast tumor progression in that it is: a) hormone independent (i.e., estrogen receptor negative), b) resistant to seemingly unrelated chemotherapeutic drugs (i.e., multidrug (MDR) resistant), and c) highly metastatic (Fairchild *et al.*, 1987; Vickers *et al.*, 1989). This cell line will form proliferating tumors in the presence or absence of estrogen (reviewed in Leonessa *et al.*, 1992). The inclusion of *ras/gpt* transfected MCF-7 cells, and their *gpt* transfected controls, needs little explanation; transfected *ras* oncogenes induce metastatic properties in some cells. The MCF-7 breast cancer cell system is also excellent from a cell culture perspective. All MCF-7 and MCF-7/Adr^R

cell lines are easy to manipulate in culture, have similar growth parameters (e.g., Table 2, Results for MCF-7 and MCF-7/Adr^R), demonstrate high viability, and are stable with respect to genotypes and phenotypes.

Relative to the MCF-7 parent, increased levels of cathepsins B (11 to 25 fold) and L (11 fold) were observed in the adriamycin resistant MCF-7 cell line but not in either the gpt-transfected or the *ras/gpt*-transfected MCF-7 cell lines. In contrast, levels of cathepsin H in MCF-7/Adr^R, MCF-7/*gpt*, and MCF-7/*ras/gpt* were similar or lower than those found in the MCF-7 cell line. Although the absolute values differ, the relative order of these results are consistent with those of Lah *et al.*, (1992b) who reported a 18.5 fold and 52.5 fold increases in cathepsins B and L, respectively, in 70 matched pairs of breast carcinoma. The relative order of increased [B and L] activities in MCF-7/Adr^R also supports the close relationship between the cysteine proteases and the major excreted protein, MEP, observed in other malignantly transformed cells (Denhardt *et al.*, 1986; Gal and Gottesman, 1986; Chambers *et al.*, 1992).

The results with *ras* transfected MCF-7 were of particular interest since transfected *ras* oncogenes have been shown to induce metastatic properties in both murine and human cells. However, it is clear from the literature that not all cells respond in the same way to expression of oncogenic *ras*. Transfection of murine NIH 3T3 fibroblast cells with v-*ras*H, for example, resulted in metastasis and increased levels of cathepsin B (Joseph *et al.*, 1987; Hill *et al.*, 1988; Chambers *et al.*, 1990; Chambers *et al.*, 1992). Murine LTA fibroblast-like cells, on the other hand, were *ras*-resistant (i.e., resistant to transformation) and remained tumorigenic but nonmetastatic after transfection with v-*ras*H (reviewed in Chambers, 1992). In contrast, transfection of

human MCF-7 breast cancer cells with the v-*ras*H oncogene resulted in metastasis without an increase in cathepsin B (Kasid *et al.*, 1985). The derivative MCF-7/*ras* cell line, unlike the original estrogen positive MCF-7 cell line, no longer required estrogen supplementation to form tumors in nude mice. In addition, although these cells demonstrated cellular and secreted polypeptides that were in common, the patterns were not identical, most notably with respect to the secretion of cathepsin D, an estrogen regulated gene in MCF-7 (Rochefort *et al.*, 1987; Leonessa *et al.*, 1992; Brünner *et al.*, 1993). The human cathepsin D gene has been mapped to chromosome 11p15, while lysosomal cathepsins H and L have been mapped to 15q25-q25 and 9q21-q22 respectively (reviewed in Fong *et al.*, 1992). The human cathepsin B gene (CTSB) was recently localized between the p22 and p23.1 regions of chromosome 8 (Fong *et al.*, 1992). Interestingly, Emi *et al.* (1992) noted frequent loss of heterozygosity for various human tumors at region 8p21.3 to 8p23.1, indicating that one or more tumor suppressor genes may be present in that region.

Adriamycin can also induce metastatic properties in MCF-7 cells. This appears to be the result of a multidrug resistant (MDR) phenotype selected against adriamycin which compounds genetic alterations accompanying the progression of MCF-7 to a hormone-independent, metastatic phenotype *in vitro* (Clarke *et al.*, 1986; Vickers *et al.*, 1988; Vickers *et al.*, 1989; Hochhauser and Harris, 1991; Pastan and Gottesman, 1991; Yeh *et al.*, 1992; Ferguson and Baguley, 1993). Although this also occurs *in vivo*, it is not known if tumor cells acquire the MDR phenotype naturally during their malignant progression, or if particular cells intrinsically possessing the MDR phenotype are selected for during chemotherapeutic therapy (Riordan *et al.*, 1985;

Batist *et al.*, 1986; Beck, 1987). Regardless of this uncertainty, it is clear that adriamycin resistant MCF-7 cells, unlike their estrogen stimulated or *ras* transfected counterparts, are estrogen receptor negative. The acquisition of adriamycin resistant and estrogen receptor negative phenotypes, however, does not appear linked since selection for hormone-independence does not alter MCF-7's sensitivity to cytotoxic drugs (Leonessa *et al.*, 1992). Based on our results, adriamycin resistant MCF-7 cells also demonstrate higher levels of cathepsins B and L than wild type or *ras* transfected MCF-7 cells. Taken together, these observations suggest that: 1) factors contributing to disturbances in antiestrogen and cytotoxic drug sensitivities, hormone-dependent growth, metastatic potential, and tumorigenicity can act independently of one other, and 2) *ras* transfected, estrogen receptor positive, MCF-7 cells acquire their malignant phenotype via a different mechanism than that for the adriamycin resistant, estrogen receptor negative, MCF-7/Adr^R cells -a mechanism not involving an obvious elevation of lysosomal cysteine proteinases. Adriamycin induces P-gp (p170), a 170 kDa M.W. glycoprotein which is the putative drug efflux pump (Kartner *et al.*, 1983; Yeh *et al.*, 1992); *ras* induces p21, a 21 kDa protein that presumably acts as a signal transducing molecule (reviewed in Cantly *et al.*, 1991). These differences, of course, do not preclude a possible link between the induction of p21 and the induction of p170. The overexpression of p170, for example, can occur by several mechanisms including multiple gene copies, positive transcriptional control, and post translational modifications such as phosphorylation through protein kinase C and phosphatidinositol pathways (Riordan *et al.*, 1985; reviewed in Ferguson and Baguley, 1991; Schecter *et al.*, 1991). Given our expanding understanding of p21 function (e.g., guanine

nucleotide binding protein with GTPase activity and cysteine protease inhibitor-like properties), it is possible that *ras*, or *ras*-related products, participate in this overexpression. The analysis of an adriamycin resistant, *ras* transfected MCF-7 cell line would be helpful in this regard.

The complexity of the metastatic process is further suggested by our results concerning expression of cysteine protease activities in a somatic cell hybrid between the poorly metastatic, MCF-7 wild type parent and its highly metastatic, adriamycin resistant variant, MCF-7/Adr^R. The M:A hybrids resemble the MCF-7 parent. In contrast, Tuck reported that somatic cell hybrids between LTA cells and NIH3T3 cells, both with and without H-*ras* supplied by one or the other parent, were always metastatic (Tuck *et al.*, 1991). Interestingly, hybrids from control crosses with neither parent supplying a transfected *ras* oncogene were also metastatic, a result which puts the role of *ras* in the metastatic phenotype observed in the other LTA x NIH3T3 hybrids in question. At any rate, the explanations for expression of the metastatic phenotype in these murine cell hybrids -complementation of (a) factor(s), and intracellular reciprocity of the *ras* product- are clearly not operative in our human cell hybrids.

Elevated levels of cathepsin B activity in body fluids have been reported in clinical cases for pancreatic, colon, liver, and breast tumors (reviewed in Sloane *et al.*, 1987; Petrovà-Skalkovà *et al.*, 1987). In cell and tissue culture, Recklies *et al.*, (1980, 1982), and Mort and Recklies (1986), reported 11 fold greater cathepsin B activity in the culture media of explants of malignant breast tumors than in the media of explants of normal breast tissue or non-malignant tumors. Studies of extracellular media from

ascitis cells demonstrated that this activity reflected high levels of a 41 kDa, inactive pro-cathepsin B which was subsequently activated by the aspartic protease pepsin, at acid pH, to a 33 kDa single chain active form that was immunologically related to the lysosomal cathepsin B (Mort *et al.*, 1981). Nishimura subsequently identified a 39 kDa cathepsin B proform in hepatic endoplasmic lumen (Nishimura *et al.*, 1988). This proform could be activated in several ways including incubation for 36 hours at pH 3.8, conditions simulating the lysosomal environment, or treatment with pepsin or cathepsin D at acid pH. This latter result supported a processing role for cathepsin D, a major aspartic protease, in the proteolytic maturation of pro-cathepsin B. Although there is limited evidence for the secretion of a second cathepsin B proform which is active against low molecular weight synthetic substrates at alkaline pH (Mort and Recklies, 1986), it is generally agreed that the inactive (i.e., latent) proform is the form significant to metastasis. Despite this progress, research toward understanding the relationship between synthesized and secreted protease activities over time has been restricted by serum present in tissue samples or that added to cell culture environment to maintain growth (reviewed in Freshney, 1990). Serum contains undefined components (e.g., inhibitors, activators) which can interfere with the measurement of protease activity. Mort and Leduc (1984), for example, demonstrated the presence of two enzymes present in human serum that mimicked the proteolytic action of cathepsin B against synthetic substrates. Research in our laboratory has further demonstrated that serum components interfere with levels of cysteine protease activity synthesized in, and secreted from, human breast cancers cells in culture (Scaddan and Dufresne, 1993b). The problem has been addressed historically by omitting serum from the

growth medium for short periods (e.g., 12-24 h) prior to measurement. This is not a solution since the viability of the cells invariably decreases as a result of "culture shock", which may in turn, result in the release of cellular components which can interfere with levels of protease activity. This study made use of a defined, serum-free medium which permits analysis of levels of intracellular active and extracellular activatable levels of cathepsin B over time without compromising the viability or growth parameters of the cells (Dufresne *et al.*, 1993b). Results from these studies provide evidence for the secretion of a latent, activatable proform of cathepsin B by MCF-7/Adr^R (Petrova-Skalkova *et al.*, 1987). Increases in protease activity, detected under the conditions optimal for cathepsin B, were observed in extracellular fractions of MCF-7/Adr^R only after pepsin activation at acid pH, and could be inhibited by the omission of cysteine, and by the addition of either pepstatin, an aspartic protease inhibitor, or E-64, a cysteine protease inhibitor.

The results also suggest that increases in extracellular levels result from increases in intracellular levels and are related to cell growth. Specifically, the specific activity of active cathepsin B in intracellular fractions of MCF-7/Adr^R increased modestly while that of extracellular fractions remained relatively constant during the initial lag and early exponential phases of cell growth (i.e., Days 2-4). During this time, levels of activity in intracellular fractions of MCF-7/Adr^R were consistently greater than those of MCF-7, while levels of activatable activity in extracellular fractions were similar in both cell lines. Within the next 24 hours (i.e., Day 5) -a time corresponding to the late exponential phase of growth- the specific activity in intracellular fractions of MCF-7/Adr^R markedly increased and peaked. This was followed by a concomitant

increase in activatable activity detected in extracellular fractions (i.e., Day 6), at which time levels of activity in intracellular fractions decreased. Although a similar pattern of expression may occur in fractions of the M:A somatic cell hybrid, levels remained comparable to the low levels observed in the MCF-7 wild type parent over time. This result is in agreement with our previous observations, and suggests that the low levels of latent activity in extracellular fractions are the result of suppression of the MCF-7 /Adr^R cysteine protease phenotype in the hybrid cell.

For reasons already discussed, the current literature does not yet contain results concerning the expression of cysteine proteases over time. However, while our results can't be confirmed at present, there are compelling reasons to support their significance. First, similar patterns of expression have been reported for other malignant phenotypes such as the initial up regulation and subsequent down regulation of *fos* and *jun* oncogenes following *ras* transformation of murine cells (Stacey *et al.*, 1987; Sistonen *et al.*, 1989; Chambers, 1992). Second, similar patterns of expression have been reported for protease activity levels in clinical studies. Veksler, for example, reported that the activity of proteases increased during the growth of tumors, reached peak levels when metastases were beginning to form, then decreased (Veksler *et al.*, 1987). Third, our results can be explained in terms of available data concerning tumor progression and protease effects. Cysteine proteases are known to act as growth factors by exerting mitogenic effects on cells (reviewed in Scher, 1987; Scott, 1992; Scott and Tse, 1992). Therefore, the increased expression of active intracellular cathepsin B, and subsequent secretion of activatable procathepsin B, during mid to late exponential growth of MCF-7/Adr^R cells could help the cells overcome contact

inhibition and density limited growth at later stages of growth. Some proteolytic mitogens have, in fact, been implicated in stimulating the release of other proteases from the cell. In this role, these proteases may initiate part of a proteolytic cascade, which could result in the degradation of extracellular matrices and promote metastasis (He *et al.*, 1989).

In normal cells, cysteine proteases are targeted to lysosomes, but when they are overexpressed in malignant cells they also become associated with the plasma membrane and are secreted (Chambers *et al.*, 1992). While alterations in cellular localization no doubt contributes to the differences in intracellular and extracellular levels of cathepsin B between MCF-7 and MCF-7/Adr^R, they may not be the only contributing factor. Both cell lines are tumorigenic and one is derived from the other. Therefore a change in cellular localization alone might be expected to result in a "flip-flop" in the levels of intracellular and extracellular cathepsin B activity between each cell line (e.g., MCF-7 expressing high levels of intracellular activity, MCF-7/Adr^R expressing high levels of extracellular activity). In our studies, levels of activity in both intracellular and extracellular fractions of MCF-7/Adr^R were generally greater than those of MCF-7. This, together with the multifactorial nature of metastasis, supports the involvement of other mechanisms (reviewed in: Sloane, 1990, and Chambers, 1992).

The cysteine proteases are inhibited by a group of heat and alkaline-stable proteins known as cysteine protease inhibitors (CPIs), or cystatins. Members of this family are found both intracellularly and extracellularly where they bind in a one-to-one stoichiometric fashion to their target enzymes with varying affinities (Green *et al.*,

1984; reviewed in Chambers, 1992). The high affinity of these inhibitors for the cysteine proteases has led to the speculation that their major cellular function is to protect cells against the uncontrolled activity of these enzymes. It follows that the net proteolytic activity that would permit cells to invade through the extracellular matrix and metastasize could ultimately depend on the balance between proteases and their inhibitors.

The biological significance of the protease-to-inhibitor balance was immediately recognized and resulted in intense research activity. The basic approach to this research has been articulated by Sloane who suggests that any assay for cysteine proteases activity in biological fluids, homogenates, or culture medium will be affected by the presence of cysteine protease inhibitors (CPIs), and will therefore reflect the concentration and efficiency of CPIs in the sample (Sloane, 1990). This approach also defines a practical problem in the quantification of inhibitor or protease activities in a sample; inhibitors bound to proteases are by definition biologically inactive. Historically, this problem has been approached by combining immunological techniques with biological assays, or by exposing samples to extreme heat, a treatment which presumably permits assay for total inhibitor by inactivating the heat sensitive proteases and dissociating them from the heat resistant inhibitors (Green *et al.*, 1984). This approach was not entirely successful for homogenates of MCF-7 or MCF-7/Adr^{R}. While cathepsin B activity in both cell lines was similarly sensitive to heat inactivation -a result that tells us nothing about the mechanism of inactivation but does support structural and functional similarity- changes in inhibitor activity could not be detected regardless of the conditions tested. This immediately suggested that inhibitors were

either not a factor in the MCF-7, MCF-7/Adr^R differences, or that assay of free inhibitor in heated samples was somehow being obstructed (e.g., nonspecific binding to denatured protein). Evidence for the latter was provided by fractionation of non-heated and heated, intracellular fractions on a Superose 12 HR 10/30 FPLC column which had previously been shown to separate pork liver cathepsin B from commercial cystatin without affecting their biological activity.

Trends of cathepsins B, H, and L activities within and between MCF-7 and MCF-7 /Adr^R homogenates after FPLC fractionation were similar to those observed before fractionation. In short, levels of cathepsins B and L activities were greatest in MCF-7/ Adr^R, while levels of cathepsin H activity were comparably low in both cell lines. Cathepsins B and L activities eluted at a position corresponding to an approximate molecular weight of 31.0 kDa. This is within the 24.0 kDa to 35.0 kDa range (i.e. 24.0 kDa, 27.0 kDa, 29.0 kDa, 33.0 kDa, and 35.0 kDa) reported by others for these enzyme activities in human tumors (Sloane, 1990) and transformed, human fibroblast cells (Hanewinkel *et al.*, 1987). The elution position of cathepsin H activity in both cell lines, 27.5 kDa, is also consistent with the 28.0 kDa form of cathepsin H activity reported by other investigators (Nishimura *et al.*, 1990). There is some consensus that the variation in reported values for the different cathepsins reflects the cell or tumor type, the extent of intracellular processing, and variations in technique. Regardless of the positions, the activities of all cathepsins for both cell lines were eliminated by treatment of homogenates at 80°C for five minutes before application to the FPLC column. Differences in A_{280nm} absorbance profiles at molecular weight positions

greater than 23.0 kDa between non-heat and heat-treated fractions support this inactivation.

After FPLC, two distinct regions of inhibition against cathepsin B activity were detected at similar elution positions, 15.0 kDa and 25.0 kDa, in non-heated homogenates of both MCF-7 and MCF-7/Adr^R. Under these conditions, levels of inhibition were similar in both cell lines (i.e. less than 3% difference in total inhibition), suggesting that differences in levels of "free" protease activity (i.e., cathepsins B and L) are not accompanied by differences in "free" inhibitor.

In contrast to its effect on protease activity, heat treatment of homogenates from both MCF-7 and MCF-7/Adr^R resulted in an increase in levels of inhibition compared to levels measured in non-heated homogenates. This increase reflected inhibition at three similar regions; however, the increases at each region were greater for MCF-7 cells. The first, corresponding to an approximate molecular weight of 45.0 kDa, was not observed in non-heated samples of either cell line, and contributes to most of the additional inhibitory activity in MCF-7/Adr^R. Levels of inhibition (i.e., average % inhibition measured against pork liver cathepsin B) at this region were 2 to 3 fold greater in MCF-7 fractions than in MCF-7/Adr^R samples. The second region of inhibition, 24.0 kDa-28.5 kDa, and the third region of inhibition, 11.0 kDa-19.0 kDa, appear to correspond to the 25.0 kDa and 15.0 kDa regions of inhibition, respectively, observed in non-heated fractions of both cell lines. However, in heat-treated fractions, levels of inhibition at both these regions were almost 2 fold greater in MCF-7 fractions compared to MCF-7/Adr^R.

Direct evidence for the molecular identity of inhibitory regions observed in non-heated and heated fractions of both cell lines is not yet available. However, the number and molecular weight positions of these regions are consistent with multiple forms of cystatin-like, cysteine protease inhibitors previously reported. Endogenous cysteine protease inhibitors, such as cystatins and stefins, have molecular weights ranging from 11.0 kDa to 15.0 kDa (reviewed in Sloane, 1990; Moin *et al.*, 1992). Certain forms of these inhibitors can aggregate -by virtue of disulfide bonds- to form dimers, trimers and even aggregates of up to a 70.0 kDa (Matsuishi *et al.*, 1988; Lah *et al.*, 1989b; Moin *et al.*, 1992). Interestingly, heat treatment of many mammalian cells has been reported to increase the number of multiple molecular weight forms of the same inhibitory activity (Katanuma *et al.*, 1983). Recent results from collaborative research, involving our laboratory and Dr. David Cotter's of the University of Windsor, support multiple forms of cystatin-like CPIs in the MCF-7 breast cancer cell system. On SDS gels, protein from each region of inhibition migrates as a single band corresponding to the position of our cystatin control. On native gels, both the standard cystatin and the inhibitory fractions migrate as multiple bands at similar positions (Ioannidis *et al.*, 1993). While these results suggest that the different regions of inhibition contain cystatin-like inhibitors, they do not prove that these inhibitors are identical. Cystatin-like cysteine protease inhibitors from human liver treated at 80°C, for example, reflect at least two immunologically distinct 12.4 kDa inhibitors, each in several molecular forms (Green *et al.*, 1984). Until the molecular identity of inhibitory activity in each region is established for each cell line, contributions from

other cystatin inhibitors (i.e. higher molecular weight kininogens), as well as non-cystatin inhibitors, cannot be ruled out (Lah *et al.*, 1992a).

Regardless of the molecular identity of cathepsin B inhibition, the increase in levels of inhibition is consistent with that reported by most other researchers for heat-treated, unfractionated samples from human and murine tissues/cells (Lah *et al.*, 1989b; Rozhin *et al.*, 1990; Sloane *et al.*, 1992; Chambers *et al.*, 1992). Interpretation of our results, however, is not restricted to a comparison of "total" inhibitor in heated samples to "free" protease in non-heated samples. Comparisons of this type exclude the contribution of heat-inactivated proteases in the bound form (i.e., inhibitor complexed with protease) and assume that they will not interfere with the measurement of "total" inhibitor. Our results suggest that these restrictions may in fact interfere with the interpretation of results since differences between total inhibitory activity between MCF-7 and MCF-7/Adr^R become apparent in heated fractions only after FPLC column fractionation. Moreover, a comparison of ratios of levels of inhibition in heat-treated to levels of inhibition in non-heated fractionated homogenates between these two cell lines (i.e. 2.0-2.5 for MCF-7; 1.3 for MCF-7/Adr^R), suggests that MCF-7 samples retain better than half of their total cysteine protease inhibitor activity in a protease-bound form -an interpretation in keeping with the low levels of free cathepsin B activity detected in fractionated and unfractionated homogenates of this cell line. MCF-7/Adr^R, on the other hand, not only expresses lower levels of total inhibition than MCF-7, but appears to maintain little of this activity in a protease-bound form. There are several alterations which could explain this result including altered transcription and translation of normal inhibitor, or transcription and translation

of an altered inhibitor. Physicochemical studies, including kinetic analyses to determine binding affinities, would be helpful in this regard. Nonetheless, these results -together with the peak average cathepsin B activities for MCF-7 and MCF-7/AdrR cells- suggest an imbalance in the levels of functional cysteine protease inhibitor which favors increased cathepsin B activity in the adriamycin resistant, estrogen receptor negative, MCF-7/Adr^R cell line.

As discussed previously, cystatins are reported to bind in a one-to-one stoichiometric fashion to their target enzymes with varying affinities. If this is also the stoichiometry observed in the MCF-7 cell system, the decrease in functional inhibitory potential in the MCF-7/Adr^R cell line may not be sufficient to account for the 4-5 fold increase observed in its cathepsin B activity. Other factors may contribute to increased proteolysis (Lah *et al.*, 1992b). Cathepsin D, for example, activates cathepsin B, while inactivating its endogenous, low molecular weight cystatin inhibitors (Lenarcic *et al.*, 1988). In addition, feed-back mechanisms for the reciprocal proteolytic maturation of both cathepsins B and D have been reported (Sohar and Katona, 1992; Lah *et al.*, 1992b). Since cathepsin D is regulated by estrogen in MCF-7 cells and is up regulated in ER negative cell lines (Brünner *et al.*, 1993), it is possible that it contributes to increased levels of cathepsin B activity in the MCF-7/AdrR breast cancer cell line. The inhibition of activation by the aspartic protease pepstatin, in our extracellular studies, supports this possibility, however analyses of intracellular and extracellular levels of cathepsin D are required to confirm it.

Recent reports also suggest that cysteine protease inhibitors can affect levels of protease indirectly. Cysteine protease inhibitors are known to inhibit cellular

proliferation, for example, by inhibition of protein turnover, or of protein processing (Scott, 1992). However, when levels of inhibitor are low (i.e., at sub-inhibitory levels) they appear to be mitogenic -presumably by contributing to increased levels of active protease available for tumor progression (Shaw and Dean, 1980). Thus, an alteration in the relationship between proteases and their inhibitors could provide a molecular "catch 22" which is optimal for metastasis. This is consistent with current literature. For example, a number of researchers have observed that highly elevated levels of cathepsins B and L, and decreased levels of endogenous cysteine protease inhibitors (CPI) are often associated with poorly differentiated carcinomas and those with negative estrogen receptor values (Krepela *et al.*, 1989; Lah *et al.*, 1990). Research has also demonstrated that the increased levels of activatable cathepsins B and L activities in intracellular and extracellular fractions of MCF-7/Adr^R could reflect alterations at several levels including: increased transcription and translation, altered processing and subcellular localization, increased secretion and activation, or changes in its regulation by endogenous CPIs. Current data neither excludes nor identifies any one of these possibilities, rather it suggests the involvement of various mechanisms (reviewed in Sloane, 1990). Nonetheless, evidence from this thesis research suggests that the balance of functional cystatin-like cysteine protease inhibitors to cysteine proteases is significant to tumor progression in human breast cancer cells. It also supports an association between the loss of estrogen receptors, increased cysteine protease activity, and metastasis.

The biological significance of any cell culture study must be considered carefully since results can reflect artifacts of the experimental system or design. This possibility

seems minimal in our studies since the results do not reflect: 1) the specificity of the substrate, since increases in activities were specifically inhibited by the addition of commercial cysteine protease inhibitors; 2) the efficiency or sensitivity of the assay procedure, since the reaction was linear over time and the results were similar using either colorimetric or fluorometric substrates; 3) differences in cell growth or viability, since an equal number of viable cells were used to prepare homogenates for each cell line; 4) differences in the original cell population, since all MCF-7 variants were derived from the same MCF-7 parent; 5) results not relevant to adriamycin resistance or *ras* transfection, since MCF-7 cells were exposed to adriamycin alone, and the effect of *ras* was isolated by analyzing MCF-7 transfected with *gpt* alone; 6) reversion of variant genotypes and phenotypes, since cells retained all genetic markers; 7) undefined interactions between inhibitors and proteases, since their activities in both non heat-treated and heat-treated samples were separated on FPLC columns; or 8) undefined reactions to changes in culture environments, since a defined, serum-free medium was used in this study.

The possibility that our results do not present a complete picture is real. Although the low molecular weight inhibitors are primarily found in the cytoplasm or the extracellular fluid (reviewed in Sloane, 1990), different research groups have identified cysteine protease inhibitor activities, including the CPI-related activity of p21, with the lysosomal or plasma membranes (Pontremoli *et al.*, 1983, Hiwasa *et al.*, 1990; Moin *et al.*, 1992). Since cell homogenates were clarified of particulate matter by microcentrifugation in our studies, it is probable that inhibitory activities associated with these membranes would have been missed. Although all cells were treated the

same way, inclusion of membrane analyses would have provided a more complete picture.

Lastly, and most importantly for those working in cell culture, the results obtained in cultured cells must reflect what is happening at the level of the original tumor. Since the human breast cancer cell line, MCF-7, was transformed in vivo prior to being established in culture, the potential for selection inherent in the evolution of any cell line in culture is minimized (reviewed in Freshney, 1990). Nevertheless, MCF-7 cell lines represent a two dimensional array of cells with similar genotypes and phenotypes. Tumors, on the other hand, represent a three dimensional array of different subpopulations of cells, each subpopulation exhibiting diverse genotypes and phenotypes for many characteristics, including morphology, growth characteristics, immunogenicity, tumorigenicity, drug sensitivity, and metastatic potential (Miller, 1983; Kerbel *et al.*, 1988). This heterogeneity in tumor cells can provide additional, and perhaps unique, mechanisms for neoplastic progression, (e.g. acquisition of new characteristics during neoplastic development) which contribute to the development of a highly invasive subclone (Heppner *et al.*, 1983; Stetler-Stevenson, 1990). Therefore the physiological and biochemical characteristics of an individual tumor cell in vivo are likely the consequence of complex intracellular and intercellular interactions which, in turn, reflect both the intracellular and extracellular environments of each cell. However, this complexity which makes in culture/in vivo correlations difficult, also makes in culture studies necessary.

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