A cell culture analysis of the expression of cathepsin D activity during myogenesis and tumor progression.

Jason Andrew Keller

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A Cell Culture Analysis of the Expression of Cathepsin D Activity during Myogenesis and Tumor Progression

by

Jason Andrew Keller

A Thesis
Submitted to the Faculty of Graduate Studies and
Research through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for the
Degree of Master of Science

University of Windsor

Windsor, Ontario, Canada

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Abstract

There is considerable evidence supporting the involvement of acidic lysosomal proteases in the catabolic processes associated with developmental programs and with the pathologies that arise when these programs fail. However, for the aspartyl protease cathepsin D (catD) this evidence is limited and equivocal. In view of this, I examined levels of catD activity in cell and media fractions prepared from developmental (i.e., myogenensis) and pathological (i.e., tumor progression) cell culture model systems. The differentiation of mononucleated L6 myoblasts to form multinucleated myotubes, was accompanied by a fusion-related increase in catD activity, a pattern not observed in fusion-deficient L6D3 myoblasts or in LM fibroblasts. In prefusion myoblasts, this activity eluted at 34 kDa, a molecular mass consistent with the fully processed double chain form of the protease. In fused myoblasts, a second peak of activity appeared at 48 kDa, consistent with the less processed, single chain form of catD. In contrast, activity in fusion-deficient L6D3 myoblasts and in LM fibroblasts eluted at 48 kDa at all stages of growth. These results suggest that catD expression is up-regulated during myogenesis. Unlike myogenesis, the progression of low metastatic MCF-7 breast cancer cells to highly metastatic, adriamycin-resistant ones was accompanied by a decrease in catD activity that could not be increased with estradiol treatment. Levels were similarly lower in highly metastatic MDA-231 and MDA-468 breast cancer cells than in lowly metastatic ZR75 and HS578T ones, and lower in malignant HPL-R human lung fibroblasts than in WI38 normal ones. These results suggest that the
expression of catD activity is down regulated during the progression of normal cells to highly metastatic ones. The functional significance of catD secreted from malignant cells was addressed by examining the effect of protease inhibitors on levels of acid activatable procathepsin B (procatB) in the media of two cell lines with contrasting extracellular protease levels: HepG2 human hepatoma cells (high catD, low procatB) and HPL-R human sarcoma cells (low catD, high procatB). Acid activation of procatB in the media of both cell lines was related to catD concentration and was inhibited by the catD inhibitor, pepstatin, but not by inhibitors of serine or metallo proteases. When HepG2 and HPL-R media were combined, activatable catB levels were greater than predicted additive levels. These results suggest that catD secreted in the extracellular environment may play an initial role in a proteolytic cascade that begins with the activation of procatB and leads to the increased activity of other proteases associated with the highly metastatic phenotype.
to my mother, family, and friends
Acknowledgements

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>Adr&lt;sup&gt;R&lt;/sup&gt;</td>
<td>adriamycin resistant</td>
</tr>
<tr>
<td>alpha MEM or α-MEM</td>
<td>alpha modification of Eagle's minimal medium</td>
</tr>
<tr>
<td>AP-2</td>
<td>&quot;basic leucine zipper&quot; transcription factor</td>
</tr>
<tr>
<td>AU</td>
<td>Anson Units</td>
</tr>
<tr>
<td>B.C.E</td>
<td>before current era</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cat</td>
<td>cathepsin</td>
</tr>
<tr>
<td>CpG islands</td>
<td>groupings of cytosine and guanine nucleotides</td>
</tr>
<tr>
<td>CPI</td>
<td>cysteine proteinase inhibitor</td>
</tr>
<tr>
<td>CPK</td>
<td>creatine phosphokinase</td>
</tr>
<tr>
<td>DCC-FBS</td>
<td>dextran coated charcoal-fetal bovine serum</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E-64</td>
<td>L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen responsive element</td>
</tr>
<tr>
<td>EU</td>
<td>enzyme unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>GC boxes</td>
<td>guanine-cytosine nucleotide repeat boxes</td>
</tr>
<tr>
<td>Hep</td>
<td>human hepatoma</td>
</tr>
<tr>
<td>HLH</td>
<td>“helix-loop-helix” motif</td>
</tr>
<tr>
<td>HPL</td>
<td>human primary lung</td>
</tr>
<tr>
<td>$K_{av}$</td>
<td>separation coefficient, or proportion of stationary gel volume available for diffusion of a given solute</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>M-6-P</td>
<td>mannose-6-phosphate</td>
</tr>
<tr>
<td>MCF</td>
<td>Michigan Cancer Foundation</td>
</tr>
<tr>
<td>MDR</td>
<td>multi drug resistance</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>MPR</td>
<td>mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>Myc</td>
<td>cell cycle regulating transcription factor</td>
</tr>
<tr>
<td>MyoD or myoD</td>
<td>myogenesis determinant gene</td>
</tr>
<tr>
<td>n.d.</td>
<td>not detected</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PepA</td>
<td>pepstatin A</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pH</td>
<td>negative log hydrogen ion concentration</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylflouride</td>
</tr>
<tr>
<td>pro-Cat</td>
<td>pro-cathepsin</td>
</tr>
<tr>
<td>ras</td>
<td>rat sarcoma</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>S.D.</td>
<td>saturation density</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sp1</td>
<td>promotor binding factor</td>
</tr>
<tr>
<td>$T_D$</td>
<td>population doubling time</td>
</tr>
<tr>
<td>Tween</td>
<td>polyoxyethylene-sorbitan</td>
</tr>
<tr>
<td>Z-arg-arg-NMec</td>
<td>Nα-CBZ-argininyl-argininyl 7-amido-4-methyl-coumarin.HCL</td>
</tr>
<tr>
<td>Z-phe-arg-NMec</td>
<td>Nα-CBZ-phenylalanyl-argininyl 7-amido-4-methyl-coumarin.HCL</td>
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</tbody>
</table>
Introduction

1.1 Historical Review

The functional significance of enzymes capable of degrading protein (i.e., proteases) has probably been known for millennia. In the Libyan Sahara, cave paintings dating to 5200 B.C.E depict milk processing using the protease, rennet, also known as chymosin. There is also evidence that other early cultures including the Sumerians (3500-2800 B.C.E.), Egyptians (3000-2800 B.C.E.), Chinese (1028-220 B.C.E.) and the Greeks (−800 B.C.E.) used proteases in food preparation, processing and preservation (Scecsi et al., 1992). However, experimentation into the biochemistry of protease action is relatively recent, beginning with studies on digestion and tissue decomposition in the early 1800s. These studies identified proteases such as Pepsin as requiring an acidic environment. Since then many proteases with broad environmental requirements have been identified. These proteases have been classified into four mechanistic classes: aspartyl proteases, cysteine proteases, metalloproteases and serine proteases. While the aspartyl proteases are not the most numerous, they include such biologically important enzymes as renin involved in vasoconstriction and nephrola functioning, pepsin, gastric and chymosin involved in digestion, fungal and microbial proteases involved in disease pathology, HIV retroviral protease involved in retroviral maturation, cathepsin E involved in erythrocyte function, and cathepsin D involved in numerous biological and pathological processes. The current study focuses on this latter protease.
1.2 Cathepsin D

In 1960, Jacoby partially purified the cellular acid protease we now recognize as cathepsin D. Since then, physio-chemical studies have demonstrated that cathepsin D, like other aspartyl proteases (e.g., pepsin and penicillopepsin), is bilobal with two domains separated by a pronounced cleft (Figure 1). The two lobes have been shown to exhibit twofold symmetry (James and Sielecki, 1983; Andreeva et al., 1984). It has been suggested that this structure reflects duplication of the primordial gene (Tang et al., 1978). Throughout evolution of the aspartyl protease gene, aspects of this duplication have been blurred in most parts of the primary structure, but maintained in the tertiary structure. Indeed, gene duplication is most evident in the active site of the enzyme where evolution is most restricted (Polgar, 1989).

Biological studies have demonstrated that cathepsin D comprises approximately 10% of the total protein in the lysosome and is widely distributed in animal and human tissues. Despite its ubiquitous distribution, there are organ-, tissue- and cell-specific qualitative and quantitative differences in cathepsin D activity (Whitaker and Rodhes, 1983; Reid et al., 1986). The molecular explanations for these differences remain poorly understood but are likely related to genetic regulation in concert with the numerous physiological functions associated with the protease.

1.2.1 Genetic Regulation of Cathepsin D

The primary and arguably most important control of cathepsin D activity occurs at the level of transcription by the binding of transcription factors to binding sites in the
FIGURE 1

Structure and Symmetry of Cathepsin D

Cathepsin D is bilobal with two domains separated by a pronounced active site cleft. The two lobe have been shown to exhibit twofold symmetry reflecting duplication of the primordial gene.
promoter region of the cathepsin D gene (Figure 2). In humans, the majority of the promoter's activity is associated with four Sp1 binding sites downstream from position -124 which recognize the 85 kDa, Sp1 transcription factor. The Sp1 sites, defined by the consensus sequence 5'-GGGCGG, are flanked by CpG islands of about a thousand or so nucleotide base pairs in length. It is generally agreed that these islands mark the 5' ends of complete transcription units or complete genes and may be involved in gene regulation via DNA methylation (Bird, 1986).

The presence of CpG islands and Sp1 sites within the cathepsin D gene, as well as the gene's ubiquitous distribution, are general properties of “housekeeping” genes (Bird, 1987). However, other structural characteristics suggest that a component cathepsin D gene transcription is under specific regulatory control; for example, the presence of a putative TATA box (Redecker et al., 1991) and of a 5'-CCCCAGGC consensus binding site for AP-2, an element of the SV40 enhancer, at positions -24, -186, +708 and +1089 (Michell et al., 1987). Cell surface signaling molecules, including insulin-like growth factor 1, epidermal growth factor, and basic fibroblast growth factor, which induce the AP-2 system via protein C-kinase activation, have also been shown to induce cathepsin D mRNA (Rochefort et al., 1989).

At least two human cell systems are known in which the transcription of the cathepsin D gene is induced by hormones. In MCF-7 breast cancer cells it is directly stimulated by estrogens via an estrogen receptor (Westly and May, 1987; Cavailles et al., 1989) and indirectly in U937 promonocytic cells by calcitriol via the vitamin D receptor (Redecker et al., 1989). The cathepsin D gene contains no consensus vitamin
FIGURE 2

Location and Structure of the Cathepsin D Gene

The cathepsin D gene is located on the short arm of chromosome 11. Numerous regulator binding sites flank the transcription initiation site, including: SP-1 sites (5'-GGGCGG), CpG islands, AP-2 binding sites (5'-CCCCAGGC), and E-box elements (5'-CANNTG). After transcription nine exons are used from the structural portion of the gene to make up the complete mRNA transcript.
D responsive elements (Kerner et al., 1989); however, characteristic components of hormone responsive elements have been identified in a number of hormone responsive cell lines. These include 48 TGA and 27 TCA motifs associated with diad symmetry promoter elements. Moreover, while the cathepsin D gene does not contain a classical palindromic estrogen responsive element (ERE) (Krishnan et al., 1994), it does contain an imperfect palindromic sequence, 5'-CGGGTCAGCTGATCCG, near to the transcription initiation site at position -20. Redecker et al. suggest that this sequence is the ERE equivalent (Redecker et al., 1991); Krishnan et al., on the other hand, argue that a putative ER-Sp1 like sequence, 5'-GGGCAGG(n)23ACGGG, from position -199 to -165, is responsible for estrogen dependent transcription (Krishnan et al., 1994).

Other structural elements of the cathepsin D promoter support specific control of cathepsin D expression. The 5'-CANNTG sequence, commonly termed the E-box element, present at positions -729, -714, -643, -356, -47, -15, +134, +1,400, and + 2,287 of the cathepsin D promoter is of particular interest (Redecker et al., 1991). This element is associated with the Myc family of cell cycle regulators, and the MyoD family of muscle specific transcription factors involved in pathological, developmental and/or myogenic programs (reviewed in Olsen, 1993; Vandorpe et al., 1997). Recent evidence from our laboratory and that of our collaborator suggest that E-box elements in the promoter of cathepsin B are linked to the regulated expression of muscle-specific phenotypes during myogenesis. A similar argument may apply for the E-box elements in the cathepsin D promoter.
1.2.2 Targeting of Cathepsin D to Lysosomes

Specific alterations in cathepsin D activity could also reflect translational and post-translational mechanisms. Cathepsin D, like other lysosomal enzymes, is synthesized on membrane bound polysomes in the rough endoplasmic reticulum then targeted to the lysosomes (Figure 3). Each newly synthesized protein contains a hydrophobic amino terminal signal peptide which interacts with an 11S ribonucleoprotein recognition particle, initiating the transport of the nascent protein across the endoplasmic reticulum membrane into the lumen of that organelle (Erickson et al., 1981; Erickson et al., 1983). In the lumen, transported proteins undergo cotranslational glycosylation of select asparagine (Asn) residues (Kornfield and Kornfield, 1985). The signal peptide is then cleaved, and processing of the Asn-linked oligosaccharide begins with cleavage of its three glucose and one mannose residue. The proteins then move, via vesicular transport, to the Golgi stack where they undergo a variety of post-translational modifications and are sorted for targeting to lysosomes, secretory granules or the plasma membrane. During the movement of these proteins through the Golgi, the oligosaccharides on secretory and membrane glycoproteins are processed to sialic acid-containing units. While some of the oligosaccharides on lysosomal enzymes undergo similar processing, most undergo a different series of modifications. The critical modification is the acquisition of phosphomannosyl residues which serve as the essential component of a recognition marker for the binding to high affinity, mannose-6-phosphate (M-6-P) receptors, and for subsequent translocation to lysosomes (Kaplan et al., 1977). This recognition marker is generated by the
**FIGURE 3**

**Intracellular Sorting of Cathepsin D**

Cathepsin D is initially synthesized on polysomes of the rough endoplasmic reticulum (RER) as a 52 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 (4) in the golgi (3). The protease/receptor complex exits the golgi via a clathrin (-) 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).
sequential action of two Golgi enzymes. The first, N-acetyl-glucosaminy1-phospho-
transferase, transfers N-acety1-glucosamine-1-phosphate from uridine diphosphate-N-
acety1-glucosamine to selected mannose residues on lysosomal enzymes producing a
phosphodiester intermediate (Reitman et al., 1981, Waheed et al., 1982). The second,
N-acetyl-glucosamine-1-phosphodiester α-N-acetyl-glucosamidase, removes the N-
acetyl-glucosamine residue to expose the recognition signal (Varki and Kornfeld, 1981;
Waheed et al., 1981).

Following the generation of the phosphomannosyl residues, the lysosomal
enzymes bind to M-6-P receptors in the Golgi, effectively segregating lysosomal
enzymes from those that are destined for secretion. Most cell types contain two types
of M-6-P receptor, a cation-independent type ( Sahagian et al., 1981; Steiner and Rome,
1982; Sahagian, 1984), and a cation-dependent type (Hasilik et al., 1981; Gabel et al.,
1983; Robbins and Myerowitz, 1981; Hoflack and Kornfeld, 1985). Regardless of the
receptor type, the ligand-receptor complex exits the Golgi via a coated vesicle and is
delivered to a pre-lysosomal staging area (endosome), where dissociation of the ligand
occurs by acidification of the compartment (Gonzalez-Noriega et al., 1980). The
receptor is then recycled back to the Golgi where it can interact with another ligand
molecule, while the unbound lysosomal enzymes are packaged into vesicles to form
primary lysosomes. Not all of the unbound enzymes are delivered to the lysosomes.
Some of the secreted enzymes bind to cell surface M-6-P receptors and are delivered to
lysosomes after internalization (Willingham et al., 1981). In fibroblasts, this
"secretion-recapture" mechanism functions as a enzyme salvage pathway delivering 5-10% of total lysosomal enzymes to lysosomes (Vladutiu and Rattazzi, 1979).

While the major mechanism for lysosomal enzyme targeting is mediated by M-6-P receptors, a second pathway involving a receptor-independent mechanism has been identified (Reitman et al., 1981b). Evidence initially came from studies involving patients with I cell disease (mucolipidosis II [ML-II]) and pseudo-Hurler polydystrophy (mucolipidosis III [ML-III]), both manifestations of a phosphotransferase deficiency (Reitman et al., 1981b). As a consequence, newly synthesized lysosomal enzymes are unable to bind to M-6-P. In some cell types, including fibroblasts, cathepsin D is secreted into the extracellular milieu. However, in other cells, such as hepatocytes and leukocytes, near normal levels of activity are found to be sorted to lysosomes. This alternative sorting mechanism may have evolved very early as evidenced in the simple eukaryote Dictyostelium discoidium, an organism that sorts lysosomal enzymes in the absence of a MPR dependent mechanism. For cathepsin D this may be accomplished by specific and direct interaction with microsomal membranes (Capony et al., 1994; Rijnboutt et al., 1991; McIntyre and Erickson., 1991). Two areas of the cathepsin D molecule are being examined as recognition sites for this interaction. The first is in the pro-piece of the immature cathepsin D zymogen; the second is in the C-terminal region. In either case, if the direct association of enzymes with lysosomal membranes is disrupted by membrane destabilizers, cathepsin D is not localized to lysosomes.
1.2.3 Processing of Cathepsin D

To protect cellular organelles from degradation, aspartyl proteases are generally synthesized as inactive, preproenzymes containing an endoplasmic reticulum translocation signal and a propeptide for control of enzyme activity. The processes of activation and maturation appear to vary among the different aspartyl proteases. Studies in macrophages suggest that procathepsin D (52 kDa) is fully matured in lysosomes and pre-lysosomal endosomes (Diment and Stahl, 1985; vonFigura and Hasilik, 1986) (Figure 4). Propeptide removal results in conversion to the active single chain form of the enzyme (48 kDa) and in several species including humans, cathepsin D is further processed in lysosomes to a two-chain form (34 kDa and 14 kDa chains).

Procathepsin D, like most aspartyl proteases, undergoes in vitro autoactivation at acid pH to yield a catalytically active, incompletely processed, form of the enzyme termed pseudocathepsin D (Hasilek et al., 1981). However, unlike other aspartyl proteases, complete maturation or removal of the remainder of the cathepsin D propeptide has not been demonstrated suggesting that another protease may be required. This is supported by the observation that cysteine protease inhibitors prevent lysosomal processing of procathepsin D to the single-chain form of the enzyme in cell cultures (Rijnboutt et al., 1991). Interestingly, there is also in vitro evidence for the role of cathepsin D in the activation of other proteolytic enzymes (Nishimura et al. 1987). The cysteine proenzyme procathepsin B, for example, is cleaved by cathepsin D to an active form which is further processed by the exopeptidase, dipeptidylpeptidase I, to yield the mature form commonly observed in cells.
FIGURE 4

Processing and Maturation of Cathepsin D

Cathepsin D is initially synthesized as a 52 kDa pro-enzyme. The removal of the pro-piece opens the active site and forms a 48 kDa mature enzyme. Processing is completed when amino acids 98 thru 106 are cleaved from the protein resulting in non-covalent association of a light (14 kDa) and heavy (34 kDa) double chain form.
1.3 The Role of Cathepsin D in Biological and Pathological Systems

Cathepsin D activity has been associated with an increasing number of biological functions including the processing, secretion, activation and catabolism of other proteolytic enzymes and hormones (Dean, 1975, Tanaka et al., 1981, Barrett, 1977, Nishimura et al., 1990), the stimulation of DNA synthesis and mitosis during tissue regeneration (Morioka and Terayama, 1982), and the mobilization of leukocytes and the permeabilization of vessel walls during the immune response (Barret, 1977). Cathepsin D has been implicated in complex processes of development and cellular differentiation (Collela et al., 1986; Stein et al., 1987; Huet et al., 1994). Not surprisingly then, alterations in cathepsin D activity have also been associated with a number of pathological processes including ischemic myocardial injury, muscular dystrophy, liver disease, and inflammation (Barret, 1977; Wildenthal, 1978; Kalra et al., 1988; McGowan et al., 1977; Ryvnyak et al., 1970; Gopalan et al., 1987). More recently alterations of cathepsin D activity have been implicated in the complex process of malignant tumor progression (reviewed in Ren and Sloane, 1996; Leto et al., 1992).

The work described in this thesis examines the role of cathepsin D in cytodifferentiation and tumor progression using the differentiating L6 skeletal muscle and the MCF-7: MCF-7/Adr\textsuperscript{R} breast cancer cell culture model systems.
1.3.1 Cytodifferentiation: Myoblast Differentiation (Myogenesis) Model (Figure 5)

The formation of myofibrils from muscle precursor cells has become a paradigm for understanding the molecular basis of how cell lineages are established and how stem cells become specialized. In the process of skeletal muscle differentiation (myogenesis) at each stage of development some cells divide yielding replicating myogenic cells which retain the capacity to differentiate (presumptive myoblasts), and non-replicating cells (postmitotic myoblasts) which undergo a series of changes leading to their differentiation into multinucleated myotubes which are irreversibly withdrawn from the cell cycle (Yaffe, 1971; Yeoh and Holtzer, 1977). At the level of myotube formation, two types of events have been recognized, those which allow aligned myoblasts to recognize each other and adhere, and those which allow adhered myoblasts to fuse with each other (Nameroff and Manor, 1976; Dufresne et al., 1976; Knudsen and Horwitz, 1977; Wakelam, 1988). The recognition events appear to be mediated by cell membrane glycoproteins, while the fusion events appear to be mediated by extensive reorganization of membrane components to form protein-free areas of phospholipids (Prives and Shinitzky, 1977; Wakelam, 1988).

1.3.2 Proteolysis in Myoblast Differentiation

The catabolic restructuring implicit in recognition and fusion related membrane reorganization, in restructuring of the cytoskeleton and in the turnover of myofibrillar components, argue for the involvement of cellular proteases both within and outside of differentiating myoblasts. The evidence supports this argument; for example: 1) the
FIGURE 5

Myoblast Differentiation

When isolated myoblasts are placed in a suitable culture medium (low mitogen medium), they divide rapidly and after a few days of growth, migrate towards each other, align, adhere, and fuse to form multinucleated myotubes which synthesize the muscle-specific proteins necessary for mature muscle structure and function.
nonlysosomal protease, calpain, demonstrates increased activity (Kaur and Sanwal, 1981), peripheral distribution (Schollmeyer 1986), and membrane localization (Brustis et al., 1994) in differentiating myoblasts; 2) nonlysosomal metalloproteases are secreted from differentiating myoblasts (Matrisian and Hogan, 1990; Guerin and Holland, 1995); and 3) lysosomal cysteine proteases demonstrate increased activity in fused myoblasts (Kirschke et al., 1983) and degrade muscle-specific proteins in vitro (Matsukshi et al., 1992). Recent biochemical, genetic and immunocytochemical studies in our laboratory have provided evidence supporting a role for the specific cysteine protease, cathepsin B, in myogenesis (Jane and Dufresne, 1994; Jane et al., 1998a and b). Since the expression of cathepsin B during differentiation of cultured L6 myoblasts is differentially regulated and fusion-related, it is unlikely that this role is restricted to "housekeeping" activities previously associated with this protease. Similar studies have not been conducted for cathepsin D. This is surprising for several reasons. First, lysosomes isolated from whole muscle homogenates contain cathepsin D along with myofilament, myosin and actin fragments (Bird et al., 1978; Libelius et al., 1978; Gerard and Schneider, 1979). Second, cathepsin D degrades purified, native and denatured actin and myosin in vitro (Schwartz and Bird, 1977; Bird et al., 1980). Third, cathepsin D has been implicated in the activation of cysteine proteases (Nishimura et al., 1987). And fourth, the cathepsin D promoter, like that of cathepsin B, contains E-box elements associated with regulated expression (Redecker et al., 1991; reviewed in Olsen, 1993)
1.3.3 Tumor Progression (Figure 6)

The formation of a malignant tumor is a multi-step, multi-factorial process that begins with the transformation of normal cells by mutations into cells that reproduce and differentiate abnormally. As tumor formation progresses, genetic and epigenetic mechanisms can lead to the acquisition of additional phenotypes in dividing transformed cells within the tumor. Phenotypic diversity at the cellular level ultimately increases cellular diversity at the tumor level. Since 85% of cancer deaths are due to complications arising from secondary and tertiary tumors, those cells which can readily invade surrounding tissue and metastasize (i.e., escape from the primary tumor, disseminate through blood and lymph vessels, evade host immune defense, and home to secondary, specific target organs) are generally considered to be the most malignant (Schirrmacher, 1985; Hill, 1987; Thompson et al., 1992). Not surprisingly, the acquisition, action and interaction of phenotypes associated with these highly malignant cells has become the focus of considerable international research. This research is relatively recent but has provided some insights. For example, Liotta and colleagues observed that the expression of phenotypes associated with invasion and metastasis requires a set of effector genes different from those for transformation of normal cells to immortality (Liotta et al., 1991). Such studies suggest that the expression of different phenotypes is under different genetic controls. There is also some consensus that decreased cell-to-cell adhesion and increased cell motility are important malignancy-associated phenotypes. It has been suggested that these phenotypes might result from changes that occur in the structure of the cell membrane of normal founder
FIGURE 6

Cellular Components of Tumor Tissue

Mutations in genes that regulate the cell cycle can transform a normal cell into a malignant one. As transformed cells divide they acquire diverse phenotypes by means of genetic and epigenetic mechanisms (Panel A). Phenotypic diversity at the cellular level ultimately increases cellular diversity at the tumor level. Those cells in the tumor that acquire the ability to invade surrounding tissue and metastasize are considered to be the most highly malignant (Panel B).
or cancer cells. Alternatively, they may result from changes in the ability of a cancer cell to degrade and invade through the basement membrane (i.e., the extracellular matrix) (reviewed in: Recklies et al., 1980; Nicolson, 1991; Liotta, 1992). In either case, the action of proteases both within and outside of malignant cells is logically implicated.

1.3.4 Proteases in Tumor Progression

A variety of degradative enzymes, including heparanases, collagenases and lysosomal cysteine- and aspartyl- proteases, have been reported to contribute to the metastatic cascade, a series of interrelated phenotypes that lead to increased invasive and metastatic potentials (Sloane, 1990; Liotta et al., 1991; Chambers et al., 1992; Ren and Sloane, 1996). Of these, alterations in the activities of the cysteine proteases, cathepsin B and L, and of the aspartyl protease, cathepsin D, appear to be particularly significant (Ren and Sloane, 1996, Rochefort et al., 1987; Rochefort, 1992). These alterations have been correlated with the processes of extracellular matrix degradation during invasion (Mountcourrier et al., 1994).

The mechanism or mechanisms by which cathepsin D facilitates tumor invasion and metastasis are currently under investigation by a number of laboratories. Cathepsin D may play a direct role in this process, for example by degrading a number of basement membrane molecules (Briosso et al., 1994; Montcourrier et al., 1994). Alternatively, it may play an indirect role, for example by activating other proteases which in turn degrade basement membrane molecules. In 1987, Nishimura suggested
such an indirect mechanism for the activation of procathepsin B by cellular cathepsin D in lysosomes (Nishimura et al., 1987, Nishimura et al., 1989, Nishimura et al., 1990). Based on this model, I predicted a similar role for cathepsin D in the extracellular environment.

1.3.5 The MCF-7 - MCF-7/AdrR Tumor Progression Model

Much of what we know concerning the relationship between protease activities and malignancy has been obtained from analysis of rodent and human malignant tumors. While these studies are important, their interpretation is limited by the inherent complexity of malignant transformation, tumor progression, tumor composition and host-tumor interactions (Kerbel et al., 1988; Miller et al., 1989; Stetler-Stevenson 1990; Liotta et al., 1991). The use of cell culture has reduced this complexity by permitting the controlled establishment and characterization of homogeneous populations and subpopulations of mortal and immortal cell lines (reviewed in Freshney, 1990). Cell lines established from breast cancers are particularly good for analyses of malignant phenotypes since breast cancers usually metastasize early in tumor progression. One of the most relevant, versatile and well studied breast cancer cell lines is MCF-7 (Vickers et al., 1989; Scaddan and Dufresne, 1995). The MCF-7 wild type cell population is characteristic of “early” breast tumor progression in that it is: a) fully hormone dependent (i.e., estrogen receptor (ER) positive), b) sensitive to chemotherapeutic drugs, and c) poorly (lowly) metastatic. MCF-7 cells rarely form tumors without the influence of hormones and require
physiological levels of estrogen for tumor formation in nude mice (Kasid et al., 1985; 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., ER negative), 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 forms proliferating tumors in the presence or absence of estrogen (reviewed in Leonessa et al., 1992). 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).

The MCF-7 - MCF-7/Adr^R iso-system is also excellent from a cell culture perspective. Both cell lines are easy to manipulate in culture, have similar growth parameters and viability, and can be maintained in culture for extended periods of time without alterations in their genotypes or phenotypes. Lastly, the MCF-7 - MCF-7/Adr^R iso-system was used previously in this laboratory to study the expression and regulation of cysteine protease activities (Scaddan and Dufresne, 1995).

1.4 Research Description and Objectives

The following work describes an analysis of the activity of the lysosomal aspartyl protease, cathepsin D, during muscle cell differentiation and during tumor progression. The specific objectives of the study are:
I) to examine the levels of cathepsin D activity in cell and medium fractions prepared during differentiation of L6 rat myoblasts in culture, and to compare these levels with those obtained in:

i) L6D3, a fusion deficient variant of L6,

ii) LM skeletal muscle fibroblasts,

II) to examine the levels and biological significance of cathepsin D activity in cell and media fractions prepared during growth of lowly metastatic, MCF-7 and highly metastatic, MCF-7/Adr\textsuperscript{R} human breast cancer cells, and to compare these levels with those obtained in:

i) four other human breast cancer cell lines: ZR75, HS578T, MDA-231, and MDA-468, and

ii) two human lung cell lines: WI38 normal, lung fibroblast cells, and HPL-R highly metastatic, lung sarcoma cells

(Manjunath and Dufresne, 1989).

iii) HepG2, a differentiating, malignant human liver cell line
Materials and Methods

2.1 Materials

Superose 12 HR 10/30 FPLC columns were purchased from Pharmacia (Upsala, Sweden). The protein assay kit was purchased from Bio-Rad Laboratories (Mississauga, ON). Medium (alpha-, E-, or Dulbecco-MEM), horse and fetal bovine sera, gentamycin sulfate, trypsin-EDTA, ethylenediaminetetra acetic acid (EDTA), and tissue culture plasticware were purchased from GIBCO Laboratories (Burlington, ON). Bovine serum albumin (BSA, fraction V), sodium selenite, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), pepstatin A, 7-amino 4-methylcoumarin, pepsin, estradiol, tamoxifen, bovine cathepsin D, human cathepsin D, murine cathepsin B, hemoglobin, and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). N-CBZ-argininyl-argininyl 7-amido-4-methyl-coumarin HCl (Z-arg-arg-NMec), and N-CBZ-L-phenylalanyl-L-argininyl-7-amido-4-methyl coumarin HCl (Z-phe-arg-NMec) were purchased from Bachem California (Torrance, CA). Phenylmethanesulphonyl flouride (PMSF) was purchased from Boehringer Mannheim Canada (Laval, QU). The cathepsin B selective inhibitor, CA074, was a generous gift from Dr. B. Sloane (Pharmacology, Wayne State University, Detroit, MI). All other chemicals were purchased from Sigma and Fisher Scientific Co. (Fair Lawn, NJ).
2.2 Cell Culture Maintenance, Collection and Treatment

2.2.1 Cell Cultures

The differentiating rat myoblast cell line, L6, was originally established from rat fetal skeletal muscle by Dr. D. Yaffe (Yaffe, 1968), then cloned and characterized by Dr. M. Dufresne (Dufresne et al., 1976). A 5-azacytidine resistant, fusion deficient (i.e. incapable of forming myotubes) variant of L6, L6D3, was generously provided by Dr. B. Sanwal (Department of Biochemistry, University of Western Ontario, London, ON). Resistance in this variant reflected an alteration of the component of two target pathways of this drug, the de novo pyrimidine pathway, and an undefined sequence leading to the synthesis of membrane components (Ng et al., 1976). The LM mouse fibroblast cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD).

The human breast cancer cell line, MCF-7, was clonally derived from the original cell line established from a breast explant of an adult female with primary breast carcinoma. MCF-7/ADR^R, an adriamycin resistant variant of MCF-7, was established by Dr. G. Batiste (Batist et al., 1986; Fairchild et al., 1987) and subsequently characterized in this laboratory (Labrizzo and Dufresne, 1989; Scaddan and Dufresne, 1995). Both MCF-7 cell lines, along with four additional well studied human breast cancer cell lines, ZR-75, MDA-468, MDA-231, and HS578T (Engel et al., 1978; Pathak et al., 1979; Cailleau et al., 1974; Hacket et al., 1977), were generously supplied by Dr. K. Cowan (National Institute of Health, Bethesda, MD).
The human primary lung sarcoma cell line, HPL-R4, was established in our laboratory from a primary lung tumor biopsy (Manjunath and Dufresne, 1989). The differentiating human hepatoma cell line, HepG2, was originally established from a liver biopsy of a child with primary hepatoblastoma (Aden et al., 1979; Knowles et al., 1980), and generously supplied by its originators, Dr. B. Knowles and Dr. D. Aden (Wistar Institute of Anatomy and Biology, Philadelphia, PA). The mortal, human lung fibroblast cell line, WI38, was purchased from ATCC (Rockville, MD).

2.2.2 Maintenance and Transfer of Cell Cultures

All cell cultures were maintained at 37°C in an atmosphere of 5% CO₂: 95% air in either: α-MEM supplemented with 5% heat-inactivated FBS (MCF-7, MCF-7/ADR^R, ZR-75, HS578T, MDA-231, MDA-468, HPL-R4, HepG2), E-MEM supplemented with 10% heat-inactivated FBS and 1 mg/mL pyruvate (WI38), or α-MEM supplemented with 10% heat-inactivated FBS and 4.5 g/L D-glucose (L6, L6-D3, LM) all containing 50 μg/mL gentamicin sulphate. Cells were maintained in culture for extended periods of time by serial passage (Freshney, 1987). Near confluent or, in some cases, confluent cells were washed with 37°C citrate saline (15 mM trisodium citrate, 134 mM potassium chloride, pH 7.8), trypsinized (0.25% trypsin-EDTA), collected, suspended in 5x the volume of serum-containing growth medium, and centrifuged at low speed (1500 x g) for 5-10 minutes. The cell pellet was resuspended in serum-containing medium and plated into tissue culture plasticware at a concentration of 10^4 to 10^5 cells/mL medium. Neither the biological nor biochemical properties of the cell lines used in this study were affected by the transfer procedure.
2.2.3 Cell Cloning

Cells were cloned in 96-well Linbro microtest plates (6 mm) as follows. Cells in exponential growth were dissociated from flasks with trypsin, centrifuged, resuspended in growth medium at a concentration of 5 cells/mL growth medium, and plated into microtest plates (0.2 mL of the cell suspension/well). The following day, wells were examined for single cells and those that in time gave rise to single colonies of at least 20 cells were trypsinized and serially cultured into 24 well plates, 6 well plates, 25 cm² tissue culture flasks, and finally 75 cm² flasks. To guard against their loss through contamination and technical/mechanical failures, and to maintain their genotypic and phenotypic integrity, these cells were stored in dimethylsulfoxide (DMSO)/serum medium at -70°C in a Revco Ultralow Freezer (Revco Inc., West Columbia, SC) and at -195°C in liquid nitrogen.

2.2.4 Cell Storage

Near confluent cells were washed with citrate saline, trypsinized using 0.25% trypsin-EDTA, collected and suspended in 5x the volume of serum-containing growth medium, then centrifuged at low speed (1000 x g) for 5 minutes. The cell pellet was resuspended at a concentration of 2 x 10⁶ cells/mL in freezing solution consisting of 70% α-MEM, 10% DMSO and fetal calf serum (v/v). One mL aliquots were transferred into Nunc cryotube vials, placed at -20°C for 30 minutes then stored in an ultra-cold freezer at -70°C and in liquid nitrogen at -195°C. Approximately one week after freezing, sample vials were checked for contamination and cell viability. Cells
frozen in this manner remained viable without loss of or alterations in relevant
genotypes and phenotypes for a number of years.

2.2.5 Preparation and Collection of Cells and Conditioned Medium

Cells were routinely maintained in 25 cm² culture flasks, but were passaged to
larger 75 cm² culture flasks to provide sufficient numbers for plating in 100 mm tissue
culture dishes. Dishes were plated with between $10^5$ to $5 \times 10^5$ cells depending on the
cell line and incubated at 37°C. At each appropriate time point (i.e., day/phase of
growth), medium was decanted and examined for cellular debris. Cells on the surface
of the dishes were collected on ice in 1 mL cold (4°C) phosphate buffered saline (PBS:
0.027 mM KCl, 1.5 mM KH₂PO₄, 150 mM Na₂HPO₄·7H₂O, and 140 mM NaCl, pH
7.6) using a rubber policeman. Cells were then diluted in cold PBS and centrifuged at
1000 x g for 5 minutes. The cell pellet was suspended in 10 mL cold PBS and
centrifuged again at 1000 x g for 5 minutes. After two additional PBS/centrifugation
cycles, the final pellet was frozen down at -20°C. Prior to biochemical analyses, the
cell pellet was thawed and resuspended in either 600 ul of homogenization buffer (135
mM KCl, 0.1 mM ETDA, 0.1% tween, and 15 mM KH₂PO₄. pH 6.0) for protease
activity assays, or in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM Na₂EDTA, 1 mM
PMSF, 0.5 % SDS, and 0.5% BME) for SDS-PAGE studies. Cell suspensions were
then homogenized on ice with a Brinkman Polytron PT-10 (Brinkman Instruments,
Oakville, ON) on setting 4 for three, 10 second pulses. Homogenates were clarified by
microcentrifugation for 5 minutes at 4°C using a Fisher Model 59 microfuge.
For coordinate analysis of protease activities present in and secreted from cells during their growth, medium on plates was replaced with serum-free, defined medium 24 hours prior to collection (Dufresne et al., 1993). This transfer did not affect the viability, growth parameters, phenotypes or karyotypes of any of the cell lines examined. Moreover, all cell lines were anchorage-dependent and the proportion of cells liberated into regular or defined medium, even at saturation, was negligible. At each time point, the serum-free medium added to cells 24 h prior to collection was removed, centrifuged in the cold at 1500 x g for 5 minutes and either was used directly or was concentrated by Amicon filtration. Concentration of medium was performed in a 4°C coldroom using N₂ gas positive pressure filtration through a Ymb3 Amicon filter (selective for molecular weights greater than 10 kDa). Routinely 20 fold concentration was achieved (e.g., 40 mL to 2 mL). In this manuscript, fractions prepared from cell homogenates and their corresponding media are referred to as cell or intracellular, and media or extracellular fractions, respectively.

2.2.6 Treatment of Cells with β-Estradiol and Tamoxifen

Cells were seeded at 2x10⁵ cells per 100 mm dish in growth medium containing 3% dextran coated charcoal FBS (DCC-FBS) and incubated at 37°C according to a published protocol (Coopman et al., 1994). After two days of growth, the medium on each dish was decanted and fresh 3% DCC-FBS medium containing either 10 nM β-estradiol, 100 nM tamoxifen, or DMSO (solvent control) was added. The cells were incubated in this medium for 24 hours at 37°C, then exposed to serum free, defined medium containing the corresponding treatments. After another 24 hours at 37°C, both
the cells and their corresponding serum-free media were collected as previously described.

2.3 Protease Activity Assays

2.3.1 Cathepsin B Activity

Cathepsin B activity was measured in intracellular and extracellular fractions using the appropriate fluorometric, 4-methylcoumaryl-amide substrates according to a modified procedure of Barrett and Kirschke (Jane and Dufresne, 1994; Barrett and Kirschke, 1981). In a final reaction volume of 1 mL, a 500 μL aliquot of fraction containing 0.1 μg protein diluted in 0.1% Brij 35 solution in water, or 150 μL of extracellular media, was preincubated for 5 minutes at 37°C with 250 μL cathepsin B activation buffer (352 mM KH₂PO₄, 48 mM Na₂HPO₄, 4 mM disodium EDTA, and 8 mM dithiothreitol, pH 6.0). The reaction was started by the addition of 250 μL of 0.02 mM substrate solution (cathepsin B: Z-Arg-Arg-NMec, Z-Phe-Arg-NMec). After 10 minutes incubation at 37°C, 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). Since both cathepsin B and L-type proteases recognize the Z-Phe-Arg-NMec substrate, cathepsin B activity was measured with Z-Phe-Arg-NMec in the presence and absence of the cathepsin B selective inhibitor CA074 (1 x 10⁻⁷ M).

The fluorescence of free aminomethylcoumarin in each reaction tube was measured 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,
CA). Fluorescence readings were standardized with 0.5 μM 7-amino-4-methylcumarin standard 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.

Levels of acid/pepsin activatable cathepsin B (i.e. latent procathepsin) activity were determined in extracellular fractions using a modified procedure of Mort published in Jane and Dufresne (Jane and Dufresne, 1994; Mort et al., 1981). For each reaction, 150 μL of conditioned media 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 adjusted to 6.0 with 60 μL of 0.2 M potassium phosphate buffer, pH 9.0. The reaction was initiated by adding 250 μL of substrate, terminated with 1 mL of stopping reagent, and the liberated 7-amino-4-methylcumarin measured as described above.

2.3.2 Cathepsin D Activity

Cathepsin D activity was measured in intracellular and extracellular fractions using 8.0% w/v hemoglobin as substrate according to a modified version of the standard general protease assay of Turk et al. (Turk et al., 1984). [Modifications to the cathepsin D assay were based upon results obtained and presented in the appendix.] In a final reaction volume of 1 mL, 500 μL of diluted sample was added to 250 μL of acetic acid assay buffer (1.35 M acetic acid, 20 mM NH₄SO₄, pH 3.5;) and 250 μL of bovine hemoglobin substrate (8.0% w/v hemoglobin). The reaction was started by
incubating the reaction tubes at 37°C. After 60-120 min of incubation the reaction was terminated with the addition of 2 mL of 3% (w/v) trichloroacetic acid which precipitated non-digested hemoglobin and dissolved liberated peptide fragments. After an additional 15 minutes incubation, this reaction mix was centrifuged for 10 minutes at 4000 rpm and to 1 mL of clear supernatent was added to 2 mL of modified biuret reagent and allowed to stand for an additional 15 minutes at room temperature. Absorbance at 750 nm was determined after the addition of 600 μL Folin-Ciocalteau reagent. One Anson Unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute as measured by Folin-Ciocalteau reagent. The linear relationship between absorbance and increasing concentrations of a tyrosine standard is presented in Figure 7. All activities were measured concurrently in the presence and absence of the microbial pentapeptide, Pepstatin A, and represent total activity minus Pepstatin insensitive activity measured concurrently from a second aliquot of the sample. Pepstatin strongly inhibits cathepsin D activity prepared from a variety of sources including swine liver, rabbit liver and human liver, bovine lung, rabbit lung, rabbit alveolar macrophages and porcine brain.

2.4 Fast Protein Liquid Chromatography (FPLC) Analyses

2.4.1 FPLC Selectivity Curve and Molecular Mass Determination

Molecular mass standards were run on a Superose HR 10/30 FPLC column and their elution volumes recorded. Blue Dextran (V₀), as well a bovine albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), and cytochrome C (12.4
**FIGURE 7**

**Tyrosine Standard Curve**

Tyrosine standards of 0.01-1 μM concentrations in 1 mL volumes of 3% w/v TCA were prepared from a 1 mM stock. Absorbance at 750 nm was measured after addition of 2 mL modified biuret reagent and 600 μL Folin-Ciocalteau reagent as described in Methods 2.3.2.
kDa), were used to construct a selectivity curve for the approximation of molecular mass and is presented in Results: Figure 11. Separation coefficients \( (K_{av}) \) were calculated using the following formula:

\[
K_{av} = \frac{V_e - V_o}{V_t - V_o}
\]

where \( V_e \) is the elution volume, \( V_o \) is the void volume, and \( V_t \) is the total volume.

2.4.2 FPLC Fractionation of Intracellular and Extracellular Cathepsin D

After centrifugation \((12,000 \times g, 5 \text{ min})\), 100-200 \( \mu \text{g} \) of protein from the appropriate cell or media fraction was loaded in 500 \( \mu \text{L} \) of homogenization buffer (135 mM potassium chloride, 0.1 mM EDTA, 0.1% Tween 80, 15 mM potassium phosphate, pH 6.0) on a Superose 12 HR 10/30 FPLC column (Pharmacia, Upsala, Sweden). Fractions were 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 0.5 mL/min. Eluted column fractions were divided in two and assayed in the presence and absence of Pepstatin A. Activities measured in the presence of inhibitor were subtracted from the activity measured in the absence of inhibitor to give cathepsin D specific activity.

2.5 SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modified method of Laemmli (1970) using 4% (w/v) stacking and 10-12% (w/v) resolving gels (Dufresne et al., 1993). Samples were prepared by first mixing a volume containing 12 \( \mu \text{g} \) sample protein with an equal volume of sample buffer (125 mM Tris-HCL, pH 6.8, 10% bME,
20% glycerol, 4% SDS, and 0.02% bromophenol blue) then heating in a boiling water bath for 5 minutes. After application to the gel, samples were electrophoresed at 60 volts until samples migrated through the stacking gel and entered the resolving gel. Once the samples were in the resolving gel the voltage was increased to 110 volts and this voltage was maintained until the dye front reached the bottom of the gel. The gels were then fixed and stained in 0.2% Coomassie Brilliant Blue G-250 (in 10% (v/v) acetic acid and 40% (v/v) methanol). The relative molecular mass of bands was determined by reference to proteins of known molecular mass: carbonic anhydrase (29.0 kDa), egg albumin (45.0 kDa), bovine albumin (66.0 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116.0 kDa), and myosin (205.0 kDa).

2.6 Protein Determination

Protein content was determined according to a modified version of Bradford (Bradford, 1976; Jane and Dufresne, 1994). This assay is based on the linear colour change of Bio-Rad Coomassie brilliant blue G-250 dye in response to increasing concentrations of protein. When the dye binds to protein, there is a shift in the maximal absorption from 465 nm to 595 nm. The standard used for determination of protein concentration was bovine serum albumin (BSA). This was prepared by dissolving 29 mg of BSA in 10 mL of nano-pure water. Fifty μL of this stock was then added to 1.4 mL of nano-pure water to give a second stock concentration of 100 ug/mL BSA. One mL of this solution was diluted with 4 mL water to give a working concentration of 20 μg/mL.
Protein standards and samples were prepared as follows:

<table>
<thead>
<tr>
<th>protein amount (µg)</th>
<th>stock volume (µL)</th>
<th>water volume (µL)</th>
<th>dye volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>100</td>
<td>700</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
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<tr>
<td>4</td>
<td>200</td>
<td>600</td>
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<td>5</td>
<td>250</td>
<td>550</td>
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</tr>
<tr>
<td>8</td>
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<td>200</td>
</tr>
<tr>
<td>12</td>
<td>600</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>standard blank</td>
<td>0</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td>cell sample</td>
<td>30</td>
<td>770</td>
<td>200</td>
</tr>
<tr>
<td>cell sample blank</td>
<td>30</td>
<td>770</td>
<td>200</td>
</tr>
<tr>
<td>medium sample</td>
<td>150</td>
<td>650</td>
<td>200</td>
</tr>
<tr>
<td>medium blank</td>
<td>150</td>
<td>650</td>
<td>200</td>
</tr>
</tbody>
</table>

Cell samples were suspended in homogenization buffer; in this case the sample blank was homogenization buffer. The medium sample blank was fresh serum free medium.

Standards and samples were analyzed with a visible light wavelength of 595 nm with a Sargent-Welch PU 8610 spectrophotometer.

2.7 Statistical Analyses

Calculation of standard deviation, mean and mode are described in Mendenhall et al., (1974).
Results

3.1 The Expression of Cathepsin D Activity in Differentiating Myoblast Cells

3.1.1 Cathepsin D Activity during L6 Myoblast Differentiation Follows an “Up-Down-Up” Pattern and is Fusion-Related: The expression of muscle-specific proteins during muscle differentiation has been shown to be temporally related to the fusion of myoblast cells into syncytial myotubes (Dufresne et al., 1976). Alterations in the expression of some cellular proteases, including the nonlysosomal, calcium-activated neutral proteases or calpains (Kaur and Sanwal, 1981) and the lysosomal cysteine proteases (Bird et al., 1981; Jane and Dufresne, 1994), also appear to be fusion-related. There is early evidence suggesting that a similar association may exist for the lysosomal aspartyl protease, cathepsin D (Bird et al., 1981). I examined this possibility by measuring cathepsin D activity in L6 myoblasts, their fusion-deficient variants, L6D3, and in LM fibroblasts. Cells were plated in medium containing 10% horse serum, conditions which promote myoblast differentiation in L6 (Jane and Dufresne, 1994). Each day, cells were analyzed for cathepsin D activity, fusion index measurements and/or cell number (Figures 8-10).

Cathepsin D activity was initially high in presumptive L6 myoblasts (Figure 8). The activity decreased as cells exited the cell cycle and increased as postmitotic myoblasts aligned and fused into myotubes. This “up-down-up” pattern of activity is consistent with that reported for the expression of intracellular cathepsin B activity during differentiation of L6 rat myoblasts (Jane and Dufresne, 1994) and of cysteine
FIGURE 8

Levels of Cathepsin D Activity in Intracellular Fractions of L6 Cells During Growth and Differentiation

Cells ($5 \times 10^5$) were seeded and grown in serum-supplemented medium. During growth in culture, cells were collected each day after plating and a representative plate fixed and stained with Giemsa. Fusion index (closed circles), or the percent ratio of number of nuclei in myotubes to total number of nuclei was determined by the method of Morris and Cole (1972). Cathepsin D activity (hatched bars) was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
proteases in general during the myoblast-myotube differentiation in primary cultures of calf muscle cells (Bechet, et al., 1991). Significantly, this pattern was not observed during growth of fusion-deficient L6D3 myoblasts (Figure 9) or LM fibroblasts (Figure 10). Activities in both these cell lines were related to cell number and increased until saturation density was achieved then decreased.

3.1.2 Cathepsin D Activity and Molecular Mass Determination in Fast Protein Liquid Chromatographed (FPLC) Cell Homogenates:

Differences in cathepsin D activity during differentiation and between cell lines could be explained by several mechanisms including alterations in transcription and translation, and/or alterations in processing and cellular localization (reviewed in Sloane, 1990; and Chambers et al., 1992). They could also reflect, undefined, complex interactions occurring in crude cell homogenates that could artificially mask or enhance activity (Green et al., 1984; Barret et al., 1986; Chambers et al., 1992). To minimize this potential artifact and to correlate cathepsin D activity with molecular masses, cell homogenates collected at different stages of cell growth/differentiation in culture were fractionated on a Superose 12 HR 10/30 column. This column has been shown to efficiently separate proteins with molecular masses between 10 kDa and 300 kDa. This range includes the reported molecular masses for all pro- and mature forms of cathepsin D (Rochefort et al., 1989).

3.1.3 Commercial Cathepsin D Activity Elutes at Positions Corresponding to Molecular Masses of 34 kDa and/or 48 kDa: For molecular mass
FIGURE 9

Levels of Cathepsin D Activity in Intracellular Fractions of L6D3 Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). Cathepsin D activity (hatched bars) was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
FIGURE 10

Levels of Cathepsin D Activity in Intracellular Fractions of LM Cells During Growth

Cells ($5 \times 10^5$) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). Cathepsin D activity (hatched bars) was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg protein ($\text{mAnson Units/mg protein}$) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
determination, a selectivity curve (Figure 11) was prepared by calculating separation coefficients, \( K_{av} \), for different molecular mass standards as described in Methods. For reference, Figure 11 includes the positions on the selectivity curve for the mature double (i.e., 34 kDa) and single (i.e., 48 kDa) chain forms of cathepsin D, and for procathepsin D (i.e., 52 kDa). This curve agrees with the separation curve provided by the manufacturer.

The FPLC column used to generate the selectivity curve also permitted the reproducible separation of both human liver cathepsin D and bovine spleen cathepsin D without loss of their biological activities (Figure 12). Commercial human liver cathepsin D activity eluted at a position corresponding to an apparent molecular mass of 34 kDa, while bovine cathepsin D eluted at positions corresponding to apparent molecular masses of 34 kDa and 48 kDa. Molecular masses of cathepsin D activity positions determined from selectivity curves were confirmed by concurrent analysis using SDS-PAGE electrophoresis (Figure 13).

3.1.4 Fused Myoblasts Exhibit Cathepsin D Activity at 34 kDa and 48 kDa Positions: Cell homogenates were prepared from growing cultures of presumptive, post-mitotic and fused L6 myoblasts. Similar amounts of protein from each homogenate sample (i.e., 500 \( \mu \)g protein) in homogenization buffer (pH 6.0) were injected separately onto a Superose 12 HR 10/30 FPLC column. Five hundred \( \mu \)L fractions were collected, placed on ice, then assayed for cathepsin D activity (Figures 14, 15 and 16). At each stage of differentiation analyzed, cathepsin D activity eluted at a position corresponding to an apparent molecular mass of 34 kDa. However, in fused
FIGURE 11

Superose 12 HR10/30 FPLC Column Standard Curve

Molecular mass standards (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 12.4 kDa) were applied to and eluted from a Superose 12 HR 10/30 FPLC column (Methods: 2.4.1). The void volume was determined with Blue Dextran and used, together with the elution volume for each molecular mass standard, to calculate $K_a v$ values. A plot of $K_a v$ versus molecular mass (kDa) was constructed and used to obtain an approximate molecular mass for cathepsin D activities using hemoglobin as substrate (Methods: 2.4.2).
Approximately 50 Anson Units of commercially purified human cathepsin D in 500 µL or 100 Anson Units of commercially purified bovine cathepsin D in 500 µL was applied to, and eluted in 500 µL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (closed circles) was determined using hemoglobin as substrate and represents the activity in 250 µL of fraction minus the residual activity of the second 250 µL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
| Molecular Mass (kDa) | 66 | 45 | 29 | 14.2 |

**FIGURE 13**

**SDS-Polyacrylamide Gel Electrophoresis of Bovine Cathepsin D**

Commercial purified bovine Cathepsin D was loaded on a 10% polyacrylamide gel and separated using electrophoresis according to Methods 2.5.
FIGURE 14

FPLC Separated Cathepsin D Activity in Presumptive L6 Myoblasts

Cells were initially grown in 10% FCS supplemented media to inhibit precocious fusion. These same cells were later seeded (5 x 10^5) in 10% HS (a fusion permissive medium) for experiments. Centrifuged homogenates were prepared from L6 cells collected during presumptive myoblast growth. Approximately 200 μg was applied to, and eluted in 500 μL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (closed circles) was determined using hemoglobin as substrate and represents the activity in 250 μL of fraction minus the residual activity of the second 250 μL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
FIGURE 15

FPLC Separated Cathepsin D Activity in Post-Mitotic L6 Myoblasts

Cells were initially grown in 10% FCS supplemented-media to inhibit precocious fusion. These same cells were later seeded (5 x 10^5) in 10% HS (a fusion permissive medium) for experiments. Centrifuged homogenates were prepared from post-mitotic L6 cells, which had exited the cell cycle and begun to align. Approximately 200 µg was applied to, and eluted in 500 µL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (closed circles) was determined using hemoglobin as substrate and represents the activity in 250 µL of fraction minus the residual activity of the second 250 µL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
FIGURE 16

FPLC Separated Cathepsin D Activity in Fused L6 Myotubes

Cells were initially grown in 10% FCS supplemented-media to inhibit precocious fusion. These same cells were later seeded (5 x 10^5) in 10% HS (a fusion permissive medium) for experiments. Centrifuged homogenates were prepared from fused L6 myotubes. Approximately 200 μg was applied to, and eluted in 500 μL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (closed circles) was determined using hemoglobin as substrate and represents the activity in 250 μL of fraction minus the residual activity of the second 250 μL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
myoblasts (Figure 16), a second peak of activity was detected at a molecular mass position of 48 kDa. Interestingly, FPLC analysis of homogenates prepared from fusion-deficient, L6D3 myoblasts (Figure 17) and from LM fibroblasts (Figure 18) demonstrated a single peak of cathepsin D activity at 48 kDa.

3.2 The Expression of Cathepsin D Activity in Malignant Cells

Alterations in cathepsin D activity have been implicated in tumour cell invasion and metastasis and are being examined as potential biochemical indicators for treatment efficiency and patient survival (reviewed in Ren and Sloane, 1996). Not surprisingly, much of this research is directed at breast and lung cancers, the two most common cancers in North America.

3.2.1 Cathepsin D Activity in Breast Cancer Progression: As discussed in the introduction, the lowly metastatic MCF-7-highly metastatic MCF-7/AdrR cell culture system has become the experimental system of choice for many researchers examining phenotypes associated with tumor progression (section 1.3.5). To complement the data obtained in this laboratory for cysteine proteases, (Scadden and Dufresne, 1995), I measured activity levels of the aspartyl protease, cathepsin D, in MCF-7 and MCF-7/AdrR cell and media fractions. Furthermore, I compared these levels to those obtained in four other breast cancer cell lines routinely used to study malignant phenotypes: ZR75, HS578T, MDA-231 and MDA-468. For convenient reference, relevant properties of the six breast cancer cell lines used in the present study have been compiled in Table 1.
FIGURE 17

FPLC Separated Intracellular Cathepsin D Activity in Non-Fusing L6D3 Myoblasts

Centrifuged homogenates were prepared from L6-D3 myoblasts collected during early exponential growth. Approximately 200 µg was applied to, and eluted in 500 µL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (closed circles) was determined using hemoglobin as substrate and represents the activity in 250 µL of fraction minus the residual activity of the second 250 µL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
FIGURE 18

FPLC Separated Intracellular Cathepsin D Activity in LM Fibroblasts

Centrifuged homogenates were prepared from LM fibroblasts collected during early exponential growth. Approximately 200 µg was applied to, and eluted in 500 µL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 mL/min. Cathepsin D activity in each fraction (closed circles) was determined using hemoglobin as substrate and represents the activity in 250 µL of fraction minus the residual activity of the second 250 µL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Classification (^a)</th>
<th>Source (^b)</th>
<th>Initial Patient Treatment (^c)</th>
<th>Estrogen Receptor (^d)</th>
<th>Tumor Formation (^e)</th>
<th>Doubling Time (hr) (^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>IDAC</td>
<td>PE</td>
<td>R H</td>
<td>195000</td>
<td>N+</td>
<td>22</td>
</tr>
<tr>
<td>MCF-7/Adr(^R)</td>
<td>IDAC</td>
<td>MCF-7</td>
<td>R H (MCF-7) A selection</td>
<td>&lt;1000</td>
<td>N+</td>
<td>23</td>
</tr>
<tr>
<td>ZR75</td>
<td>IDC</td>
<td>A</td>
<td>E, T FL</td>
<td>72000</td>
<td>N+</td>
<td>19</td>
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<tr>
<td>HS578T</td>
<td>CS</td>
<td>O</td>
<td>R</td>
<td>&lt;1000</td>
<td>1-</td>
<td>18</td>
</tr>
<tr>
<td>MDA231</td>
<td>ADC</td>
<td>PE</td>
<td>O, F, A C M, M P</td>
<td>&lt;1000</td>
<td>N+</td>
<td>17</td>
</tr>
<tr>
<td>MDA468</td>
<td>ADC</td>
<td>PE</td>
<td>R, FAC, S, FMT</td>
<td>&lt;1000</td>
<td>N+</td>
<td>14</td>
</tr>
</tbody>
</table>

\(^a\) Classification: (IDAC) Infiltrating Ductal Adenocarcinoma, (IDC) Infiltrating Ductal Carcinoma, (CS) Carcosarcoma, (ADC) Adenocarcinoma (Engel and Young, 1978)

\(^b\) Source: (PE) Pleural Effusion, (A) Ascites Fluid, (O) Original Tumour Adenocarcinoma (Engel and Young, 1978)

\(^c\) Treatment: commas separate treatment regimes, including (R) radiation therapy, (F) 5-Fluorouracil, (A) Adriamycin, (C) Cytoxin, (M) Methotrexate, (T) Tamoxifen, (P) Prednisone, (E) receiving estrogen therapy, (Fl) Fluoxymestrone, (H) undefined hormone therapy, (S) surgery, (O) oophorectomy (Engel and Young, 1978)

\(^d\) Estrogen Receptor: content fmol/mg protein (Vickers et al., 1989)

\(^e\) Tumor Formation: (N) nude mouse, (I) immunosuppressed mouse (Engel and Young, 1978)

\(^f\) Doubling Time: calculated from growth curves presented in this text
3.2.1A: Levels of Cathepsin D Activity in MCF-7 are Higher than

Levels in MCF-7/AdrR at Each Stage of Growth: Each breast cancer cell line was plated and analyzed daily for intracellular cathepsin D activity and cell number (Figures 19 - 24). The limitations in sensitivity of the hemoglobin assay necessitated concentration of medium for detection of extracellular activity. Since the concentration of medium for each day of growth of six different breast cancer cell lines, in triplicate, was neither practical nor feasible, extracellular levels of cathepsin D were measured in the concentrated medium collected from exponentially growing (i.e., day 4) cells. During growth of MCF-7 cells in culture, intracellular levels of cathepsin D initially increased then decreased as cells became more confluent (i.e., day 3/4) (Figure 19). In contrast, intracellular levels of cathepsin D activity increased throughout growth of MCF-7/AdrR until confluence (i.e., day 4/5) (Figure 20). At each time point, levels of cathepsin D activity in MCF-7 homogenates were reproducibly higher than those in MCF-7/AdrR. Extracellular levels of cathepsin D activity in concentrated medium from day 4 MCF-7 cells were also reproducibly higher than those in MCF-7/AdrR.

The expression of cathepsin D activity in the four other breast cancer cell lines followed two general patterns. The first, an increase until confluence (ZR75B: Figure 21, and HS578T: Figure 22), was reproducibly similar to that observed in MCF-7/AdrR. The second, no obvious time-related increase in activity (MDA231: Figure 23, and MDA468: Figure 24), appeared to be unique to the two MDA cell lines. Levels of cathepsin D activity in ZR75 and HS578T homogenates were generally
FIGURE 19

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of MCF-7 Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
FIGURE 20

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of MCF-7/AdrR Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 µL of fraction minus the residual activity of a second 100 µL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.

FIGURE 21

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of ZR75 Cells During Growth
FIGURE 22

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of HS578T Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 µL of fraction minus the residual activity of a second 100 µL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
FIGURE 23

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of MDA-231 Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 µL of fraction minus the residual activity of a second 100 µL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
higher than those in MCF-7/Adr\(^R\) and lower than those in MCF-7 at each time point during growth. In contrast, levels of cathepsin D activity in MDA231 and MDA468 homogenates were generally lower than those in either MCF-7 cell line. The relative order of cathepsin D activity in concentrated medium from day four cells paralleled that observed in cell homogenates in the case of ZR75B and MDA468 cells, but not MDA231 or HS578T. Specifically, extracellular levels of cathepsin D activity in ZR75 and MDA231 were greater than those observed in MCF-7/Adr\(^R\), while levels in MDA468 and HS578T were lower. To facilitate comparison, a rank summary of the levels of cathepsin D activity in cells and media prepared from day 4 breast cancer cell cultures is presented in Table 2. Two general trends are worth noting. First, highly metastatic cancer cell lines (i.e., MCF-7/Adr\(^R\), MDA231 and MDA468) appear to demonstrate lower levels of intracellular cathepsin D activity than moderately metastatic (i.e., ZR75) or lowly metastatic (i.e., MCF-7 and HS578T) cancer cell lines. Second, the progression from lowly metastatic MCF-7 cells to their highly metastatic MCF-7/Adr\(^R\) cells appears to be associated with a decrease in both intracellular and extracellular levels of cathepsin D activity. These decreases are in direct contrast to the increases in levels of cysteine protease activities (e.g., cathepsin B, L and H) associated with increased metastatic potential (Ren and Sloane, 1996), MCF-7 \(\rightarrow\) MCF-7/Adr\(^R\) tumor progression (Scaddan and Dufresne, 1995) and cytodifferentiation (Jane and Dufresne, 1994). The decreases also contrast with the increases in cathepsin D activity associated with myoblast-myotube differentiation (this thesis: section 3.1.1).
Table 2. Summary and Rank of Cathepsin D Activities in Cells and Medium of Breast Cancer Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Metastatic Potential (^a)</th>
<th>Fraction  (^b)</th>
<th>Cathepsin D mAnson Units/mg</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
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<td>Cells</td>
<td>2073</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>373</td>
<td>1</td>
</tr>
<tr>
<td>MCF-7/Adr(^R)</td>
<td>High</td>
<td>Cells</td>
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<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>166</td>
<td>4</td>
</tr>
<tr>
<td>ZR75</td>
<td>Low</td>
<td>Cells</td>
<td>870</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>245</td>
<td>2</td>
</tr>
<tr>
<td>HS578T</td>
<td>Low</td>
<td>Cells</td>
<td>606</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>97</td>
<td>5</td>
</tr>
<tr>
<td>MDA-231</td>
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<td>Cells</td>
<td>261</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>188</td>
<td>3</td>
</tr>
<tr>
<td>MDA-468</td>
<td>High</td>
<td>Cells</td>
<td>230</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>49</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) Low (low metastatic potential), High (high metastatic potential) (Engel and Young, 1978)

\(^b\) Fraction refers to cells or concentrated conditioned medium collected during exponential growth
3.2.1B: Cathepsin D activity in Estrogen Receptor (ER)-Positive Breast Cancer Cell Lines is Induced by Estradiol: The reported regression of breast tumors in young women following ovariectomy in 1896 provided the first evidence for the estrogen responsiveness of some breast cancers (Beatson, 1896). To-day, the mitogenic action of estrogens is thought to be the most significant tumor promoting agent during early breast tumor progression (May and Westly, 1995). It is well established that the mitogenic action of estrogens occurs by direct mechanisms, for example by affecting cell cycle regulators such as c-myc (Bradbury, 1996). However, more recent evidence suggests that estrogens can also exert their mitogenic activity by indirect mechanisms, for example by the induction of secondary mitogens such as cathepsin D (Stewart et al., 1994). With this indirect mechanism in mind, I examined the effect of estrogen (i.e., β-estradiol) on levels of cathepsin D activity in the MCF-7 -> MCF-7/AdrR tumor progression culture system. Because of its use in the prevention and treatment of breast cancer, I also examined the effect of tamoxifen, a nonsteroidal anti-estrogen which inhibits a dimerization necessary for the binding of estrogen to the receptor (Howell et al., 1996).

MCF-7 and its variant MCF-7/AdrR were treated with estradiol and tamoxifen. Cells were grown for three days in the presence of either $1 \times 10^{-8}$ M estradiol, $1 \times 10^{-7}$ M tamoxifen or DMSO control. [NOTE: The concentrations used were determined from standard estradiol and tamoxifen concentration experiments.] Twenty four hours before collection of the cells and their corresponding medium, the growth medium was changed to serum free, defined medium containing the appropriate concentration of
drug. Cell homogenates (intracellular fractions) were prepared and medium was concentrated (extracellular fractions) from exponentially growing cells; both fractions were then assayed for cathepsin D activity (Figure 25).

Treatment of estrogen receptor (ER) positive MCF-7 cells with estradiol resulted in a reproducible greater than 2 fold increase in intracellular (e.g., 2.3 fold) and extracellular (e.g., 2.7 fold) levels of cathepsin D activity relative to solvent controls. Not surprisingly, treatment of ER negative MCF-7/Adr\textsuperscript{R} cells with estradiol did not induce cathepsin D activity in either fraction. However, the significant increase in cathepsin D activity in intracellular fractions prepared from tamoxifen treated MCF-7 cells was somewhat surprising. Induction by this anti-estrogen, although lower than that observed for estradiol, was reproducible and may reflect a partial agonistic effect of tamoxifen with the estrogen receptor (Howell \textit{et al.}, 1996). The fact that tamoxifen did not affect intracellular or extracellular levels of cathepsin D activity in ER negative MCF-7/Adr\textsuperscript{R} is consistent with this possibility.

The intracellular results obtained in estradiol treated MCF-7 and MCF-7/Adr\textsuperscript{R} cells culture system paralleled those obtained in the four other breast cancer cell lines (Figure 26). Estradiol induced cathepsin D activity in ER positive ZR75 cells approximately 2 fold, but had no effect on ER negative HS578T, MDA231 or MDA468 cells. Tamoxifen treatment, on the other hand, had no significant effect on intracellular levels of cathepsin D activity in any of the four breast cancer cell lines. Taken together, these results suggest that estradiol induces cathepsin D activity in ER
FIGURE 25

Effects of β-Estradiol and Tamoxifen on the Induction of Cathepsin D Activity in MCF-7/MCF-7/Adr^R Cell Lines

Cells were seeded at (2x10^5) and allowed to grow for two days in 3% dextran coated charcoal treated FCS (DCC-FCS). On the second day, the media was removed, the cells washed, and fresh 3% DCC-FCS containing either 10^{-8} M β-estradiol, 10^{-7} M tamoxifen or DMSO solvent control was added. After the third day of incubation the media was removed once again, the cells washed, and fresh 3% DCC-FCS with treatment was added. On the fourth day the cells and corresponding conditioned media were collected for assay. Cathepsin D activity in MCF-7 and MCF-7/Adr^R, solvent control (solid bars), β-estradiol (hatched bars) and tamoxifen (open bars) treated samples, was measured using hemoglobin as substrate and represents the activity in 100 µL of fraction minus the residual activity of a second 100 µL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
FIGURE 26

Effects of β-Estradiol and Tamoxifen on the Induction of Cathepsin D Activity in Breast Cancer Cell Lines

Cells were seeded at (2x10^5) and allowed to grow for two days in 3% dextran coated charcoal treated FCS (DCC-FCS). On the second day, the media was removed, the cells washed, and fresh 3% DCC-FCS containing either 10^-8 M β-estradiol, 10^-7 M tamoxifen or DMSO solvent control was added. After the third day of incubation the media was removed once again, the cells washed, and fresh 3% DCC-FCS with treatment was added. On the fourth day the cells and corresponding conditioned media were collected for assay. Cathepsin D activity in ZR75, HS578T, MDA-231 and MDA-468, solvent control (solid bars), β-estradiol (hatched bars) and tamoxifen (open bars) treated samples, was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg of protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
positive cell lines, and that the estradiol induction phenotype is lost during MCF-7 ->
MCF-7/Adr^R tumor progression.

3.2.2 Cathepsin D Activity in Normal and Metastatic Human Lung

Fibroblast Cell Lines: Most human cell culture systems used to study
tumor progression begin with immortal cells established from tumor biopsies
and focus on comparisons among cancer cell populations with varying metastatic
potential. While these studies have provided considerable insight into the acquisition of
the highly malignant phenotype in lowly metastatic cancer cells, they ignore the initial
"normal cell to malignant cell" transformation event (see Introduction Figure 6) and
consequently tell us nothing about the initial acquisition of the malignant phenotype in
normal cells. There are two major reasons for this research omission (Freshney, 1989). First, it is exceedingly difficult to establish and maintain normal cells from
normal tissue biopsies. Therefore, tumor model systems such as MCF-7 have no
normal cell line component. Second, when normal cells are successfully cultured from
human tissue, attempts to transform them to immortality and malignancy have been
universally unsuccessful. Therefore, comparisons of normal cells and their malignant
counterparts are lacking. The availability of the "normal" human lung fibroblast cell
line, WI38, and the establishment (in this laboratory) of the "malignant" human lung
fibroblast (sarcoma) cell line, HPL-R, allowed me to address these problems.
Specifically, I examined levels of cathepsin D activity in crude and FPLC
chromatographed cell and medium fractions prepared from human WI38 and HPL-R
fibroblasts growing in culture.
3.2.2A: Levels of Cathepsin D Activity in Normal WI38 Lung

Fibroblasts are Higher than Levels in Malignant HPL-R

Lung Fibroblasts: Both WI38 (Figure 27) and HPL-R (Figure 28) fibroblasts demonstrated a cell number related increase in intracellular levels of cathepsin D activity during exponential growth. These levels plateaued as cells reached confluency. Increases in cathepsin D activity during growth of WI38 were gradual compared to those for HPL-R. This is consistent with the lower growth rate (i.e., longer population doubling time) of WI38 relative to HPL-R. Extracellular levels of cathepsin D activity in medium collected and concentrated from exponentially growing normal WI38 cells were also higher than those observed in the medium of malignant HPL-R cells. At this point during exponential growth, levels of cathepsin D in fractions prepared from WI38 were reproducibly between 2 and 3 fold greater (e.g., 2.3 and 2.7 fold for intracellular and extracellular fractions, respectively) than those measured in HPL-R.

3.2.2B: The Major Peak of Cathepsin D Activity in Normal WI38 and Malignant HPL-R Cell and Medium Fractions Elutes at 34 kDa:

Since nothing is known concerning the properties of cathepsin D activity in the normal WI38/malignant HPL-R human lung fibroblast system, I used FPLC chromatography to examine the molecular masses associated with cathepsin D activities in cell and medium fractions prepared from exponentially growing cells. FPLC analysis of homogenates prepared from WI38 fibroblasts demonstrated a single peak of cathepsin D activity at a position corresponding to an
**FIGURE 27**

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of WI38 Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned media for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This media was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
FIGURE 28

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of HPL-R4 Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This medium was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 μL of fraction minus the residual activity of a second 100 μL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAnson Units per mg protein (mAnson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
apparent molecular mass of 34 kDa (Figure 29). In contrast, two peaks of activity were detected in HPL-R: a major peak at 34 kDa and a minor peak at 48 kDa. The ratio of areas under these two peaks is approximately 3:1. FPLC analysis of concentrated medium collected from exponentially growing WI38 fibroblasts demonstrated two peaks of cathepsin D activity at two positions: a major peak at 34 kDa and a minor peak at 52 kDa (Figure 30). In contrast, only a 34 kDa peak of activity was detected in HPL-R. The predominance of the 34 kDa (mature enzyme) relative to the higher molecular weight (pro-enzyme) forms suggests that cathepsin D may be secreted in its active form. It is interesting to note that this result, together with the relative lower levels of cathepsin D activity in malignant HPL-R cells, contradict the belief held by some researchers that cancer cells in general secrete copious amounts of procathepsin D (Rochefort et al., 1987).

3.2.3 Cathepsin D Activity in Differentiating, Malignant HepG2 Human Liver Cells: Alterations in levels of lysosomal proteases have been implicated in end-point differentiation of normal cells (Campbell et al., 1991), as well as in metastasis of malignantly transformed cells (Veksler et al., 1987). Since the liver hepatoma cell line, HepG2, expresses both differentiation (i.e., liver-specific) and malignant phenotypes, it presents an interesting chimeric system for the study of protease expression in both biological and pathological systems (Dufresne et al., 1993). For this reason, I examined the expression of cathepsin D activity during growth of this well studied cell line.
**FIGURE 29**

**FPLC Separated Intracellular Cathepsin D Activity in WI38 and HPL-R4 Cells**

Centrifuged homogenates were prepared from WI38 (open circles) and HPL-R4 (closed circles) cells collected during early exponential growth. Approximately 200 µg was applied to, and eluted in 500 µL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction was determined using hemoglobin as substrate and represents the activity in 250 µL of fraction minus the residual activity of the second 250 µL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 µmol of free tyrosine per minute.
FIGURE 30

FPLC Separated Extracellular Cathepsin D Activity in WI38 and HPL-R4 Cells

Concentrated condition medium was prepared from WI38 (open circles) and HPL-R4 (closed circles) cells collected during early exponential growth. Approximately 200 μg was applied to, and eluted in 500 μL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction was determined using hemoglobin as substrate and represents the activity in 250 μL of fraction minus the residual activity of the second 250 μL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 μmol of free tyrosine per minute.
3.2.3A: HepG2 Cells Demonstrate a Biphasic Pattern of Cathepsin D Activity that Parallels their Biphasic Pattern of Growth:

HepG2 demonstrated two phases of growth (Figure 31): phase I, from day 1 to day 5, reflected a relatively short cell cycle time, and phase II, from day 5 to day 9, reflected a relatively longer cell cycle time. This two phase pattern of growth is consistent with previous reports and has been shown to reflect a modulation of the liver phenotype which occurs during fetal/adult development or during liver regeneration (Kelly and Darlington, 1989; Dufresne et al., 1993). The pattern of cathepsin D activity paralleled the biphasic growth pattern in that it increased during phase I of growth, leveled off, then increased again during phase II of growth. This pattern of expression is similar to that previously reported for cathepsin B during HepG2 growth (Dufresne et al., 1993). Moreover, the increases in cathepsin D activity occurring during HepG2 growth are consistent with those reported for other developmental systems including monocyte-macrophage differentiation of HL-60 cells and mucinous differentiation of HT-29 colon carcinoma cells (Atkins and Troen, 1995; Huet et al., 1994; Rossman et al., 1990).

Levels of cathepsin D activity in medium collected and concentrated from exponentially growing HepG2 cells were reproducibly lower (i.e., 2-3 fold) than levels in the corresponding cell homogenates. It is interesting to note that relative to all the normal, differentiating, and malignant cell lines examined in this thesis research, HepG2 demonstrated the highest levels of cathepsin D activity (Table 3).
FIGURE 31

Levels of Cathepsin D Activity in Intracellular and Extracellular Fractions of HepG2 Cells During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. Each day after plating, representative cells were collected for assay and counted to form growth curves (closed circles). To obtain conditioned medium for examination of extracellular cathepsin D activity, cells in exponential growth were changed to serum free medium 24 hours before collection. This medium was concentrated as outlined in Methods 2.2.5. Intracellular (hatched bars) and extracellular (solid bar) cathepsin D activity was measured using hemoglobin as substrate and represents the activity in 100 µL of fraction minus the residual activity of a second 100 µL of fraction assayed in the presence of Pepstatin A. Enzyme Activity is expressed in mAson Units per mg protein (mAson Units/mg protein) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 umol of free tyrosine per minute.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Source</th>
<th>Species</th>
<th>Metastatic Potential</th>
<th>Differentiating</th>
<th>Intracellular Activity (mAU/mg)</th>
<th>Extracellular Activity (mAU/mg)</th>
<th>Ratio Intra-/Extracellular</th>
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</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>epithelial</td>
<td>liver</td>
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<td>yes</td>
<td>2245</td>
<td>618</td>
<td>3.6</td>
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<tr>
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<td>breast</td>
<td>human</td>
<td>low</td>
<td>no</td>
<td>2073</td>
<td>373</td>
<td>5.5</td>
</tr>
<tr>
<td>L6</td>
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<td>muscle</td>
<td>rat</td>
<td>N/A</td>
<td>yes</td>
<td>1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ZR75</td>
<td>epithelial</td>
<td>breast</td>
<td>human</td>
<td>low</td>
<td>no</td>
<td>870</td>
<td>245</td>
<td>3.5</td>
</tr>
<tr>
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<td>connective tissue</td>
<td>mouse</td>
<td>N/A</td>
<td>no</td>
<td>720</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HS578T</td>
<td>fibroblastic</td>
<td>breast</td>
<td>human</td>
<td>low</td>
<td>no</td>
<td>606</td>
<td>97</td>
<td>6.2</td>
</tr>
<tr>
<td>L6D3</td>
<td>myoblast</td>
<td>L6</td>
<td>rat</td>
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<td>no</td>
<td>480</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>lung</td>
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<td>low</td>
<td>no</td>
<td>468</td>
<td>200</td>
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</tr>
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<td>human</td>
<td>high</td>
<td>no</td>
<td>435</td>
<td>166</td>
<td>2.6</td>
</tr>
<tr>
<td>MDA-231</td>
<td>epithelial</td>
<td>breast</td>
<td>human</td>
<td>high</td>
<td>no</td>
<td>261</td>
<td>188</td>
<td>1.4</td>
</tr>
<tr>
<td>MDA-468</td>
<td>epithelial</td>
<td>breast</td>
<td>human</td>
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<td>no</td>
<td>230</td>
<td>49</td>
<td>4.7</td>
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<td>human</td>
<td>high</td>
<td>no</td>
<td>191</td>
<td>74</td>
<td>2.6</td>
</tr>
</tbody>
</table>
3.2.3B: The Major Peak of Cathepsin D Activity in Differentiating, Malignant HepG2 Cell and Medium Fractions Elutes at 34 kDa: Since HepG2 is both a differentiating and a malignant cell line, I was interested in determining the molecular mass positions of its cathepsin D activity using FPLC chromatography. In general, the molecular mass:cathepsin D activity associations in HepG2 resembled those observed in HPL-R malignant lung fibroblasts. FPLC analysis of homogenates prepared from HepG2 cells demonstrated two peaks of cathepsin D activity (Figure 32): a major peak at 34 kDa and a minor peak at 48 kDa. In contrast, a single 34 kDa peak of activity was detected in HepG2 medium (Figure 33). A summary of the molecular mass:cathepsin D activity associations for many of the differentiating and malignant cell lines examined in this thesis research is presented in Table 4.

3.3 The Biological Role of Cathepsin D

There is abundant and convincing evidence for the involvement of many cysteine, serine, metallo and aspartyl proteases -including cathepsin D- in tumor progression. In most, if not all, cases reported in the literature, this role is based on a direct relationship between increased levels of protease activity, increased tissue degradation, and consequently increased metastatic potential (Matrisian et al., 1995; Scaddan and Dufresne, 1995; Ren and Sloane, 1996; Lah et al., 1997). However, my results suggest that such a direct relationship may not explain the role of cathepsin D. These leads to an obvious question: How can decreased levels of cathepsin D activity
FIGURE 32

FPLC Separated Intracellular Cathepsin D Activity in HepG2 Cells

Centrifuged homogenates were prepared from HepG2 cells collected during early exponential growth. Approximately 200 ug was applied to, and eluted in 500 uL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (open circles) was determined using hemoglobin as substrate and represents the activity in 250 uL of fraction minus the residual activity of the second 250 uL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 umol of free tyrosine per minute.
FIGURE 33
FPLC Separated Extracellular Cathepsin D Activity in HepG2 Cells

Concentrated condition medium was prepared from HepG2 cells collected during early exponential growth. Approximately 200 μg was applied to, and eluted in 500 μL fractions from a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin D activity in each fraction (open circles) was determined using hemoglobin as substrate and represents the activity in 250 μL of fraction minus the residual activity of the second 250 μL of fraction in the presence of Pepstatin A. Enzyme Activity is expressed in Anson Units (Anson Units) where one Anson unit is defined as the amount of protease able to liberate, through degradation of hemoglobin, 1 umol of free tyrosine per minute.
Table 4. Apparent Molecular Masses of Cathepsin D Activities in Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Fraction</th>
<th>Cathepsin D Molecular Mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>Presumptive Cells</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Postmitotic Cells</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Fused Cells</td>
<td>34, 48</td>
</tr>
<tr>
<td>L6D3</td>
<td>Cells</td>
<td>48</td>
</tr>
<tr>
<td>LM</td>
<td>Cells</td>
<td>48</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Cells</td>
<td>34, 48, 52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>34, 52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HPL-R</td>
<td>Cells</td>
<td>34, 48</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>34</td>
</tr>
<tr>
<td>WI38</td>
<td>Cells</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>34, 52</td>
</tr>
<tr>
<td>HepG2</td>
<td>Cells</td>
<td>34, 48</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>34</td>
</tr>
</tbody>
</table>

<sup>a</sup> Laury-Kleintop et al., 1995.
<sup>b</sup> (data not shown)
contribute to increased metastatic potential? Given that: 1) tumor progression has been associated with increased secretion of inactive, procathepsin B (Scaddan and Dufresne, 1995; Sloane et al., 1997), 2) cathepsin D has been implicated in the intracellular maturation of procathepsin B (Nishimura et al., 1987; Nishimura et al., 1988; Nishimura et al., 1990), and 3) cathepsin D, while active at acidic pH, is stable at neutral pH (Baldwin et al., 1993), it is reasonable to predict that low levels of cathepsin D are sufficient to activate increasingly high levels of procathepsin B in the appropriate extracellular microenvironment. The experiments described next were designed to begin addressing this prediction.

3.3.1 Acid Activation of Latent Procathepsin B: Barret was the first to successfully use acid conditions for the maturation of latent procathepsin B to an active, measurable form in vitro (Barret, 1994; Barret and Kirschke, 1981). Since then, researchers have determined that the addition of pepsin, a gastric aspartyl protease, enhances maturation by cleavage of the propeptide (Barret, 1994). Since pepsin is added exogenously and is not native to any of the cell lines used in this and most other studies, my first approach to examining the role of cathepsin D was to determine: 1) whether the media of cell lines I used demonstrated acid activatable cathepsin B activity in the absence of pepsin, and 2) if so, whether inhibition of cathepsin D activity inhibited this activation. I examined these questions in two human cell lines that demonstrated contrasting levels of cathepsin D activity in their media: HepG2, which demonstrated high levels of cathepsin D in its medium, and HPL-R, which demonstrated low levels of cathepsin D in its medium.
3.3.1A: Media from HepG2 and HPL-R Cells Demonstrate Time-Dependent, Acid-Activatable Cathepsin B Activity: Media from exponentially growing HepG2 and HPL-R cells lines were collected, concentrated and measured for levels of acid activatable cathepsin B activity every 6 hours over a 24 hour period according to established procedures (Jane and Dufresne, 1994). The results presented in Figure 34 demonstrate a time-dependent increase in acid activatable cathepsin B activity for both cell lines examined. While this figure presents relative data, it is relevant to point out that at each time point examined, absolute levels of cathepsin B activity in HPL-R medium were approximately 4 fold greater than those observed in HepG2. No significant increase in activity was observed at non-acid pH. These results are consistent with a time-dependent, “inactive procathepsin B-to-active mature cathepsin B” conversion reported by other researchers (Jane and Dufresne, 1994; Scaddan and Dufresne, 1995).

3.3.1B: The Aspartyl Protease Inhibitor, Pepstatin, Decreases Levels of Acid-Activatable Cathepsin B Activity Detected in HepG2 and HPL-R Media: To determine what, if any, class of endogenous protease was contributing to the acid activation of procathepsin B in the media of HepG2 and HPL-R cells, media was incubated for 24 hours at pH 3.5 in the presence of protease class-specific commercial inhibitors. Three inhibitors were available: EDTA, which inhibits metalloprotease activity by chelating divalent cations, PMSF, which inhibits serine proteases by binding to their active site, and Pepstatin, which inhibits aspartyl proteases by binding to their active site (Beynon and Bond,
FIGURE 34

The Effects of Time on Acid Dependent Pro-Cathepsin B Enzyme Activation

Serum free conditioned medium was collected from cells during exponential growth. One hundred and fifty microlitres of media was combined with 30 μL cathepsin D buffer (pH=3.5) and incubated for 0, 6, 12, 18, or 24 hr. Subsequently, the samples were adjusted to pH 6.5 and assayed for cathepsin B activity (Methods 2.3.1). Concurrently, pepsin acid activated cathepsin B activity (total activity) was measured from fresh serum free conditioned media. Percent activation is defined as the amount of activity produced by acid activation divided by the total amount pepsin activated activity.
1989). The results presented in Figure 35 demonstrate a clear and significant decrease in acid-activatable cathepsin B activity in the presence of pepstatin, but not EDTA or PMSF. This activity was reproducibly reduced by 80-85% in HepG2 medium, and by 60-70% in HPL-R medium. In each cell line, the extent of pepstatin-inhibited, acid activatable cathepsin B activity was correlated to the level of cathepsin D in the medium. These results are consistent with a role for endogenous cathepsin D in the activation of procathepsin B in the medium of HepG2 and HPL-R.

3.3.1C: Medium from HepG2 Cells Increases Levels of Acid Activatable Cathepsin B Activity in the Medium of HPL-R

Cells: As indicated in Table 3 and Figure 34, HepG2 demonstrated high levels of cathepsin D and low levels of activatable cathepsin B activities in its medium. In contrast, HPL-R demonstrated low levels of cathepsin D and high levels of activatable cathepsin B in its medium. Given this complementarity and the results suggesting a role for cathepsin D (Figure 35), and to determine if cathepsin D endogenous to one cell type could affect the activation of procathepsin B secreted by another, I measured levels of activatable cathepsin B activity in HepG2 and HPL-R media alone, and in combinations of varying proportions (i.e., %HepG2:%HPL-R = 25:75, 50:50, 75:25). The results are presented in Figure 36. In this figure the y-axis label "Percent Above Predicted Combined Activity" refers to the difference between the activity measured in the combined medium samples and the predicted activity calculated by adding the two appropriate percent activities measured in each medium alone (see Figure Legend). For each combination tested, levels of
FIGURE 35
Effect of Commercial Class Specific Inhibitors on Acid Dependent Cathepsin B Pro-Enzyme Activation

Serum free conditioned media was collected from cells during exponential growth. One hundred and fifty microlitres of media was combined with 30 μL cathepsin D buffer (pH=3.5) and incubated for 24 hours with or without one of either Pepstatin A, PMSF or EDTA. The samples were then adjusted to pH 6.5 and assayed for cathepsin B activity (Methods 2.3.1) Percent inhibition is defined as the percentage of pro-cathepsin B activation inhibited by the protease class specific inhibitors Pepstatin A (solid bars), PMSF (hatched bars), and EDTA (open bars) and was calculated for HepG2 and HPL-R4.
FIGURE 36

Acid Activatable Activity in Combinations of HepG2 and HPL-R4

Serum free conditioned media was collected from HepG2 and HPL-R4 cells during exponential growth. Varying proportions (i.e., %HepG2:%HPL-R4 = 100:0, 75:25, 50:50, 25:75, 0:100) were combined to form 150 μL. Thirty microlitres of cathepsin D buffer (pH=3.5) was added to these combinations and incubated for 24 hr at 37°C. After incubation samples were adjusted to pH 6.5 and assayed for cathepsin B activity (Methods 2.3.1). Percent Above Predicted Combined Activity is defined as the difference between the activity measured in the combined medium samples and the predicted activity calculated by adding the two appropriate percent activities measured in each medium alone.
cathepsin B activity were greater than those predicted from levels in each medium, suggesting a synergistic effect. Moreover, the effect was related to the percent HPL-R medium in the combination such that 25% HepG2:75% HPL-R combination produced the greatest increase in cathepsin B activity.

3.3.1D: Activation of Latent Procathepsin B by Cathepsin D in HepG2:HPL-R Media Combinations is Inhibited by the Aspartyl Protease Inhibitor, Pepstatin: To confirm that activation of procathepsin B in HepG2: HPL-R media combinations was the result of endogenous cathepsin D activity, the combination experiment was repeated in the presence of the aspartyl protease inhibitor, pepstatin. The results of this experiment are presented in Figure 37. In each case, pepstatin inhibited cathepsin B activity by approximately 80%, a level comparable to that observed in HepG2 medium alone. Taken together, the results described in 3.3.1C and 3.3.1D suggest that endogenous cathepsin D in HepG2:HPL-R media combinations is responsible for the activation of procathepsin B, and that activation by cathepsin D is not cell or tissue type specific.

3.3.1E: Activation of Procathepsin B in HepG2 and HPL-R Media is Dependent on the Concentration of Purified, Commercial Cathepsin D: The results obtained in the combination experiments suggest that the activation of endogenous procathepsin B is related to the concentration of endogenous cathepsin D. The final experiment in this first approach to examine the role of cathepsin D, was to see if this relationship could be confirmed using purified cathepsin D. For this experiment, various concentrations of pure,
FIGURE 37

Effects of Pepstatin on Acid Dependent Cathepsin B Pro-Enzyme Activation in Combinations of HepG2 and HPL-R4

Serum free conditioned media was collected from HepG2 and HPL-R4 cells during exponential growth. Varying proportions (i.e., %HepG2:%HPL-R4 = 100:0, 75:25, 50:50, 25:75, 0:100) were combined to form 150 µL. Thirty microlitres of cathepsin D buffer (pH=3.5) was added to these combinations and incubated for 24 hr at 37°C. After incubation samples were adjusted to pH 6.5 and assayed for cathepsin B activity (Methods 2.3.1). Percent inhibition is defined as the percentage of pro-cathepsin B activation inhibited by the protease class specific inhibitor Pepstatin A.
bovine cathepsin D were incubated in with conditioned media from HepG2 and HPL-R cells at pH 3.5. The results, presented in Figure 38, demonstrate a cathepsin D concentration-related increase in levels of activatable cathepsin B activity in the medium of each cell line. Since the source of the purified cathepsin D in this study was bovine, these results not only confirm the involvement of cathepsin D in activation of procathepsin B, but suggest that it is not species specific. The initial prediction is once again supported.
Serum free conditioned media was collected from HepG2 and HPL-R4 cells during exponential growth. Increasing concentrations of Cathepsin D (mM) were added to these concentrated media. Thirty microlitres of cathepsin D buffer (pH=3.5) was added to these combinations and the mixtures incubated for 24 hr at 37°C. After incubation samples were adjusted to pH 6.5 and assayed for cathepsin B activity (Methods 2.3.1). Fold Activity is defined as the fold difference in acid activatable cathepsin B activity of samples with cathepsin D over acid controls.
Discussion

Cathepsin D is ubiquitously distributed in nearly every mammalian tissue. For this, and other reasons detailed in the introduction, it is reasonable to predict that its regulated expression plays a role in normal development and in maintenance of homeostasis. It is equally reasonable to predict that a breakdown in this regulated expression could have serious pathological consequences. Despite these predictions, essentially nothing is known concerning the role of cathepsin D in mammalian developmental processes, while contradictions exist concerning its role in pathological processes. My first approach to this problem was to examine the expression of cathepsin D in a model developmental process, myogenesis -the progression of undifferentiated muscle cells to differentiated ones, and in a model pathological process, tumorogenesis -the progression of lowly metastatic cancer cells to highly metastatic ones.

Differentiation of L6 rat myoblasts in culture is associated with alterations in the activities of non-lysosomal proteases, such as calcium activated neutral protease (CANP) (Kaur and Sanwal, 1981), and of lysosomal proteases, such as the cysteine proteases, cathepsin B and L (Jane and Dufresne, 1994). In general, these alterations involve increases that are related to the fusion of myoblasts to form myotubes. Reductions in the fusion ability of myoblasts negatively affects these fusion-related increases (Jane and Dufresne, 1994); conversely, reductions in the levels of protease activities in differentiating myoblasts negatively affects myoblast fusion (Gogos et al.,
1996; Jane et al., 1997). My results suggest that the expression of cathepsin D activity during differentiation of L6 rat myoblasts in culture is also fusion-related. Moreover, this expression parallels that reported for cathepsin B and L activities in the same system (Jane and Dufresne, 1994). Specifically, cathepsin D activity was initially high in cycling L6 myoblasts (i.e., “up”), then decreased as the majority of these cells exited the cell cycle, aligned and adhered in preparation for fusion (i.e., “down”), then increased as postmitotic myoblasts fused to form myotubes (i.e., “up”). This “up-down-up” pattern has been observed in a second myoblast system, C2C12 mouse myoblasts, (Jane et al., 1997), but was not observed in fusion-deficient L6D3 myoblasts or in LM fibroblasts. Moreover, increases in levels of activities after FPLC chromatography of presumptive, postmitotic and fused L6 myoblast fractions paralleled those observed in crude cell homogenates. Therefore, the changes in cathepsin D activity I observed during L6 differentiation are more likely to reflect the differentiation program (i.e., biologically significant) rather than the molecular heterogeneity of crude homogenates (i.e., artifact) (Kirschke et al., 1983; Lah et al., 1989; Chambers et al., 1992; Lah et al., 1992).

A precedent for the involvement of cathepsin D has been established in other cytodifferentiation systems. For example, in HT-29, a heterogeneous colon carcinoma cell line consisting of undifferentiated cells and a small proportion of mucous-secreting and columnar absorptive cells (Lesuffleur et al., 1990), selection for resistance to methotrexate resulted in a population of cells expressing differentiated phenotypes including mucous secretion and the synthesis of “brush border” dipeptidylpeptidase IV
hydrolase (Huet et al., 1994). Selection to the more differentiated state was accompanied by a 2-fold increase in levels of cathepsin D. The maturation of macrophages from monocyte precursor cells has also been associated with increases in levels of cathepsin D activity (Rossman et al., 1990). In this system, isolated human blood macrophages demonstrated a 33% increase in cathepsin D activity over peripheral blood monocytes.

Several mechanisms could explain alterations in levels of cathepsin D activity associated with cytodifferentiation in general, and with myoblast differentiation specifically. These include: 1) differences in the distribution of lysosomes and in the localization of cathepsin D containing vesicles (Portez et al., 1980), 2) differences in gene transcription, translation, and in post-translational processing, and 3) differences in targeting to lysosomes and prelysosomal endosomes (Bird et al., 1981; Collela et al., 1986; Nishimura et al., 1990). For example, U937 and HL-60 promonocyte cell lines can be stimulated to differentiate into monocytes/macrophages in culture by calcitriol (Stein et al., 1987). Differentiation in these cell lines is accompanied by an increase in cathepsin D mRNA synthesis. Since induction of gene transcription takes several days and requires protein synthesis, it has been suggested that cathepsin D induction is mediated by the differentiation program or by a second transcription factor (Atkins and Troen., 1995). The presence of E-box elements in the promoter region of the cathepsin D gene suggests that the MyoD family of muscle specific transcription factors could function in this latter capacity (detailed in the Introduction). Recent research in our laboratory, in collaboration with Bonnie Sloane’s, supports this
possibility for cathepsin B, a cysteine protease which is upregulated during the course of muscle cell differentiation (Jane and Dufresne, 1994; Jane et al., 1998a, and 1998b). The promoter of cathepsin B, like cathepsin D, contains E-box elements. Studies into the regulatory significance of E-box elements in cathepsin B should be extended to cathepsin D.

Analysis of the molecular masses associated with cathepsin D activity during L6 differentiation provides insight into the contribution of the various active forms, and into the possible contribution of other mechanisms to the “up-down-up” pattern of activity I observed in crude cell homogenates. After FPLC, presumptive, post-mitotic and fused myoblasts demonstrated a peak of cathepsin D activity at a position corresponding to an apparent molecular mass of 34 kDa. This mass is consistent with the highly processed, mature double chain, active form of the enzyme (Richo and Conner, 1994; Diment and Stahl, 1985). Fused myoblasts demonstrated a second, minor peak of activity at 48 kDa. This is consistent with a single chain active form of the enzyme (Richo and Conner, 1994). The demonstration of a single 48 kDa peak of cathepsin D activity at all stages of growth in LM fibroblasts is not surprising since processing to the 34 kDa form does not occur in this system (Richo and Conner, 1994). However, the demonstration of a single 48 kDa peak of activity in L6D3 is interesting, and together with the L6 results, suggests a role for the 34 kDa activity during L6 differentiation. Since the 34 kDa form is the only activity detected in presumptive and postmitotic myoblasts, it is reasonable to predict that this role occurs prior to and/or during fusion. Once noncycling myoblasts fuse, the need for the 34 kDa cathepsin D
activity would be expected to diminish. This diminished need could be realized by alterations in cathepsin D processing or metabolism. A decrease in levels of the 34 kDa form would not necessarily be accompanied by an alteration in overall cathepsin D activity since both the 34 kDa and 48 kDa forms of the protease are active. However, accumulation of the 52 kDa inactive proform and/or alterations in the cells metabolism leading to decreased synthesis or increased degradation of the 34 kDa and 48 kDa active forms would.

Toward examining these various mechanistic possibilities, it would be interesting to compare the levels of the 34 kDa and the 48 kDa activities in fused myoblasts over time. It would also be interesting to quantify levels of the various cathepsin D forms using Western Blot analysis, and to localize these forms during myogenesis using immunocytochemical techniques.

Finally, the appearance of the 48 kDa activity in fused myoblasts could reflect alterations in levels of cathepsin B activity. Several observations support this possibility (Rijnboult et al., 1991; Jane and Dufresne, 1994) First, the pattern of expression of cathepsin D activity I report parallels that reported by others. Second, the level of cathepsin B activity has been shown to decrease in fused myoblasts. Third, procathepsin D does not appear to undergo autoactivation in vivo. Fourth, the addition of cysteine protease inhibitors has been shown to prevent cathepsin D processing in vitro. Cathepsin D/cathepsin B colocalization studies, together with inclusion of the cathepsin B selective inhibitor, CAO74, in the activity/localization experiments suggested in the previous paragraph, should be informative in this regard.
Regardless of the mechanisms contributing to alterations in cathepsin D activity, it is worth restating that my results support a link between the regulated expression of cathepsin D and differentiation. Therefore, it is not surprising that alterations in this regulated expression have been associated with the cellular dedifferentiation that characterizes tumor progression (Capony et al., 1994). What is surprising is the obvious controversy concerning the expression of cathepsin D in cancer cells, the role of cathepsin D in tumor progression, and the models predicted to explain this role.

Studies conducted in the 1970s and 1980s on whole tissue samples suggested that, in general, metastatic tumors exhibited higher levels of protease activity than their non-metastatic tissue counterparts (reviewed in Ren and Sloane, 1996). Numerous proteases contributing to this increased activity have been identified including metalloproteases (e.g., type IV collagenase)(Stetler-Stevenson et al., 1990), serine proteases (e.g., plasminogen activator)(Dalet-Fumeron et al., 1993), cysteine proteases (e.g., cathepsin B and L)(Sloane et al., 1994), and more recently aspartyl proteases (i.e., cathepsin D) (Garcia et al., 1987). At the same time, studies in cultured cells suggested that these higher levels of activities were a consequence of increased synthesis and secretion of proteases by the most metastatic cells within the tumor population (reviewed in Ren and Sloane, 1996; Sloane et al., 1994). Among these studies was one reported by Rochefort examining levels of cathepsin D transcription during growth of the human breast cancer cell line, MCF-7 (Rochefort et al., 1987). At first glance, the results are clear and their significance obvious: synthesis and secretion of the 52 kDa proform of cathepsin D in MCF-7 cells is high in vitro, and
these high levels are directly involved in the degradation of extracellular matrix components in vivo (Briozzo et al., 1988; Rochefort, 1992). He further states that: "ER-negative (estrogen nonresponsive, breast cancer) cells constitutively overexpress Cath-D……" and suggests that this overexpression is related to the more aggressive, highly metastatic nature of these cells relative to ER positive cells (Sheikh et al., 1995). However, it should be pointed out that Rochefort's MCF-7 experiments were carried out in the presence of estrogen, a hormone that he acknowledges up-regulates the expression of cathepsin D in estrogen receptor (ER) positive cells (Rochefort et al., 1987). My results with estradiol and those of others using MCF-7 (Ratajczak et al., 1990) are consistent with estrogen-induced over-expression. Moreover, in a series of unrelated breast carcinoma cell lines with increasing invasive potential, other researchers have reported an apparent inverse relationship between the secreted concentration of the 52 kDa proform of cathepsin D and in vitro cell invasiveness (Johnson et al., 1993; Lah et al., 1996). My results are consistent with this latter possibility.

Specifically, in the MCF-7 -> MCF-7/Adr tumor progression model, selection for adriamycin resistance in vitro resulted in a complete loss of detectable estrogen receptor and a significant loss of cathepsin D activity in cell and medium fractions. This trend was paralleled in the four other unrelated breast cancer cells lines examined; levels of cathepsin D activity in cell and medium fractions prepared from lowly metastatic ZR75 and HS578T cells were significantly higher than those in highly metastatic MDA-231 and MDA-468 cells. It is interesting to note that of these four
cell lines, only ZR75 is estrogen receptor positive containing about half the receptor content of MCF-7. In contrast, MDA-231 and MDA-468, like MCF-7/AdrR, are estrogen receptor negative and may have lost this differentiated phenotype as a result of drug selection in vivo (i.e., patient treatment with adriamycin). Taken together, these results suggest that for adenocarcinomas of the breast, dedifferentiation to the highly malignant state is accompanied by a decrease in cathepsin D expression; these phenotypes, in turn, may be correlated to the loss of the estrogen receptor positive differentiated phenotype. [NOTE: HS578T must be excluded from this latter correlation since it was established from a sarcoma (i.e., fibroblast cell) rather than a carcinoma (i.e., epithelial cell) like the other breast cancer cell lines used in this study. Fibroblast cells do not express estrogen receptor.]

My results support an inverse relationship between metastatic potential and cathepsin D expression during progression from a lowly metastatic tumor to a highly metastatic one. It is reasonable to predict that similar inverse relationship could exist during the initial transformation from a normal cell to a malignant one in vivo. Unfortunately this prediction has been difficult to examine since there are no in vitro transformation model systems that complement tumor progression (see Introduction: 1.3). For example, there are no normal counterparts to any of the breast cancer cell lines used in research. The availability of normal human lung WI38 fibroblasts and the establishment of malignant human lung HPL-R fibroblasts in this laboratory provided "a next best" alternative model -one free of the potentially confounding effects of estrogen receptor. When I measured cathepsin D activity in cell and medium fractions
prepared from these two fibroblast cell lines, levels in normal WI38 were reproducibly 2-3 fold greater than those measured in malignant HPL-R. Interestingly, coordinate measurements of cathepsin B activity in these two cell lines demonstrated a contrasting trend. Specifically, levels of cathepsin B activity in fractions prepared from HPL-R were approximately 2 fold greater than those in WI38. This is consistent with the 5 fold increase in cathepsin B activity reported in the MCF-7 -> MCF-7/AdrR tumor progression model (Scadden and Dufresne, 1995), compared to the 5 fold decrease in cathepsin D activity I report. It is also consistent with trends reported in other model systems. Lah, for example, reported a 2 fold decrease in intracellular cathepsin D and a 3-4 fold increase in cathepsin B and L protein and activity levels in highly metastatic c-Ha-ras (oncogene) transfected MCF-10 breast cancer cells relative to controls (Lah et al., 1996). Taken together, the results of others and those I present for two related, lowly metastatic and highly metastatic MCF-7 breast cancer cell lines, four unrelated breast cancer cell lines with different metastatic potential, and for normal and metastatic lung fibroblast cells suggest: 1) that levels of cathepsin D are lowest in the most malignant cancer cells, and highest in normal cells, 2) that the expression of cathepsin D is down-regulated during malignant transformation and tumor progression, 3) that aspartyl (i.e., cathepsin D) and cysteine proteases (e.g., cathepsin B) are differentially expressed in cancer cell lines with highly invasive phenotypes, and consequently, 4) that the expression of different classes of proteases may be under different controls. These suggestions, in turn, challenge the Rochefort paradigm for the direct role of cathepsin D in tumor progression in vivo.
While there is controversy regarding the relationship between levels of cathepsin D and metastatic potential, it is generally agreed that the secreted form of cathepsin D plays a role in tumor progression. Moreover, since Rochefort's work (Rochefort et al., 1987), there has been evidence suggesting that cancer cells secrete exclusively the inactive 52 kDa proform of the protease. However, most if not all of these investigations have been done in inducer-stimulated cells and/or using anti-cathepsin D antibody that recognizes both pro- and mature forms of the enzyme (Rochefort, 1990; Johnson et al., 1993). This raises questions concerning the exclusivity of the proform, and consequently the mechanisms underlying secretion in cancer cells. For example, since maturation of cathepsin D takes place primarily in prelysosomal (endosomal) and lysosomal compartments, secretion of only the 52 kDa proform would suggest a disruption in the lysosomal targeting and/or processing mechanisms. Gabel provides support for such a mechanism and suggests it reflects an inefficient interaction between lysosomal precursors and mannose-6-phosphate receptors in the trans Golgi network (Gabel et al., 1983). On the other hand, secretion of the 48 kDa and/or 34 kDa mature forms of cathepsin D would suggest localization of the processed forms of cathepsin D to the membrane by means of lysosomal vesicles, followed by secretion. My analysis of the molecular masses associated with cathepsin D protein/activity in the medium of cells using FPLC chromatography provides support for this mechanism. In all the malignant cell lines I examined, both intracellular and extracellular cell fractions demonstrated cathepsin D activity at positions corresponding to molecular masses of 48 kDa and 34 kDa, consistent with the
mature single chain and highly processed double chain forms respectively. Moreover, based on the area of each peak, the 34 kDa form appears to be the dominant one. Interestingly, the only time I detected activity at the 52 kDa proform position was in preliminary experiments in estrogen-stimulated MCF-7 breast cancer cells (data not presented) and in experiments reported in this thesis in WI38 normal lung fibroblasts. It can be argued that detection of the active forms of cathepsin D in my experiments reflects processing of the proform after secretion and prior to or during FPLC chromatography. Two lines of evidence argue against this possibility. First, the pH of medium, 7.4, does not support activation (i.e., 3.5). Second, acid activation of the fractions results in a decrease, rather than an increase, in cathepsin D activity over time. Still, coordinate FPLC and immunoprecipitation analyses in the presence of appropriate protease inhibitors would confirm this. Taken together, these results suggest: 1) that secretion of the 52 kDa proform by some cancer cells may be the consequence of estrogen treatment, 2) that secretion of the 52 kDa proform occurs in normal cells, 3) that many cancer cells secrete the fully processed 34 kDa form of cathepsin D, and consequently 4) that lysosomes play an active role in both the processing and secretion of cathepsin D from many cancer cells.

Finally, there is considerable evidence that under the same conditions I used to detect the highly processed 34 kDa secreted form of cathepsin D, the cysteine proteases, cathepsins B and L, are secreted as high molecular mass proforms (Jane and Dufresne, 1994; Scaddan and Dufresne, 1995; Jane et al., 1997). This, together with the intracellular and extracellular activity data, suggests that the extracellular
environment of these cells *in vivo* would consist of a "protease cocktail" consisting of low levels of the fully processed active form of cathepsin D and high levels of the unprocessed inactive forms of other proteases such as cathepsin B. It is my prediction that under the appropriate acidic microenvironment these low levels of cathepsin D are sufficient to initiate activation of the proform pool. According to this prediction, cathepsin D affects metastatic potential indirectly by increasing the activity of other proteases which then degrade extracellular matrix components, rather than directly, as Rochefort suggests, by degrading extracellular matrix components on their own. Does the extracellular environment *in vivo* permit activation by cathepsin D?

Cathepsin D has a pH optimum of 3.5 and demonstrates no activity above pH 5.5 (results not shown). Consequently, it is not surprising that the digestion of the extracellular matrix, which was believed to occur at physiological pH (e.g., 6.8 - 7.4), was first thought to be initiated by neutral proteases such as urokinase-type plasminogen activator, and metallo-proteinases acting at the plasma membrane level (Dano *et al.*, 1985; Liotta *et al.*, 1991). Tumors however are acidic in nature and there is increasing evidence that this reflects a decreased extracellular pH brought about by various mechanisms including the migration of large acidic vesicles (LAVs) to the surface of the cell, the action of H⁺-ATPase pumps, and the secretion of lactic acid into the extracellular medium (Baron *et al.*, 1985; Silver *et al.*, 1988; Griffiths, 1991, Mountcourrier *et al.*, 1994). This acidic environment would favor the involvement of acidic proteases rather than neutral ones in tissue degradation *in vivo*. *In vitro* enzyme inhibition studies not only confirm this possibility but show that inhibition of cathepsin
B by anti-human cathepsin B and inhibition of cathepsin D by pepstatin reduce degradation in acidified extracellular medium by 21% and 74% respectively (Young and Spevacek, 1993). Taken together, these results suggest that the acidic extracellular environment of cancer cells is suitable for proteolytic activity by acidic proteases such as cathepsin B and cathepsin D. But how could the low extracellular levels of cathepsin D activity I observe contribute significantly to the increased proteolytic activity associated with the metastatic phenotype?

Both direct and indirect mechanisms have been hypothesized to explain the role of cathepsin D in tumor progression. In general, direct mechanisms predict the involvement of secreted, active or activatable cathepsin D in remodeling of the extracellular matrix. This prediction is based largely on increased expression and secretion of the cathepsin D in the most malignant cells (Rochefort et al., 1987; Briozzo et al., 1988; Rochefort, 1990). Indirect mechanisms, on the other hand, predict the involvement of secreted, active or activatable cathepsin D in increasing the activity of other metallo-, serine- and cysteine proteases whose activities are directly related to metastatic potential. This could occur by the cathepsin D-mediated degradation of inhibitors of these proteases in the extracellular environment, for example the cystatin-like inhibitors of cysteine proteases (Lenarcic et al., 1988). However, studies in vivo (Lah et al., 1996) and in vitro (Scaddan and Dufresne, 1995) provide convincing evidence that alterations in levels of endogenous protease inhibitors are not sufficient to explain the increases in extracellular levels of protease activities associated with increased metastatic potential. An increase in the activity of other
proteases could also occur by the activation of secreted, inactive proforms of these proteases (Eeckhout and Vaes, 1977; Nishimura et al., 1990; Kobayashi et al., 1991). My results are consistent with this latter possibility. They also identify procathepsin B in the extracellular environment as a substrate for cathepsin D.

Acidification of media from HepG2 (a low metastatic, differentiating human cell that secretes relatively low levels of procathepsin B and high levels of cathepsin D) and from HPL-R, (a highly metastatic, undifferentiated human cell line that secretes relatively high levels of procathepsin B and low levels of cathepsin D) was sufficient to activate latent procathepsin B. Addition of commercial inhibitors against serine and metallo proteases had no affect on this activation. In contrast, addition of the cathepsin D inhibitor, pepstatin reduced activation in HepG2 and HPL-R by 80-90 % and 50-60%, respectively. Interestingly, inhibition of procathepsin B activation by pepstatin was reproducibly greatest in medium from HepG2, the cell line with the highest levels of cathepsin D activity. Activation of procathepsin B was also observed when the media from HepG2 and HPL-R were combined in different proportions, a situation that more closely reflects the heterogeneity of malignant phenotypes in vivo. In general, levels of activation in combined medium samples were greater than the predicted additive levels calculated from single medium (i.e., HPL-R or HepG2 media) samples. Once again, activation was specifically inhibited by the addition of the cathepsin D inhibitor, pepstatin.

The medium activation experiments not only support a role for cathepsin D in the activation of procathepsin B, but suggest that cells with different metastatic
potentials can interact to enhance this activation, and ultimately to further increase the metastatic potential of the tumor. If this is the case, and I suspect it is, then the protease contribution of the cancer cell's neighbor becomes just as important as the contribution of the cancer cell itself. Normal cells are not excluded from this consideration. Stromal fibroblasts, for example, express many of the more common proteases associated with tumor progression, as well the specific protease, stromelysin-3. A direct relationship between stromelysin-3 activity in tumors and metastatic potential has been reported (Linder et al., 1997; Tetu et al., 1993). This relationship can be linked to cathepsin D if a cathepsin D-initiated proteolytic cascade is considered (Figure 39). The initial step in this cascade, the cathepsin D-initiated activation of procathepsin B, has already been discussed. Active cathepsin B can then contribute to its own activation (i.e., autoactivation) (Rowan et al., 1992), or to that of other proteases such as procollagenase IV (Murphey et al., 1992) and pro-urikinase type plasminogen activator (pro-uPa) (Kobayashi et al., 1991). Finally, active uPa is known to convert plasminogen to plasmin, a potent activator of a number of prometalloproteases, including procollagenase, progelatinase B, and prostromelysin.

Thus, it is possible for low levels of cathepsin D in an acidic extracellular environment to initiate a complex, proteolytic cascade that ultimately leads to the extensive matrix remodeling associated with highly malignant tumors. In view of this, I suspect that the controlled expression of cathepsin D will be similarly linked to the extensive matrix remodeling associated with normal cytodifferentiation processes such as myogenesis. Time and research will tell. But one point is immediately apparent
FIGURE 39

Model for a Role for Cathepsin D in Invasion and Metastasis

The initial step in this cascade is the cathepsin D-initiated activation of procathepsin B. The source for this cathepsin D in the extracellular environment may be both cancer cells and normal cells in and around the tumor. Active cathepsin B can then contribute to its own activation (i.e., autoactivation) (Rowan et al., 1992), or to that of other proteases such as procollagenase IV (Murphey et al., 1992) and prourikinase type plasminogen activator (pro-uPa) (Kobayashi et al., 1991). Once active uPa is known to convert plasminogen to plasmin, a potent activator of a number of prometalloproteases, including procollagenase, progelatinase B, and prostromelysin. Thus, it is possible for low levels of cathepsin D in an acidic extracellular environment to initiate a complex, proteolytic cascade that ultimately leads to the extensive matrix remodeling associated with highly malignant tumors.
from my research: both the progression to the fully differentiated state and the
progression to the fully malignantly transformed state are associated with the
progressive acquisition of phenotypes. During differentiation, one of these phenotypes
is an increase in cathepsin D activity; whereas during malignant transformation, one of
these phenotypes is a decrease in cathepsin D activity. Both types of alterations are
likely to have functional significance.
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