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EXPRESSION OF LYSOSOMAL CYSTEINE PROTEASE ACTIVITIES IN DIFFERENTIATING HEPATOCYTE AND MYOBLAST CELLS IN CULTURE

by

Derek Thomas Jane

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

1993
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Abstract

The expression of the lysosomal cysteine proteases, cathepsins B, H and L and cysteine protease inhibitors in intracellular and extracellular (media) fractions prepared from the differentiating malignantly transformed human hepatoma cell line HepG2, the differentiating rat myoblast cell line L6, and the L6-D3 non-fusing variant, were examined using synthetic substrates. HepG2 cells were established in serum-free medium for greater than 100 generations and demonstrated biological (e.g., growth characteristics) and biochemical (e.g., the synthesis and secretion of apolipoprotein B and cathepsin B) parameters similar to their serum-grown counterparts. These serum-free culture conditions also supported the maintenance of L6 and L6-D3 rat myoblasts without affecting their growth and differentiating properties. HepG2 cells expressed high constant levels of intracellular cathepsins B and L activity as well as a growth related increase in the secretion of latent activatable cathepsin L. L6 differentiating myoblasts demonstrated high levels of intracellular cathepsins B and L activities, and low levels of intracellular cathepsin H activity. L6 cells also exhibited low levels of latent activatable cathepsin activity. There was a fusion related increase in the intracellular activity of all three cathepsins as well as a fusion related increase in extracellular latent cathepsin L activity. Fusion was not a prerequisite for cathepsin expression as the L6-D3 non-fusing variant demonstrated high constant levels of intracellular cathepsins B and L activities, low levels of cathepsin H activity and high constant levels of extracellular latent cathepsin L activity during phase growth. Since HepG2 and L6-D3 exhibited a similar pattern of cathepsin expression, this suggests that
L6-D3 demonstrate malignant transformation rather than differentiation. Although L6-D3 demonstrated levels of cathepsin B significantly higher than L6, the ratio of cathepsin B activity to cathepsin B inhibitory activity as well as the level of cathepsin B inhibitory activity remained constant during growth. In contrast there was a three-fold increase in the ratio of cathepsin B enzyme activity to cathepsin B inhibitor activity during fusion of L6 cells. This does not reflect alterations in the total cysteine protease inhibitor activity as heat-treated fractions did not demonstrate altered levels of cathepsin B inhibition. These results support a role for intracellular and extracellular activity of cysteine proteases during growth and differentiation of L6 and HepG2 cells.
to my parents
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Firstly, I would like to thank my parents for their support and patience during these last three years.

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<tbody>
<tr>
<td>Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>AdriR</td>
<td>Adriamycin resistant</td>
</tr>
<tr>
<td>AHH</td>
<td>aryl hydrocarbon hydroxylase</td>
</tr>
<tr>
<td>alpha MEM</td>
<td>alpha modification of Eagle's minimal medium</td>
</tr>
<tr>
<td>ApoB</td>
<td>apolipoprotein B100</td>
</tr>
<tr>
<td>AzaR</td>
<td>5-azacytidine resistant</td>
</tr>
<tr>
<td>Cbz-arg-arg</td>
<td>Nα-CBZ-argininyl-argininyl 4-methoxy-β-napthylamide</td>
</tr>
<tr>
<td>auto-nano-water</td>
<td>autoclaved nanopure water</td>
</tr>
<tr>
<td>B[a]P</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>βME</td>
<td>beta-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBZ</td>
<td>carboxybenzyl</td>
</tr>
<tr>
<td>CP</td>
<td>cysteine protease</td>
</tr>
<tr>
<td>CPI</td>
<td>cysteine protease inhibitor</td>
</tr>
<tr>
<td>CURL</td>
<td>compartment for uncoupling of receptor and ligand</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DEA</td>
<td>diethanolamine</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>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</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>E.O.P.</td>
<td>efficiency of plating</td>
</tr>
<tr>
<td>EU</td>
<td>enzyme unit</td>
</tr>
<tr>
<td>ExtrAvidin-ALP</td>
<td>ExtrAvidine-alkaline phosphate</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>gpt</td>
<td>guanine phosphotransferase</td>
</tr>
<tr>
<td>Hep</td>
<td>human hepatoma</td>
</tr>
<tr>
<td>KIU</td>
<td>kallikrien inactivating unit</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>$K_{cat}$</td>
<td>enzymatic rate constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant</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>Mb</td>
<td>Myoblast</td>
</tr>
<tr>
<td>MCF</td>
<td>Michigan Cancer Foundation</td>
</tr>
<tr>
<td>MEPr</td>
<td>metalloprotease</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>myoD</td>
<td>myogenesis determinant gene</td>
</tr>
</tbody>
</table>
myf  myogenesis factor
min  minute
M.W.  molecular weight
n.d.  not detected
PAH  polycyclic aromatic hydrocarbon
PBS  phosphate buffered saline
PMSF  phenylmethylsulfonfylfluoride
PNPP  para-nitrophenylphosphate
ras  rat sarcoma
RER  rough endoplasmic reticulum
RNA  ribonucleic acid
S.D.  saturation density
SDS-PAGE  sodium dodecylsulfate polyacrylamide gel electrophoresis
TCDD  2,3,7,8-tetrachlorodibenzo-p-dioxin
TD  population doubling time
Tween 20  polyoxyethylene-sorbitan
UV  ultraviolet
Z-arg-arg-NMec  Nα-CBZ-argininyl-argininyl 7-amido-4 methylcoumarin.HCl
Z-arg-NMec  N-α-CBZ-L-argininyl-7-amido-4-methylcoumarin. HCl
Z-phe-arg-NMec  N-CBZ-L-phenylalanyl-L-argininyl-7-amido-4-methylcoumarin.HCl
Introduction

1.1 Historical Perspective

The goal of cell differentiation and embryology, a discipline founded by Aristotle in the fourth century B.C., is to understand the processes by means of which the genes direct the formation of cellular structures and components in the successive cycles of cell growth and division. The first coherent theory of cell differentiation was developed by Weismann during the 1890s when he elaborated the chromosomal theory of inheritance that led to the subsequent rediscovery of Mendel’s laws. Weismann proposed that cell differentiation was the consequence of an unequal partition of the hereditary determinants in successive cell divisions, that is to say, the consequence of nuclear differentiation which was, in turn, the consequence of a progressive loss of parts of the parental genome. Although classical genetics demonstrated in the first part of the 20th century that every somatic cell has exactly the same chromosome complement, no real critical evidence against nuclear differentiation was reported until the 1960s when nucleus transplantation techniques were developed. Using these techniques, J.B. Gurdon was able to show that normal adult frogs could be obtained from frog eggs into which the nuclei of differentiated cells of the tadpole intestine had been transplanted. These elegant studies demonstrated that the nucleus of the differentiated intestine cell still carried all the genes necessary for instructing the frog egg how to produce a whole frog. Thus it was concluded that cell differentiation is not the consequence of a permanent change in the character of the cell genome, that is nuclear differentiation, but must instead derive from a differential expression of the
genes embodied by that genome. The focus of subsequent research to explain differentiation, at the cellular and organismic levels, consequently shifted to understanding the mechanisms of the regulation of gene function.

1.2 Muscle Development: Myogenesis

Much of the initial research concerning gene regulation was conducted in prokaryotes and simple eukaryotes. Their small size, simple structure, minimal growth requirements, and relatively short generation times permitted biochemical and genetic manipulations. Technological advancements and establishment of reliable cell and tissue culture techniques in the late 1960s, permitted such analyses to be extended to the more complex avian and mammalian cytodifferentiating systems (Freshney, 1987). Of these, myogenesis, the differentiation of progenitor skeletal muscle cells into nondividing muscle fibers, is arguably the most intensely researched (reviewed in Stockdale, 1992).

All skeletal muscle in the body is derived from the somites or, in the case of some head muscles, from the prechordal plate which has a similar mesodermal origin (Buckingham, et al., 1992). The first somites appear at about 8 days in the mouse embryo as a result of segmentation of the paraxial mesoderm, and resemble balls of epithelial-like cells. These rapidly differentiate into dermomyotome and sclerotome, which contribute mainly to skin and muscle, and skeletal structures, respectively. At this stage, from avian and murine studies, precursor cells appear to migrate from the dermomyotome, probably from the ventral, lateral edge, to found premuscle masses in the limbs and elsewhere. The cells that migrate are already determined in the sense that they only form muscle and no muscle forms in their absence. The myotome, the first
skeletal muscle to form in the embryo, forms in the central region of the somite as a result of migration of cells from the dermomyotome, principally from the cranial/dorsal edge adjacent to the neural tube.

In the early embryo, muscle masses do not show fiber type specialization. Rather, muscle genes are expressed uniformly in any given muscle at any given time. On the other hand, in the fetus, when muscle fibers are in general innervated by numerous neurons, fiber type specialization can already be detected in muscle masses in terms of muscle gene expression. This partly reflects the presence of secondary fibers which may result from the fusion of a population of secondary myoblasts that proliferate rapidly, perhaps in response to neuronal or humoral factors (Miller, 1991). Secondary (2°) fibers can be distinguished from pre-existing primary (1°) fibers on the basis of their morphology and their patterns of myosin gene expression (Figure 1).

The adult muscle fiber types are established in the perinatal period and their phenotypes depend directly on the motor neuron that contacts the muscle and on thyroid hormone, although the intracellular mechanisms of these contributions is not yet understood. Adult skeletal muscle is a highly specialized tissue composed of differentiated muscle fibers containing organized arrays of muscle sarcomeres. Different fiber types, within the same or different muscles, have different contractile properties, reflecting the fact that their sarcomeres contain different isoforms of myosin together with different isoform combinations of other structural and soluble muscle proteins (Miller, 1991). These isoforms, generated either from separate genes or by differential splicing of the same gene, reflect products of multigene families.

Expression of different members of multigene families begins at different times,
Figure 1. Representation of Myogenic Lineage During Higher Vertebrate Muscle Development

Embryonic myoblasts in developing axial and appendicular muscles of higher vertebrates predominate in the embryo until morphogenesis of the limbs is fully complete. Fetal myoblasts predominate in the muscles of the fetus and undergo marked growth through new fiber formation (secondary fiber formation), and increase in girth, length, and nucleation of existing fibers. The transition from embryonic to fetal myoblasts coincides with innervation of the limb. Adult myoblasts, important in fiber growth and repair, appear in the limb muscles at mid fetal life and are the predominate myoblast type prior to hatching or birth. Within each of these three categories in mammals and birds there can be subtypes of myoblasts that in vitro form only specific fiber types.
modified from Stockdale, 1992

FIGURE 1
and subsequent changes in their expression occur independently for each gene (Buckingham et al., 1992). Among the earliest muscle genes to be expressed in the myotome are the desmin, titin, skeletal α-actin, and cardiac α-actin, the major actin isoform at this stage, genes. In the mouse, these genes are expressed at least 12 hours and 24 hours, respectively, before the first myosin heavy chains (MHC_{emb}). Expression of the fetal heavy chain myosin isoform (MHC_{ph}) begins soon after and continues throughout embryonic and fetal development. At different times within the fetal stage of development, most isoforms of the major structural genes (i.e., actin, myosin heavy chain, myosin light chain, and creatine phosphokinase) are expressed in muscle. Some of these (i.e., skeletal α-actin, myosin light chains: MCL1_{F}, MCL3_{F}, MCF1_{F}, and creatine phosphokinase, M-CK) continue to be expressed after birth. Adult myosin heavy chain (MHC_{IIB}), on the other hand appears to be expressed only after birth. Although these results reflect studies in murine systems, the emerging consensus for avian and mammalian systems in general is that: a) the definitive phenotype of an adult skeletal muscle fiber type (i.e. the number and types of isoforms it has) is the result of events that begin in the embryo and are continuously modulated and redefined throughout the life of the organism; and b) the formation of myofibrils from progenitor myoblasts, and hence of potentially functional muscle, at any stage, depends on the expression of the appropriate genes from each multigene family pool.

1.3 Regulation of Myogenesis

A. Myotube Formation

In the process of skeletal muscle differentiation at each stage of development, some
cells divide yielding replicating myogenic cells which retain the capacity to differentiate (presumptive myoblasts), while others (postmitotic myoblasts) undergo a series of changes leading to their fusion into syncytial myotubes which are irreversibly withdrawn from the cell cycle (Yaffe, 1971; Yeoh and Holtzer, 1977). The fusion of postmitotic myoblasts is preceded by cell alignment, cell recognition, and cell adhesion (Nameroff and Manor, 1976; Dufresne et al., 1976; Knudsen and Horwitz, 1977; Wakelam, 1988). It is achieved by the union of myoblast lipid bilayers involving modifications of the lipid content and of the fluidity of the plasma membrane (Kalderon and Gilula, 1977; Prives and Shinitzky, 1977; Wakelam, 1988). Two adhesion systems coexisting at the surface of myoblasts are implicated in myogenesis (Gibraltar and Turner, 1985). One is Ca\(^{2+}\) independent (e.g., N-CAM) (Dickson et al., 1990; Knudsen et al., 1990a); the other is Ca\(^{2+}\) dependent (e.g., N-cadherin) (Knudsen et al., 1990b). Both N-CAM and N-cadherin belong to the CAM (cell adhesion molecules) class of cell surface glycoproteins mediating cell-cell adhesion (Anderson, 1990). N-CAM is expressed in somites and in myotomes (Thiery et al., 1982), then on myoblasts, myotubes, and muscle fibers until innervation (Rieger et al., 1985). N-cadherin belongs to the cadherin family, a class of structurally related CAMs mediating calcium dependent, homophilic cell adhesion (Takeichi, 1988). In early embryogenesis, N-cadherin is expressed in the ectoderm that will give rise to the neural plate in response to neural induction (Hatta and Takeichi, 1986; Duband et al., 1988). It is also expressed in somites formed by the condensation of the mesenchymal cells of the mesoderm (Duband et al., 1987) and persists in myotomes and in embryonic muscles where it mediates myoblast fusion (Hatta et al., 1987; Duband et al., 1987). Although
N-CAM and N-cadherin coaccumulate at the area of contact between fusing myogenic cells, there is evidence that they may act independently in the cascade of events leading to myotube formation. For example, inhibition of N-CAM with monoclonal antibodies had no effect on N-cadherin-mediated myotube formation of chicken myoblasts (Mege et al., 1992).

Several lines of evidence point to the role of other molecules as part of the signaling mechanism in myotube formation. Extracellular matrix molecules, for example, appear to be important since: a) coating tissue culture dishes with collagen enhanced the differentiation of primary avian myoblasts (De La Haba et al., 1975); b) treating myoblasts with antibody to cell surface matrix receptors inhibited fusion (Menko and Boettiger, 1987); and c) addition of exogenous fibronectin decreased fusion (Podleski et al., 1979). Procollagen synthesis and/or processing in skeletal myoblasts also appears to be needed as an early event in myogenesis since treatment of cultured myoblast cells before alignment with ethyl-3,4-dihydroxybenzoate, a specific inhibitor of procollagen synthesis, decreased the production of a collagen-binding glycoprotein (gp46) present in the endoplasmic reticulum, and myotube formation (Nandan, et al., 1990).

Insulin also appears to be needed for myotube formation; however this need may reflect insulin breakdown since inhibition of insulin degrading enzyme (IDE)-mediated catabolism of insulin inhibited myotube formation in primary and immortal L6 skeletal muscle cells (Couch and Strittmatter, 1983; Kayalar et al., 1989). These types of results led to the hypothesis that insulin fragments act as inducers of myotube formation while intact insulin acts as a repressor (Kayalar et al., 1989).
B. Expression of Muscle-Specific Proteins

Myotube formation at each stage of skeletal muscle development is accompanied by variable alterations in the expression of muscle-specific regulatory and structural genes (Nameroff and Manor, 1976; Dufresne et al., 1976). The challenge of determining the complex regulation of expression of these genes was first addressed in whole animal tissue studies and subsequently complemented with those in mortal and immortal, homogeneous myoblast cell lines (Figure 2). It is not clear what physiological factors cause different genes to be activated at different times. However, the data suggest that both intrinsic and extrinsic factors appear to contribute to the "continuum" of fiber phenotypes expressed, including the types of myoblasts within and between the different developmental stages (i.e., embryonic, fetal, and adult), innervation, work load, hormones, and immediate environmental factors (Pette and Staron, 1990; Sutherland et al., 1991). Although fiber type, fiber function, and patterns of fiber distribution are largely dependent on the expression of fast and slow isoforms of MHC within a fiber, it is generally agreed that mechanisms for regulation of patterns of expression of muscle proteins other than MHC are important because those mechanisms must, in some fashion, be integrated into the overriding pattern of fiber type distribution that characterizes every muscle at each stage of its development (Sutherland et al., 1991). The recent isolation of the MyoD family of myogenesis determinant genes has provided a breakthrough in our understanding of myoblast growth and division, myotube formation, and expression of muscle specific proteins.

Four muscle-specific, nuclear transcription factors, MyoD1, myogenin, Myf-5, and Myf-6 (also known as MRF-4 and herculin), are expressed in myoblasts and
FIGURE 2

Evolution of a Cell Line

The vertical axis represents total cell growth (assuming no reduction in cell number at each passage); the horizontal axis represents time (weeks) in culture for a hypothetical cell culture. Although an immortal cell line is depicted as arising at 12½ weeks, times for transformation, as well as times for senescence, vary with the type of cell.

FIGURE 2

Total Cell Yield (Theoretical)

Weeks in Culture
skeletal muscle. These factors have the ability to activate myogenisis in nondifferentiating cells (e.g., transfected C3H-10T1/2 cells) and appear to regulate muscle differentiation by binding to a **CANNTG** consensus sequence (the E box) in the promoters or enhancers of many muscle genes (Olson, 1990; Weintraub et al., 1991). The MyoD family of nuclear transcription factors share a basic DNA-binding motif and a helix-loop-helix (HLH) dimerization domain. As members of the HLH superfamily of transcriptional regulators, they can potentially form homodimers and heterodimers with each other and with other members of this family. In the case of the muscle creatine phosphokinase (M-CK) gene, binding is more efficient when the myogenic proteins forms a heterodimer with the universal proteins E12 or E47, products of the E2A gene. In muscle cell cultures, this is the preferred combination (Weintraub et al., 1991).

Members of the MyoD family of proteins act as transcriptional activators (Braun et al., 1990; Weintraub et al., 1991). In muscle cell culture systems, different muscle-specific structural genes appear to be activated similarly by the products of the four myogenic regulatory genes. However, the expression of these regulatory genes, while restricted to skeletal muscle cells, appears to differ both within a muscle cell line and between muscle cell lines of different origins (Olson, 1990). For example, high levels of myoD mRNA transcripts are present in the myoblasts of several mouse lines. Other muscle cell lines do not contain myoD transcripts, and these lines tend to contain more myf-5 transcripts. Myogenin transcripts are always expressed when muscle cells differentiate, and myf-6 transcripts usually accumulate only in older differentiated
cultures at a time when structural genes characteristic of more mature fibers are activated (Montarras et al., 1991).

The expression of myogenic determinant genes has also been examined during development in vivo. Each myogenic gene has a different pattern of expression in the mouse embryo and this differs between myotomes and premyotome masses in the limb. The only myogenic transcript detectable in the mouse embryo somite before myotome formation is myf-5 mRNA. Its expression in the epithelial-like cells of the somite as it forms suggests that it may be involved in a determination step that directs precursor cells to the myogenic lineage. In late fetal skeletal muscle all three transcripts are abundant (Bober et al., 1991; Hinterberger et al., 1991) while the major myogenic transcript in postnatal muscles is myf-6 suggesting that it plays a role in the maintenance of muscle gene transcription (Rhodes and Konieczny, 1989). Myogenin and myoD transcripts are also detectable, but at low levels.

It has been suggested that the differential expression of myogenic determinant genes may reflect negative regulation by another category of HLH muscle regulators which do not have a DNA binding region. These proteins, exemplified by the protein Id, will form heterodimers with E12, for example, and prevent E12 from binding to the myogenic regulators (Weintraub et al., 1991). This ultimately reduces the activation efficiency of these regulators. This is not the only molecular explanation for differential expression of regulator activity during myogenesis. For example, the protooncogene c-fos, implicated in the regulation of cell proliferation and differentiation, as well as activated forms of ras and jun, are reported to inhibit muscle differentiation by inhibiting expression of myoD1 (Olson et al., 1987; Lasser et al.,
1989a & 1989b). This suggests that nuclear proteins other than Id may act as negative regulators.

An additional molecular explanation for differential expression of myogenic genes reflects the finding that the MyoD family of factors can be post-translationally phosphorylated. Since transcription of serum-inducible genes occurs within minutes of stimulation of cell surface receptors for growth factors (Angel et al., 1988; Muller et al., 1984), it is likely that growth factor dependent, intracellular signal transduction pathways lead to the phosphorylation of transcription factors by various kinases. Inhibitor studies have been conducted to examine the role of specific phosphatases involved in the signal transduction pathway leading to terminal differentiation of skeletal muscle cells. In one such study, Okadaic acid, an inhibitor of type 1 and type 2A phosphatases, was used to enhance phosphorylation by allowing the unopposed activity of protein kinases constitutively present in the cell. This treatment inhibited skeletal muscle cell differentiation and the expression of myoD1, but induced expression of the Id negative regulator (Kim et al., 1992). Sustained phosphorylation of a specific site on Histone H1 has also been shown to block growth factor-induced differentiation of C2 mouse myoblasts, presumably by interfering with the binding of nuclear factor to its response element. Together, these data suggest that phosphorylation may act as a negative regulator of myoblast differentiation and may permit modulation of function by growth factors and second messenger pathways (Olson, 1990; Weintraub et al., 1991).

The MyoD family of transcriptional factors bind to the E box element. Another DNA regulatory sequence, CC(A/T)\textsubscript{6}CG, (the CArG box) has been identified in a
number of muscle-specific genes including cardiac and skeletal muscle α-actins (Minty and Kedes, 1986), dystrophin (Klamut et al., 1990), and myosin light chain (Ernst et al., 1991). The CArG box motif is related to the serum response element (SRE) first identified in the upstream region of the c-fos gene and implicated in the induction of c-fos transcription in response to stimulation of cell growth and division by serum or mitogens. An ubiquitous 67-kDa nuclear factor, termed the serum response factor, p67SRF, interacts with the c-fos SRE (Treisman, 1987 and 1990) and the CArG box in vitro, suggesting that these two motifs bind the same factor(s) in vivo (Taylor et al., 1989). This is supported by the finding that anti-p67SRF antibodies microinjected into rat L6 or mouse C2 myoblasts specifically inhibited myoblast-myotube differentiation, as well as the expression of the myogenic nuclear factor, myogenin, and the skeletal muscle-specific protein, troponin T (Vandromme et al., 1992). There is evidence that the CArG and SRE regulatory elements may bind factors other than p67SRF, for example, the muscle actin factors MAPF2, a 35 kDa protein restricted to muscle cells and present in the nuclear fractions of L6 rat and C2 mouse myoblasts, and MAPF1 (Walsh and Schimmel, 1987).

Other factors affect and reflect altered expression of muscle genes during differentiation, for example, the activation of the interferon system (Birnbaum et al., 1990), and those to accommodate the high energy demands associated with contraction of striated muscle fibers including: a) the differential expression of adenine nucleotide translocator (ANT) isoforms (Stepien et al., 1992), b) the differential expression of the GLUT1 and GLUT4 glucose transporters (Mitsumoto et al., 1991), and c) changes in phosphofructokinase isozymes (Wills and Mansour, 1990). For practical reasons, these
changes, and others, are not addressed in the text but are included in Table 1 which attempts to chronicle the major advances in muscle research during the last 25 years.

1.4 The Role of Proteolysis in the Regulation of Myogenesis

Owing largely to the work done on structural proteins of the differentiating myoblasts, the consensus has emerged that muscle proteins are regulated at the level of transcription or translation. However, as early as 1981, some researchers pointed out that there was no reason to believe that all proteins of the muscle are regulated at the level of synthesis. Kaur and Sanwal, for example, suggested that control of activity of some muscle proteins occurs by alterations of the rates of their degradation (Kaur and Sanwal, 1981). In support of their hypothesis, they reported a fusion-related expression of a calcium activated neutral protease (CANP), a nonlysosomal protease which had previously been implicated in the turnover of some myofibrillar components in homogenates of various muscle tissue (Reddy et al., 1975; Dayton et al., 1976). Soon after, evidence for the involvement of another endogenous nonlysosomal protease, the metalloprotease MEPr, in muscle differentiation was reported. Inhibitors of MEPr, when added to the culture medium, inhibited myotube formation and expression of muscle-specific proteins in primary and immortal L6 skeletal muscle cells (Couch et al., 1983; Kayalar et al., 1989). Since the MEPr inhibitors also inhibited the enzyme-mediated degradation of insulin, it was suggested that MEPr was the 110 kDa insulin degrading enzyme, IDE (Kayalar et al., 1989).

Although initial studies suggested that myofibrillar protein breakdown was mainly regulated by nonlysosomal pathways, a role for lysosomal proteases (Table 2) was
Table 1. Biochemical Gene Expression of Differentiating Murine Myoblasts in Culture

<table>
<thead>
<tr>
<th>Products of Structural Genes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pattern(I/D)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference (1st Author, Year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen phosphorylase</td>
<td>I</td>
<td>de la Haba, 1968</td>
</tr>
<tr>
<td>Myosin heavy chain</td>
<td>I</td>
<td>Coleman, 1968</td>
</tr>
<tr>
<td>Acetylcholine receptor</td>
<td>I</td>
<td>Dryden, 1970</td>
</tr>
<tr>
<td>Creatine phosphokinase</td>
<td>I</td>
<td>Shainberg, 1971</td>
</tr>
<tr>
<td>Glycogen synthetase</td>
<td>I</td>
<td>Luzzati, 1972</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>I</td>
<td>Wahrmann, 1973</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>I</td>
<td>Fluck, 1973</td>
</tr>
<tr>
<td>Myosin light chains</td>
<td>I</td>
<td>Emerson, 1975</td>
</tr>
<tr>
<td>Adenyl cyclase</td>
<td>I</td>
<td>Dufresne, 1976</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-dependent neutral protease</td>
<td>I</td>
<td>Kaur, 1981</td>
</tr>
<tr>
<td>Metalloprotease (MEPr)</td>
<td>I</td>
<td>Couch, 1983</td>
</tr>
<tr>
<td>Cathepsins B, H, and L</td>
<td>I</td>
<td>Kirschke, 1983</td>
</tr>
<tr>
<td>Cysteine Protease Inhibitors</td>
<td>I</td>
<td>Kirschke, 1983</td>
</tr>
<tr>
<td>Fructose di-P aldolase</td>
<td>I</td>
<td>Turner, 1983</td>
</tr>
<tr>
<td>N-cadherin (adhesion protein)</td>
<td>I</td>
<td>Hatta, 1986</td>
</tr>
<tr>
<td>Insulin GF binding protein</td>
<td>D</td>
<td>McClusker, 1986</td>
</tr>
<tr>
<td>P40 polypeptide</td>
<td>D</td>
<td>Meadus, 1989</td>
</tr>
<tr>
<td>N-CAM (adhesion glycoprotein)</td>
<td>I</td>
<td>Dickson, 1990</td>
</tr>
<tr>
<td>Procollagen</td>
<td>I</td>
<td>Nandan, 1990</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>I</td>
<td>Wills, 1990</td>
</tr>
<tr>
<td>Histone H4</td>
<td>D</td>
<td>Larson, 1991</td>
</tr>
<tr>
<td>Glucose Transporters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT1</td>
<td>D</td>
<td>Mitsumoto, 1991</td>
</tr>
<tr>
<td>GLUT4</td>
<td>I</td>
<td>Mitsumoto, 1991</td>
</tr>
<tr>
<td>Adenine Nuclear Translocators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ANT isofroms)</td>
<td>I</td>
<td>Stepien, 1992</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Products of Regulatory Genes</th>
<th>Mode(+/-)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p67SRF</td>
<td>-</td>
<td>Treisman, 1987</td>
</tr>
<tr>
<td>Muscle Actin Factors (MAPF-1 &amp; 2)</td>
<td>-</td>
<td>Walsh, 1987</td>
</tr>
<tr>
<td>HelixLoopHelix Myogenic Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyoD1 protein</td>
<td>+</td>
<td>Davis, 1987</td>
</tr>
<tr>
<td>Myogenin</td>
<td>+</td>
<td>Wright, 1988</td>
</tr>
<tr>
<td>Myf-5 protein</td>
<td>+</td>
<td>Braun, 1989</td>
</tr>
<tr>
<td>Myf-6 (MRF-4) herculcin</td>
<td>+</td>
<td>Braun, 1990</td>
</tr>
<tr>
<td>Id protein</td>
<td>-</td>
<td>Weintraub, 1991</td>
</tr>
</tbody>
</table>

<sup>a</sup> Includes both muscle-specific proteins and myogenesis-specific patterns of proteins not specific to muscle.

<sup>b</sup> I (increase), D (decrease) in protein (iso)forms; + (positive) regulation, - (negative) regulation of gene expression.

<sup>c</sup>
supported by studies in whole muscle homogenates. As early as 1970, two or more populations of lysosomes in muscle could be isolated by centrifugation techniques on the basis of equilibrium density (Canonico and Bird, 1970; Stauber and Bird, 1974). Using cytochemical techniques, isolated lysosomes were shown to contain a complement of proteolytic enzymes, including cathepsins (Bird et al., 1978), as well as fragments of myofilaments in secondary lysosomes (Libelius et al., 1979), and immunoprecipitates of actin and myosin fragments in muscle lysosomes treated with antibody to actinomyosin fragments (Gerard and Schneider, 1979). The concomitant finding that the lysosomal aspartic protease, cathepsin D, and the lysosomal cysteine proteases, cathepsin B, cathepsin H, and cathepin L, degraded native or denatured purified actin and myosin (Schwartz and Bird, 1977; Bird et al., 1980), provided further substantial support for the hypothesis that the lysosomal apparatus of muscle cells is involved in the terminal degradation of myofibrillar proteins in normal as well as pathological tissues (Bird, 1975).

1.5 Proteolysis in Muscle and Other Differentiating Systems

Although changes in proteolysis in muscle tissue were well documented by 1980, overall levels in skeletal muscle were extremely low (Bird et al., 1968; Bird, 1975). These levels were thought to reflect the low average turnover of muscle proteins in normal tissue (Bohley and Seglen, 1992). This rather ordinary interpretation of protease data had the potential of diminishing research interest into the cellular basis, molecular mechanism, and biological significance of degradation of myofibrillar
<table>
<thead>
<tr>
<th>CLASS</th>
<th>EC NUMBER</th>
<th>EXAMPLES</th>
<th>pH RANGE for activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>3.4.21.-</td>
<td>trypsin</td>
<td>7-9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chymotrypsin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>plasmin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>plasminogen activator</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cathepsin G</td>
<td></td>
</tr>
<tr>
<td>Cysteine or thiol</td>
<td>3.4.22.-</td>
<td>cathepsin B</td>
<td>3-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cathepsin H</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cathepsin L</td>
<td></td>
</tr>
<tr>
<td>Aspartic or acid</td>
<td>3.4.23.-</td>
<td>pepsin</td>
<td>2-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cathepsin D</td>
<td></td>
</tr>
<tr>
<td>Metallo-</td>
<td>3.4.24.-</td>
<td>collagenases</td>
<td>7-9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gelatinase</td>
<td>neutral</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stromolysin</td>
<td>neutral</td>
</tr>
</tbody>
</table>

\(^a\) from Barrett and Kirschke, 1981.
proteins. In retrospect, this interpretation reflected a limitation of the experimental system. In studies utilizing muscle homogenates, it is difficult to assign a specific role in the degradation of myofibrillar proteins to a particular cell type or enzyme because of the cellular heterogeneity of muscle tissue. This includes fibroblasts, nerve fragments, and epithelial cells, which like myoblasts, do not normally secrete proteolytic enzymes, and macrophages, which secrete proteolytic enzymes in response to induction (reviewed in Rozhin et al., 1990). Bird and colleagues examined lysosomal protease activities in primary cell cultures prepared from chick embryonic skeletal muscle and in prefusion and postfusion populations of the differentiating L6 rat myoblasts, a myogenic cell line established from trypsin-suspended skeletal muscle cells from normal, fetal skeletal muscle (Yaffe, 1968; Dufresne et al., 1976; Kaur and Sanwal, 1981). They found that levels of cysteine proteases, using azocasein as a substrate, were at least two orders of magnitude higher in cultured cells as compared with muscle homogenates. They also reported that cathepsin enzyme activities increased 6 to 7-fold after fusion of the myoblasts (Bird et al., 1981). With the development of sensitive fluorometric synthetic substrates and conditions to assay the different cathepsins (Barrett and Kirschke, 1981), this fusion-related increase was found to reflect a 5-fold increase in cathepsin L, a 2-fold increase in cathepsin B, and a 7-fold increase in cathepsin H (Kirschke et al., 1983).

The studies in L6 rat myoblasts were the first to demonstrate that a fully functional lysosomal apparatus was present in cultured avian and murine muscle cells, and that increases in specific activities of the lysosomal catabolic enzymes paralleled myoblast-myotube differentiation. Since then, differences in levels of lysosomal
proteases have been reported in other cell and tissue differentiating systems. In general these studies demonstrate some tissue-specific and species-specific differences in the expression of lysosomal cathepsins (Béchet et al., 1986). For example, increased levels of cathepsin B and cathepsin L, but not cathepsin H, were reported during fetal calf myoblast-myotube differentiation using cDNA probes (Béchet et al., 1991). In cultures of rat epidermal keratinocytes, on the other hand, increased levels of all three cathepsins were observed during differentiation using immunoblotting procedure (Tanabe et al., 1991). Yet another variation in cathepsin expression occurs during granulocyte-macrophage colony-stimulating factor (GMCSF)-induced differentiation of the human promonocytic cell line, U937. During this process, increased levels of the neutrophil enzyme, cathepsin G, the serine protease, human leukocyte elastase (HLE), and the cysteine protease, cathepsin B, were observed (Ward et al., 1990). Unlike the previous differentiating systems discussed, the active form of cathepsin B also appears to be targeted to the membrane and into the extracellular fluid. It has been postulated that this pattern of expression contributes to the increased phagocytic activity of the differentiated, monocyte-like cells.

The differences in expression of cathepsin activities reported in the literature are paralleled with differences concerning their biological function. For example, cathepsin B in homogenates of murine skeletal muscle (Schwartz and Bird, 1977; Noda et al., 1980), and monkey skeletal and cardiac muscle (Hirao et al., 1984), was reported to degrade actin, myosin, tropomyosin and troponin. Cathepsin B in homogenates of rabbit skeletal muscle, on the other hand, was reported to degrade myosin heavy chain, actin, and troponin T, but not α-actinin, tropomyosin, troponin I or troponin C.
(Matsuishi et al., 1992). These studies, despite the differences, supported an important role of cathepsin B in differentiation. Other studies, however, did not. For example, injection of synthetic derivatives of E-64, a powerful inhibitor of cysteine proteases such as cathepsin B, into male Wistar rats had little effect on degradation of endogenous and exogenous proteins by liver homogenate cathepsins (Kominami et al., 1991). Other studies suggested that cathepsin L, rather than cathepsin B (and cathepsin D), plays the major role in lysosomal degradation. These alternate explanations are not mutually exclusive. While it is true that levels of cathepsin L are generally greater than levels of cathepsins B and H in most biological and pathological process studied, and that rabbit skeletal muscle cathepsin L had previously been shown to degrade heavy chain myosin, actin, α-actinin, and troponin (Matsukura et al., 1981), the results obtained for the liver cathepsins do not necessarily imply that the same results will be found for skeletal muscle cathepsins. Moreover, the complexity of synthesis, sorting, activation, and regulation of proteases, as well as the diversity of protein isoforms and cell types associated with differentiation, make it unlikely that the level of any one cathepsin is the sole determinant in gene regulation (Buckingham, 1992).

1.6 Regulation of Lysosomal Protease Activity During Myogenesis

Based on the literature, there is little doubt that lysosomal proteases, in particular cathepsins B, H, and L, and cathepsin D, play an important role in both pathological and non-pathological (i.e., biological) processes (Neurath, 1989; Polgár, 1989; Sloane, 1990, 1990a and 1990b; Chambers, 1992). However, because of an obvious research emphasis on pathological processes, such as muscular dystrophy (Gopalan et
al., 1986 and 1987) and tumor progression (Chambers et al., 1992; Scadden and Dufresne, 1993), the mechanisms by which cathepsin expression is controlled during non-pathological processes, such as muscle development, are poorly understood. This problem reflects the complexity of cathepsin expression in vivo (Figure 3) (Nishimura et al., 1990; Mach et al., 1992).

There are three general mechanisms of control addressed in the literature: a) transcriptional activation (e.g. up-regulation at enhancer/promoter sequences, activation of cathepsin isozymes) (Colella et al., 1986; Béchet et al., 1991), b) post-transcriptional modifications (e.g., alterations in processing and cell targeting (Matsuishi et al., 1992; Sohar and Katona, 1992; Mach et al., 1992), and c) regulation by endogenous inhibitors (Bode et al., 1990; Tanabe et al., 1991; Warner and Sonnenfeld-Karcz, 1992). Of the three, the latter mechanism has received considerable attention in studies of differentiating systems including myogenesis. For example, Kaur and Sanwal reported the presence of a potent endogenous inhibitor of nonlysosomal, calcium-dependent proteinase II in prefusion but not fused L6 myoblasts (Kaur and Sanwal, 1981). Kirschke and colleagues subsequently reported the presence of endogenous inhibitors of cysteine proteases in both myoblasts and myotubes of L6 rat myoblasts (Kirschke et al., 1983). In these studies, cathepsin L and H inhibitor activities in heat-treated and alkaline-treated cell homogenates increased after myotube formation while cathepsin B inhibitor activity did not. More recently, Matsuishi identified three peaks of cystatin-like inhibitor activity in rabbit skeletal muscle homogenates but could not determine the cellular source or the pattern of expression.
FIGURE 3

Intracellular Sorting of Cathepsin B

Cathepsin B is initially synthesized on polysomes of the rough endoplasmic reticulum (RER) as a 39-44 kDa pro-form that is co-translationally N-glycosylated (1). This pro-form then follows a secretory route into the golgi (2), where the majority of the protease acquires a phosphomannosyl residue (P). This residue serves as a high affinity ligand for binding to mannose-6-phosphate receptors (→) in the golgi (3). The protease/receptor complex exits the golgi via a 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).
from Dahms et al., 1989
modified by J. Kappos

**Intracellular Sorting of Cathepsin B**

1. RER
2. Golgi Apparatus
3. Late Endosome
4. Procathepsin B
5. Early Endosome
6. Lysosome
7. Active 29 kDa Cat. B
8. Plasma Membrane
9. Secretory pathway

Initially synthesized as 39-44 Kd Procathepsin B N-Glycosylated

Processing to the mature form

**FIGURE 3**
during muscle differentiation (Matsuishi et al., 1992). Thus, while there is considerable evidence that members of the cystatin superfamily are endogenous inhibitors of cysteine proteases in differentiating cells (Barrett et al., 1986), their role in the differentiation process remains unclear. This uncertainty is not surprising given the previously discussed uncertainty concerning the role of lysosomal proteases in differentiation. Both may reflect limitations in the experimental system and in the methods used to measure protease and protease inhibitor levels in a biological sample.

1.7 Measurement of Cysteine Proteases and Their Endogenous Inhibitors

Levels of protease are determined in two general ways: 1) activity against a natural or synthetic substrate in a reaction mix or in solid-phase, for example gel electrophoresis and plate assays (reviewed in Sarath et al., 1989), and 2) immunoassay (Recklies and Mort, 1982). The former measures only active enzyme and is limited by the sensitivity of the assay, the specificity of the substrate, and by interference from components present within cell/tissue homogenates and extracellular fluids (Kirschke et al., 1983; Scaddan and Dufresne, 1993). Some of the problems have been minimized by using more sensitive fluorometric substrates, synthetic inhibitors of nonrelevant proteases, and stringent assay conditions, for example pH (reviewed in Polgár, 1989). Immunoassay, while more sensitive, does not readily discriminate between active and inactive proforms, active mature, or endogenous inhibitor-inactivated proteases (Recklies and Mort, 1982; Gabrijelic et al., 1992; Higashiyama et al., 1993).

Levels of inhibitor are also measured in two ways: 1) assay of percent inhibition against a standard, exogenous protease (e.g., the purified plant cysteine protease
papain, or purified mammalian cathepsin B) in a reaction mix, or more recently, embedded in a gel (Rozhin et al., 1990; Ioannidis et al., 1993), and 2) immunoassay. These methods present limitations similar to those for assay of protease levels. Under physiological conditions, for example, the inhibitor can exist in two forms which reflect protease regulation at the simplest level, bound (i.e. inhibitor-inactivated protease), and free. Assay of inhibitor levels under these conditions reflects free rather than total inhibitor. Moreover, since inhibitor levels are routinely determined by measuring percent inhibition against a purified exogenous protease, endogenous protease present in the sample could confound the interpretation of results. One approach to this problem utilizes the heat and/or alkaline sensitivity of the lysosomal cysteine proteases and the resistance of their endogenous inhibitors (reviewed in Sloane, 1990). In a representative assay to measure cysteine protease inhibitors (CPIs), for example, cell or tissue homogenates are treated at 100°C for 5 minutes prior to addition of the exogenous protease (Rozhin et al., 1990; Chambers et al., 1992; Sloane et al., 1992). Theoretically, this treatment eliminates endogenous protease activity and dissociates heat-resistant CPIs from the protease:inhibitor complex. The effectiveness of this approach, however, is based on several assumptions, for example: 1) that elimination of protease activity and dissociation of inhibitor are complete; 2) that treatment eliminates protease activities but does not alter the structure, and therefore the biological activity, of either free or initially bound inhibitor populations; 3) that the experimental conditions do not vary over time, or between experimental systems; and 4) that inhibitors do not reassociate with the inactivated proteases or their fragments. At present, none of these assumptions have not been proven. Moreover, since there is
no direct method to measure the inhibitor concentrations in the presence of the enzyme, or in the presence of serum, it is impossible to calculate the recovery of endogenous inhibitor after alkaline or heat treatment of cells/tissues and their extracellular fluids. Thus, the significance of determinations of inhibitor-to-protease levels in both cell/tissue homogenates and extracellular fluids remains questionable as does their relevance to the differentiation process. The experiments described in this thesis were designed to address these limitations to obtain a better understanding of the role and regulation of lysosomal cysteine proteases during differentiation.

1.8 Research Description and Objectives

The following work describes an analysis of the expression of three lysosomal cysteine protease activities, cathepsin B, cathepsin H, and cathepsin L within differentiating liver and muscle cells and their extracellular media. The specific objectives of the study were:

a) to establish serum-free, defined culture conditions to support short-term and long-term maintenance of cells without affecting their growth and differentiation properties;

b) to examine and compare intracellular and extracellular levels of cathepsins B, H, and L during growth of the human differentiating hepatoma cell line, HepG2;

c) to examine and compare intracellular and extracellular levels of cathepsins B, H, and L, during growth of the differentiating rat myoblast cell line, L6, and its 5-azacytidine-resistant, fusion-deficient variant, L6-D3;
d) to examine and compare levels of cathepsins B, H, and L, and levels of endogenous inhibitor to purified cathepsin B, in homogenates prepared from differentiating L6 and fusion-deficient L6-D3 cells at different phases of growth, after fractionation on a Superose 12, H/R 10/30 FPLC column;

e) to examine and compare the ratio of cathepsin B to endogenous cathepsin B inhibitor activities in FPLC fractions of heat-treated and control homogenates prepared from prefusion L6, postfusion L6, and near-confluent L6-D3 cells.
Materials and Methods

2.1 Materials

Medium (alpha MEM), horse, calf, and fetal calf sera, gentamicin sulfate, trypsin EDTA and tissue culture plasticware were purchased from Gibco Laboratories (Burlington, Ontario). All other chemical were purchased from Sigma Chemical Co. (St. Louis, Missouri), or Fisher Scientific Co. (Fair Lawn, New Jersey), and were of reagent grade or better. Fast gallai CBC salt, L-cysteine, bovine serum albumin (fraction V), electrophoresis reagents (e.g., acrylamide and SDS), p-hydroxymercuribenzoic acid, Nα-CBZ-arginine-arginine 4-methoxy-β-napthylamide, Nα-CBZ-arginine-arginine 7-amido-4-methylcoumarin·HCl, E-64, leupeptin, pepstatin, 7-amino 4-methylcoumarin, pepsin, sodium selenite, biotin, ExtrAvidin, and low molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, Missouri). N-α-CBZ-L-arginine-7-amido-4-methylcoumarin·HCl, and N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin·HCl were purchased from Bachem California (Torrance, California). Apolipoprotein B (apoB) antibody was purchased from Medix Biotech IN. (Foster City, CA). ApoB standards were purchased from Atlantic Antibodies (Scarborough, MN) and para-nitrophenyl phosphate (PNPP) was purchased from Chemlog (South Plainfield, NJ). Nytran S+S was purchased from Mandel Scientific Co. (Guelph, ON). Purified pork liver cathepsin B and Trayslol (aprotinin) were generous gifts from Dr. A. Warner (Biological Sciences, University of Windsor) and Dr. G. Stojanovic (Arizona State, Arizona) respectively. PMSF was purchased
purchased from Boehringer Mannheim Canada (Laval, Quebec). Superose 12 HR
10/30 FPLC columns were purchased from Pharmacia (Uppsala, Sweden). The protein
assay kit was purchased from Bio-Rad Laboratories (Mississauga, Ontario).

2.2 Cell Cultures

A large number of human and murine, transformed and malignantly transformed, immortal cell lines were used in these studies including: 1) the malignantly transformed human breast cancer line MCF-7, its adriamycin resistant variant, MCF-7/AdrR, and its gpt- and ras/gpt- transfected variants MCF-7/gpt and MCF-7/ras gpt; 2) the malignantly transformed breast cancer cell lines HS5-78T, MDA-231, MDA-468, and ZR-75B; 3) the malignantly transformed human primary lung carcinoma cell line, HPL-R4; 4) the malignantly transformed human hepatoma cell line, HepG2, 5) the mouse hepatoma cell line, Hepa 1cl-9, 6) the mouse fibroblast cell line, LMTK−, and 7) the differentiating rat myoblast cell line, L6, and its 5 azacytidine resistant, fusion-deficient mutant, L6-D3. The human immortal breast cancer cell lines were originally obtained from Dr. Kenneth Cowan (National Institute of Health, Bethesda, Maryland, 20205). The human primary lung, mouse fibroblast, and mouse hepatoma cell lines were established and/or characterized in this laboratory (Dufresne and Dosescu, 1985; Manjunath and Dufresne, 1989). The original human hepatoma cell line, HepG2, was obtained from Drs. B. Knowles and D. Aden (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Clonal populations demonstrating similar phenotypes as the parent HepG2 cells were used in these studies (Labruzzo et al., 1989).
The differentiating rat myoblast cell line, L6B, was originally established from rat fetal skeletal muscle by Yaffe (Yaffe, 1968), then cloned and characterized by Dufresne (Dufresne et al., 1976). A 5-azacytidine resistant, fusion-deficient (i.e. incapable of forming myotubes) variant of L6, L6-D3, was generously provided by Dr. B. Sanwal (Department of Biochemistry, University of Western Ontario, London). Resistance in this variant reflected an alteration of the components 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).

All cell lines, with the exception of L6 and L6-D3 rat myoblasts, were maintained in alpha-MEM medium supplemented with heat-inactivated serum (5% consisting of 6 parts calf serum to 4 parts fetal calf serum, v/v) and 50 µg/ml gentamicin sulfate. The rat myoblast cell lines were maintained in alpha-MEM supplemented with either 10% horse serum (heat-inactivated) or 10% fetal calf serum (heat inactivated). In L6 cell cultures, the ratio of fusion-restricted, non-presumptive myoblasts, to fusion-capable, postmitotic myoblast subpopulations in the myogenic lineage was serum-controlled; medium supplemented with 10% fetal calf serum favors the former, while medium supplemented with 10% horse serum favors the latter (Yeoh and Holtzer, 1977).

2.3 Establishment of HepG2 in Serum-Free Medium

HepG2 cells growing exponentially in serum-supplemented medium were used to establish cells in serum-free, defined medium. The procedure used is summarized in Figure 4. The serum-free medium used in this study was a modified version of Darlington's (Darlington et al., 1987) modified by Adeli and Sinkevitch (1990). It
FIGURE 4

Establishment of HepG2 Cells in Serum-Free Medium

Cells growing exponentially in serum-supplemented medium were trypsinized, centrifuged, and resuspended in serum-free, defined medium at a concentration of $2 \times 10^5$ cells/ml. Cells were grown to near confluency before each subsequent subculture.
ESTABLISHMENT OF HEPG2 IN SERUM-FREE MEDIUM

HEPG2 IN 5% SERUM near confluency

TRANSFER CELLS INTO SERUM-FREE MEDIUM
- trypsinization
- syringe separation
- centrifugation
- resuspension

PLATE CELLS INTO SERUM-FREE MEDIUM
(10^6 CELLS/25 CM^2 FLASK)

FEED CELLS EVERY 24 HOURS

WHEN NEAR CONFLUENT, TRANSFER
(X3=putative, established populations)

CHARACTERIZE
- biological parameters
- biochemical parameters

FIGURE 4
consisted of 3 parts alpha MEM and one part Waymouth's MB752/1 supplemented with: 2 mM L-glutamine, 30 nM sodium selenite, 1.0 mg/L l-inositol, 8.0 mg/L thymidine, 0.05 mg/L CuSO₄·5H₂O, 0.016 mg/L MnSO₄·H₂O, 0.03 mg/L ZnSO₄·7H₂O, 0.024 mg/L Mo₇O₂₄·4H₂O, 0.022 mg/L CoCl₂·6H₂O and antibiotic-antimycotic solution consisting of either gentamicin sulfate, or penicillin, streptomycin, amphotericin B and fungizone. [NOTE: This serum-free medium was also used in studies to measure levels of lysosomal cysteine protease activities in extracellular fractions of cells since serum interferes with measurements (Freshney, 1987).]

2.4 Cell Transfers (Subculture of Cells)

All cell cultures used in this study were anchorage dependent and were therefore serially passaged (i.e., also termed cell subcultures and cell transfers) using 0.25% trypsin-EDTA after washing with warm citrate saline solution (15 mM trisodium citrate, 134 mM potassium chloride, pH 7.8) (Freshney, 1987). Trypsinization did not affect the biological or biochemical properties of any of the cells examined. All cell cultures were maintained at 37°C in an atmosphere of 5% CO₂ and 95% air.

2.5 Cell Storage

Long term storage of cells is a routine cell culture procedure used to prevent loss of cells in culture due to factors such as contamination and incubator malfunction, and to minimize changes in genotypes and phenotypes which may occur with prolonged maintenance in culture. Trypsinized cells were diluted in regular alpha medium
supplemented with 5% serum and centrifuged at 1000 rpm for 5 minutes to remove trypsin. Cells were then resuspended in freezing solution (7 parts alpha medium, 2 parts fetal calf sera, 1 part dimethylsulfoxide, v/v) and transferred into Nunc cryotubes at 2x10^6 cells per vial. Vials were transferred to -20°C for 2 hours prior to storage in both liquid nitrogen (-195°C) and freezer (-80°C) (Revco Inc. West Columbia, S.C., 29169) conditions. One to two weeks after freezing, cell samples were checked for contamination and viability. Cells stored under these conditions retained genotypic and phenotypic characteristics for at least three years.

2.6 Biological Characterization of Cells in Culture

i) Population Doubling Time and Saturation Density

The population doubling time (T_D) and the saturation density (S_D) were determined from growth curves constructed for each cell line. After trypsinization, centrifugation, and resuspension, cells were plated into 60 mm tissue culture dishes at a concentration of 10^5 to 10^6 cells per plate and incubated at 37°C. Once plated, the number of cells in each of two dishes was determined at two time points each day using a hemacytometer. The cell counts were then plotted on a semi-logarithmic scale against the appropriate time point. The population doubling time was calculated during exponential growth phase while saturation density was calculated from the plateau phase and expressed as cells/cm².

ii) Cell staining and Fusion Index

A quantitative estimation of myoblast fusion, referred to as fusion index (i.e., %
fusion), was obtained using the procedure of Morris and Cole (1972). Cells are plated in tissue culture dishes and incubated at 37°C. At various times after incubation the medium was removed, cells were washed with 0.4% citrate saline (pH 7), and fixed by addition of 95% ethanol. After drying, the cells were stained with Giemsa (6%) stain for 20 minutes, rinsed with tap water, air dried, and examined with an inverted microscope. The total number of nuclei and the number of nuclei within myotubes was determined and the ratio of the latter to the former expressed as a percentage. A cell was scored as a myotube if it contained 3 or more nuclei within the same cell membrane. Since binucleate cells were not included in myotube counts, estimates of fusion index usually reflect minimum levels.

2.7 Preparation of Cell Homogenates and Media Fractions

Cells were routinely maintained in 25 cm² culture flasks, and were transferred to larger 75 cm² culture flasks to provide sufficient numbers for plating (e.g., "seeding"). Each 100 mm tissue culture dish was seeded with 2x10⁵, 5x10⁵, or 10⁶ cells, depending on the cell line, and incubated at 37°C. At each appropriate time point (i.e., phase of growth), medium was removed and examined for cellular debris. Cells on the surface of the plate were then collected, on ice, in phosphate buffered saline (PBS: 0.027 mM KCl, 1.5 mM KH₂PO₄, 150 mM Na₂HPO₄·7H₂O, and 140 mM NaCl, pH 7.4) using a rubber policeman, and transferred into polypropylene tubes at approximately 5x10⁶ cells/tube. After three cycles of PBS suspension followed by low speed centrifugation (1200, 5 min) the final cell pellets were assayed immediately or stored at -20°C until time of enzyme assay. Prior to assay of cysteine protease and
cysteine protease inhibitor activities, the final cell pellet was resuspended in 600 µl of homogenization buffer (135 mM KCl, 0.1 mM EDTA, 0.1% Tween 80, and 15 mM KH₂PO₄, pH 6.0); for SDS-PAGE, each pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM Na₂EDTA, 1 mM PMSF, 0.5% SDS, and 0.5% βME). Cell suspensions were then homogenized on ice with a Brinkman Polytron PT-10 (Brinkmann Instruments, Oakville, ONT) on setting 4 for two, 10 second pulses. Homogenates were clarified by microcentrifugation for 5 minutes at 4°C using a Fisher Model 59 microfuge.

In some experiments, medium on cells was replaced with serum-free medium 24 h before collection. In assays involving both cell homogenates and their corresponding media, medium removed from cells was centrifuged at 1200 rpm for 5 minutes at 4°C and used directly in, or concentrated for, subsequent experiments. In this thesis, cell homogenate and its corresponding medium are referred to as intracellular and extracellular fractions respectively.

2.8 SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modified method of Laemmli (1970) using 5% (w/v) stacking and 7% (w/v) resolving gels (Dufresne et al., 1993). Intracellular and extracellular samples were prepared by first mixing a volume containing 12 µg sample protein with an equal volume of sample buffer (125 mM Tris-HCl, pH 6.8, 10% βME, 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 56 volts for 18 hours. The gels were then fixed and stained in
FIGURE 5
Cathepsin B Activity and Cell Protein

Centrifuged Homogenates were prepared from rat myoblast (▲) and human breast (●) cells. For each cell population, different amounts of protein (μg) were assayed at 15 min and 30 min with the colorimetric substrate, Cbz-arg-arg. The conditions used were optimal for cathepsin B activity. Activity is expressed as enzyme units (EU), where one enzyme unit (EU) is defined as that amount of enzyme which gives rise to an absorbance unit of 0.016 in one minute at a wavelength 520 nm. Error bars are not included but values for each point differed by less than 5% within and between experiments. The vertical arrow on the x-axis identifies the protein used per reaction tube in subsequent analysis. This corresponds to an adjusted protein concentration in the homogenate of 0.16 mg/ml.
0.2% Coomassie Brilliant Blue G-250 (in 10% (v/v) acetic acid and 40% (v/v) methanol), and destained in 10% (v/v) acetic acid and 40% (v/v) methanol. Each lane of each gel was then scanned on a dual-wavelength, Shimadzu TLC Scanner at a wavelength of 595 nm. The relative molecular weights of bands was determined by reference to proteins of known molecular weight: 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).

2.9 Enzyme Assays

Enzyme assays using colorimetric (β-naphthylamide) and/or fluorometric (4-methylcoumarin) substrates were conducted on both unFractionated and FPLC-fractionated cell homogenates (intracellular fractions), as well as on serum-free cell medium collected from cells (extracellular fractions). For unFractionated homogenates, the protein concentration was adjusted down to 0.16 mg/ml, a concentration at which the reaction was linear under the conditions used (Figure 5).

A. UnFractionated Homogenate

i) Assay with Colorimetric Substrate

Cathepsin B was assayed in unFractionated homogenates using the colorimetric substrate, Nα-CBZ-arg-arg-4-methoxy β-naphthylamide (arg-arg), according to a inodified procedure of Barrett (1972). The pre-reaction mix contained, in a final volume of 300 µl: 200 µl of intracellular or extracellular fraction (0.16 mg/ml) and 100 µl of incubation buffer (1.38 mM Na₂EDTA, 88 mM KH₂PO₄, 14 mM
Na₂HPO₄·7H₂O, pH 6.0), with 0.32 mg/ml L-cysteine). This was incubated at room temperature for 10 minutes before addition of 20 μl of (0.016 mM) arg-arg. A 50 μl aliquot of the reaction mix was immediately transferred to a stopper tube (on ice) containing 50 μl of coupling reagent (3.57 g p-hydroxy-mercuribenzoic acid in 120 ml 0.5 M sodium hydroxide, with 50 mM Na₂EDTA in 1 liter, pH 6.0). The reaction mix was then transferred to a 37°C water bath. At 15 and 30 minute time intervals after transfer, 50 μl of reaction mix was removed and added to a stopper tube (on ice) containing 50 μl of coupling reagent. Upon completion of the last transfer, 50 μl of fast-garnet-Brij solution (2.5 mg fast garnet salt in 4.8 ml auto-nano-water with 200 μl 4% Brij 35 in water) was added to each stopper tube. After a minimum of 10 minutes at room temperature, 1 ml of butanol was added to each. Tubes were vortexed vigorously, then centrifuged in a Precision Vari-Hi-Speed Centricone (Precision Scientific Co., Chicago, Illinois) at high speed for 5 minutes. The liberated 2-naphthalamine contained in the top butanol layer was then measured at 520 nm with a Beckman DU-64 Spectrophotometer equipped with a Quant II Quad Soft Pack Module. One unit of proteolytic activity was defined as that amount of enzyme which gives rise to an absorbance of 0.016 in 1 minute at a wavelength of 520 nm. Specific Activity was usually expressed as enzyme units (EU) per milligram of total protein.

ii) **Assay with Fluorometric Substrates**

Cathepsins B, H, and L activities were measured in un fractionated samples using fluorometric, 4-methylcoumaryl-amide substrates (Table 3) according to a modified procedure of Barrett (Barrett, 1980; Barrett and Kirschke, 1981). While these
Table 3  A Comparison of the Properties of Lysosomal\(^a\)
Cysteine Proteases Cathepsins B, H, and L

<table>
<thead>
<tr>
<th></th>
<th>Cathepsin B</th>
<th>Cathepsin H</th>
<th>Cathepsin L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recomended test substrate (pH optimum)</td>
<td>Z-Arg-Arg-NMec (pH 6.0)</td>
<td>Arg-NMec (pH 6.8)</td>
<td>Z-Phe-Arg-NMec (pH 5.5)</td>
</tr>
<tr>
<td>Aminopeptidase activity</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Endopeptidase activity</td>
<td>Moderate</td>
<td>Variable</td>
<td>High</td>
</tr>
<tr>
<td>Peptidylpeptidase activity</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Aldolase inactivation</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Leupeptin sensitivity</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Z-Phe-Phe-CHN(_2) sensitivity</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>pI</td>
<td>4.5-5.5</td>
<td>6.0-7.1</td>
<td>5.5-6.1</td>
</tr>
<tr>
<td>Concanavalin A - Sepharose</td>
<td>Not bound</td>
<td>Bound</td>
<td>Bound</td>
</tr>
</tbody>
</table>

\(^a\) from Barrett and Kirschke, 1981.
substrates can be prohibitively expensive, they are reported to be safer (napthalamide is a suspected carcinogen) and more sensitive than colorimetric substrates (Barrett and Kirschke, 1981). Their sensitivity reflects the liberation of 7-amino-4-methylcoumarin which can be measured using a fluorometer. The linear relationship between fluorescence and increasing concentrations of an aminomethylcoumarin standard is presented in Figure 6.

In all relevant experiments described in this thesis, cell samples and synthetic substrates were kept on ice prior to addition to reaction mix. A 500 µl aliquot of homogenate sample, diluted in 0.1% Brij 35 solution in water, was preincubated for 2 minutes at 37°C with 250 µl activation buffer (cathepsin B: 352 mM KH₂PO₄, 48 mM Na₂HPO₄, 4 mM disodium EDTA, and 8 mM dithiothreitol, pH 6.0; cathepsin H: 200 mM KH₂PO₄, 200 mM Na₂HPO₄, 4 mM disodium EDTA, and 40 mM dithiothreitol, pH 6.8; cathepsin L: 340 mM sodium acetate, 60 mM acetic acid, 8 mM dithiothreitol, and 4 mM disodium EDTA, pH 5.5). The assays were started by the addition of 250 µl of 0.02 mM substrate solution (cathepsin B: Nα-CBZ-arginine-arginine 7-amido-4-methyl-coumarin.HCl, or Z-Arg-Arg-NMec; cathepsin H: Nα-CBZ-L-arginine-7-amido-4-methylcoumarin.HCl, or Z-Arg-NMec; cathepsin L: N-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin.HCl, or Z-Phe-Arg-NMec). All substrate solutions were originally prepared as 1 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at 4°C. Stock solutions were diluted to 0.02 mM working strength solutions with nanopure water (ie. 200 µl stock into 10 ml nanopure water). After 10 minutes incubation at 37°C, the reactions were terminated with the addition of 1 ml stopping reagent (100 mM sodium monochloroacetate, 30
FIGURE 6
7-Amino 4-Methylcoumarin Standard Curve

7-amino 4-methylcoumarin standards of 0.1-0.5 μM concentration were prepared from a 1.0 mM stock solution in DMSO by diluting with a 1:1 solution of activation buffer and stopping reagent. Fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm as described in Materials and Methods, section 2.9 A-ii.
mM sodium acetate, and 70 mM acetic acid, pH 4.3). [NOTE: The reaction blank was as described above except that 1 ml of stopping reagent was added before the activation buffer.] Since the specific activities for cathepsins B and L are of similar order -as are their $K_{cal}/K_m$ values- and since both proteases recognize the Z-Phe-Arg-NMec substrate, a correction was made for cathepsin L activity. Cathepsin B activity was determined with Z-Arg-Arg-NMec under conditions specific for cathepsin L (i.e., pH 5.5). These values were subtracted from the values obtained with the Z-Phe-Arg-NMec substrate to provide a corrected activity for cathepsin L (Nishimura et al., 1988). The fluorescence of free aminomethylcoumarin in each reaction tube was then measured at room temperature at an excitation wavelength of 370 nm and an emission wavelength of 460 nm with a Turner Model 430 Spectrofluorometer (G.K. Turner Associates, Palo Alto, California). Fluorescence readings were standardized with 0.5 $\mu$M 7-amino-4-methylcoumarin standard (prepared from 1 mM stock in DMSO diluted to 0.5 $\mu$M with a 1:1 mixture of activation buffer and stopping reagent) set at 1000. The spectrofluorometer was adjusted so that 1000 arbitrary units corresponded to the release of 1 nmol of product. One milliunit of proteolytic activity was defined as the quantity of enzyme releasing 1 nmol of aminomethylcoumarin per minute. For the 10 minute assay, a reading of 1000 therefore corresponds to 0.1 milliunits of activity in the tube.

B. FPLC-Fractionated Homogenate

Clarified homogenates were fractionated on a Superose 12 HR 10/30 FPLC column (Pharmacia, Uppsala, Sweden). For each fraction, 200 $\mu$g (in the case of
fluorometric substrates) or 500 μg (in the case of the colorimetric substrate) of protein was loaded in 500 μl homogenization buffer (135 mM potassium chloride, 0.1 mM EDTA, 0.1% Tween 80, 15 mM potassium phosphate, pH 6.0). 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 1.0 ml/min with a Beckman Gradient Liquid Chromatograph. Absorbance during elution was monitored using a Beckman Analytical Optical Unit at a UV wavelength of 280 nm. Sensitivity was generally between .08 and .32 absorbance units full scale (AUFS). Elution profiles were recorded on an Kipp and Zonen Chart Recorder at a chart speed of 1.0 cm/min. Two hundred and fifty μl fractions (4 fractions/minute) in the case of arg-arg, and 500 μl fractions (2 fractions/minute) in the case of fluorometric substrates, were collected with a Pharmacia Frac-100 Fraction Collector, and fractions were stored on ice until assay.

i) Assay of FPLC Fractions with Colorimetric Substrate

For assays involving the colorimetric substrate, arg-arg, the procedure and buffers used were identical to that described for assay of unfractionated homogenates (see 2.9 A-i). In brief, 25 μl from each fraction tube was preincubated with 50 μl of incubation buffer, pH 6.0, containing 3X cysteine (i.e., 0.96 mg/ml L-cysteine) and 75 μl of incubation buffer, pH 6.0, without cysteine for 10 minutes. The reaction was started by the addition of 10 μl arg-arg and -after removing 50 μl reaction mix and adding it to 50 μl of coupling reagent at pH 6.0- allowed to proceed for 15 minutes. The reaction was terminated with the addition of 50 μl of reaction mix to 50 μl coupling reagent.
Following the addition of fast-garnet-Brij solution and butanol, 0 minute and 15 minute sample tubes were vortexed, and the liberated 2-naphthylamine measured at a wavelength of 520 nm as previously described (2.9 A-i).

ii) Assay of FPLC Fractions with Fluorometric Substrates

For assays of cathepsins B, H, and L using their respective fluorometric substrates, the procedures and buffers used were identical to those described for assay of unfractionated homogenates (see 2.9 A-ii). In brief, 50 µl of fraction was diluted with 450 µl 0.1% Brij solution in nanopure water. Diluted fractions were then preincubated for 5 minutes at 37°C with activation buffer at the appropriate pH (cathepsin B, pH 6.0; cathepsin H, pH 6.8; cathepsin L, pH 5.5). The reactions were started by the addition of 250 µl of 0.02 mM substrate. After 10 minutes incubation at 37°C, the reactions were terminated by the addition of 1 ml stopping reagent. Blank and sample reaction tubes were prepared and the fluorescence of free aminomethylcoumarin in each measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm (2.9 A-ii).

C. Extracellular Medium

Levels of activatable cathepsins B, H and L (i.e. procathepsin) were determined using a procedure modified from that developed by Mort (Mort et al., 1981). For each reaction, 150 µl of extracellular fraction prepared from medium of cells, was activated with 30 µl of 0.5 mg/ml pepsin in an acid buffer (0.8 M anhydrous sodium acetate, pH 3.8) at 40°C for 1 hour. The pH was returned to near 6.0 with 60 µl of 0.2 M
potassium phosphate buffer (pH 9.0). The reaction was initiated with the addition of 250 μl the appropriate aminomethylcoumarin substrate, terminated at 0, 30, or 60 minute time points with 1 ml of stopping reagent, and the liberated 7-amino-4-methylcoumarin measured as previously described (2.9 A-ii).

2.10 Inhibitor Assays

A. Commercial Inhibitors for Exogenous Inhibitor Assays

i) Preparation of Commercial Inhibitors (Bond, 1989)

L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) inhibits cysteine proteases, has a molecular weight of 367.4, and an effective concentration of 1-10 μM. For 10X effective stock, 2.28 mg E-64 was dissolved in 2 ml auto-nano-water. This was stable for up to two months at -20°C. This was diluted by one tenth just prior to use.

Leupeptin inhibits trypsin-like serine and some cysteine proteases, has a molecular weight of 426.6, and an effective concentration of 10-100μM. For a 10X effective stock, 8.53 mg leupeptin was dissolved in 2 ml auto-nano-water. This was stable for one month at -20°C. This was diluted by one tenth just prior to use.

Phenylmethylsulphonyl fluoride (PMSF) inhibits serine proteases, has a molecular weight of 174.2, and an effective concentration of 0.1-1mM. A 10X effective stock was prepared by dissolving 5.6 mg PMSF in 2 ml 100% methanol. This was stable for nine months at 4°C. This was diluted by one tenth just prior to use.

Ethylenediamine tetraacetic acid (EDTA) inhibits metallo-proteases, has a molecular weight of 372.24, and an effective concentration of 1-10 mM. A 10X
effective stock was prepared by adding 1.6 ml EDTA (0.5 M) to 3.4 ml auto-nano-water. This was diluted by one tenth just prior to use.

Trayslol (aprotinin) inhibits serine proteases, has a molecular weight of 6500, and an effective concentration of 100 KIU (kallikrein inactivating unit). This is diluted from 10,000 KIU/ml manufacturers stock solution just prior to use.

Pepstatin inhibits aspartic proteases, and has an effective concentration of 1μM. A 10X effective stock was prepared by dissolving 2 mg pepstatin in 2 ml 100% methanol. This was diluted by one tenth just prior to use.

ii) **Commercial Inhibitor Assay**

The commercial inhibitor assay was performed exactly as the assay for cathepsin B activity in unfractionated homogenates (2.9 A-i and ii) with the exception of the following. For assay involving the colorimetric substrate, arg-arg, the reaction mix, in a final volume of 320 μl, consisted of 190 μl homogenate, 10 μl commercial inhibitor, 100 μl cysteine/incubation buffer, and 20 μl arg-arg. For assays involving fluorometric substrates, the reaction mix, in a final volume of 1 ml, contained 475 μl diluted homogenate (100 μl homogenate + 0.1% Brij solution), 25 μl commercial inhibitor, 250 μl activation buffer at the appropriate pH (cathepsin B, pH = 6.0; cathepsin H, pH 6.8; cathepsin L, pH = 5.5), and 250 μl of the appropriate fluorometric substrate.

B. **Assay of Endogenous Inhibitor Levels**

i) **Using Colorimetric Substrate for Cathepsin B**
The cathepsin B inhibitor assay using the colorimetric substrate, Cbz-arg-arg, was performed exactly as the assay for cathepsin B in fractionated samples (see 2.9 B-i), with the following exceptions. The reaction mix, in a final volume of 160 µl, consisted of 50 µl 3X cysteine/incubation buffer, 50 µl of column fraction, 50 µl of pork liver cathepsin B, and 10 µl arg-arg. Control vessels contained 50 µl 3X cysteine/incubation buffer, 50 µl column buffer, 50 µl pork liver cathepsin B, and 10 µl arg-arg. Control levels of proteolytic activity for pork liver cathepsin B were approximately 0.1 delta absorbance units at 520 nm.

ii) Using a Fluorometric Substrate for Cathepsin B

The cathepsin B inhibitor assay using Z-arg-arg NMec was performed exactly as the assay for cathepsin B activity in fractionated samples (see 2.9 B-ii), with the exception of the following. The reaction mix, in a final volume of 750 µl, contained 100 µl of homogenate diluted with 100 µl 0.1% Brij solution, 50 µl pork liver cathepsin B (1-1.5 µEu), 250 µl activation buffer, pH 6.0, and 250 µl of 0.02 mM Z-arg-arg-NMec. The enzyme control reaction mix consisted of 50 µl cathepsin B, 200 µl 0.1% Brij solution, 250 µl activation buffer, pH 6.0, and 250 µl of Z-arg-arg-NMec.

iii) Assay of Total Protease Inhibitor

Levels of total protease inhibitor against cathepsin B were determined using a procedure described by Green et al. (1984), and slightly modified from Rozhin et al. (1990). This involved heat treatment of clarified (i.e. centrifuged) homogenate, adjusted to a protein concentration of 500 µg/ml, at 80°C for 5 minutes.
The heat-treated sample was then centrifuged at 12,000 rpm for 5 minutes to sediment debris and applied to an HR-10/30 Sepharose 12B column. Fractions were collected and assayed for inhibitor activity as previously described (2.10 B-ii).

iv) Units for the Expression of Inhibitor Activity

Inhibitory levels were defined in two ways. In the first, one unit (i.e. milliunit or microunit) of inhibitor activity (i.e., mIU or μIU) is defined as that amount of protein that inhibits 1 corresponding unit of pork liver cathepsin B activity (i.e. mU or μU). In the second, levels of inhibition are presented as percent inhibition, which is defined as the percent change in the averaged activity of purified pork liver cathepsin B in FPLC sample fractions, relative to control levels of cathepsin B activity.

2.11 ELISA Analysis of Apolipoprotein B in HepG2 Media

Levels of apolipoprotein B in the medium of cells grown in defined, serum-free medium were measured by the procedure of Macri and Adeli (1993) in collaboration with Mr. André Theriault (Department of Chemistry and Biochemistry, University of Windsor). Microtiter plate wells were coated with an affinity-purified, polyclonal, monospecific apoB-antibody that was diluted in carbonate buffer (2 mg antibody/L 50 mmole/L carbonate buffer, pH 9.6) and incubated overnight at 4°C. After incubation, the antibody solution was aspirated and the wells were washed three times with PBST (0.5 ml/L Tween 20 in PBS) using an ELISA plate washer, and treated with PBS solution containing 10 g/L bovine serum albumin. Samples (i.e., apoB standards, controls, and extracellular fractions) were added to wells and
incubated for 2 h at room temperature. Following incubation, the wells were washed
with PBST, incubated for 1 h at room temperature in 100 µl/well ExtrAvidin-ALP
diluted in Tris (0.1 mol/L, pH 8.0, containing 0.5 mmol/L MgCl₂). After washing
with PBST, 100 µl of substrate solution (4 g/L PNPP dissolved in 1 mol/L DEA, pH
9.8, containing 0.5 mmol/L MgCl₂) was added to each well. After 1 h at room
temperature, the wells were washed and the reaction stopped by the addition of 100 µl
of NaOH (0.3 mol/L). The resulting colour reactions were read immediately on a
Hyperion MicroReader III at a wavelength of 405 nm.

2.12 Selectivity Curve Using FPLC

Molecular weight standards were run on a Superose 12 HR 10/30 FPLC and
their elution volumes recorded. Utilizing Blue Dextran to elicit the void volume (V₀),
as well as bovine albumin (mw 66.2 kDa), ovalbumin (mw 42.7 kDa), chymotrypsin
(mw 25.0 kDa) and cytochrome C (mw 13.0 kDa), a standard curve for the
approximation of molecular weight was produced and is presented in: Results Figure
14. Separation coefficients (Kₐᵥ) were calculated using the following formula:

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

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

2.13 Protein Determination

The standard protein microassay described by Bradford in 1976 and prepared
by Bio-Rad was followed. Bovine stock albumin, fraction V, was prepared by
dissolving 29 mg BSA in 10 ml of autoclaved nano-pure water (auto-nano-water). 50
μl of this stock solution was then added to 1400 μl of auto-nano-water to give a concentration of 100 μg/ml BSA. 1 ml of this solution was diluted with 4 ml auto-nano-water to give a final stock solution concentration of 20 μg/ml. Protein standards and samples were prepared as follows:

<table>
<thead>
<tr>
<th>protein amount</th>
<th>vol. stock</th>
<th>vol. water</th>
<th>vol. dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 μg</td>
<td>100 μl</td>
<td>700 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>3 μg</td>
<td>150 μl</td>
<td>650 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>4 μg</td>
<td>200 μl</td>
<td>600 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>5 μg</td>
<td>250 μl</td>
<td>550 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>8 μg</td>
<td>400 μl</td>
<td>400 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>12 μg</td>
<td>600 μl</td>
<td>200 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>blank</td>
<td>0 μl</td>
<td>800 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>sample</td>
<td>30 μl</td>
<td>770 μl</td>
<td>200 μl</td>
</tr>
</tbody>
</table>

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

2.14 Statistical Analyses

Calculation of standard deviations, and statistical tests, including the Students t-test (when number of observations were less than 30) are described in Mendenhall et al., (1974). Analyses (e.g. "t" test, Spearman rank correlation) were routinely computer-generated.
Results

3.1 Cathepsin B Activity in Various Cell lines

Cell culture systems provide a powerful tool to examine the role of proteolysis in both biological and pathological processes (reviewed in Polgár, 1989). However, most of the current cell culture research has focused on pathological processes, for example, metastasis of malignantly transformed cells and progression of muscular dystrophy. Such studies have demonstrated a positive correlation between the pathological state and increased protease activities, in particular the lysosomal cysteine proteases (Gopalan et al., 1986; Gopalan et al., 1987; Sloane, 1990; Greaves et al., 1991; Chambers et al., 1992). This specific correlation has been interpreted by some to suggest that changes in expression of proteases are peculiar to pathological processes, and are such that activities in cells from diseased tissues are always higher than those in cells from normal tissues. Since this has important practical implications for the study of biological processes, levels of protease activity were examined in various human and murine, normal and malignantly transformed cell lines using the synthetic substrate Cbz-arg-arg. The conditions used were those reported to be optimal for cathepsin B (Barrett and Kirschke, 1981; Rohzin et al., 1990). The results of this analysis, presented in Table 4, demonstrated that levels of activities in normal, differentiating cell populations (i.e. L6B and L6-D3) were always greater than levels detected in malignantly transformed, differentiating cell populations (i.e., HepG2), which, in turn, were greater than levels in malignantly transformed cells (e.g., HPL.
Table 4. Relative Levels of Cathepsin B Activity in Various Normal and Malignantly Transformed Cell Lines

<table>
<thead>
<tr>
<th>Cell Line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source</th>
<th>Biological/Pathological Process</th>
<th>Relative&lt;sup&gt;c&lt;/sup&gt; Cathepsin B Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>rat muscle</td>
<td>[N] myogenesis</td>
<td>44.0</td>
</tr>
<tr>
<td>L6-D3</td>
<td>rat muscle</td>
<td>[N] blocked myogenesis</td>
<td>41.7</td>
</tr>
<tr>
<td>HepG2</td>
<td>human liver</td>
<td>[CA] cytodifferentiation</td>
<td>32.3</td>
</tr>
<tr>
<td>HPL R4</td>
<td>human lung</td>
<td>[CA] malignant carcinoma</td>
<td>10.0</td>
</tr>
<tr>
<td>Hepa cl-9</td>
<td>mouse liver</td>
<td>[CA] malignant hepatoma</td>
<td>8.7</td>
</tr>
<tr>
<td>HST-78T</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>8.0</td>
</tr>
<tr>
<td>MDA-231</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>7.0</td>
</tr>
<tr>
<td>MCF-7/Adr&lt;sup&gt;*&lt;/sup&gt;</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>5.7</td>
</tr>
<tr>
<td>MDA-468</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>5.3</td>
</tr>
<tr>
<td>ZR-75B</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>2.3</td>
</tr>
<tr>
<td>MCF-7/ras&lt;sup&gt;*&lt;/sup&gt;</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>2.0</td>
</tr>
<tr>
<td>MCF-7/WT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>1.3</td>
</tr>
<tr>
<td>MCF-7/gpt&lt;sup&gt;*&lt;/sup&gt;</td>
<td>human breast</td>
<td>[CA] malignant adenocarcinoma</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> All cell lines are transformed (i.e. immortal). The MCF-7 cell lines<sup>*</sup> were all derived from the MCF-7/WT (wild type) as follows: MCF-7/gpt-MCF-7 cells transfected with the selectable marker pSV2gpt; MCF-7/ras-MCF-7 cells cotransfected with the transforming DNA from Harvey murine sarcoma virus and the selectable marker pSV2gpt; MCF-7/Adr<sup>R</sup>-adriamycin resistant variants of MCF-7 cells (Worland et al., 1989; Vickers et al., 1989). The remaining breast cancer cell lines represent lines established from explants from different patients (Vickers et al., 1989). The rat myoblast cell line, L6-D3, is an 5-azacytidine resistant, non-fusing variant of L6 (Ng et al., 1976). Characterization of all cell lines has been done in this laboratory.

<sup>b</sup> N = normal tissue; CA = cancerous tissue

<sup>c</sup> Cathepsin B specific activities, expressed as units/mg protein were determined using the synthetic substrate, Cbz-arg-arg (see Methods). Levels in cell lines are expressed relative to the cell line which consistently demonstrated the lowest activity (N=3).
R4 and the various breast cancer cell lines). While levels of cathepsin B activity were lower in malignantly transformed cells, the relative order of activities within the malignantly transformed cells (e.g., human breast cancer cells) was consistent with the literature; cells with greater metastatic potential (e.g., HS5-78T and MCF-7/AdrR) demonstrated greater levels of activity than those with low metastatic potential (e.g., ZR-75B and MCF-7 WT)(Vickers et al., 1989).

While it was difficult to establish the absolute specificity of Cbz-arg-arg for cathepsin B (addressed in Discussion), it was possible to test if this substrate was specific for the cysteine family of proteases. In brief, homogenates from various cell lines were assayed under conditions specific for cathepsin B, in the absence and presence of different commercially available inhibitors. The results of this analysis for the differentiating cell lines relevant to this thesis (i.e., L6B, L6-D3, and HepG2), are presented in Table 5. In all cases, E-64 (L-3-trans-epoxysuccinyl-leucyl amide-(4-guanidino)-butane: 1 μM - 10 μM) and leupeptin (10 μM - 100 μM), known inhibitors of cysteine proteases (Beynon and Bond, 1989; Barrett et al., 1982), eliminated activity detected using Cbz-arg-arg. In contrast, inhibitors of the serine-, aspartic-, or metallo-proteases had no effect on levels of activity.

3.2 Establishment of Serum-Free Conditions

The survey of cathepsin B levels in various cell lines suggested that the role of proteolysis in biological processes, specifically cytodifferentiation, could be examined in cell culture. Before these studies could proceed, however, it was necessary to address the problem of serum in the culture environment. Serum is necessary for
Table 5. Effect of Commercially Available Inhibitors on Cathepsin \(^c\) B, H and L Activity of L6, L6-D3, and HepG2 Cell Lines

<table>
<thead>
<tr>
<th>Inhibitor (^a)</th>
<th>Target</th>
<th>Effective Conc.</th>
<th>Intracellular Active</th>
<th>Extracellular Pepsin Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>----</td>
<td>----</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E-64</td>
<td>cysteine</td>
<td>10 uM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trayslol</td>
<td>serine</td>
<td>100 KIU</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PMSF</td>
<td>serine</td>
<td>50 uM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>trypsin, some cysteine</td>
<td>50 uM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EDTA</td>
<td>metallo</td>
<td>5 uM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>aspartic</td>
<td>1 uM</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) E-64 = L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
PMSF = phenylmethanesulphonyl fluoride
EDTA = ethylenediamine tetraacetic acid
KIU = kallikrein inactivating unit

\(^b\) determined as complete elimination of intracellular enzyme activity, as described in materials and methods

\(^c\) modified from Polgár, 1989.
growth and maintenance of cells in culture (reviewed in Freshney, 1987). However, serum contains undefined components which can interfere with expression and secretion of lysosomal cysteine proteases (Bottenstein et al., 1988; McFarland et al., 1991; Teofili et al., 1992). In terms of experimental design, the serum problem has been approached in several ways (Mort and Recklies, 1986), however the most promising involved the exposure of cells grown in medium containing serum to defined, serum-free medium. Coincidentally, at the beginning of this thesis research, a serum-free, defined medium which supported growth and proliferation of, and expression of differentiated functions in, HepG2 cells for up to 14 days was reported in the literature (Adeli and Sinkevitch, 1990). This reported success for short maintenance of the HepG2 line, together with its availability (i.e., it had been characterized by Labrutto and Dufresne, 1989) its suitability (i.e., it represented a link between biological and pathological processes and demonstrated relatively high levels of cathepsin B activity), and its relevance (i.e., the HepG2 cell culture system has been used to study the activation of procathepsin B: Scher 1987), made it an obvious choice to examine the potential of establishing serum-free conditions which would permit the long-term maintenance of differentiating cells in culture.

The procedure used to establish HepG2 cells in serum-free, defined medium and the composition of the medium has been presented in Methods (section 2.3, and Figure 4). In brief, HepG2 cells growing exponentially in serum-supplemented medium were transferred to serum-free, defined medium at high cell densities. When these cells were confluent, they were subcultured into serum-free medium; this is defined as subculture 1. Cells plated in serum-free medium, together with parallel cultures in
serum-supplemented medium, were serially transferred 10 times over a 15 week period. Biological (i.e., growth) and biochemical (i.e., intracellular and/or extracellular levels of: total protein, liver-specific apolipoprotein B, active and acid/pepsin activatable cathepsin B) parameters were examined during and after the 10 subculture period for cells maintained in both serum-free and serum-supplemented medium.

3.3 Analysis of HepG2 Maintained in Serum-Free Medium

i) Growth Parameters

Since it is well established that changes in the environment can lead to selection-related problems in characterization (Freshney, 1987), it was important to examine and compare the growth parameters of cells maintained in serum-free medium with those obtained in parallel cultures maintained in serum-supplemented medium (Methods: section 2.6). These results are summarized in Table 6. The saturation densities of both cell populations were virtually the same. In addition, both demonstrated two stages of exponential growth, a pattern previously reported for HepG2 cells growing in serum-supplemented medium (Kelly and Darlington, 1989). The first stage generation times, 20 and 27 hours, were similar in serum-supplemented and serum-free cells. However, the second stage, longer generation times were reproducibly different. Nonetheless, all growth parameter values remained stable with time in culture.

ii) SDS-PAGE Analysis of Intracellular Protein

The effect of serum-free conditions on protein synthesis in HepG2 cells was
Table 6  Growth Parameters of HepG2 Cells Maintained in Serum-Supplemented and Serum-Free Media

<table>
<thead>
<tr>
<th>CELLS</th>
<th>GENERATION TIME $^a$ (Hours) AT STAGE</th>
<th>SATURATION DENSITY $^b$ ($\times 10^5$ Cells/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2 (Serum)</td>
<td>20.4 ± 1.2</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>HepG2 (Serum-Free)</td>
<td>27.6 ± 2.4</td>
<td>0.95 ± 0.11</td>
</tr>
</tbody>
</table>

$^a$ Generation reflects the time it takes for an entire culture to double in number while in exponential growth. Stage 1 generation time reflects the mean ± standard deviations ($N=6$). The values for stage 2 growth reflect minimum times measured.

$^b$ Saturation density is defined as the maximum number of cells/cm$^2$ to which a given cell culture is capable of packing.
examined using SDS-PAGE electrophoresis. At and after initial transfer of HepG2 into serum-free medium, cells were collected at each of 10 consecutive passages (i.e., subcultures 1 through 10) and each used to prepare intracellular fractions for the analysis on 7% resolving gels containing SDS (Methods: 2.7, 2.8). These gels, along with those obtained for control fractions prepared from parallel cultures of HepG2 cells maintained in serum-supplemented medium, were stained and subsequently scanned on a Shimadzu TLC Scanner at a wavelength of 595 nm. Given the number of gels and scans, it was not practical to present all the data obtained. However, the types of absorbance profiles obtained throughout the course of the experiment are represented in Figure 7. This figure presents the gels and the gel scan profiles for subcultures 4 and 7, for serum-free (Figs 7A and 7B), and serum supplemented (Figs 7C and 7D). Intracellular fractions. Both qualitative and quantitative differences were observed in the absorbance profiles between subcultures for each cell population (e.g., compare Figs 7A and 7B), and between cell populations for each subculture (e.g., compare Figs 7A and 7C). Despite these differences, variable levels of at least thirteen peaks (identified on Figure 7) were reproducibly identified at similar molecular weight positions in gel scans of both serum-free and serum-supplemented cell preparations, at each of the 10 subcultures examined.

iii) **SDS-PAGE Analysis of Extracellular Proteins**

Part of the differentiated phenotype of the human hepatoma cell line is the synthesis and secretion of a broad spectrum of plasma proteins (Moses et al., 1983; Dashti et al., 1987). In the past, this property has been difficult to examine since
FIGURE 7
Comparison of SDS-PAGE Profiles of HepG2 Cells Grown in Serum-Free and Serum-Supplemented Media

Cell homogenates were prepared at each of 10 consecutive subcultures. Twelve μg of protein from each homogenate was applied to 7% resolving gels containing SDS. After electrophoresis, gels were stained and analyzed at a wavelength of 595 nm with a densitometer. This figure presents gels and profile scans for subcultures 4 and 7 for serum-free (A and B) and serum-supplemented (C and D) intracellular fractions (homogenates). The relative molecular weights of bands (e.g. positions 8 and 11) are compared to those listed on the x axis for protein standards (described in Materials and Methods: section 2.8).
FIGURE 7

Absorbance 595 nm

Molecular Weight (kDa)
serum, necessary for long-term growth of HepG2 cells, interferes with experimental analysis of secreted proteins in the medium (Freshney, 1987). The establishment of HepG2 cells in serum-free medium allowed us to examine the secretion of proteins into the culture medium by HepG2 cells. Medium was collected from cells at each of 10 consecutive subcultures after initial transfer to serum-free conditions, and, after concentration, applied onto 7% resolving gels containing SDS. These gels, along with those obtained for control fractions, were first stained then scanned on a Shimadzu TLC Scanner at a wavelength of 595 nm. Once again, the number of gels and the number and complexity of their scans precluded inclusion of all the data. However, the types of absorbance profiles obtained throughout the course of the study are represented in Figure 8 for media collected from cells at subculture 4 (Fig. 8B), and subculture 5 (Fig. 8C), as well as for serum-free medium not exposed to cells (Fig. 8A). No bands were detected in control gels (Fig 8A); however, variable levels of six major absorbing peaks were identified at similar positions in all other gel scans. These analyses also indicated that the expression at some peak positions increased slightly or remained relatively constant over time, while that of others decreased. The ratio of percent area under peak 5 at subculture 4 (Fig. 8B) to that at subculture 8 (Fig. 8C), for example, was approximately 1.0, while the same ratio for percent area under peak 6 was reproducibly 0.5.

iv) **Expression of Cathepsin B in HepG2 Cells**

The expression of cathepsin B activity in homogenates prepared from cells grown in serum-supplemented and serum-free medium over a 10 subculture period using the
FIGURE 8
Comparison of SDS-PAGE Profiles of Fractions Prepared from Serum-Free Growth Medium Collected Over Time

At each subculture, medium was collected and concentrated (extracellular fractions). Twelve μg of protein from each fraction was applied to each lane of a 7% resolving SDS-polyacrylamide gel. After electrophoresis, gels were stained and analyzed at a wavelength of 595 nm with a densitometer. This figure presents gels and profile scans for medium not exposed to cells (A) and medium collected from cells at subcultures 4 (B) and 7 (C). Molecular weight markers (Materials and Methods: section 2.8) and major band positions (e.g., 5 and 6) are indicated.
synthetic colorimetric substrate, Cbz-arg-arg, was examined. The results of this study are presented in Figure 9. Cells maintained in serum-free medium retained the ability to express variable levels of cathepsin B activity over time. These levels contrasted to the sometimes lower, less variable levels observed in cells maintained in serum-supplemented medium.

v) Secretion of Apolipoprotein B by HepG2 Cells

Previous studies concerning the expression of liver-specific phenotypes in HepG2 cells were restricted to short term measurements within one subculture (Adeli and Sinkevitch, 1990; Macri and Adeli, 1993). The obvious advantage of serum-free medium for long-term measurements prompted us to examine the secretion of apolipoprotein B (ApoB) into the medium over the 10 subculture period of the experiment using an enzyme-linked immunosorbant analysis (ELISA) procedure developed by Macri and Adeli (1993). Although all the samples were prepared by, and the data analyzed by the author, the ELISA analyses were conducted in collaboration with André Theriault (Chemistry and Biochemistry, University of Windsor). The results, summarized in Figure 10, indicated that HepG2 cells, established and maintained in serum-free medium, retained the ability to secrete apoB into the medium throughout the entire study period. Levels of apoB in the medium ranged from 2.85 to 5.93 µg/mg of total protein with a mean value of 4.86 ± a standard deviation of 0.97.

vi) Expression of Cathepsins During Growth of HepG2 Cells

Earlier in this section, growth parameters of HepG2 maintained in serum-free
FIGURE 9
Comparison of Cathepsin B Activity in HepG2 Cells Grown in Serum-Free Medium Over Time

At each subculture, cell homogenates were prepared and protein adjusted to 0.16 mg/ml. Protease activity was determined in each using the synthetic substrate, arg-arg, and conditions which favor cathepsin B activity. One unit of activity (EU) is defined as that amount of enzyme which gives rise to an absorbance of 0.016 at a wavelength of 520 nm. Specific activity is expressed as enzyme units (EU) per mg protein. Each value, with error bar, represents the mean ($\bar{x}$) of 4 measurements ($N = 4$) and standard deviations ($\bar{x} \pm$ s.d.) for values at each subculture (sc) were as follows: 5% serum (solid bars): sc1: (24.6 ± 1.4); sc2: (26.4 ± 2.3); sc3: (27.8 ± 2.1); sc4: (17.5 ± 1.1); sc5: (19.2 ± 1.4); sc6: (21.2 ± 2.8); sc8: (18.2 ± 1.5); sc9: (19.0 ± 1.6); sc10: (21.7 ± 2.9). Serum-free (hatched bars): sc1: (60.0 ± 2.1); sc2: (30.6 ± 8.1); sc3: (38.3 ± 2.3); sc5: (23.8 ± 2.3); sc6: (26.2 ± 2.6); sc8: (65.3 ± 6.3); sc9: (61.3 ± 2.2); sc10: (26.7 ± 1.2).
FIGURE 10
Comparison of Apolipoprotein B in the Media of HepG2
Cells Grown in Serum-Free Medium Over Time

Medium was collected from cells at each subculture and used to prepare extracellular fractions for ELISA analysis (Macri and Adeli, 1993). Extracellular fractions were incubated with apoB-biotin conjugated with extravidin-alkaline phosphatase. The colour intensity in each sample, after treatment with paranitrophenylphosphate and NaOH, was measured at a wavelength of 405 nm and converted to µg apoB/mg of total protein (solid bars). Activity, with error bar, is expressed as a mean value (N = 3) for each of the subcultures examined. Standard deviations varied considerably from subcultures (sc) 1 through 10 as follows: sc1 = 2.85 ± 0.31; sc2 = 4.41 ± 0.44; sc4 = 5.75 ± 0.06; sc5 = 4.48 ± 0.61; sc6 = 5.18 ± 0.32; sc7 = 5.28 ± 0.07; sc9 = 5.93 ± 1.19; sc10 = 5.03 ± 0.73.
FIGURE 10

SUBCULTURE NO.

μg APOB/mg PROTEIN
and serum-supplemented media were presented. In both culture environments, HepG2 demonstrated two stages of growth, the first reflecting a relatively short cell cycle and the second reflecting an extended cell cycle. This two-stage pattern of growth has been reported to reflect a modulation of the liver phenotype which occurs during fetal/adult development or during liver regeneration (Kelly and Darlington, 1989). Since alterations in levels of lysosomal proteases have been implicated in end-point differentiation of normal cells (Campbell et al., 1991), as well as metastasis of malignantly transformed cells (Veksler et al., 1987), the expression of lysosomal cysteine proteases was measured in fractions prepared from cells (intracellular fractions) and their media (extracellular fractions) during the second stage of HepG2 growth. Three fluorometric, methylcoumarin substrates were used to increase the sensitivity and range of the study (reviewed in Methods: 2.9 A-ii and Table 3). In brief, cells were seeded and grown in serum-free, defined medium. At 24 hour intervals after initial seeding, the medium was removed from cells and replaced with fresh serum-free medium. Cells were then incubated at 37°C for an additional 24 hours after which cell counts were determined, and cells and their medium were collected and used to prepare intracellular and extracellular fractions respectively (Methods: 2.7). Extracellular fractions were treated with acid (pH 3.8), and with the aspartic protease, pepsin, at pH 3.8 to activate latent proenzyme (Methods: 2.9 C.).

The combined expression of all three cathepsins [B+H+L] in intracellular and extracellular fractions of HepG2 at each phase of stage 2 growth is presented in Figure 11, while the expression of each cathepsin in each fraction at each phase is summarized in Table 7. The population doubling time for HepG2 cells was approximately 72
FIGURE 11
Combined Levels of Cathepsins [B+H+L] in Intracellular and
Extracellular Fractions of HepG2 Cells During Second Stage Growth

Cells (10^6) were seeded and grown in serum-free medium. At 24 h intervals, the medium was removed from cells and replaced with fresh serum-free medium. Cells were then incubated at 37°C for an additional 24 h after which cell counts (closed circles) were determined, and cells and their medium were collected and used to prepare intracellular and extracellular fractions respectively. Protease activity in intracellular (hatched bars), acid-treated (pH 3.8 24 h) extracellular (parallel lined bars), and pepsin-acid-treated (pH 3.8, 1 h) extracellular (open bars) was measured using Z-arg-arg, Z-arg, and Z-phe-arg aminomethylcoumarin substrates under conditions optimal for cathepsins B, H, and L, respectively. Enzyme activity is expressed as combined mEU/mlts [cathepsin B + cathepsin H + cathepsin L] per mg protein (mEU/mg) where one mEU is defined as that quantity of protease that liberates 1 nmol of aminomethylcoumarin per min. Each value, with error bar, represents the mean (x). These mean values and their standard deviations (x ± S.D.) are presented in table 6.
<table>
<thead>
<tr>
<th>Fraction/Cathepsin</th>
<th>Specific Activity in Phase&lt;sup&gt;a&lt;/sup&gt; (uEUnits/mg protein)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-lag</td>
<td>2-Early</td>
<td>3-Late</td>
<td>4-Plateau</td>
</tr>
<tr>
<td>HepG2-Intra</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>445 ± 36</td>
<td>469 ± 44 (1.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500 ± 26 (0.7)</td>
<td>456 ± 141 (1.2)</td>
</tr>
<tr>
<td>L</td>
<td>525 ± 70</td>
<td>579 ± 23 (1.1)</td>
<td>688 ± 392 (0.7)</td>
<td>584 ± 65 (1.3)</td>
</tr>
<tr>
<td>H</td>
<td>60 ± 51</td>
<td>49 ± 29 (0.8)</td>
<td>43 ± 37 (0.9)</td>
<td>61 ± 23 (1.4)</td>
</tr>
<tr>
<td>HepG2-Extra&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>63 ± 16</td>
<td>58 ± 15 (0.9)</td>
<td>56 ± 11 (1.0)</td>
<td>36 ± 9 (0.6)</td>
</tr>
<tr>
<td>L</td>
<td>133 ± 7</td>
<td>257 ± 19 (1.9)</td>
<td>289 ± 2 (1.1)</td>
<td>174 ± 23 (0.6)</td>
</tr>
<tr>
<td>H</td>
<td>21</td>
<td>--</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific activity was determined as described in Methods. The term **phases** refers to the four phases defined by Freshney (1987) for growth of cells in culture: 1 = lag - adhesion and initiation of growth and division; 2 = early exponential - initial period of linear, population doubling; 3 = late exponential - end period of linear population doubling; 4 = plateau - saturation reflecting contact inhibition.

<sup>b</sup> Numbers in parentheses reflect the ratio of the activity at one phase of growth to that measured at the preceding phase of growth.

<sup>c</sup> Activity in extracellular fractions reflects enzyme activated by pepsin treatment at acid pH (Methods).
hours, a time consistent with second stage growth. During this time, levels of total
cathepsin activities in the cells were relatively similar at each phase of growth. These
levels were greater (i.e. 4-5 fold) than the levels of total cathepsin activities detected in
extracellular fractions treated with acid alone, or with pepsin at acid pH. Levels of
total cathepsin activities in pepsin/acid-treated fractions (i.e., pepsin at pH 3.8, 1 h)
were always greater than those detected in acid-treated fractions (pH 3.8) even after 36
hours. The greater sensitivity of mammalian procathepsins (e.g., B and L) to \textit{in vitro}
acid/pepsin activation is well documented (Nishimura \textit{et al.}, 1988; Nishimura \textit{et al.},
1990). A comparison of the expression of each cathepsin (Table 7) suggested that the
relative order of cathepsin activities in all fractions from highest to lowest, was
cathepsin L, cathepsin B, and cathepsin H. This is consistent with earlier studies
characterizing cathepsins in rat liver lysosomes (Kirschke \textit{et al.}, 1977) and more recent
studies establishing the major excreted protein (MEP) with procathepsin L (Troen \textit{et al.},
1991). While levels of activity varied between cathepsins, levels for each
cathepsin, in both intracellular and pepsin/acid-treated extracellular fractions, remained
relatively constant during growth with the exception of extracellular levels of cathepsin L
which almost doubled from lag to early exponential phases of growth (i.e., the ratio of
cathepsin L activity at early exponential phase of growth to that at lag phase was 1.8
while the corresponding ratios for intracellular levels of cathepsin L, and intra and
extracellular levels of cathepsin B were 1.0, 1.0, and 0.9 respectively). These results
are consistent with previous reports suggesting that latent procathepsin L is the
dominant secreted form (i.e. M.E.P.) in malignantly transformed cells (Troen \textit{et al.},
3.4 Analysis of Differentiating L6 Rat Myoblasts

When L6 myoblasts were transferred from alpha MEM supplemented with 10% horse serum to alpha MEM without serum for 24 hours, the plating efficiency and fusion index of the resulting population decreased dramatically. In contrast, when the same cells were transferred to serum-free, defined medium, plating efficiency and fusion index remained at control levels. Thus, the serum-free, defined medium used in the analysis of HepG2 liver cells provided a more reliable culture environment to examine the expression and secretion of lysosomal cysteine proteases in the more complex L6 muscle cells, a model system for myogenic differentiation.

As discussed in the introduction, muscle differentiation involves changes in the phenotypes of progenitor myoblast cells which lead to their fusion into synehtial myotubes (i.e., morphological differentiation) and a concomitant expression of muscle-specific proteins (Dufresne et al., 1976; Kaur and Sanwal, 1981). For some time, it has been reported that these changes are temporally related and are associated with altered expression of calcium-activated neutral proteases (Kaur and Sanwal, 1981), and lysosomal cysteine proteases (Bird et al., 1981). These studies, however, were restricted to analysis of levels of proteases inside the cell. The establishment of serum-free conditions, and the availability of a fusion-deficient variant of L6, L6-D3, allowed us to extend these studies to include: 1) a comparison of the expression of intracellular and extracellular levels of lysosomal cysteine proteases during differentiation of L6 myoblasts, and 2) the relationship of the myoblast-myotube fusion process to this expression.
Table 8. Properties of Differentiating L6 Rat Myoblasts and Their Non-Fusing Variant, L6-D3a

<table>
<thead>
<tr>
<th>Growth Property</th>
<th>L6</th>
<th>Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphologyb</td>
<td>normal bipolar mononucleated myoblasts</td>
<td>flat with long long thread-like extensions mononucleated myoblasts</td>
</tr>
<tr>
<td>Early</td>
<td>![Image of early morphology]</td>
<td>![Image of early morphology]</td>
</tr>
<tr>
<td>Late</td>
<td>multinucleated myotubes</td>
<td>mononucleated myoblasts</td>
</tr>
<tr>
<td>![Image of late morphology]</td>
<td>![Image of late morphology]</td>
<td></td>
</tr>
</tbody>
</table>

Growth Parametersc

<table>
<thead>
<tr>
<th>Parameter</th>
<th>L6</th>
<th>L6-D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling Time</td>
<td>23.1 hours</td>
<td>22.2 hours</td>
</tr>
<tr>
<td>E.O.P.</td>
<td>&gt; 80%</td>
<td>&gt; 80%</td>
</tr>
<tr>
<td>Modal Chromosome No.</td>
<td>60 ± 3</td>
<td>62 ± 6</td>
</tr>
<tr>
<td>Anchorage Dependence</td>
<td>High</td>
<td>high</td>
</tr>
<tr>
<td>Viability in Serum-Free Medium</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Genetic Markers</td>
<td>---</td>
<td>8-AzacytidineR</td>
</tr>
</tbody>
</table>

a The rat myoblast cell line, L6-D3, is a 5-azacytidine resistant, non-fusing variant of L6 (Ng et al., 1976)
b Cells were stained with Giemsa, as described in Methods.
c This data is taken from Dufresne et al, 1976, and Ng et al, 1976.
Levels of Cathepsins B, H and L in L6 and L6-D3 Cell Fractions

Some biological characteristics of L6 and L6-D3 are presented in Table 8. These characteristics reflected the suitability of the L6 myoblast cell system. First, both L6 and L6-D3 demonstrated similar growth parameters and similar phases of growth (i.e., lag, exponential, and plateau). Second, both cell lines were highly anchorage dependent (i.e., require a surface for growth). Third, both were stable with respect to their phenotypes and karyotypes over time. Fourth, both could be transferred to serum-free, defined medium without loss of viability. Fifth, both were derived from the same L6B rat myoblast cell lines; and sixth, despite their common myoblast derivation, L6-D3 had lost the ability to form myotubes.

L6 and L6-D3 myoblasts were plated in medium containing 10% horse serum, conditions which enhance myoblast-myotube fusion in L6. For each of four phases of growth (i.e., lag (=1), exponential- early (=2) and late (=3), and plateau (=4)), medium was removed from cells and replaced with serum-free, defined medium. Cells were then incubated at 37°C for 24 hours after which cell counts (L6-D3) or fusion index (L6) were determined, and cells (intracellular fractions) and their medium (extracellular fractions) were collected. Levels of cathepsins B, H, and L, were then determined in intracellular fractions and acid/pepsin treated extracellular fractions using the appropriate fluorometric, methylcounarin substrate (Methods: 2.7 and 2.9 C.). To facilitate comparison, the results of these analyses are presented in Figures 12 (L6) and 13 (L6-D3), and detailed and summarized in Table 9.

Within each cell line, intracellular levels of total cathepsins (i.e., [B+H+L]) at each phase of growth were greater than levels of activated cathepsins in extracellular
FIGURE 12

Combined Levels of Cathepsins [B+H+L] in Intracellular and Extracellular Fractions of L6 Myoblasts During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. At four phases characteristic of growth of cells in culture (1 = lag, 2 = early exponential, 3 = late exponential, 4 = plateau) the medium was removed from cells and replaced with fresh serum-free medium. Cells were then incubated at 37°C for an additional 24 h after which fusion index (closed circles), the percent ratio of number of nuclei in myotubes to total number of nuclei was determined by the method of Morris and Cole (1972). Cells and their medium were collected and used to prepare intracellular and extracellular fractions, respectively (Methods: 2.7). Protease activity in each intracellular (hatched bars), and pepsin-treated (pH 3.8, 1h) extracellular (open bars) fraction was measured using Z-arg-arg, Z-arg, and Z-phe-arg aminomethylcoumarin substrates under conditions optimal for cathepsins B, H, and L, respectively. Enzyme activity is expressed as combined mEUnits of [cathepsin B + cathepsin H + cathepsin L] per mg protein (mEU/mg) where one mEU is defined as that quantity of protease that liberates 1 nmol of aminomethylcoumarin per minute. Each value, with error bar, represents the mean (X). Mean values ± standard deviations (X ± S.D.) are presented in Table 8.
FIGURE 13
Combined Levels of Cathepsins [B+H+L] in Intracellular and
Extracellular Fractions of L6-D3 During Growth

Cells (5 x 10^5) were seeded and grown in serum-supplemented medium. At four phases characteristic of growth of cells in culture (1 = lag, 2 = early exponential, 3 = late exponential, and 4 = plateau), the medium was removed from cells and replaced with fresh serum-free medium. [NOTE: Fusion was never observed in L6-D3.] Cells were then incubated at 37°C for an additional 24 h after which cell counts (closed circles) were determined, and cells and their medium were collected and used to prepare intracellular and extracellular fractions, respectively. Protease activity in each intracellular (hatched bars) and pepsin/acid-treated (pH 3.8, 1 h) extracellular (open bars) fraction was determined using Z-arg-arg, Z-arg, and Z-phe-arg aminomethylcoumarin substrates under conditions optimal for cathepsins B, H, and L, respectively. Enzyme activity is expressed as combined mEU units of [cathepsin B + cathepsin H + cathepsin L] per mg protein (mEU/mg) where one mEU is defined as that quantity of enzyme that liberates 1 nmol of aminomethylcoumarin per minute. Each value, with error bar, represents the mean (\bar{x}). These mean values and their standard deviations (\bar{x} \pm S.D.) are presented in Table 9.
<table>
<thead>
<tr>
<th>Fraction/Cathepsin</th>
<th>Specific Activity in Phase(^a) (uEUnits/mg protein)</th>
<th>1-Lag</th>
<th>2-Early</th>
<th>3-Late</th>
<th>4-Plateau</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L6-Intra/</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>216 ± 33</td>
<td>77 ± 8 (0.4)</td>
<td>326 ± 6 (4.2)</td>
<td>262 ± 2 (0.8)</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>283 ± 99</td>
<td>102 ± 21 (0.4)</td>
<td>509 ± 22 (5.0)</td>
<td>87 ± 1 (0.8)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>5 ± 2</td>
<td>2 ± 1 (0.4)</td>
<td>27 ± 6 (13.5)</td>
<td>20 ± 2 (0.7)</td>
<td></td>
</tr>
<tr>
<td><strong>L6-D3-Intra/</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>--</td>
<td>206 ± 21</td>
<td>212 ± 17 (1.0)</td>
<td>255 ± 80 (1.2)</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>--</td>
<td>838</td>
<td>1040 (1.2)</td>
<td>1050 (1.0)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>--</td>
<td>6</td>
<td>9 (1.5)</td>
<td>17 (1.9)</td>
<td></td>
</tr>
<tr>
<td><strong>L6-Extra/</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>33 ± 2</td>
<td>34 ± 1 (1.0)</td>
<td>26 ± 2 (0.8)</td>
<td>21 ± 1 (0.8)</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>22 ± 6</td>
<td>56 ± 4 (2.5)</td>
<td>125 ± 2 (2.2)</td>
<td>103 ± 7 (0.8)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>3 ± 4</td>
<td>4 ± 1 (1.3)</td>
<td>4 ± 1 (1.0)</td>
<td>3 ± 1 (0.8)</td>
<td></td>
</tr>
<tr>
<td><strong>L6-D3-Extra/</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>--</td>
<td>9 ± 2</td>
<td>11 ± 2 (2.0)</td>
<td>22 ± 4 (0.8)</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>--</td>
<td>416 ± 9</td>
<td>664 ± 54 (1.6)</td>
<td>819 ± 27 (1.2)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>--</td>
<td>4 ± 1</td>
<td>1 ± 1 (0.3)</td>
<td>4 ± 1 (4.0)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Specific activity was determined as described in Methods. The term phases refers to the four phases defined by Freshney (1987) for growth of cells in culture: 1 = lag - adhesion and initiation of growth and division; 2 = early exponential - initial period of linear, population doubling; 3 = late exponential - end period of linear population doubling; 4 = plateau - saturation reflecting contact inhibition. In the case of L6, the corresponding fusion index values (i.e., % nuclei in myotubes relative to total number of nuclei in a field) at each of these four stages are: phase 1 = 0%, 2 = < 5%, 3 = 70% - 80%, 4 = < 70%.

\(^b\) Numbers in parentheses reflect the ratio of the activity at one phase of growth to that measured at the preceding phase of growth.

\(^c\) Activity in extracellular fractions reflects enzyme activated by pepsin treatment at acid pH (Methods).
fractions (Figures 12 and 13). Absolute levels of activities in L6-D3 fractions, however, were reproducibly greater than corresponding phase-related levels in L6 fractions. This is consistent with the high levels of cysteine proteases detected in homogenates prepared - presumably- from near confluent populations of a non-fusing subclone of L6 compared to prefusion myoblasts (Bird et al., 1980).

The pattern of expression of total cathepsins, with time in culture, also differed in L6 and L6-D3. Levels of cathepsins [B+H+L] detected at the earliest phase of L6-D3 growth were high and remained relatively constant thereafter. Interestingly, this pattern was also observed in HepG2 (Figure 11, Table 7). In contrast, a fusion-related increase in intracellular levels of cathepsins [B+H+L] was observed in L6 during exponential growth (compare phase 2 and 3 in Figures 12 and 13). This pattern of expression is consistent with that reported for the expression of intracellular lysosomal cysteine proteases during keratinocyte differentiation (Tanabe et al., 1981), myoblast-myotube differentiation in L6 rat myoblasts (Bird et al., 1980; Kirschke et al., 1983; Gopalan et al., 1987), and myoblast-myotube differentiation in primary cultures of calf muscle cells (Béchet, et al., 1991).

The contribution of each cathepsin to the L6 and L6-D3 patterns is presented in Table 9. In all fractions of both cell lines, levels of cathepsin L activity were generally greater than cathepsin B which, in turn, were greater than levels of cathepsin H. The fusion-related increase in L6 intracellular cathepsin activities, described in Figure 12, reflected increases in all three cathepsins (Table 9: L6-Intra : compare ratio of activity at phase 3 to activity at phase 2 for cathepsins B, H, and L). In contrast, the fusion-related increases in the lower levels of activatable cathepsin activity in extracellular
fractions of L6 were restricted to cathepsin L (Table 9: L6-Extra). In L6-D3 fractions, the constant relatively high intracellular levels of cathepsin activities appeared to reflect the constant levels of both cathepsins B and cathepsin L (Table 9: L6-D3 Intra), while increases in the lower levels of activatable cathepsin activity in extracellular fractions were restricted to cathepsin L (Table 9: L6-D3-Extra). Comparing cathepsin L activities between fractions of L6 and L6-D3, levels of extracellular cathepsin L represented about 80% of maximum intracellular in L6-D3 compared to 20% for L6. Although there is an obvious lack of literature dealing with both intracellular and extracellular levels of cysteine proteases in differentiating cell culture systems, the biological significance of the L6, L6-D3 results was supported by three additional observations. First, increases in levels of acid/pepsin-activatable cathepsin activities were eliminated by preincubating the extracellular fractions with either the cysteine protease inhibitor, E-64, or the aspartic protease inhibitor, pepstatin. Second, while the absolute activities of the cathepsins in fractions of L6 and L6-D3 varied between experiments, the relative activities remained the same. Third, a similar increase in pepsin-activatable cathepsin L activity was observed for hepatic differentiation in HepG2 (Figure 11, Table 7).

**ii) FPLC Fractionation of L6 and L6-D3 Cell Fractions**

The fusion-related increase in cathepsins B, H, and L activities during myoblast-myotube differentiation in L6, and the differences in expression between L6 and the fusion-deficient variant L6-D3 could be explained by several mechanisms including alterations in transcription and translation, alterations in processing and
cellular localization, and alterations in regulation by endogenous inhibitors (reviewed in Sloane, 1990; and Chambers, 1992). They could also reflect undefined, complex interactions occurring in crude cell homogenates; for example, interactions between proteases and their endogenous inhibitors (Green et al., 1984; Barrett et al., 1986; Chambers, 1992). As a first approach to unraveling these possibilities, L6 and L6-D3 homogenates were fractionated on a Superose 12 HR 10/30 column. This column permits the efficient separation of molecules with molecular weights between 10 kDa and 300 kDa, a range that includes various lysosomal cysteine proteases, and cystatin-like, cysteine protease inhibitors (Barrett et al., 1986). A selectivity curve was prepared by calculating separation coefficients, $K_{av}$, (Methods: 2.12) for different molecular weight standards (Figure 14). This curve was in excellent agreement with the separation curve provided by the manufacturer and demonstrated reproducible separation of both exogenous pork liver cathepsin B and commercial egg white cystatin preparations without loss of their biological activities. For reference, Figure 14 also includes the apparent molecular weight positions at which the endogenous inhibitor I (against purified cathepsin B), and endogenous protease (i.e., cathepsins B, H, and L) activities, from L6 and L6-D3 cells, were detected. These positions, discussed next, were identical for all three HR 10/30 columns used during the course of this thesis research.

Cell homogenates were prepared from growing cultures of L6 and L6-D3 and similar amounts of protein from each (i.e., 500 µg for assays with arg-arg, 200 µg for assays with methylcoumarin substrates) in homogenization buffer (pH 6.0) were injected separately onto a Superose 12 HR 10/30 FPLC column at room temperature.
FIGURE 14
Superose 12 HR 10/30 FPLC Column Standard Curve

Molecular weight standards (bovine albumin, 66.2 kDa; ovalbumin, 42.7 kDa; chymotrypsin, 25.0 kDa; and cytochrome c, 13.0 kDa) were applied to and eluted from a Superose 12 HR 10/30 FPLC column (Methods: 2.11). The void volume was determined with Blue Dextran and used, together with the elution volume for each molecular weight standard, to calculate $K_a$ values. A plot of $K_a$ versus molecular weight was constructed and used to obtain an approximate molecular weight for cathepsin B, cathepsin H, and cathepsin L activities (B, H, and L) using aminomethylcoumarin substrates (Methods: 2.9 B-ii), and inhibitory activity (I) against purified pork liver cathepsin B (Methods: 2.10 B-ii).
Fractions were eluted at room temperature with phosphate column buffer, pH 6.0, at a flow rate of 1.0 ml/min. Absorbance at 280 nm was monitored with a Beckman Analytical Optical Unit and recorded on a Kipp and Zonen chart recorder. Depending on the experiment, 250 µl and 500 µl fractions were collected, stored on ice then assayed for cysteine protease activity using arg-arg (for cathepsin B) or the three methylcoumarin (for cathepsins B, H, and L) substrates. Inhibitor activity was measured against purified, pork liver cathepsin B (Methods: 2.9 B.). Representative elution and activity profiles for L6 and L6-D3 cells at three different stages of growth, early- exponential, mid-exponential, and late-exponential/plateau are presented in Figures 15, 16, and 17, respectively. In each figure, the top profile (i.e., A) is that for L6 while the bottom (i.e., B) is that for L6-D3.

Distinct regions of protease and inhibitor activities were observed at similar positions for both cell populations at all phases of growth. Based on the standard FPLC separation curve (Figure 14), cathepsin B and cathepsin L peak activities both eluted at a position corresponding to an apparent molecular weight of 31.0 kDa. Cathepsin H, when detected, eluted at a position corresponding to a molecular weight of 27.5 kDa. These values are consistent with values previously reported for cathepsin B from primary cultures of calf muscle cells (Béchet et al., 1991), and for cathepsin H from rat hepatocytes (Nishimura and Kato, 1988). The positions for cathepsins B, H, and L did not change during the growth of either cell line.

The relative order of cathepsin activities in fractions collected from FPLC paralleled the order of activities obtained in unfractionated cell preparations, with cathepsin L (corrected) expressing the greatest activity, followed by cathepsin B.
FIGURE 15
Levels of Protease and Protease Inhibitor Activities in FPLC Fractions of L6 and L6-D3 During Early Exponential Growth

Centrifuged homogenates were prepared from L6 and L6-D3 cells collected during early exponential growth. Two hundred μg of protein from each 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. Protease activity in each fraction of L6 (A) and L6-D3 (B) was determined using Z-arg-arg, Z-arg, and Z-phe-arg aminomethylcoumarin substrates under conditions optimal for cathepsin B, cathepsin H, and cathepsin L, respectively. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm (Methods: 2.9 B-ii). Inhibitor activity in each fraction of L6 (A) and L6-D3 (B) was measured against purified pork cathepsin B. Protease activity is expressed as μEUnits (○), where 1 μEU is defined as the quantity of protease that liberates 1 pmoles of aminomethylcoumarin per minute. Inhibitor activity (-▲-) is expressed as percent (%) inhibition which is defined as the percentage change in the averaged activity of purified pork liver cathepsin B in sample fractions relative to control levels of cathepsin B activity.
FIGURE 15
Centrifuged homogenates were prepared from L6 and L6-D3 cells during mid-late exponential growth. Two hundred μg of protein from each 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. Protease activity in each L6 (A) and L6-D3 (B) fraction was determined using Z-arg-arg, Z-arg, and Z-phe-arg aminomethylcoumarin substrates under conditions optimal for cathepsins B, H, and L, respectively. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm (Methods: 2.9 B-ii). Inhibitor activity in each fraction of L6 (A) and L6-D3 (B) was measured against purified pork liver cathepsin B. Protease activity (○) is expressed as μEUnits where 1 μEU is defined as the quantity of protease that liberates 1 pmole of aminomethylcoumarin per min. Inhibitor activity (▲) is expressed as percent (%) inhibition which is defined as the percentage change in the averaged activity of purified pork liver cathepsin B in sample fractions relative to control levels of cathepsin B activity.
Centrifuged homogenates were prepared from L6 and L6-D3 cells during late/plateau growth. Two hundred µg of protein from each fraction 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. Protease activity (○) in each fraction of L6 (A) and L6-D3 (B) was determined using Z-arg-arg, Z-arg, and Z-phe-arg aminomethylcoumarin substrates under conditions optimal for cathepsin B, cathepsin H, and cathepsin L, respectively. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm (Methods: 2.9 B-ii). Inhibitor activity (▲) in each L6 (A) and L6-D3 (B) fraction was measured against purified pork liver cathepsin B. Protease activity is expressed in µEUnits where 1 µEU is defined as the quantity of protease that liberates 1 pmole of aminomethylcoumarin per min. Inhibitor activity is expressed as percent (%) inhibition which is defined as the percentage change in the averaged activity of purified pork liver cathepsin B in sample fractions relative to control cathepsin B activity.
FIGURE 17
Cathepsin H activity, although not always detected (e.g., L6-D3), was consistently the lowest. In both cell lines at both phases of growth, peak average levels of cathepsin L (i.e., the calculated average of enzyme activity at each region) were greater than peak average levels of cathepsin B. However, in L6-D3, the ratio of cathepsin L to cathepsin B remained relatively similar over time (e.g., early exponential = 4.4; late exponential = 5.0) whereas the comparable ratios in L6 decreased (i.e., early exponential = 9.0; late exponential = 2.0). Within L6, levels of cathepsins B and L increased by about 11 and 3 fold, respectively, from early to late exponential phases of growth. In contrast, levels of cathepsins B and L activities from early to late exponential phases of growth for L6-D3 remained relatively similar (Table 10).

Inhibitor activity against cathepsin B for both L6 and L6-D3, at each phase of growth, eluted after endogenous cysteine protease activities within a 12.0 kDa to 25.0 kDa molecular weight range. Within this range, two distinct inhibitory regions were reproducibly observed at average molecular weight positions corresponding to 12.5 kDa and 23.0 kDa in profiles of L6. Similar regions were identified in L6-D3 at positions corresponding to 12.0 kDa, and 20.0 kDa. These results are consistent with those reported for cysteine protease inhibitors in rat, bovine, and rabbit skeletal muscle systems (Schwartz and Bird, 1977; Matsumoto et al., 1983; Bige et al., 1985; Matshuishi et al., 1988). A comparison of total average levels of inhibition—the sum of inhibitor activities, in μIU, against cathepsin B at both regions, demonstrated similar levels in both L6 and L6-D3 cells at all phases of growth. Taken together, these results translated into a 10-13 fold increase in the ratio of cathepsin B to inhibitor activities during growth and differentiation of L6 cells. This increase was fusion-
Table 10. Summary of Cathepsin B Enzyme and Cathepsin B Inhibitor Levels in FPLC Fractions of Non-Heated and Heated Homogenates of L6 and L6-D3 Cells

<table>
<thead>
<tr>
<th>CELLS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CATHEPSINC&lt;sup&gt;c&lt;/sup&gt; (µEUnits x 10)</th>
<th>INHIBITOR&lt;sup&gt;d&lt;/sup&gt; (µIUunits x 10)</th>
<th>RATIO&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PERCENT&lt;sup&gt;f&lt;/sup&gt; INHIBITION [N.H.] [H]&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6 myoblasts</td>
<td>19.0</td>
<td>2.5</td>
<td>0.8</td>
<td>8.7</td>
</tr>
<tr>
<td>L6 myotubes</td>
<td>49.0</td>
<td>2.2</td>
<td>10.5</td>
<td>7.7</td>
</tr>
<tr>
<td>L6-D3 myoblasts (E)</td>
<td>22.0</td>
<td>2.3</td>
<td>2.2</td>
<td>16.6</td>
</tr>
<tr>
<td>L6-D3 myoblasts (L)</td>
<td>35.0</td>
<td>3.1</td>
<td>1.6</td>
<td>22.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> For each cell population, 200 µg of non-heat treated or heat-treated (80°C, 5 min) protein was applied to the FPLC HR-10/30 column and eluted as described in Methods.

<sup>b</sup> Three cell populations were examined: L6 myoblast, L6 myotube, and L6-D3 homogenates were prepared from cells maintained in alpha-MEM supplemented with 10% horse serum. In the case of L6 myoblast and L6 myotube fractions, the corresponding fusion index values (i.e., % nuclei in myotubes relative to total number of nuclei in a field) were 0% and >60% respectively. In the case of L6-D3 cells, (E) and (L) refer to early exponential and late exponential phases of growth respectively.

<sup>c</sup> Cathepsin activity, in µEUnits was measured using the fluorometric substrates, Z-arg-arg-NMec, and Z-phe-arg-NMec, for cathepsins B and L respectively, as described in Methods. The values presented here represent the mean averaged peak activity where N=2. Cathepsin activity was not detected in heated samples.

<sup>d</sup> Inhibitor activity, in µIUunits was measured against a constant amount of purified pork liver cathepsin B. One µIUunit of inhibitor activity, µIU, is defined as that amount of protein that inhibits 1 µEUnit of cathepsin B. The values presented here represent the mean averaged peak activity where N=2.

<sup>e</sup> Ratio refers to the ratio of averaged cathepsin B activity (µEUnits) to averaged inhibitor activity (µIUunits) calculated for each cell population.

<sup>f</sup> Percent Inhibition is the percentage change in the averaged activity of purified pork liver cathepsin B in sample fractions relative to control levels of cathepsin B activity.

<sup>g</sup> [N.H.] refers to non-heated samples; [H] refers to heated samples.
related and was not observed during growth of L6-D3 where ratios of cathepsin to inhibitor were reproducibly about 2.

Although the ratio of cathepsin B to inhibitor activities increased during growth and differentiation of L6, inhibitor levels (µIU units x 10) remained relatively constant. Levels of inhibitor activity during growth of L6-D3, although higher than those measured in L6, also remained relatively constant. The expression of constant levels of inhibitor activity, presented as percent inhibition to facilitate comparison with the literature, were somewhat lower (i.e., 8%) but consistent with those reported for inhibition of cathepsin B in prefusion (i.e., 17%) and postfusion (i.e., 16%) L6 cells (Kirschke and Bird, 1983).

Heat treatment of myoblast and myotube fractions of L6 at 80°C for 5 minutes, a treatment which eliminates cathepsin activities in both L6 and L6-D3 (Figure 18) and presumably dissociates active inhibitor from protease (Green et al., 1984), had some effect on the $A_{280}$ profile and molecular weight positions of inhibitory activities in L6 myoblast (Figure 19A) and L6 myotube (Figure 19B) FPLC fractions, but not in levels of inhibitor activities (Table 10). Interestingly, heat treatment of L6-D3 fractions appeared to result in a decrease in inhibitor activity (Table 10). Although the biological and experimental significance of this observation is unclear, the overall results of the FPLC studies (i.e., Figures 10, 11, 12, 14 and Table 7) are consistent with the differences in levels of cathepsin B activities, and the similarities in levels of cathepsin B inhibitory activities within and between L6 and L6-D3 cell lines.
FIGURE 18
Sensitivity of Cathepsin B Activity in L6 and L6-D3
Homogenates to Increasing Temperatures

Cell homogenates were prepared from L6 and L6-D3 cells and, after centrifugation, adjusted to a protein concentration of 0.016 mg/ml. Two hundred and fifty µl aliquots were then treated for 5 minutes at different temperatures ranging from 25°C to 100°C. After microfuge centrifugation, protease activity in L6 early fusion (▲), L6 late fusion (●), and L6-D3 (○) samples were determined using arg-arg and conditions optimal for cathepsin B. Protease activity is expressed as enzyme units where 1 EU is defined as that amount of enzyme which gives rise to an absorbance of 0.016 in one minute at a wavelength of 520 nm. Specific activities at 15 min and 30 min time points for each temperature were identical to the third decimal place.
FIGURE 19
Levels of Cathepsin B Enzyme and Cathepsin B Inhibitor Activities in Heat-Treated Homogenates of L6 Cells After FPLC Fractionation

Cell homogenates were prepared from L6 myoblasts and L6 myotubes and, after centrifugation, adjusted to a protein concentration of 0.5 mg/ml. Homogenates were then heat-treated for 5 minutes at 80°C, centrifuged, and 200 µg of protein from each homogenate applied to, and eluted from, in a volume of 500 µl, a Sepharose 12 HR 10/30 FPLC column at a flow rate of 1.0 ml/min. Cathepsin B activity in each L6 myoblast (A) and L6 myotube (B) fraction was assayed using the aminomethylcoumarin substrate, Z-arg-arg-NMec. The fluorescence of free aminomethylcoumarin was determined at an excitation wavelength of 370 nm and an emission wavelength of 460 nm (Methods: 2.9 B-ii). Cathepsin B activity (●) is expressed in µEUnits where 1 µEU is defined as the quantity of protease that liberates 1 pmole of aminomethylcoumarin (per min). Inhibitor activity (▲) in each L6 myoblast (A) and L6 myotube (B) fraction was measured against purified pork liver cathepsin B. Inhibitor activity is expressed as percent (%) inhibition which is defined as the percentage change in the averaged activity of purified pork liver cathepsin B in sample fractions relative to control cathepsin B activity.
FIGURE 19
iii) Significance of Cathepsin B and Inhibitor Activities

The significance of the cathepsin B to inhibitor activities in FPLC fractions of L6 cells was examined by statistical analysis (Student "t" test, Spearman rank correlation) of the results from six different FPLC studies conducted over a 12 month period (Table 11). The activities reported in this table for cathepsin B (mU) and inhibitor (mIU) were measured using arg-arg, since this was the substrate used during the first year of this thesis research. Although the increases were not as large as those observed with the fluorometric substrate, levels of cathepsin B increased approximately 3-fold during growth and differentiation of L6 cells. While this increase was arguably not significant on the basis of the student "t" test (p = 0.1), the consistent ranking of myotube levels of cathepsins B and L greater than those of myoblast levels was highly significant (p < 0.005). This latter statistical criterion has been used to establish significance for differences in protease and protease inhibitor activities between other cell populations (Chambers et al., 1992). In contrast, differences in inhibitor levels between L6 myoblasts and L6 myotubes were clearly not significant (p > 0.5). Moreover, while there was a 4-fold increase in the ratio of cathepsin B to inhibitor activities during myoblast-myotube differentiation, no increase in inhibitor levels, expressed in mIU units or as percent inhibition, was observed.

Levels of cathepsin B activity in L6-D3 myoblasts were significantly higher than those of either L6 myoblasts (p < .05) or L6 myotubes (p = 0.05). Levels of inhibitor (mIU) in L6-D3 were also significantly higher than levels in either L6 myoblasts (p < .05) or L6 myotubes (p < .01). These results are consistent with those obtained in this thesis research for unfraccionated samples of L6 and L6-D3, and with the published
Table 11. Summary of Cathepsin B Enzyme and Cathepsin B Inhibitor Levels in FPLC Fractions of L6 and L6-D3 Cells Assayed Over a Twelve Month Period

<table>
<thead>
<tr>
<th>Cells LS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cathepsin B&lt;sup&gt;c&lt;/sup&gt; (mEUnits) [x±SD]</th>
<th>Inhibitor&lt;sup&gt;d&lt;/sup&gt; (mIUUnits) [x±SD]</th>
<th>Ratio&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Percent&lt;sup&gt;f&lt;/sup&gt; Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6 Myoblasts</td>
<td>63 ± 6</td>
<td>75 ± 35</td>
<td>0.8</td>
<td>16.2</td>
</tr>
<tr>
<td>L6 Myotubes</td>
<td>190 ± 136</td>
<td>57 ± 46</td>
<td>3.3</td>
<td>10.8</td>
</tr>
<tr>
<td>L6-D3 Myoblasts</td>
<td>490 ± 232</td>
<td>156 ± 54</td>
<td>3.1</td>
<td>34.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> For each cell population, 500 µg of protein was applied to the FPLC HR-10/30 column and eluted as described in Methods. Protein concentrations of combined fractions containing cathepsin B activity (e.g., 20-30 µg/ml) were generally 10 fold greater than those for combined fractions containing inhibitor activity (e.g., 2-3 µg/ml).

<sup>b</sup> Three cell populations were examined: L6 myoblast, L6 myotube, and L6-D3 homogenates were prepared from cells maintained in alpha-MEM supplemented with 10% horse serum. In the case of L6 myoblast and L6 myotube fractions, the corresponding fusion index values (i.e., % nuclei in myotubes relative to total number of nuclei in a field) were 0% and >60% respectively.

<sup>c</sup> Cathepsin B activity, in mEUnits was measured using the synthetic substrate, Cbz-arg-arg, as described in Methods. The values presented here represent the mean averaged peak activity ± standard deviation (x ± SD) where N=6.

<sup>d</sup> Inhibitor activity, in mIUunits was measured against a constant amount of purified pork liver cathepsin B. One mIU of inhibitor activity, mIU, is defined as that amount of protein that inhibits 1 mUnit of cathepsin B. The values presented here represent the mean averaged peak activity ± standard deviation (x ± SD) where N=6.

<sup>e</sup> Ratio refers to the ratio of averaged cathepsin B activity (mEUnits) to averaged inhibitor activity (mIUunits) calculated for each phase of growth of each cell line.

<sup>f</sup> Percent Inhibition is the percentage change in the averaged activity of purified pork liver cathepsin in sample fractions relative to control levels of cathepsin B activity.
data for avian, bovine, and other murine skeletal muscle systems (Bird et al., 1981; Kirschke et al., 1983; Béchet et al., 1991).
Discussion

Without exception, cell culture studies concerning the role of lysosomal proteases during differentiation have focused on levels of activity within the cell. This appears to reflect an apparently reasonable assumption based on reported differences concerning proteolytic processing and cellular localization between cells representative of biological processes and those representative of pathological processes. While lysosomal proteases in both normal and abnormal (e.g. malignantly transformed) cells are synthesized as latent proenzymes that can be activated either in an acidic prelyosomal compartment (Gieselmann et al., 1983), or as an early event in the lysosomes (Nishimura and Kato, 1987), increased secretion of active and activatable proforms is primarily a pathological response (Mort et al., 1981; Recklies and Mort, 1982; Achkar et al., 1990; Chambers et al., 1992). This has been extrapolated by some researchers to suggest that secretion of proenzymes into the extracellular fluid of normal cells is minimal and occurs by default (Hanewinkel et al., 1987; Reilly et al., 1989). While this may be true, it is not sufficient scientific reason to discount the biological significance of proteolytic activity in the medium of normal cells. However, in retrospect, it does provide a convenient means to avoid the difficulties inherent in measuring levels of proteases in the extracellular fluid.

Historically, success in the establishment of cells in culture has been coupled to serum since it represents a simple way of providing a complex set of factors necessary for growth and reproduction of cells (reviewed in Freshney, 1987). Serum, however,
contains undefined components (e.g., inhibitors, activators) which can interfere with the measurement of protease activity. Mort and Leduc (1984), for example, demonstrated the presence of two enzymes present in human serum that mimicked the proteolytic action of cathepsin B against synthetic substrates. Other researchers have reported the presence of low and high molecular weight thiol protease inhibitors (e.g., kininogens) in bovine, rat, and human sera (Sueyoshi et al., 1985; Müller-Esterl et al., 1985).

While the identity of some serum factors is known, others remain undefined. This biological complexity can interfere with some types of analyses and confound the interpretation of others (Bjare, 1992). Research in our laboratory, for example, has demonstrated that serum components interfere with levels of cysteine protease activity synthesized in, and secreted from, human breast cancers cells (Scadden and Dufresne, 1993). The serum-problem has often been addressed by omitting extracellular studies or by omitting serum from the usual growth medium during studies. The latter solution, while scientifically more correct, may not be appropriate for all cell types. Transfer of L6 myoblasts growing in MEM alpha medium supplemented with 10% horse serum, to MEM alpha medium without serum, for example, affected both the growth and expression of differentiated phenotypes. This latter observation suggests that both replicating presumptive myoblasts and postmitotic myoblasts are affected. Regardless of the cellular target, transfer ultimately resulted in cell death which resulted in the release of cellular components which, in turn, interfered with measurements of protease activities.

A more successful approach to the serum-problem in differentiating cell systems
has been the development of serum-free, "defined" media (Allen et al., 1985; Bjare, 1992; Hirobe, 1992). However, it also has limitations including maintenance of short
term growth and reproduction of cells (Darlington et al., 1987; Dashti and
Wolfbaurer, 1987; McFarland et al., 1991) and selection of a subpopulation of cells
that differs from the original in terms of composition and phenotypic properties (Kiss
et al., 1989; de Launoit et al., 1991). The human hepatoma cell line, HepG2, provided
an excellent opportunity to establish a serum-free, defined culture environment which
would both minimize these problems and provide a reliable system to study
intracellular and extracellular levels of lysosomal proteases during differentiation.
First, it was available and had been extensively characterized in this laboratory
(Labruzzo and Dufresne, 1989). Second, it was relevant in that it represented a link
between pathological (i.e., malignant hepatoma) and biological (i.e., hepatic
differentiation) processes and had been used to examine both the secretion and
differentiation of plasma proteins, and the proteolytic activation of lysosomal cysteine
proteases (Darlington et al., 1987; Dashti et al., 1987; Kelly and Darlington, 1989;
Adeli and Sinkevitch, 1990). Third, it was convenient in that a serum-free; defined
medium which supported short-term growth, proliferation, and expression of
differentiated phenotypes in HepG2 had been reported (Adeli and Sinkevitch, 1990).

HepG2 cells established and maintained in a serum-free, defined medium for
greater than 100 generations retained phenotypes expressed in their serum-
supplemented counterparts. Specifically, growth characteristics of serum-free cells
were similar to HepG2 grown in serum-supplemented medium in this laboratory and
others (Kelly and Darlington, 1989). The value for the second stage population
doubling time was an exception to this and may reflect the composition of the original cells at the time of initial subculture into serum-free conditions (Kelly and Darlington, 1989: Bjare, 1992). At any rate, the direction of change in the second stage population time (shorter rather than longer) and the retention of the two stage growth pattern in serum-free cells, suggest that serum-free cells grow at least as well as serum-supplemented cells.

HepG2 cells grown in serum-free medium demonstrated similar biochemical properties as their serum-supplemented counterparts by a number of experimental criteria. In SDS-Page analyses of intracellular and extracellular fractions, HepG2 cells grown in serum-free medium continually expressed a wide range of intracellular and plasma proteins of various molecular weights over the 10 subculture period of the study. The positions of major absorbing peaks was similar to that found in fractions prepared from cells grown in serum-supplemented medium. In gels of extracellular fractions, the predominant peak at position 5 may correspond to albumin, the HepG2 serum protein previously identified by Kelly and Darlington (1989). The relatively stable levels of peak 5 protein over time are contrasted by the initial increase and subsequent decrease in peak 6 protein. While the identity of proteins present in extracellular fractions is not known, the lack of detectable protein at any position of control gels containing concentrated serum-free medium not previously exposed to cells suggests that they are cellular in origin. Furthermore, since serum-free medium did not affect the viability of HepG2 cells, and since the media collected from cells were virtually devoid of floating cells or cellular debris, it is likely that the proteins originated from growing and dividing cells.
Establishment of HepG2 cells in serum-free medium did not affect the expression of cathepsin B, a phenotype previously studied in serum-supplemented HepG2 cells (Mach et al., 1992). Variable levels of cysteine protease activity, assayed with arg-arg under conditions optimal for cathepsin B, were observed in both serum-free and serum-supplemented intracellular fractions over time. For some subcultures, levels of activity in serum-supplemented fractions were lower than those measured in serum-free fractions. This may reflect contamination of serum-grown cell samples with serum which is known to inhibit cathepsin B activity (Bottenstein, et al., 1988; McFarland et al., 1991; Teofili et al., 1992; Scadden and Dufresne, 1993). Regardless of the molecular basis for the variation, the absence of a consistent pattern with increasing subcultures argues against selection of a different subpopulation of cells (Freshney, 1987; Forster-Gibson, et al., 1988).

The retention of hepatic differentiation properties in HepG2 cells maintained in serum-free, defined medium was supported in several ways. First, the two stage pattern of growth characterized by a dramatic lengthening of the population doubling time of the cultures at confluence, reflects a modulation of the liver phenotype which occurs during fetal/adult development (Darlington and Kelly, 1989). Second, serum-free cells continued to secrete plasma proteins such as albumin as evidenced by SDS-PAGE analysis of extracellular fractions over time. Third, and perhaps most significant, serum-free cells synthesized and secreted the liver-specific constitutive protein, apolipoprotein B. Based on ELISA analysis, levels of apoB in the media of serum-free cells ranged from 2.85 μg to 5.93 μg apoB/mg of total protein over the 10 subculture period. Absolutely no signals were obtained in control, serum-free medium not
previously exposed to cells. Moreover, the levels of apoB in this research study are comparable with those previously reported for HepG2 cells maintained for two weeks in this same medium (Adeli and Sinkevitch, 1990).

Since "add-back" experiments were not conducted with HepG2, the contribution of each of the "defined" components to the support of hepatic differentiation is not known. Nevertheless, based on the biochemistry of mammalian cells (Freshney, 1987; Stryer, 1981) it is reasonable to assume that: a) selenium, a component found in all serum-free media, assists in the removal of free radicals of oxygen; b) i-inositol, one of the most common alcohol moieties of phosphoglycerides, is used in the synthesis of phosphatidyl inositol, a membrane component of most higher organisms; and c) thymidine, a substrate for the salvage pathway for pyrimidine synthesis, stimulates cell growth and helps in the maintenance of specialized cells. Regardless of the assumptions, the results obtained with HepG2 led to the conclusion that the serum-free, defined medium used in this research study supported long-term growth, division and expression of phenotypes associated with, and specific to differentiating hepatocytes. Therefore, it could be used to examine intracellular and extracellular levels of cysteine protease activities during HepG2 hepatic differentiation.

Results from these studies provide evidence for the synthesis of high levels of cathepsins B and L during second stage growth of HepG2 cells. This is contrasted by a growth-related increase in the secretion of a latent, activatable proform of cathepsin L. This is consistent with the identification of procathepsin L with MEP in other cell systems (Troen, et al., 1991; Chambers et al., 1992). The contrast between constant levels of intracellular cathepsin and increasing levels of extracellular cathepsin L in
HepG2 further suggests that the increased levels of activatable cathepsin L previously reported in human, whole liver homogenates (Green et al., 1984) may reflect proenzyme in extracellular fluid. Alternatively, it may reflect a difference in intracellular activation and secretion between malignantly transformed and normal cells. For example, the two stage growth of HepG2 cells has been explained on the basis of normal hepatic differentiation (Kelly and Darlington, 1989). However, cysteine proteases are known to act as growth factors by exerting mitogenic effects on cells (reviewed in Scher, 1987; Scott, 1992; Scott and Tse, 1992). Therefore, the high levels of active cathepsins in, and the subsequent increased secretion of cathepsin L from HepG2 cells, could help them overcome contact inhibition and density limited growth observed in late stages of normal cell growth. Some proteolytic mitogens have in fact been implicated in stimulating the release of other proteases from the cell. In this role, these proteases may initiate part of a proteolytic cascade, which could result in the degradation of extracellular matrices and promote metastasis (He, et al., 1989). This alternative interpretation of HepG2 results is consistent with Veksler’s explanation of expression and secretion of proteases during tumor progression (Veksler et al., 1987). It is also consistent with the contrasting pattern of expression of cathepsins observed during growth of normal L6 myoblasts discussed next.

The need for serum-free conditions to study requirements for myoblast-myotube muscle differentiation has been recognized since the 1970s (McFarland et al., 1991). Omission of serum from minimal or modified minimal media is not a solution since this results in reduced viability and loss of differentiated phenotypes. In response to this, several serum-free media formulations have been developed for muscle cell types
including chick embryonic (Dollenmeirer et al., 1981), ovine (Dodson and Mathison, 1988), human (Ham et al., 1988), and rat (Florini and Roberts, 1979; Allen et al., 1985). While all these media support growth and proliferation of immortal cell lines, they also reduce the expression of differentiated phenotypes. This latter observation has been presented positively as a means to study the contribution of nonmyogenic cells. The serum-free, defined medium (Adeli and Sinkevitch) used in this thesis research to establish HepG2, on the other hand, supports the growth, division and expression of differentiated phenotypes. Thus it is well suited for the study of myogenic cells, and therefore, myogenesis.

The relationship between L6 differentiation and the expression of three lysosomal cysteine protease activities, cathepsin B, cathepsin H, and cathepsin L, was examined in intracellular fractions of differentiating L6 and nonfusing L6-D3 muscle cells. These analyses support a fusion-related increase in all three cathepsins during L6 myoblast-myotube differentiation. Although the increase in cathepsin activities during L6 differentiation is fusion-related, fusion does not appear to be a prerequisite for expression of cathepsin activities since L6-D3 cells demonstrated higher levels of cathepsin activities at each stage of growth. However, the expression of constant levels of activities in L6-D3 suggests that fusion, or fusion-related properties, may be necessary for the controlled increase during myoblast-myotube differentiation. A similar relationship has been reported for the increased expression of nonlysosomal calcium-activated neutral proteases (CANPs) during L6 myoblast-myotube differentiation compared to fusion-inhibited cells (Kaur and Sanwal, 1981).

The fusion-related increase in cathepsin B, cathepsin H, and cathepsin L activities
is consistent with that previously reported for L6 rat myoblasts (Kirschke et al., 1983. In contrast, bovine (Béchet et al., 1992), and C2 mouse (Colella et al., 1986), myoblast-myotube differentiation is accompanied by increases in cathepsins B and L activities, but not cathepsin H. These differences may reflect differences in cell types and sources (Freshney, 1987; Buckingham, 1992).

Although L6 and L6-D3 differed with respect to the growth-related expression of intracellular cathepsin activities, the order of these activities was similar in both cell lines at parallel stages of growth; levels of cathepsin L activity were greater than those for cathepsin B activity which, in turn, were greater than those for cathepsin H activity. This trend does not appear to be restricted to muscle systems since the order was also maintained in differentiating HepG2 human hepatoma cells previously discussed. These results contribute to the emerging consensus that cathepsin L may be the most powerful lysosomal protease in normal differentiating systems exhibiting more than 40% of the total proteolytic activity (Kirschke et al., 1977; Bohley et al., 1985 and 1987; Kominami et al., 1991; Tanabe et al., 1991).

Significantly, these levels are usually greater than those reported for pathological processes, a fact supported by the analysis of cathepsin B in normal and malignantly transformed cell lines in this thesis research (see Table 4), but often overlooked in the literature.

Changes in levels of cathepsin activities during myoblast-myotube differentiation can reflect several mechanisms including alterations in transcription, translation, post-translational processes, and/or interaction with endogenous inhibitors (Béchet et al., 1991). In cloned, cell culture systems, these changes may also reflect the molecular heterogeneity of cell homogenates (Kirschke et al., 1983; Lah et al., 1989; Chambers et al., 1992, Lah et al., 1992). As a first approach to examining these alternatives, intracellular fractions, prepared
from L6 and L6-D3 cells during growth, were fractionated on a Superose 12 HR 10/30 FPLC column which had previously been shown to separate pork liver cathepsin B from commercial cystatin without affecting their biological activity. Distinct regions of cathepsins B, H, and L activities were observed at similar positions at all phases of growth for both L6 and L6-D3 cells with cathepsin B, cathepsin H, and cathepsin L eluting at apparent molecular weight positions of 31.0 kDa, 27.5 kDa, and 31.0 kDa, respectively. These values are similar to those reported for these proteases in murine fibroblast cells (Hanewinkel et al., 1987), and in differentiating primary calf skeletal muscle (Béchet et al., 1991) and rat hepatocyte (Nishimura et al., 1990) cells.

Trends of cathepsins B, H, and L activities within and between L6 and L6-D3 homogenates after FPLC fractionation were similar to those observed before fractionation. However, the fold increase between myoblast and myotube populations of L6 was greater than that observed in unfractionated homogenates both in this study and those reported by others for differentiating mouse C2 and rat L6 myoblast cells (Collela et al., 1986; Béchet et al., 1991). Regardless of these differences, the fusion-related increase in cysteine protease activities during differentiation of L6, and the differences in levels of cysteine proteases between L6 and L6-D3, were statistically significant. While this does not prove biological significance, it does support it.

Reports suggest that cysteine protease inhibitors affect levels of protease by binding to their target enzymes, with varying affinities, in a one-to-one stoichiometric fashion (Green et al., 1984). The high affinity of these inhibitors for the cysteine proteases has led to the speculation that their major cellular role is to protect cells against the uncontrolled activity of these enzymes during biological processes. It follows that the differences in proteolytic activity
during L6 differentiation could reflect differences in the balance between proteases and their endogenous inhibitors.

Two regions of endogenous inhibitor activity against purified cathepsin B eluted at similar apparent molecular weight positions, 12 kDa and 25 kDa, in FPLC fractions of homogenates prepared from L6 myoblast, L6 myotube, and L6-D3 myoblast cells. This is within the 10 kDa to 25 kDa reported by others for CPIs from rat (Schwartz and Bird, 1977), rabbit (Matsumoto et al., 1983) and bovine (Bige et al., 1985) skeletal muscle cells. Direct evidence for the molecular identity of the 12 kDa and 25 kDa inhibitory regions is not yet available. However preliminary results from research initiated in our laboratory by Jason Broderick and continued in Dr. Cotter’s laboratory (Department of Biological Sciences), support multiple forms of cystatin-like CPIs in L6 and L6-D3 cell populations. On SDS gels, protein from each region of inhibition migrates as a single band corresponding to the position of our egg white cystatin control. On native gels, both the standard cystatin and the inhibitory fractions migrate as two bands at similar positions corresponding to apparent molecular weights of 12.5 kDa and 24.5 kDa. The interpretation of multiple forms is also consistent with forms of cystatin-like, protease inhibitors previously reported. Endogenous CPIs, such as cystatins and stefins, have molecular weights ranging from 11.0 kDa to 15.0 kDa (reviewed in Sloane, 1990; Moin et al., 1992). Certain forms of these inhibitors can aggregate by virtue of disulfide bonds to form aggregates. In some cases, for example rat and bovine skeletal muscle, the aggregates appear to be multiples of one molecular subunit (Schwartz and Bird, 1977; Matsumoto et al., 1983). However, in others, aggregates can reflect CPIs with similar molecular weights but different physicochemical properties. For example, two CPIs, I-S and I-M, with apparent molecular weights of 10.5 kDa have been identified in rabbit skeletal muscle
(Matsuishi et al., 1988). While both were most effective toward cathepsin L and cathepsin H, and less effective towards cathepsin B; only I-M was capable of forming trimers.

While both L6 and L6-D3 demonstrated similar regions of cathepsin B inhibitory activity, the ratio of endogenous cathepsin B to cathepsin B inhibitor activities differed. During growth of L6-D3 cells, the ratio of cathepsin B to cathepsin B inhibitor activities, as well as levels of cathepsin B inhibitor, in percent inhibition, remained constant. In contrast, a 10 to 13 fold, fusion-related increase in the ratio of cathepsin B to cathepsin B inhibitor activities was observed during growth and differentiation of L6 cells. It is unlikely, however, that the increase in CP:CPI ratio reflects changes in free CPI levels of inhibitor activity, although lower than L6-D3, also remained constant. It is also unlikely that it reflects alterations in total CPI (i.e. free and protease bound) since heat-treatment of myoblast and myotube fractions before application to the FPLC column did not alter levels of cathepsin B inhibition.

It has been suggested that alterations in levels of lysosomal cysteine proteases may reflect alterations in transcription and translation (Doherty et al., 1985). While this may be true for some proteases, there is evidence that the increase in lysosomal activities of cathepsin B is not related to any change in active concentration or to modifications in levels of cathepsin B mRNA in fetal calf (Béchet et al., 1992), L6 rat, and C2 mouse (Colella et al., 1986) myoblast-myotube differentiation systems. In these studies, alterations in cathepsin B activity are reported to reflect limited differences in cathepsin B isozymes. The isolation of different cystatin-like CPI isozymes from rabbit skeletal muscle (Matsuishi et al., 1988), and the differential expression of multigene families characteristic of muscle development (Buckingham et al., 1992) support this explanation.
While alterations in lysosomal protease activities often reflect changes at the molecular level, they can also reflect changes at the cellular level, for example, changes in the distribution of presumptive lysosomes within the cell (Poretz et al., 1980; Bird et al., 1981). Although this possibility has not been examined in our laboratory, studies in other laboratories indicated that the pattern of distribution of lysosomes within prefusion and postfusion populations of L6 cells, using acridine orange as a fluorescent marker, remains constant (Bird et al., 1981). Moreover, the pattern of lysosomal distribution in immortal L6 myoblast cells was also found to be similar to that observed in diploid, mortal primary cells in culture (Poretz et al., 1980), and in immortal, nonfusing subclones of L6 (Bird et al., 1981). However, while the L6 cells used in this thesis research were derived from the same cloned stock as those used by Bird, the nonfusing variants were not. Bird’s nonfusing subclones arose spontaneously, while Ng’s L6-D3 nonfusing subclone was selected against 5-azacytidine. Thus, although both nonfusing variants were derived from the same parent, they may have different patterns of lysosome distribution. In any case, neither mortal-to-immortal transformation nor lack of myoblast-myotube fusion affect expression of lysosomal cysteine proteases in L6 cells.

Regardless of the molecular or cellular explanation for the differences in levels of cathepsins B, H, and L activities within L6 and between L6 and L6-D3, it is unlikely that they reflect differences in cell growth or viability since equal numbers of viable cells were used to prepare homogenates for each cell line. Moreover, both L6 and L6-D3 demonstrated similar, stable growth characteristics, phenotypes, and chromosome numbers. In addition, both cell lines were highly anchorage dependent and could be transferred to serum-free medium without loss of viability. As a result, they did not liberate cells into the medium. This was routinely confirmed using erythrosin B.
In the past, it has been assumed that only small amounts of latent, lysosomal procathepsins are secreted "by default" from normal cells (Hanewinkel et al., 1987; Reilly, et al., 1989). However, evidence obtained in this thesis research challenges this view. Specifically, a fusion-related increase in low levels of pepsin/acid-activatable cathepsin L was observed in the media of L6 cells during differentiation. This was contrasted with a constant high level of activatable cathepsin L in the media of nonfusing L6-D3 cells. In both cell lines, expression was eliminated by preincubation with either E-64, a specific inhibitor of cysteine proteases, or pepstatin, a specific inhibitor of aspartic proteases. The different patterns of expression in extracellular fractions of L6 and L6-D3 may reflect different biological roles. For example, the high constant levels of extracellular cathepsin L expressed during growth of L6-D3 are consistent with high levels of procathepsin L (M.E.P.) secreted during growth of malignantly transformed cells in culture (Sloane, 1990, Rohzin et al., 1990; Chambers et al., 1992; Scaddan and Dufresne, 1993). Since L6-D3 was selected against 5-azacytidine, a known mutagen and suspected carcinogen, it is possible that expression in these cells reflect malignant transformation rather than differentiation. This interpretation is consistent with that reported for a nonfusing variant of L8, another rat skeletal muscle, myoblast cell line. This variant, termed fu-1, promoted tumors in athymic nude mice although its L8 parent did not (Kaufman and Parks, 1977). It is also consistent with the pattern of expression observed during growth of HepG2, the malignantly transformed, differentiating cell line studied in this thesis research.

While the malignancy of L6-D3 has not been determined, the nonmalignancy of differentiating L6 myoblasts has been established (Ng et al., 1976; Dufresne et al. 1976). Therefore, the fusion-related increase in activatable cathepsin L may play an important role in myoblast-myotube differentiation of L6 cells, for example, in the remodeling of extracellular
matrix (ECM) molecules during fusion. The extracellular matrix is a dense meshwork of collagen, elastin, proteoglycans, and glycoproteins. The matrix forms a three-dimensional supporting scaffold that isolates tissue compartments, mediates cell attachment, and determines tissue architecture (Terranova et al., 1980; Kleinman et al., 1981). It has been known for some time that the matrix acts as a selective macromolecular filter and influences mitogenesis, morphogenesis, and cytodifferentiation (Bernfield et al., 1972). Several secreted proteases, including plasmin, plasminogen activators, elastase, stromelysin, interstitial and type IV collagenases, as well as lysosomal cathepsins, have been reported to participate in the degradation of the EMC (reviewed in Matrisian and Hogan, 1990). It is well established that many of these matrix-degrading enzymes are expressed in a latent form and require activation (e.g., proteolytic cleavage) and specific environmental conditions (e.g., acid pH), and are inhibited by specific inhibitors (e.g., cystatins). It has been suggested that the balance of active versus inactive matrix-degrading enzymes is modulated (e.g., by growth and differentiation factors) during biological processes such as matrix remodeling, tissue morphogenesis, and embryonic development. Several lines of evidence support the hypothesis that cathepsin L acts as a matrix-degrading enzyme and functions both within L6 cells and in their extracellular fluid during differentiation: 1) the collagenolytic activity of cathepsin L is about 10-fold more active in cleaving the cross-linked extra-helical peptides from collagen than is cathepsin B (Barrett, 1980), 2) the fusion-related increase in intracellular levels of active cathepsin L during L6 myoblast-myotube differentiation are consistent with increased levels of intracellular active and extracellular activatable enzyme, 3) muscle cells contain numerous target proteins for cathepsin L activity including: myosin, troponins, calmodulin, parvalbumins, tubulin, vimentin, insulin, glucagon, and many cytosolic enzymes (Bohley and Seglen, 1992), and 4)
invasion of tissue by highly metastatic, malignantly transformed cells is associated with increased secretion of procathepsin L (i.e. M.E.P) and with increased degradation of EMC (Stearns et al., 1990; Chauhan et al., 1991).

Although these observations, and those presented in this thesis, support a role for cathepsin L in muscle differentiation, they do not exclude the involvement of other activatable cysteine proteases in the extracellular fluid. Synthetic substrates currently available to measure cathepsins B, H, and L, for example, are not specific, and cathepsin L degrades protein over a broad pH range (i.e., 3-7) in vitro. Alternatively, levels of cathepsin B and H in the extracellular fluid may be too low to detect. In any case, the development of different specific synthetic cysteine protease inhibitors, the development of more sensitive assay procedures, and the isolation of different cysteine protease-deficient variants of L6, would be useful in assessing the biological significance of activatable cathepsins during myogenesis.
References


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