Role of Calpain in Apoptosis

By

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First Dr. Gilchrist, not only my supervisor in the area of science but also my pattern in life, a real man with great personality, thanks for helping me learn science but more thanks for helping me learn patience and toleration. I will never be able to thank you enough.

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LIST OF FIGURES

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ABSTRACT

Calpains are intracellular non-lysosomal calcium-dependent cysteine proteases. They are involved in different physiological functions including cell apoptosis. Apoptosis or programmed cell death is a mechanism by which multi-cellular organisms can get rid of unwanted, damaged or mutated cells. We hypothesize that calpain is actively involved in the process of apoptosis. To test this hypothesis, we examined whether vascular smooth muscle cells (VSMCs) and HeLa (cervical cancer) cells over-expressing calpastatin (the only known specific endogenous inhibitor of calpain) are more resistant to apoptosis induced by either staurosporine or hydrogen peroxide. Our results demonstrated that well-differentiated cells like primary VSMCs are quite resistant to these apoptotic stimuli and inhibition of calpain by over-expression of calpastatin in these cells causes no significant increase in resistance. However, non-differentiated cells like HeLa cells are quite susceptible to apoptosis and inhibition of calpain does have a significant protective effect against apoptosis in these cells.

DEDICATION

To my Father

INTRODUCTION

Calpains are Ca^{2+}-dependent intracellular cysteine proteases that are classified into two groups. The first are the typical heterodimeric calpains with a highly conserved 80 kDa catalytic and a 28 kDa regulatory subunit (Arthur et al., 2000). Amongst this sub-family are the ubiquitously expressed μ- and m-calpains as well as some of the tissue specific calpains. The second are the highly variable atypical calpains conserved only in the proteolytic domain of a single catalytic subunit (Goll et al., 2003). *In vitro* experiments have revealed that m-calpain needs millimolar concentrations of Ca^{2+} to be activated whereas μ-calpain requires micromolar concentrations of Ca^{2+}. Such concentrations of Ca^{2+} are not normally present in cells but may be found transiently in the vicinity of the endoplasmic reticulum or ion channels during the apoptotic process (Lu et al., 2002). Calpastatin is the specific endogenous inhibitor of calpain that is naturally found in mammalian cells and controls proteolytic activity of calpain (Barnoy and Kosower, 2003, Barnoy et al., 1998).

Apoptosis or programmed cell death is an important mechanism through which unwanted or damaged cells are eliminated. Such a mechanism is crucial during embryonic development and also for maintaining tissue homeostasis during adult life. There are two major pathways for apoptosis. The first is the extrinsic pathway which involves activation of death receptors located on plasma membrane. The second is the intrinsic pathway which involves disruption of mitochondrial and endoplasmic reticulum function. Defective apoptosis renders individual cells susceptible to different types of cancer and autoimmune diseases while increased apoptosis is usually seen in neurodegenerative diseases such as Alzheimer's disease (Hengartner, 2000). Caspases

are cysteine proteases which are involved in the process of apoptosis. Caspase-8 and -10 are associated with the activation of death receptors like TNF-alpha and FAS. Caspase-12 is activated by endoplasmic reticulum stress and caspase-9 is activated during the intrinsic pathway of apoptosis (Kidd et al., 2000, Willis and Adams, 2005). Other proteins like Bax and Bak which are pro-apoptotic proteins can increase mitochondrial membrane permeability and promote release of cytochrome c and subsequent activation of caspase-9 (Scorrano and Korsmeyer, 2003).

Calpain has been reported to play a role in apoptosis (Blomgren et al., 2001,Carafoli and Molinari, 1998, Choi et al., 2001, Gao and Dou, 2000, Nakagawa and Yuan, 2000, Shiraishi et al., 2000, Wood and Newcomb, 1999). It has been shown that during the process of apoptosis, the precursor of calpain, named procalpain, translocates from the cytosol to intracellular membranes and becomes activated (Wood and Newcomb, 1999). Activated calpain then cleaves its potential substrates such as transcription factors, cytoskeletal proteins, membrane associated proteins, and as a result induces apoptosis (Kato et al., 2000, Wang, 2000). *in vitro* experiments have proven that calpastatin is able to interact with calpain and prevent apoptosis (Chi et al., 1999, Lu et al., 2002). Calpains can partially cleave many anti- and pro-apoptotic proteins and therefore may be actively involved in apoptosis. Proteins like p53, Bcl-2, Bcl-xl, Bid, Bax, caspase 3, 7, 8, 9, 12 and NFkB are all considered to be substrates of calpain (Gil-Parrado et al., 2002, Pariat et al., 1997). Calpain can act both as a positive and negative regulator of apoptosis. For example, calpain can cleave p53 and thus prevent DNA damage induced apoptosis whereas cleavage of Bax by calpain leads to apoptosis (Wood et al., 1998).

LITERATURE REVIEW

Calpain

Calpain Protein Structure

Calpains are intracellular, non-lysosomal, Ca^{2+}-dependent cysteine proteases. In addition to the two ubiquitously expressed μ- and m-calpains, 12 tissue-specific isoforms have been identified in mammalian cells (Dubin, 2005).

Both μ- and m- calpains are heterodimers with a large subunit of approximately 80 kDa, and a small subunit around 28 kDa (Croall and DeMartino, 1991). The 28 kDa small subunit is shared by both μ-calpain and m-calpain heterodimers and is the product of a single gene located on chromosome 19 (Ohno et al., 1990). However, the 80 kDa subunit in μ-calpain is product of a gene on chromosome 11 and is distinct from the 80 kDa subunit in m-calpain which is encoded by a different gene on chromosome 1 (Ohno et al., 1990). In these two subunits, six domains have been identified as shown in Figure 1. Domain I is the NH_2-terminal of the large 80 kDa subunit. Domain II, is a papain-like protease or catalytic domain (Strobl et al., 2000) of the large subunit containing a critical Cys residue at position 115 and 105 for μ- and m-calpain, respectively. This Cys residue along with His and Asn residues forms a catalytic triad. Other cysteine proteases like papain, cathepsin B, L and S also contain this catalytic triad (Goll et al., 2003). Domain III is a linker region between domains II and IV. This domain may play a role in binding phospholipids and also controlling calpain activity through electrostatic interactions. Domain IV has a sequence very similar to calmodulin with five EF-hand motifs.

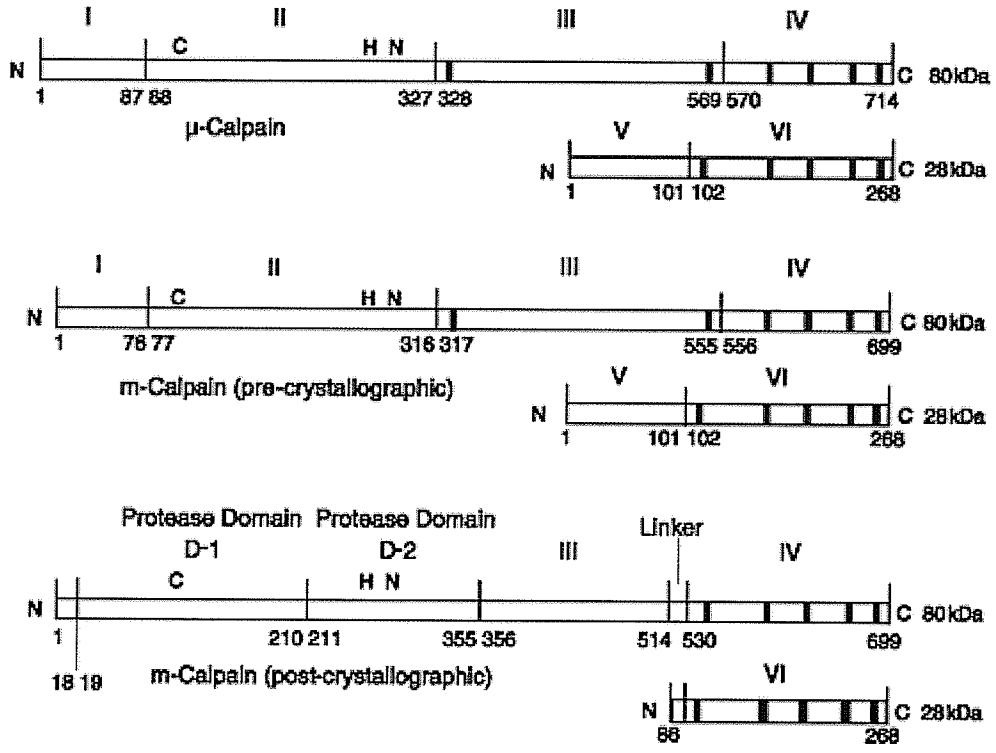

Figure 1: Schematic structure of calpain (Goll et al., 2003).

Both μ- and m-calpain are heterodimers of a large (80 kDa) and a small (28 kDa) subunits. Large and small subunits have four domains (I, II, III and IV) and two domains (V and VI) respectively.

These regions are thought to participate in Ca^{2+} binding with the fifth (COOH-terminal) EF-hand involved in dimerization of the small and large subunits. Domain V is the NH_2-terminal region of the 28 kDa subunit. This domain is Gly rich and indeed 40 out of first 64 amino acids in this domain are Gly. Also called the hydrophobic domain, this domain may interact with cell membranes (Goll et al., 2003). Domain VI is the COOH-terminal region of the 28 kDa subunit and contains five EF-hand Ca^{2+} binding sequences. Thus calpains are also part of penta-EF-hand family of proteins (Maki et al., 1997).

Calpain-Like Molecules

It has been shown that molecules which have sequence homology of less than 35% with domain II of ubiquitous calpains are frequently functionally distinct from calpain. According to this finding, molecules with sequence homology more than 20-25% have been placed in a so-called calpain-like family. Fourteen calpain-like genes have been recognized in mammals, five in *Trypanosoma brucei,* twelve in *C. elegans,* four in *Drosophila* and one transmembrane calpain in plants, and two in two species of fungi and yeast (Sorimachi and Suzuki, 2001). These thirty eight calpain-like genes can be further divided into two groups. Typical calpains have similarity in the domain structure to the 80 kDa subunit of ubiquitous calpain and especially EF-hand sequences in domain IV (eight in vertebrates and three in *Drosophila*). Atypical calpains which include the remaining twenty seven calpain-like molecules lack the EF-hand sequence in domain IV (Goll et al., 2003). In addition, six of the atypical calpains and one typical calpain do not have proteolytic activity due to the absence of one or more of the residues in the catalytic

triad. Furthermore, they function as a monomer (except for *Schistosoma* calpain) rather than a heterodimer as with the ubiquitous calpains (Goll et al., 2003).

Ca^{2+}-Binding Properties of Calpain

Ca^{2+} binding to any EF-hand except EF-4 is able to induce proteolytic activity (Dutt et al., 2000). Also EF-5 hands in both small and large subunits are active in the dimerization of calpain molecule and apparently are not involved in binding to Ca^{2+}. EF-3 shows the highest affinity for Ca^{2+}. There is an EF-hand like sequence in the boundary between domains II and III but it lacks a tertiary EF-hand structure and thus is not involved in Ca^{2+} binding (Hosfield et al., 1999). There are also two more Ca^{2+} binding sites in IIa and IIb domains which are not EF-hands but form peptide loops (Moldoveanu et al., 2002). Other studies have also shown that μ-calpain can bind 5-8 Ca^{2+} molecules which correspond to six EF-hand Ca^{2+} binding structures and two Ca^{2+} binding sites in domain IIa and IIb. However, m-calpain binds about 11-20 Ca^{2+} molecules which surprisingly exceeds its predicted Ca^{2+} binding sites (Hosfield et al., 2004, Moldoveanu et al., 2004).

Physiologic Functions of Calpain

Cell Motility:

It has been demonstrated that calpain activity is necessary for cleavage of cytoskeletal/membrane attachments that are a crucial step in the fusion of myoblasts (Temm-Grove et al., 1999). Also, fibroblasts that are derived from *Capn4*$^{-/-}$ embryos have decreased migration ability, disrupted cytoskeleton and reduced number of focal adhesions. Expression of the small 28 kDa subunit of calpain partially restores normal function and phenotype (Dourdin et al., 2001). Spreading and motility of cells depends on detachment and cleavage of focal adhesions and this is done by calpain (Glading et al.,

2002). Over-expression of calpastatin in NIH-3T3 fibroblast cells has been shown to have a negative effect on lamellipodia formation and the ability of cells to migrate (Potter et al., 1998).

Signal Transduction Pathways:

Enzymes such as kinases (e.g., PKC, ppFAK^{125}, pp60$^{c\text{-}src}$), phosphatases (e.g., tyrosine phosphatase PTP-1B), and cytoskeletal proteins (e.g., talin, filamin, paxillin, vinculin) are all considered to be substrates for calpain. All of these enzymes and proteins are involved in signal transduction pathways. Integrins and especially β-integrins are also cleaved by calpain (Pfaff et al., 1999). Calpains are also involved in disrupting integrin/cytoskeletal protein interactions as well as activation of Rac1 and inactivation of RhoA (Glading et al., 2000).

Cell Cycle:

Calpain plays an important role in the progression through G_1 to the S phase of the cell cycle (Goll et al., 2003) but fibroblasts from *Capn4*$^{-/-}$ mice can divide normally. Over-expression of calpastatin (the specific endogenous inhibitor of calpain) in chick embryo fibroblasts has been shown to have negative effects on levels of cyclin A and D, cdk2, pRb phosphorylation and transition through G_1 phase of cell cycle (Carragher et al., 2002). Also 20 μM benzyloxycarbonyl-Leu-Leu-Tyr-diazomethyl ketone (ZLLY-CHN_2), a synthetic calpain inhibitor, is able to stop the cell cycle at late G_1 in WI-38 fibroblasts and significantly increase p53 expression (Zhang et al., 1997). It seems that calpain plays a secondary or complementary role to the proteasome in the proteolytic regulation of the cell cycle (Goll et al., 2003).

Regulation of Gene Expression:

Some transcription factors like c-Jun, c-Fos (Hirai et al., 1991) and p53 (Gonen et al., 1997) are potential calpain substrates *in vitro*. However, it seems that the 26S proteasome plays a primary role in the degradation of these transcription factors.

Apoptosis:

The role of calpain in apoptosis is complicated and is dependent on the type of cells and the nature of the stimuli (Kidd et al., 2000). Caspases play a major role in apoptosis. These enzymes are cysteine proteases but compared to calpains, they are not Ca^{2+} dependent. Lysosomal cathepsins and the proteasome are also involved in apoptosis (Goll et al., 2003). In senescent neutrophils, calpains act downstream of caspases during the process of apoptosis (Knepper-Nicolai et al., 1998). However, in human platelets, apoptosis is triggered by calpain as an upstream regulator of caspases (Wolf et al., 1999).

Calpastatin

Calpastatin Protein and Genomic Structure

The only known specific endogenous inhibitor of calpain is calpastatin (Crawford et al., 1990). Calpastatin has no sequence homology to any protein identified so far. It is the product of a single gene located on chromosome 5 in humans (Hao et al., 2000). Calpastatin is a polypeptide consisting of four repeat domains (see Figure 2). These domains are marginally homologous and each domain contains approximately 140 amino acids. In each domain there are three subdomains A, B, C. In addition to the four major domains, there is a "domain L" which is also called NH_2-terminal domain (Lee et al., 1992). The N-terminal L domain has special role in the regulation of L- type Ca^{2+} channels (Hao et al., 2000). Calpastatin is a robust randomly coiled polypeptide that is

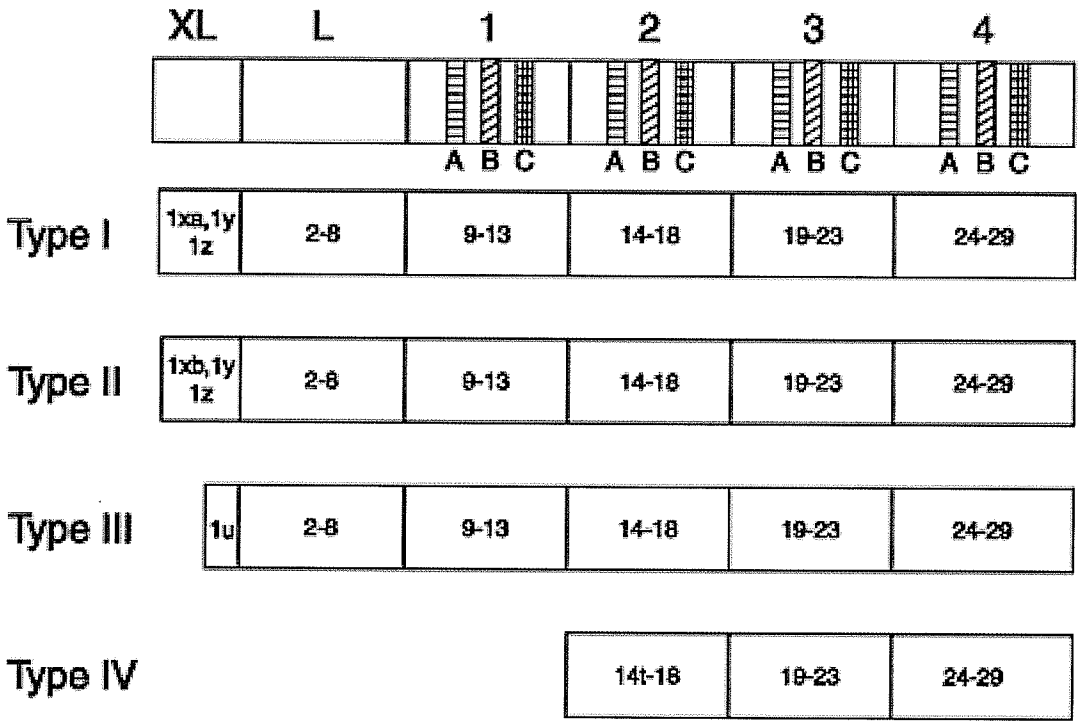

Figure 2: Schematic structure of mouse calpastatin gene (Goll et al., 2003).
Calpastatin is a polypeptide consisting of four domains each containing approximately 140 amino acids. Each domain has also three subdomains of A, B, C. There is also a NH_2- terminal domain called domain L.

resistant to different denaturing agents such as SDS, trichloroacetic acid and urea (Geesink et al., 1998).

Theoretical molecular weight of calpastatin ranges between 68-78 kDa. However, studies have revealed great variation in the predicted molecular weight of expressed calpastatin with estimates ranging between 34- and 300 kDa. Some factors are considered to be responsible for these differences and inaccuracies. First, calpastatin can be cleaved by endogenous proteolytic enzymes even in the presence of protease inhibitors (Mellgren et al., 1986). Second, calpastatin has a coil conformation and thus size exclusion chromatography may overestimate its true molecular weight (Goll et al., 2003). Third, although all isoforms of calpastatin are the product of a single gene (in humans this gene is located on chromosome 5), because of the use of different promoters and alternative splicing mechanisms, there are at least eight different known isoforms of calpastatin with molecular weights ranging from 17.5 kDa to 84 kDa (Parr et al., 2001, Takano et al., 2000). Fourth, calpastatin migrates on SDS-PAGE gel more slowly than other proteins exhibiting anomalous molecular weights ranging from 107 kDa to 172 kDa (Lepley et al., 1985). Finally, multiple calpastatin bands are commonly seen on SDS-PAGE gels but, without sequencing, it is not known whether these are proteolytic fragments of calpastatin or different isoforms of calpastatin (Takano et al., 1988).

The calpastatin gene contains 34 exons (29 coding exons plus 5 upstream exons including 1xa, 1xb, 1y, 1z, 1u). In the mouse, for example, there are four types of calpastatin cDNAs encoding four different NH_2- terminal sequences (see Figure 2):

1. Type1: Contains 1xa, 1y, 1z and is found in mouse liver, brain, testis
2. Type 2: Contains 1xb, 1y, 1z which encode XL domain. This domain includes 68 amino acids and has three PKA phosphorylation sites, identified in skeletal and cardiac muscle.
3. Type 3: Contains 1u and encodes the prototypical calpastatin which is ubiquitously identified in mouse tissues.
4. Type 4: Transcription starts at a unique exon between exons 14 and 15 and is called 14t. This calpastatin starts with domain II (Goll et al., 2003).

Inhibition of Calpain by Calpastatin

Calpastatin binds to calpain in a fashion such that the subdomain A of calpastatin attaches to domain IV of calpain and subdomain C of calpastatin attaches to domain VI of calpain. These two interactions are Ca^{2+} dependent. The third binding occurs between subdomain B in calpastatin and domain II (catalytic domain) in calpain (Goll et al., 2003). Interestingly, binding of a calpastatin molecule containing subdomain A and C to calpain, is able to prevent attachment between calpain and the plasma membrane (Kawasaki et al., 1993). This finding suggests that domain IV and VI in calpain are responsible for binding of calpain to membrane. It has been speculated that subdomain A, B, and C in calpastatin must simultaneously bind to calpain in order to provide maximum inhibition (Goll et al., 2003). However, a synthetic inhibitor mimicking only the subdomain B of calpastatin is able to inhibit calpain activity on its own (Dubin, 2005).

It has been shown that the central part of each domain of calpastatin (including the 12-residue consensus sequence common to all domains) is located on the surface of the calpastatin molecule and plays a much more important role in the inhibition of calpain

activity compared to the amino and carboxyl sections of each domain of calpastatin which form alpha-helical structures and are located in the interior of the molecule. These sides (amino and carboxyl sides) are more involved in stabilizing the tertiary structure of the domain (Maki et al., 1988). Each of the four domains of calpastatin are able to block the proteolytic activity of one calpain (μ- or m-calpain) therefore one calpastatin molecule is theoretically capable of inactivating four calpains (Emori et al., 1988).

Fragmentation of Calpastatin during Apoptosis

Calpastatin activity (calpain inhibitory potential) exceeds calpain activity in many cell types so in physiologic conditions calpain activity is apparently controlled by calpastatin efficiently (Murachi, 1990). Although it has been shown *in vitro* that calpain can cleave calpastatin, it is not exactly clear if the same is true under physiological conditions (Nagao et al., 1994). Fragmentation of calpastatin precedes cell death and it has been shown that caspases (especially caspases-1 and -3) can be responsible for this (Wang et al., 1998). When low molecular weight calpastatin is degraded by caspase-1, its potential inhibitory properties for both μ- and m-calpain are reduced by about 50 %. This is possible because caspase-1 attacks sub-domain helices A & C which interact with the Ca^{2+} binding domains but not the catalytic domains of calpain. Thus, it seems that caspase-1 can cleave calpastatin and modestly increase calpain activity during apoptosis (Wang et al., 1998).

High molecular weight (HMW) calpastatin (i.e. 110 kDa) is not significantly degraded by caspases 2, 4, 5, 6 whereas caspase-7 cleaves HMW calpastatin into two major fragments of 75- and 35-kDa. Caspase-3 cleaves calpastatin with the same pattern as caspase-7 except it also cleaves the 35 kDa fragment into 20- and 15-kDa fragments.

Interestingly caspase 1 cleaves calpastatin in many sites creating 75-, 60-, 35-, 23-, and 14-kDa fragments (Wang et al., 1998).

Apoptosis

In multicellular organisms, there are two pathways through which disrupted or unwanted cells can be removed (i.e., necrosis and apoptosis). Necrosis is characterized by plasma membrane disruption, a sharp decrease in ATP levels and also the presence of inflammation (Wyllie, 1993).

Apoptosis, however, is a more organized process in which the integrity of the cell membrane is preserved. DNA fragmentation and cell shrinkage are also characteristic features of apoptosis. Contrary to necrosis, apoptosis requires ATP (Wyllie, 1993). Apoptosis also occurs during embryogenesis. Removal of webbing and subsequent formation of digits and toes is a very well known example of apoptosis during embryogenesis (Wyllie, 1995). Abnormal apoptosis can be responsible for development of certain pathologies such as Alzheimer's disease, Parkinson's disease, Huntington's disease, cancer and cardiovascular disease (Thompson, 1995). Of these, the occurrence of apoptosis in cardiovascular pathologies like hypoxia, ischemia-reperfusion injury and myocardial infarction is more serious because adult ventricular myocytes have limited proliferation and renewal capacity (Narula et al., 1996).

There is a large super-family of homologous proteins named the Bcl-2 protein family (Yang et al., 1997). The members of this family are capable of either promoting or suppressing apoptosis. Pro-apoptotic proteins include Bax, Bak, Bad, Bid, Bim, Bik,

Bok_S BNIP3, Nix and Hrk (Sedlak et al., 1995). Anti-apoptotic protein members include Bcl-2, $Bclx_L$, Bcl-W, Bfl-1, Mcl-1 and A1 (Sedlak et al., 1995).

Role of Mitochondria in Apoptosis

Two major mitochondrial functions are energy metabolism and apoptosis. The inner mitochondrial membrane separates the matrix from the intermembrane space located between inner and outer mitochondrial membranes. The mitochondrial matrix contains the enzymes of the citric acid cycle, beta oxidation as well as mitochondrial DNA. The inner mitochondrial membrane is very convoluted which forms cristae projecting into the matrix. Cristae greatly increase the surface area of this membrane which houses the elements of the electron transport chain and the F_0F_1-ATPase proton pump involved in oxidative phosphorylation (Regula et al., 2003).

The intermembrane space is located between the inner and outer mitochondrial membranes and contains pro-caspases 2, 3, 9. The permeability of outer mitochondrial membranes can increase upon a pro-apoptotic signal and this allows the release of these enzymes into the cytoplasm (Regula et al., 2003). In addition to caspases, there are several other important mediators of apoptosis which are found in the intermembrane space of mitochondria.

Cytochrome C:

Cytochrome C is a product of the nuclear genome as apo-cytochrome *c* and lacks a heme. Once imported to the mitochondria, heme is added to it to form holo-cytochrome *c* (Yang et al., 1997).

Holo-cytochrome *c* after transferring into the cytosol actively takes part in the formation of apoptosomes. Apo-cytochrome *c* not only prevents apoptosis in cells but also blocks the apoptotic property of holo-cytochrome *c*. This finding reflects the importance of heme in the apoptosome complex (Yang et al., 1997).

Second Mitochondrial Activator of Caspase (SMAC):

SMACs neutralize the action of cytosolic inhibitors of apoptosis (IAPs) such as c-IAP1, c-IAP2 and X-IAP (Tikoo et al., 2002). Once a death signal is received, SMAC leaves the mitochondrial intermembrane space and liberates caspase 3 and 9 from the inhibitory action of IAPs and therefore induces apoptosis (Silke et al., 2002). SMAC, like cytochrome *c*, is a product of the nuclear genome and in order to be active and mediate apoptosis, its mitochondrial localization sequence needs to be deleted (Regula et al., 2003).

High temperature requirement A2 (HtrA2)

HtrA2 is a serine protease with a molecular weight of 36 kDa that functions similar to SMAC and inhibits IAPs. However it mediates a phenotypically different form of apoptosis without formation of membrane blebs and apoptotic bodies. Interestingly, HtrA2 mediated apoptosis has been shown to be caspase independent (Cilenti et al., 2003).

Apoptosis-inducing factor (AIF)

AIF is a novel mitochondrial flavoprotein which can mediate apoptosis and high-molecular weight DNA fragmentation in a caspase independent manner (Lorenzo et al., 1999).

Endonuclease G (Endo G):

Endo G functions similar to AIF and causes high-molecular-weight DNA fragmentation in a caspase independent manner. However, unlike AIF, it is a nuclease and is capable of causing DNA degradation and fragmentation on its own (Davies et al., 2003).

Permeability Transition Pore:

The Permeability Transition Pore (PTP) is a non-specific ion channel that opens once a cell is affected by a pro-apoptotic stimulus and lets molecules up to 1500 Da pass through (Crompton et al., 1999). As shown in Figure 3, PTP consists of a voltage-dependent anion channel (VDAC), an adenine nucleotide translocase (ANT) and cyclophilin D which are located in the outer membrane, inner membrane and matrix, respectively. Also a hexokinase-1 and the benzodiazapene receptor have been identified in the outer membrane (Crompton et al., 1999). Once PTP opens, an influx of H^+ ions, depolarization, and inflation of the matrix occurs. Since the inner mitochondrial membrane is highly convoluted, just the outer mitochondrial membrane bursts as a result of an increase in matrix volume. This leads to release of apoptogenic contents of intermembrane space into cytosol while the matrix remains intact, providing the cell with the energy necessary for the process of apoptosis (Bernardi et al., 1998).

Pathways of Caspase Activation

In resting cells, caspases are inactive and in order to be active they need to be cleaved. Activation of caspase occurs through two major pathways. The ***receptor-mediated pathway*** that is activated following ligation of death receptors (DR5, Fas, CD95 and TNFα receptors) and formation of death-inducing signaling complex (DISC) (Scaffidi et al., 1999). This complex plays an important role in the activation of upstream caspases

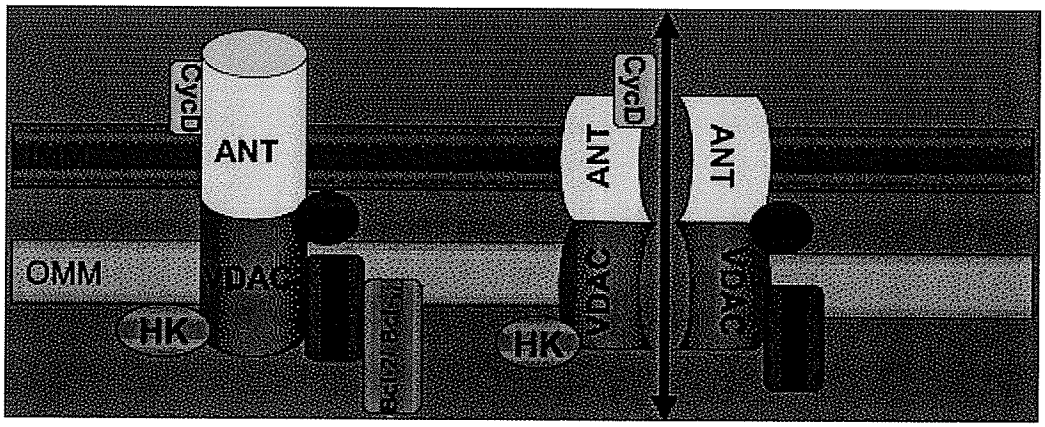

Figure 3: Regulation of the Mitochondrial Permeability Transition Pore (Regula et al., 2003)

Mitochondrial Permeability Transition Pore (PTP) is a non-specific ion channel which consists of voltage-dependent anion channel (VDAC) in outer membrane, adenine nucleotide translocase (ANT) in inner membrane and cyclophilin D (CycD) in matrix.

like caspase 8, and then distal caspases like 3, 6, 7 (Kuwana et al., 1998). In the ***mitochondrial-mediated pathway***, any pro-death signal can increase the permeability of the outer mitochondrial membrane and release of apoptogenic agents such as cytochrome *c*. Cytochrome *c* forms a complex with Apoptosis-protease-activating factor-1 (Apaf-1) and also pro-caspase 9. This complex is called the "apoptosome" and plays a crucial role in activation of caspase 9 and subsequently caspase 3 (Slee et al., 1999). Although activation of the caspase family of proteases is often seen in apoptosis, the apoptotic process can also occur without considerable caspase activity. This finding suggests that other non-caspase proteases such as calpains, granzymes and cathepsin are also involved in apoptosis (Johnson, 2000).

Role of Calpain in Apoptosis

Mechanisms of Action

Earlier studies had often made the distinction between calpains being mainly involved in necrotic-types of cell death and caspases being largely responsible for the apoptotic forms of cell death (Newcomb et al., 1999). Indeed, in recent studies of cisplatin-induced endothelial cell death, caspases were found to be involved in both apoptosis and necrosis but calpains were only found to mediate necrosis (Dursun et al., 2006). Although calpain activity is not entirely necrosis-specific, cytoskeletal damage caused by calpain is different from that mediated by caspases (Ogden et al., 2005). However, it has now become clear that in many other model systems calpain activation is indeed involved in the process of apoptosis (Nath et al., 1996).

Sustained $[Ca^{2+}]_i$ increase is considered to be an important trigger of apoptosis (Nicotera and Orrenius, 1998). Such increase can lead to activation of different Ca^{2+}

dependent enzymes like calpain, calcineurin, protein kinase II and JNK which execute apoptosis. The inhibitors of these enzymes have been shown to protect MIN6N8a cells against apoptosis (Choi et al., 2006). Compared to caspases, the role of calpain in apoptosis seems more complicated. Calpain can cleave and inactivate caspases 7, 8, 9 (Chua et al., 2000) while at the same time is capable of cleavage of procaspase-12 to produce an active caspase and also cleavage of the anti-apoptotic Bcl-X_L molecule into a pro-apoptotic molecule (Kaushal, 2003, Nakagawa and Yuan, 2000).

There are three calpain-dependent pathways for activation/augmentation of apoptosis. The first is the ligand-activated pathway involving caspase 8. The second is the mitochondrial pathway involving cytochrome C, Caspase 9 and apoptosome formation. The third is the endoplasmic reticulum stress pathway and activation of caspase 12 (Yoneda et al., 2001). ER stress can lead to calpain processing of caspase-12 which is located on the outer membrane of the endoplasmic reticulum (Nakagawa and Yuan, 2000, Nakagawa et al., 2000). Ca^{2+} homeostasis disruption can lead to m-calpain mediated cleavage of caspase 12, activation of caspase 12 and finally apoptosis. m-calpain can also cleave apoptotic inhibitor Bcl-xL. Ischemic injury, for example, can lead to disturbances in intracellular Ca^{2+} storage. Such disturbances are able to induce apoptosis via calpain-mediated caspase 12 activation and Bcl-xL inactivation (Nakagawa and Yuan, 2000). .

In addition to targeting caspases, calpains also play an important role in regulating the stability of p53 and Bcl-2 family proteins. Calpains are able to cleave p53 within its N-terminal. Inhibition of calpain causes stabilization of p53 and altered cell cycle progression (Kubbutat and Vousden, 1997). p53 and p73, both components of the DNA damage response, are transcription factors that can be cleaved by calpains. This finding

shows that calpains are also involved in the regulation of transcriptional events (Munarriz et al., 2005).

Members of the Bcl-2 family of proteins have either a positive or a negative regulatory role in apoptosis. They are also potential substrates for calpain (Shacka and Roth, 2005). For example, calpain can cleave Bax which leads to the creation of a new 18 kDa product. This fragment can easily transfer from the cytosol to the mitochondria and cause apoptosis via increasing outer mitochondrial membrane permeability and the release of cytochrome *c.* (Gao and Dou, 2000).

Figure 4 is a schematic representation of the proposed involvement of calpain during apoptosis. A stress like hypoxia can lead to an increase in the intracellular concentration of Ca^{2+} ions (Step 1) which can activate Ca^{2+}-dependent enzymes such as calpain (Step 2). Activated calpain can cleave Bax or Bid into a pro-apoptotic molecule (Step 3) which can then translocate to mitochondria (Step 4) and increase the permeability of the outer mitochondrial membrane (Wood et al., 1998). This increase in permeability can lead to the release of many mediators of apoptosis located in the intermembrane space of mitochondria including AIF (Step 5) and Endo G (Step 6) which can translocate to the nucleus and cause fragmentation of DNA. It is important to note that the release of AIF from mitochondria is dependent on its cleavage by endogenous mitochondrial calpain which again reflects another important role of calpain during apoptosis (Polster et al., 2005). Cytochrome *c* is another mediator of apoptosis which is released from mitochondria during apoptosis. It can bind to Pro-caspase-9 and Apaf-1 (Apoptosis protease-inducing factor-1) and form a complex named apoptosome (Step 7) which

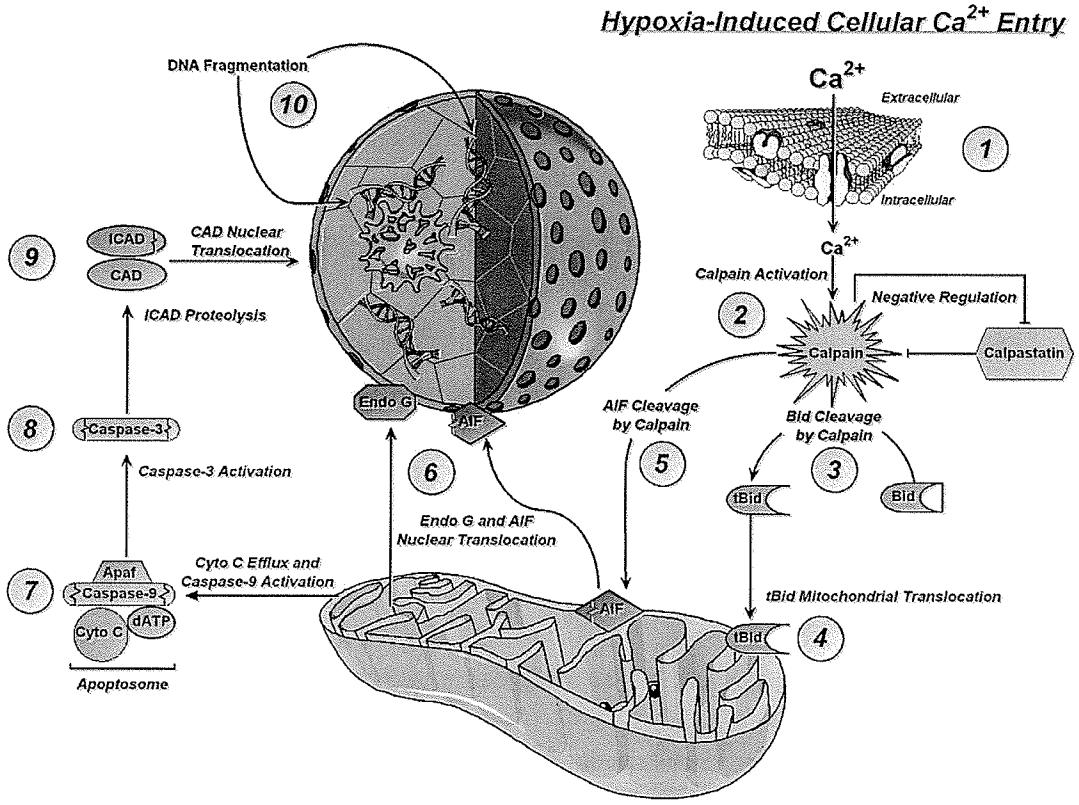

Figure 4: Scheme depicting involvement of calpain in apoptosis

A stress like hypoxia can lead to an increase in intracellular Ca^{2+} concentration (1) and activation of calpain (2). Calpain cleaves Bid to tBid (3) which then translocates to outer mitochondrial membrane (4) and increases the permeability of this membrane and subsequent release of apoptotic factors like AIF (5), Endo G (6) and cytochrome *c* (7). Cytochrome *c* is important in formation of apoptosome (7) and consequent activation of caspase-3 (8) and caspase-activated DNAase (9).

converts pro-caspase 9 to active caspase 9. Caspase-9 activation can lead to activation of caspase-3 (Step 8) which is the final executioner of apoptosis (Slee et al., 1999).

Experimental Evidence for Pro-Apoptotic Roles of Calpain

Leupeptin, a calpain inhibitor protects inner ear hair cells against gentamicin ototoxicity *in vitro* (Ding et al., 2002). BAF which is a general caspase inhibitor is not able to prevent apoptosis in outer hair cells (OHCs) and inner hair cells (IHCs) when used in conjunction with 1 mM neomycin (Momiyama et al., 2006). Leupeptin, which is one of the common inhibitors of calpain, prevents acoustic overstimulation-induced apoptosis ((Salvi et al., 1998, Wang et al., 1999) and hypoxia and neurotrophin-withdrawal induced apoptosis in auditory hair cells (Cheng et al., 1999). Leupeptin has also been shown to increase the number of cells surviving following neomycin treatment which reflects the important role of calpain rather than caspase in neomycin induced apoptosis of auditory hair cells (Momiyama et al., 2006). In agreement with the proposed roles for calpain in apoptosis, calpain inhibitors such as calpain inhibitors I and II, E64d can inhibit and stop apoptosis in some cells (Nakagawa and Yuan, 2000). Interestingly, MDL 28170, a cell permeable calpain inhibitor is capable of preventing Ca^{2+} ionophore-induced apoptosis in PC12 cells (Ray et al., 2000). MDL 28170 also provides neuroprotection for organotypic hippocampal slice cultures affected by ischemia (Cho et al., 2004).

Other studies have shown protection against apoptosis as a result of over-expression of calpastatin. A testis-specific isoform of calpastatin (tCAST) can be identified in haploid germ cells but not in spermatocytes. Ectopic expression of tCAST in

spermatocytes increased the resistance to heat-induced apoptosis in these cells (Somwaru et al., 2004). Over-expression of a calpastatin transgene prevents muscle atrophy in a murine model of muscle disuse (Tidball and Spencer, 2002) and a decrease in calpastatin level is associated with increased progression of apoptosis (Porn-Ares et al., 1998). Transgenic mice in which calpastatin expression has been increased (over-expression) or deleted (knocked out) are healthy (Takano et al., 2005). This suggests that activation of calpastatin and therefore inhibition of calpain can occur largely under pathological conditions. Cells in which calpastatin has been over-expressed, show more resistance to apoptosis whereas cells in which calpastatin has been knocked-out, are less resistant to apoptosis (Takano et al., 2005).

Apoptosis observed in AIDS patients is related to increased calpain activity. Both apoptosis and increased calpain activity are eliminated by protease inhibitor (PI) drugs (Lichtner et al., 2006). Indeed, clinical application of calpain inhibitors for rescuing of lymphocyte function in AIDS patients has been suggested. However, the major problem is the fact that known calpain inhibitors are highly toxic *in vivo* (Sarin et al., 1994).

Both aging and neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases are caused by neuronal cell apoptosis (Mattson, 2000). Many protein substrates of calpains are targeted in neurodegeneration and, as a result, calpains are thought to participate in regulating this process (Nixon, 2003). In neuronal apoptosis, calpain appears to participate in a cascade of three protease systems initiated by calpain-mediated cathepsin release, followed by cathepsin-mediated caspase activation and then finally caspase-mediated calpastatin degradation. The latter event then serves to increase

calpain activity providing feed-forward control of this protease cascade (Yamashima, 2000, Yamashima, 2004).

Activation of calpain is also involved in apoptosis mediated by treatment of MIN6N8 insulinoma cells with IFN-γ/TNF-α (Chang et al., 2004). Recent studies show that apoptosis in RGC-5 cells exposed to IMN and IFN-γ is accompanied by an increase in Bax: Bcl-2 ratio, release of cytochrome c from mitochondria, and increased activity of both calpain and caspases including caspase 8 (Das et al., 2006). Calpeptin (CP), which is a calpain inhibitor, can protect RGC-5 cells from apoptosis (Krishnamoorthy et al., 2001). It has been concluded that CP provides neuroprotection for RGC-5 cells treated with IMN or IFN-γ via prevention of calpain activation (Das et al., 2006). Pre-treatment with the calpain specific inhibitor Z-LLY-fmk has been shown to decrease the extent of TNF-α-induced apoptosis in HL-1 cells (Bajaj and Sharma, 2006).

Recent studies reveal that degradation of IκBα, which is essential for NF-κB signaling, can be achieved both by 26S proteasome and calpains. IκBα action to inhibit NF-κB is not just limited to the cytosol but also involves actions in the nucleus. NF-κB increases the production of IκBα mRNA (Chiao et al., 1994) and newly synthesized IκBα proteins migrate to the nucleus, where calpain is active, to attach and separate NF-κB from gene promoters (Arenzana-Seisdedos et al., 1995). Administration of ALLN following organ injury due to hemorrhagic shock attenuates NF-κB activation and its binding to DNA as well as binding of activated NF-κB to DNA and the degradation of IκB isoforms (McDonald et al., 2001).

Anti-Apoptotic Roles of Calpain

Despite this wealth of experimental evidence to implicate the pro-apoptotic functions of calpain, a handful of other studies suggest calpain may have anti-apoptotic functions in some experimental settings. Calpains are divided into two types, i.e. ubiquitous and tissue-specific. Calpains 1 and 2 are considered as ubiquitous or conventional calpains. Calpain 3 is the best example of a tissue-specific calpain and is mainly expressed in skeletal muscle (Sorimachi et al., 1989). Although calpain is involved in neuronal cell death (Chan and Mattson, 1999), calpain 3 is anti-apoptotic in muscular tissue (Baghdiguian et al., 1999). These ideas are supported by the pro-apoptotic properties of calpain inhibitors I and II. Calpains are thought to regulate p53 function through degrading it and earlier studies showed that calpain inhibitor I (ALLN) enhanced p53-dependent apoptosis in tumor cell lines (Atencio et al., 2000). Similarly, calpain inhibitor II (ALLM) induced apoptosis in acute lymphoblastic leukemia and non-Hodgkin's lymphoma cells which express high level of calpain. These observations suggested calpain inhibitor II and its analogues with anti-leukemic/ anti-lymphoma properties should be assessed more for future clinical use (Zhu and Uckun, 2000). Related to this dichotomous involvement of calpains in apoptosis, more recent evidence suggests that calpain may possess both pro-apoptotic and anti-apoptotic functions depending upon the nature of the stimulus and the cell type being studied. In the *Capn 4* knockout mouse, where the proteolytic activity of calpain is silenced due to the absence of expression of the regulatory subunit, activation of the Akt survival pathway was markedly suppressed following treatment of embryonic fibroblasts with different apoptotic agents (Tan et al., 2006a,Tan et al., 2006b). These studies suggested competing involvement of calpain in

pro-life and pro-death pathways and results from studies of muscle-specific calpastatin transgenic mice corroborate this idea with evidence of dramatically decreased Akt activity in these tissues (Otani et al., 2006, Otani et al., 2004).

HYPOTHESIS

Apoptosis or programmed cell death is a crucial process during embryonic development (Raynaud and Marcilhac, 2006). It is also a mechanism by which a multicellular organism can get rid of genetically mutated cells and thus has a protective role against cancers; however dysregulation of this process can lead to the formation of many diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and also cardiovascular and autoimmune pathologies (Thompson, 1995).

Members of a family of intracellular proteases, named caspases, are very well known as major mediators of biochemical processes leading to apoptosis (Raynaud and Marcilhac, 2006). On the other hand, intracellular Ca^{2+} ions have also been shown to have an important role in apoptosis. Indeed, an increase in intracellular free Ca^{2+} ion concentration can initiate and mediate apoptosis (Sergeev and Norman, 2003).
Calpains are Ca^{2+}-dependent cysteine proteases and they need Ca^{2+} at the concentration of micromolar or even milimolar to be activated (Goll et al., 2003). Calpains can also cleave some mediators of apoptosis such as transcription factors like p53 (Munarriz et al., 2005) and also members of Bcl-2 family like Bcl-X_L and Bax and Bid (Shacka and Roth, 2005). Calpains can even cleave caspases 7, 8, 9, 12 (Chua et al., 2000). Also synthetic calpain inhibitors have been shown in different experiments to be able to prevent apoptosis.

Based on these findings, we hypothesized that calpain plays an important role in apoptosis. In the majority of the experiments done so far, synthetic inhibitors of calpains

have been used which not only inhibit calpain activity but also inhibit other intracellular proteases. Therefore, interpreting the results of these experiments lack specificity and may be problematic. In the current project, I adenovirally over-expressed calpastatin in both vascular smooth muscle cells (VSMCs) and HeLa cells. Calpastatin is the only known specific endogenous inhibitor of calpain and as a result it only inhibits calpain activity while the activity of other intracellular proteases remains intact.

By specific inhibition of calpain, we tested the hypothesis that calpain is a major mediator of apoptotic process and thus its inhibition will lead to resistance to apoptosis.

MATERIALS AND METHODS

Large Scale Production of Adenoviral Particles

First amplification

Human Embryonic Kidney 293 (HEK) cells were plated at a density of 5 million cells in one 75 cm^2 flask containing 10 mL DMEM (Invitrogen) supplemented with 5% FBS (Hyclone), 1% penicillin/streptomycin (Hyclone) and 1% 200 mM L-glutamine (Hyclone) and maintained at 37°C in a NuAire Air-Jacketed and Humidified Incubator. Cells were allowed to attach for 3-4 hours and then media was removed and replaced with the mixture of 0.5 mL viral stock and 0.5 mL of media. After gentle rocking of virus over the top of cells, flask was returned to 37°C incubator and allowed to sit for 90 minutes. After 90 minutes, 9 ml of media was added to the flask and left to sit in 37°C incubator for 72 hours. After 72 hours, media was transferred to 15-ml falcon tube and three cycles of freeze/thaw were done at -20°C and +37°C respectively. First freezing was overnight but second and third freezing were for duration of two hours each. Cell debris was pelleted at 5000 RPM for 10 minutes. The supernatant was transferred into a new falcon tube.

Second amplification

HEK cells were plated in three 175 cm^2 flasks with a density of 1x 10^6 cells in each flask containing 30 ml DMEM supplemented with 5% FBS, 1% 200 mM L-glutamine, 1% penicillin/streptomycin and maintained at 37C° in a NuAire Air-Jacketed and Humidified Incubator. Cells were allowed to attach for 3-4 hours. After 3-4 hours, media was removed and replaced by a mixture of 4 mL media and 1 mL virus (from first

amplification) for each flask. Cells were then incubated in a 37°C incubator for 90 minutes. After 90 minutes 25 mL media was added to each flask and the flasks allowed to sit for 72 hours in 37°C incubator. After 72 hours, we gently transferred the lysed cells into three 50-mL falcon tubes (one falcon tube for each flask) which were centrifuged at 600xg for 5 minutes. Supernatant was removed and each pellet was resuspended in 3 mL of media and then the contents of the three 50-mL falcon tubes (9 mL) were transferred to a 15-mL falcon tube and three phases of freeze/thaw were performed as mentioned in the first amplification. Cell debris was pelleted at 5000 RPM for ten minutes and supernatant was transferred to a new 15-mL falcon tube.

Tissue Culture Infectious Dose (TCID 50) Method

10,000 HEK cells (in 100 µl media supplemented with 2% FBS, 1% 200 mM L-glutamine, 1% penicillin/streptomycin) were plated into each well of two 96-well plates and maintained at 37 °C in a NuAire Air-Jacketed and Humidified Incubator to allow cell attachment. On the same day, different dilutions of virus in media were prepared starting from 10^{-3} and ending 10^{-10} and then 100 µl of each dilution was pipetted into each well of its allocated row (for example row H was allocated to 10^{-3} dilution and row A was allocated to 10^{-10}dilution). Columns 11 and 12 of each row were left as control and were added with the media lacking virus (each well 100 µl media).

After 10 days incubation in 37 °C incubator, plates were viewed under a light microscope. The wells that showed Cytopathic Effect (CPE) were counted and titer (pfu) of virus was calculated via this formula: $T=10^{1+(s-0.5)}$

S= the sum of the ratios of the wells showing CPE to the all infected wells in the same row

Adenoviral Infection of HeLa and VSMCs

Cells were cultured in serum-starved media {(DMEM supplemented with 1% supplement (Hyclone), 1% fungizone (Invitrogen) and 1/10,000 insulin (Sigma)} on 10 cm^2 plates and maintained at 37 °C for four days in a NuAire Air-Jacketed and Humidified Incubator. The media was then changed to DMEM supplemented with 2.5% FBS for twenty four hours before infection with adenovirus. On the next day, cells were passaged into 6-well plates with density of 100,000 cells in each well containing 2 mL DMEM supplemented with 2.5% FBS. Six-well plates were returned to the 37 °C incubator for a further three hours to allow cell attachment. Following this, plates were transferred from incubator to the biological safety cabinet and 1mL media was removed from each well and the required volume of virus was added to each well according to required MOI (Multiplicity of Infection) and known pfu (plaque forming unit). 6-well plates were returned back to 37°C incubator and were left there for at least three hours to let viral transfection take place.

After three hours, 6-well plates were transferred from incubator to biological safety cabinet and 1mL media (DMEM supplemented with 2.5% FBS) was added into each well. 6-well plates were returned again to the 37 °C incubator.

Treatment of VSMCs and Hela cells with H_2O_2 and staurosporine

One day after infection, media was changed to DMEM supplemented with 2.5% fetal bovine serum (Hyclone) and 1 % fungizone (Invitrogen). Three days after that, media

again was changed to serum starvation media consisting of DMEM supplemented with 1% supplement (Hyclone), 1% fungizone (Invitrogen) and 1/10,000 insulin (Sigma). If we were to treat cells with hydrogen peroxide, this apoptogenic agent was added at this point to our new media at concentrations ranging from 250 μM to 750 μM and incubated overnight for less than 12 hours.

If staurosporine was the apoptogenic agent of choice, it was added on the next day (Day 4) at the time of changing media to the new serum starvation media at concentrations ranging from 1 μM to 4 μM and exposed the cells between one to four hours.

Microplate Protein Assay

Five different dilutions of protein standard including 0.25, 0.5, 1.0, 1.5, 2.0 mg/ml of bovine serum albumin (BSA) in RIPA buffer were prepared. Twenty μl reagent S (Bio-Rad, catalog no. 500-0115) was added to 1 mL of reagent A (Bio-Rad, Cat. #500-0113) to make working reagent A'. Five μl of standards and samples and blank (RIPA buffer) were carefully pipetted (in duplicate) into the wells of a 96-well microtiter plate. Then 25 μl of reagent A' was added to each well followed by adding 200 μl reagent B (Bio-Rad, catalog no. 500-0114). After waiting 15 minutes, absorbance at 750 nm was recorded.

Western Blot Analysis

Cells were harvested using RIPA buffer consisting of 150 mM NaCl, 50 mM Tris-HCl, 1mM EDTA, 1% Triton, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, (pH=7.5).

We added DTT and PMSF (protease inhibitor) to the RIPA buffer (immediately before harvesting cells) to a final concentration of 1 mM. The cell extract was centrifuged at maximum speed (16,000g for 5 minutes) and the supernatant transferred into a new 500µl Eppendorf tube. Lysate protein was assayed as described above and equivalent amounts of protein (20 µg) were loaded in each well of either a 7.5% or a 10% of polyacrylamide gel.

Proteins were electrophoretically resolved by SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes with transferred proteins were blocked overnight via immersing them (protein side down) in blocking buffer {5% skim milk powder in Tris-Buffered Saline (TBS)}. Membranes were incubated the next day with an appropriate dilution of primary antibody (according to the type of antibody) in blocking buffer (1-5% milk powder in TBS) for different durations ranging from two hours to an overnight incubation.

After two washes of ten minutes each with TBS containing 0.1% Tween 20 (TBST), membranes were incubated for one hour in secondary antibody (a goat anti-mouse IgG conjugated to horseradish peroxidase) diluted 5000 times in blocking buffer. After three washes with TBST, the membranes were developed using ECL Plus Western Blotting Detection System from Amersham Biosciences company (catalog no. RPN2132).

The following primary antibodies were used in our experiments.

1. p53 Antibody, 1:500 dilution and overnight incubation, MEDICORP , catalog no. MS-187-P1
2. Spectrin Antibody, 1:1000 dilution and overnight incubation, CHEMICON INTERNATIONAL, catalog no. MAB 1622

3. Actin, Smooth Muscle Antibody, 1:3000 dilution and two hours incubation, MEDICORP, catalog no. MS-113-P
4. Mouse Anti-calpastatin Monoclonal Antibody, 1:1000 dilution and two hours incubation, CHEMICON INTERNATIONAL, catalog no. MAB3084
5. Anti-flag M2 Monoclonal Antibody, 1:10,000 dilution and two hours incubation, SIGMA, catalog no. F-3165

Immunocytochemistry

Four days after infection, media was removed and cells were washed with Phosphate-buffered saline (PBS: 8.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 145 mM NaCl and 5.02 mM $MgCl_2$, pH 7.5) and then were fixed using 4% paraformaldehyde. After fixation, cells were washed for two times (10 min/wash) with PBS followed by three times with PBT (PBS+0.3% Triton). After removing PBT, coverslips were blocked with 5% Goat Serum (SIGMA, catalog no. G9023) in PBT for one hour followed by overnight incubation with Anti-Flag M2 Monoclonal Antibody (1:1000 dilution) at 4°C.

On the second day, primary antibody was removed and coverslips were washed three times (10 min/wash) in PBT and incubated for one hour in fluorescent secondary antibody diluted 1:400 in blocking buffer (5% Goat Serum in PBT).

Secondary antibody is Texas Red dye-conjugated AffiniPure Donkey Anti-Mouse IgG from Jackson ImmunoResearch LABORATORIES, INC.

After three washes with PBT, coverslips were mounted using the SlowFade Antifade Kit with DAPI (Molecular Probes, catalog no. S24635) for staining the nuclei. Slides were observed using a Zeiss Axioskope 2 fluorescent microscope

Nuclei Isolation

Cells were grown to reach confluence of at least one million cells per 10 cm^2 culture plate. After removing media, cells were washed with warm (37°C) PBS twice and then 1mL trypsin was added. Plate was then returned to the incubator for less than 5 minutes in order to detach cells from the surface of plate. After checking detachment of cells under microscope, 3 mL of inactivation solution (20% FBS in DMEM) was added and the cell suspension was transferred into a 15 ml falcon tube. The cell pellet was obtained via centrifuging the cell suspension at RCF =220 g, 23 °C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1mL cold PBS. The cells were then counted using a hemocytometer. The cells were pelleted via centrifuging at RCF =220 x g, 23 °C for 5 minutes and then the supernatant was discarded and the pellet was resuspended in one mL of ice-cold solution A per one million of counted cells. Solution A consisted of 320 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 1% Triton X-100. In one Eppendorf tube, 0.5mL of ice-cold sucrose cushion solution (1.8 M sucrose, 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) was added followed by 1 mL of the resuspended cells available in the 15mL falcon. This formed two layers in the Eppendorf tube. We then centrifuged the Eppendorf tubes at maximum speed (13200 rpm) for 15 minutes. The upper layer, which contained the cytosol, was transferred to another clean Eppendorf tube and the pellet which contained nuclei was resuspended in 100 µl of ice-cold solution A (without Triton X-100).

Statistical Analysis

To analyze differences between unpaired sets of sample means, repeated t-tests were performed using GraphPad Prism. Statistical significance was taken as a p value of less than 0.05.

RESULTS

Production of Virus

The purpose of this investigation was to determine a possible role of calpain in promoting apoptosis in both primary vascular smooth muscle cell line and in HeLa cells as an example of an immortal cell line. In order to do this, we utilized an adenovirus capable of expressing full length human calpastatin which was engineered with an antigenic C- terminal octapeptide FLAG epitope. Figure 5 shows a typical method for calculating plaque forming unit (pfu) using Tissue Culture Infectious Dose Method. PFU (the number of virus particles per unit volume) was calculated from the sum of the number of wells exhibiting cytopathic effects as a result of infection from the virus as follows Titer=$T=10^{1+d(s-0.5)}$

S= the sum of ratios +2 (because 10^{-1} and 10^{-2} are assumed to induce CPE in all wells so these concentrations were not used during experiment).

d=logarithm 10 of the dilution and (=1 in ten fold dilution).

From Figure 5, pfu is calculated as: S=1+1+1+1+1+0.2 + 2=7.2 and $T=10^{8}$

Typically, viral titers between 10^{7} and 10^{8} pfu were obtained.

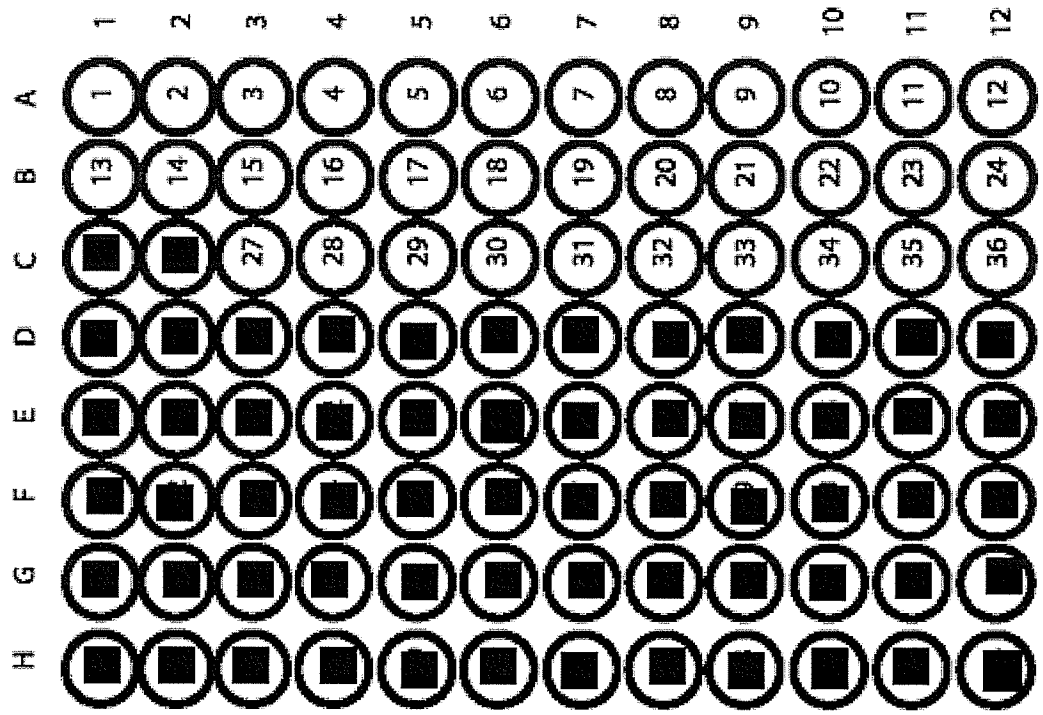

Figure 5: Obtaining Titres for Adenovirus using TCID50 method.

$TCID_{50}$ test was performed using a 96-well plate. Black squares represent wells with CPE ten days after infecting HEK cells with Ad-Calp.

Expression of Ad-Cast-FLAG in VSMCs

To determine the dose dependent pattern of expression of calpastatin-FLAG in vascular smooth muscle cells, we first performed western blots using anti-FLAG antibodies. To infect vascular smooth muscle cells, we first exposed them to a serum starved condition for four days prior to infection. Preliminary work had demonstrated that this approach facilitated the uptake of virus into vascular smooth muscle cells and gave better expression of the calpastatin-FLAG protein. Figure 6 shows that at a low MOI, FLAG reactive protein was expressed as a single band migrating in SDS-PAGE gels at around 110 kDa. This result is consistent with the expression of full-length human calpastatin which has a predicted molecular weight of around 84 kDa. Altered electrophoretic mobility of this protein in these gels has been attributed to its high charge density. It was also observed that when cells were infected at increasingly higher MOI, there was a progressive formation of lower molecular weight FLAG reactive peptides. Consistently, major peptide fragments were observed between 90 and 84 kDa, as well as an increasing concentration of a 45 kDa peptide at higher MOI. At the highest MOI of 25, a unique 28 kDa peptide was also observed. This observation suggests that when calpastatin is highly overexpressed, vascular smooth muscle cells react by proteolytically cleaving it. However, complementary enzymatic studies performed by our lab showed that although calpastatin appeared to be expressed as a fragmented protein, it was still able to potently inhibit calpain (data not shown).

During the early course of these investigations, a delay in the magnitude of calpastatin-FLAG expression in vascular smooth muscle cells was observed.

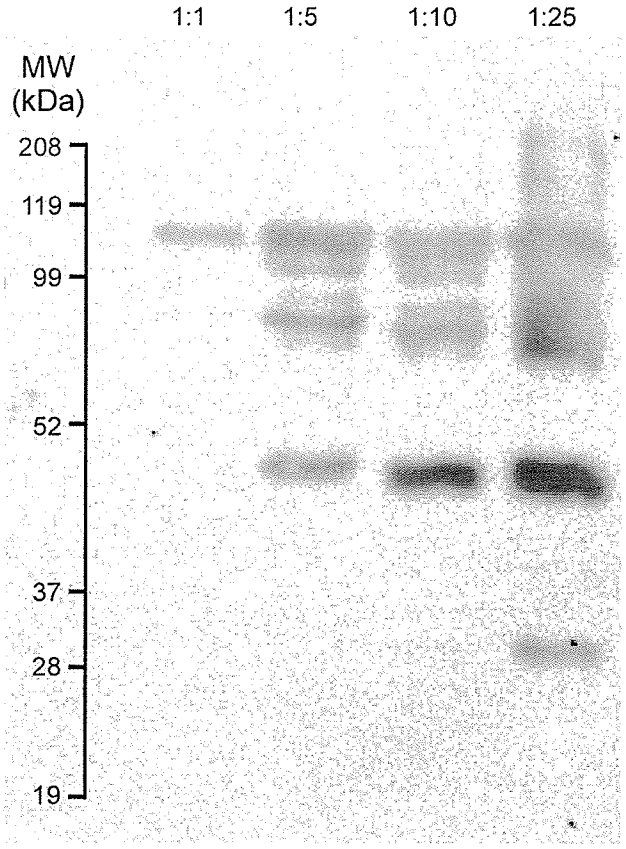

Figure 6: Effect of Multiplicity of Infection (MOI) on the level of expression of calpastatin.

This is the western blot of the 10% SDS-Polyacrylamide gel probed with anti-FLAG monoclonal antibody. Molecular weights are shown on the left side of the blot. Three days after transfection of vascular smooth muscle cells with Ad-Calp in 2.5% FBS, the pattern of expression of calpastatin varies with different MOIs of 1, 5, 10, and 25 accordingly. The first band that appears has the molecular weight of 110 kDa and is common for all MOIs. As MOI increases, different bands begin to appear. Finally at a MOI of 25, a unique band with molecular weight of 28 kDa can be visualized.

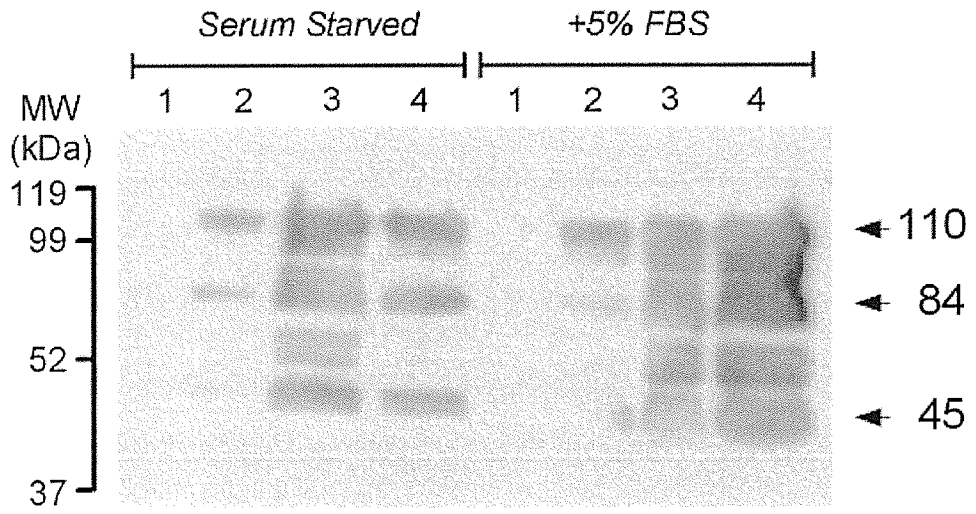

Figure 7: The relationship between time and the level of expression of calpastatin.

This is the western blot of a 10% SDS-Polyacrylamide gel probed with an anti-FLAG antibody. Molecular weights have been shown on both sides of the blot. Time (days) have been shown on the top of the blot. A delay in expression of calpastatin after infection of vascular smooth muscle cells with Ad-Calp at a MOI of 10 can be observed. In both conditions of serum starvation and 5% FBS, at least three days is required to induce the maximal expression of calpastatin

Figure 7 shows the time dependent expression of calpastatin in the days following initial infection. It was not until the second day that protein expression was detected regardless of whether these experiments were done in the absence or presence of 5% FBS. The strongest level of expression was observed after the third day following infection. It is possible that this fragmentation of calpastatin may not necessarily be due to expression of high protein levels but may be due to prolonged exposure of cells to serum starved culture conditions that were required to promote the uptake of the adenovirus.

To confirm the above dose dependent pattern of calpastatin expression, we also probed western blots with an anti-human calpastatin monoclonal antibody. Figure 8 shows that a single peptide migrating at 110 kDa was detected when vascular smooth muscle cells were infected with the adenovirus at an MOI of 1. As above, infection at a tenfold higher MOI resulted in a similar pattern of fragmentation when detected with this antibody. Thus, these data suggest that the anti-human calpastatin antibody may recognize a C-terminal epitope and that the peptides that are generated from the intact protein are due to proteolytic removal of the N-terminal portion of calpastatin.

Effects of Protease Inhibitors on Cast-FLAG Fragmentation

To investigate the possibility that the fragmentation of expressed calpastatin was due to activation of caspases which were hypothesized to be active as a result of culture

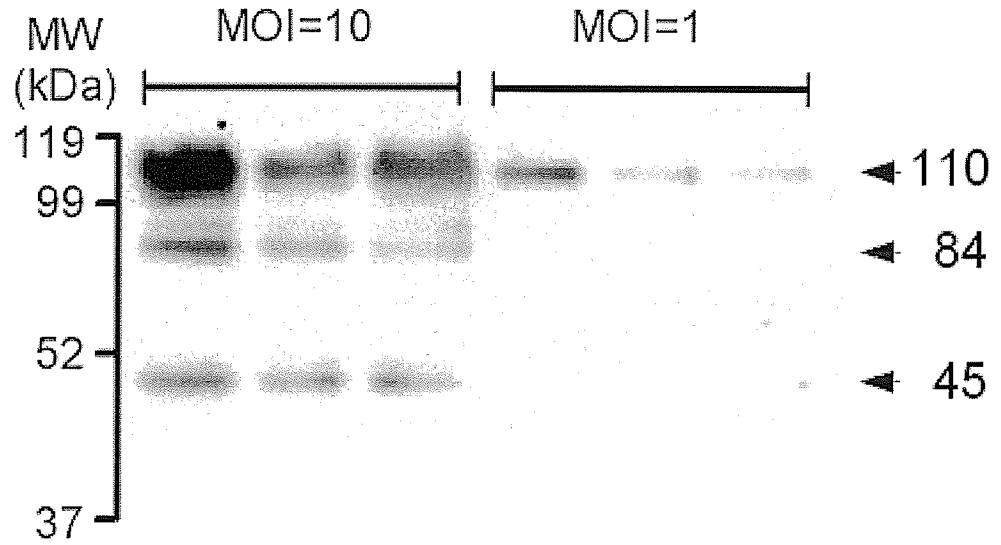

Figure 8: Confirming the pattern of expression of calpastatin using a anti-human calpastatin monoclonal antibody.

This is the western blot of a 10% SDS-Polyacrylamide gel probed with anti-human calpastatin monoclonal antibody. Molecular weights have been shown on both sides of the blot. Infection of vascular smooth muscle cells with Ad-Cast-FLAG at MOI of 1 and 10 and then probing with anti-human calpastatin monoclonal antibody shows the same pattern of fragmentation as when probed using anti-FLAG antibody.

conditions required to infect vascular smooth muscle cells with the virus, synthetic inhibitors of caspases and calpain were included in the culture media at the time of the viral infection. Figure 9 shows the results of western blots probed with anti-FLAG antibodies to investigate the effects of inhibitors at two different drug concentrations (5 and 25 μM). As shown in this figure, only a faint production of the 28 kDa anti-FLAG fragment was observed in control cells in addition to a generation of the characteristic higher molecular weight fragments. FK-012 was chosen as an inhibitor of caspase 8 in order to test the possibility that activation of the extrinsic pathways of apoptosis may contribute to this fragmentation of calpastatin. This figure shows that there was no effect of caspase 8 inhibition upon this fragmentation profile. Interestingly, the pan-caspase inhibitor ZVAD appeared to promote further fragmentation of calpastatin in vascular smooth muscle cells as observed by increased levels of 28 kDa peptide. With DEVD, there was no significant effect upon the calpastatin fragmentation profile but in the case of ALLN, there was a marked reduction in the intensity of both the fragmentation products at ~90 and 45 kDa. At a concentration of 25 uM, ALLN resulted in the appearance of a single peptide around 90 kDa whereas in all of the other conditions, a discrete peptide doublet could be observed. These data suggest that in the case of ALLN there is an inhibition of an unidentified protease which recognizes calpastatin as a substrate. ALLN is known to inhibit several intracellular proteases including calpain, the 26S proteasome and cathepsins B and L. However, our studies do not discriminate amongst these different proteases.

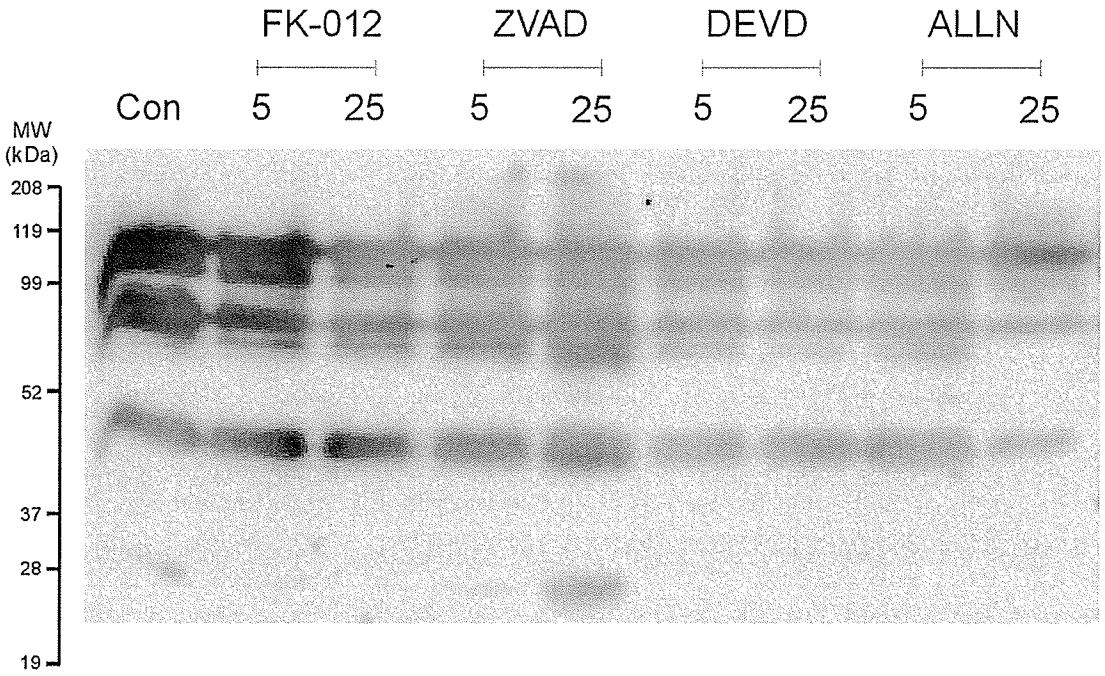

Figure 9: Effect of different caspase and calpain inhibitors on the pattern of expression of calpastatin.

This is the western blot of the 10% SDS-Polyacrylamide gel probed with an anti-FLAG antibody. Molecular weights have been shown on the left side of the blot. Vascular smooth muscle cells were treated with two different concentrations of 5μM and 25μM of Fk-012, ZVAD, DEVD, ALLN at the same time as transfection with Ad-Cast at MOI=25. Decreased intensity of 45- and 90- and 28 kDa fragments after treatment with ALLN at a concentration of 25uM is evident.

Spatial Distribution of Ad-Cast-FLAG in VSMCs

To determine the subcellular distribution of Cast-FLAG, vascular smooth muscle cells were prepared for immunocytochemical staining (see Methods) following infection at two different MOIs (5 and 25). As shown in Figure 10, calpastatin-FLAG was largely restricted to the cytosol and excluded from the nucleus at the lower MOI. This pattern of expression has been reported by others. At a five fold higher MOI, almost all cells were infected and expressed calpastatin-FLAG with many of the cells exhibiting clear nuclear anti-FLAG staining in addition to cytosolic staining (Figure 11). To confirm this dose dependent increase in nuclear calpastatin-FLAG localization, western blot studies were performed after isolation and separation of vascular smooth muscle cells into cytosolic and nuclear fractions. As shown in Figure 12, calpastatin-FLAG was predominantly expressed as a 110 kDa peptide in the cytosol with negligible staining detected in nuclei. In cytosolic fractions, minor fragmentation products were observed at 84 and 45 kDa but this pattern of expression was essentially consistent with that commonly observed at low levels of infection. At the higher MOI, extensive fragmentation of calpastatin-FLAG was observed in cytosolic fractions but, as indicated from the immunocytochemical staining above, calpastatin-FLAG expression was also detected in the nucleus as an intact 110 kDa protein.

Figure 10: Subcellular distribution of calpastatin and the morphology observed in VSMCs infected with low MOI (5) of Ad-Calp

VSMCs were plated on glass coverslips and after the appropriate time necessary for full expression of calpastatin (4 days), were fixed and immunocytochemically stained. Anti-FLAG Immunocytochemistry shows that calpastatin (red staining) is confined to cytoplasm and is not present in the nuclei stained with DAPI (blue). Cells look phenotypically normal and healthy.

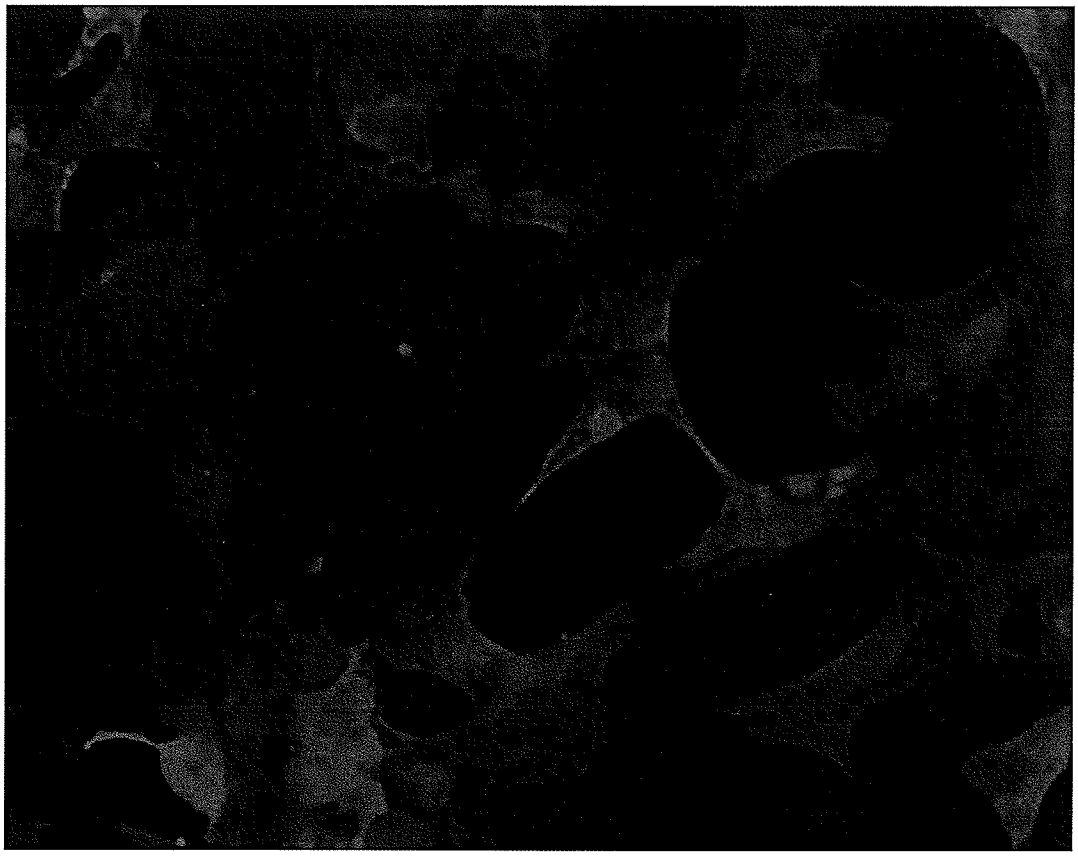

Figure 11: Distribution of calpastatin and the morphology observed in VSMCs infected with high MOI (25) of Ad-Calp

VSMCs were plated on glass coverslips and after the appropriate time necessary for full expression of calpastatin (4 days), were fixed and immunocytochemically stained. Anti-FLAG Immunocytochemistry shows that calpastatin (red staining) is present in cytosol as well as the nuclei stained with DAPI (blue). Cells often have the round shape and misshapen nuclei characteristic of apoptosis.

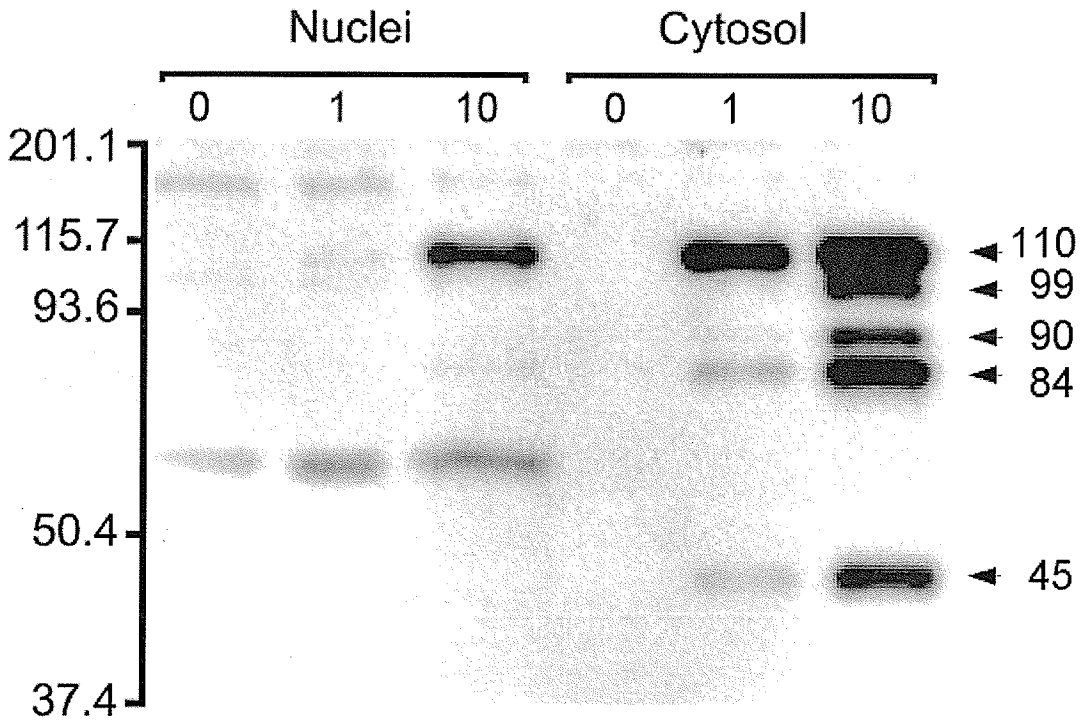

Figure 12: Nuclear and cytosolic localization of calpastatin-FLAG in low and high MOI conditions

This is the western blot of the 10% SDS-Polyacrylamide gel probed with anti-FLAG antibody. Molecular weights have been shown on both sides of the blot. Calpastatin was localized primarily to the cytosol. Both at low (1) and high (10) MOIs, calpastatin was more strongly localized to the cytosol rather than the nuclei. Calpastatin was detected in the nuclei at the high MOI (10).

Apoptosis in Ad-Cast-FLAG infected VSMCs

Effects of H_2O_2 and Staurosporine

To test the hypothesis that calpain activation may be associated with induction of caspase 3 mediated apoptosis, the effects of apoptosis-inducing reagents (H_2O_2 and staurosporine) were compared in Ad-EGFP (control) and Ad-Cast-FLAG infected vascular smooth muscle cells. As shown in Figure 13a, H_2O_2 induced a small dose-dependent increase in the formation of a 120 kDa caspase 3-specific spectrin breakdown product (SBP). A statistically significant difference was not observed between Ad-EGFP and Ad-Cast-FLAG infected VSMCs (Fig.13b), but in our experience, the effect was highly variable. In one instance, production of the 120 kDa SBP was elevated in Ad-Cast-FLAG infected VSMCs, as shown in Figure 13a. Corresponding dose-dependent increase in p53 protein expression were also observed in both sets of infected cells. Again, no statistically significant differences were observed between Ad-EGFP and Ad-Cast-FLAG infected cells but, in the same experiment described above, corresponding p53 levels appeared significantly increased in Ad-Cast-FLAG VSMCs lysates. In addition, we also observed detectable formation of a higher molecular weight product (94 kDa). This peptide was tentatively identified as a ubiquitinated p53 product although no specific test of this possibility was performed.

In general, the experiments with H_2O_2 were difficult to perform reproducibly because of the highly disruptive nature of this compound upon cell morphology. Concentrations of H_2O_2 above 500 μM caused significant cell damage raising the possibility of a loss of cytosolic proteins to the media.

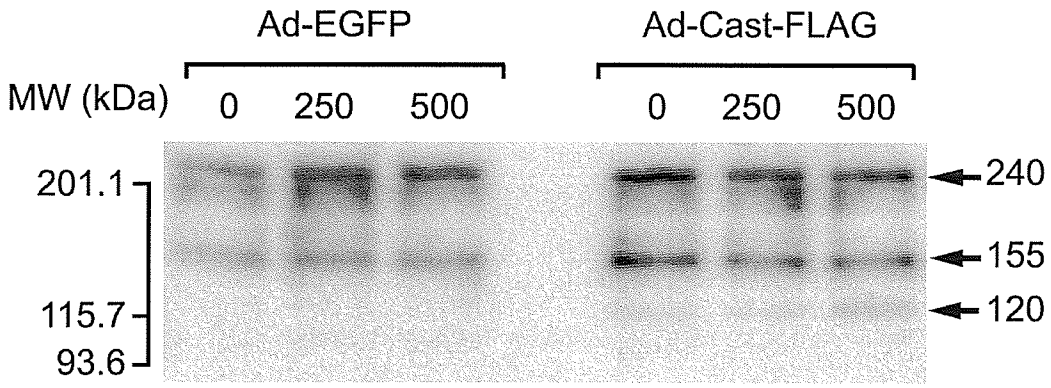

Figure 13a: Effect of calpain inhibition on the pattern of H_2O_2-induced spectrin degradation.

This is the western blot of the 7.5% SDS-Polyacrylamide gel probed with an anti-spectrin antibody. Molecular weights have been shown on both sides of the blot Vascular smooth muscle cells were exposed to 250, 500 μM of H_2O_2 for 12 hours. 120-kDa caspase 3 specific spectrin breakdown product (SBP) was relatively more expressed in Ad-Cast-FLAG infected cells compared to the control. The expression of 120-kDa fragment increased dose-dependently.

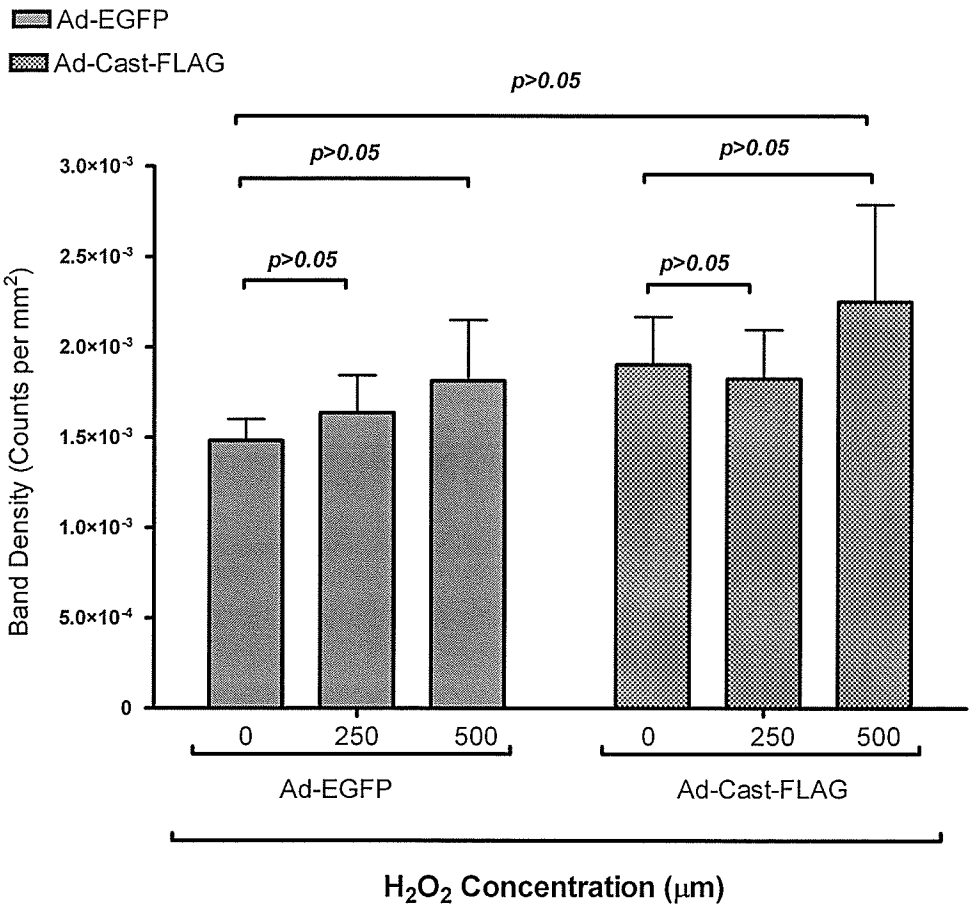

Figure 13b: Concentration-dependent increase in the level of expression of caspase-3 specific 120-kDa SBP in Ad-EGFP and Ad-Cast-FLAG infected VSMCs exposed to hydrogen peroxide.

The expression of 120-kDa fragment increased dose-dependently in both Ad-EGFP and Ad-Cast-FLAG infected VSMCs. There is not a significant increase in the resistance to hydrogen peroxide-induced apoptosis in Ad-Cast-FLAG infected VSMCs compared to the control. This bar graph represents three independent experiments.

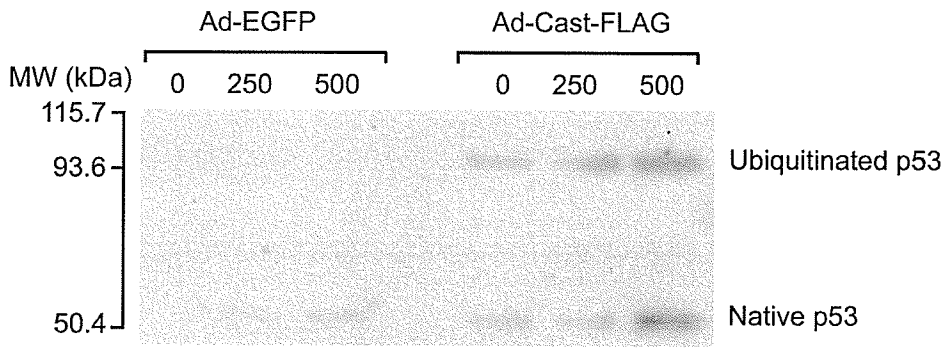

Figure 14a: Effect of calpain inhibition on the level of expression of p53.

This is the western blot of the 10% SDS-Polyacrylamide gel probed with an anti-p53 antibody. Molecular weights are shown on the left side of the blot. Vascular smooth muscle cells were exposed to 250, 500 μM concentration of H_2O_2 for 12 hours. Level of expression of p53 expression was higher in Ad-Cast-FLAG infected cells compared to the control and increased dose-dependently in both control and Ad-Cast-FLAG infected cells. We tentatively identified the ubiquitinated p53 (94 kDa).

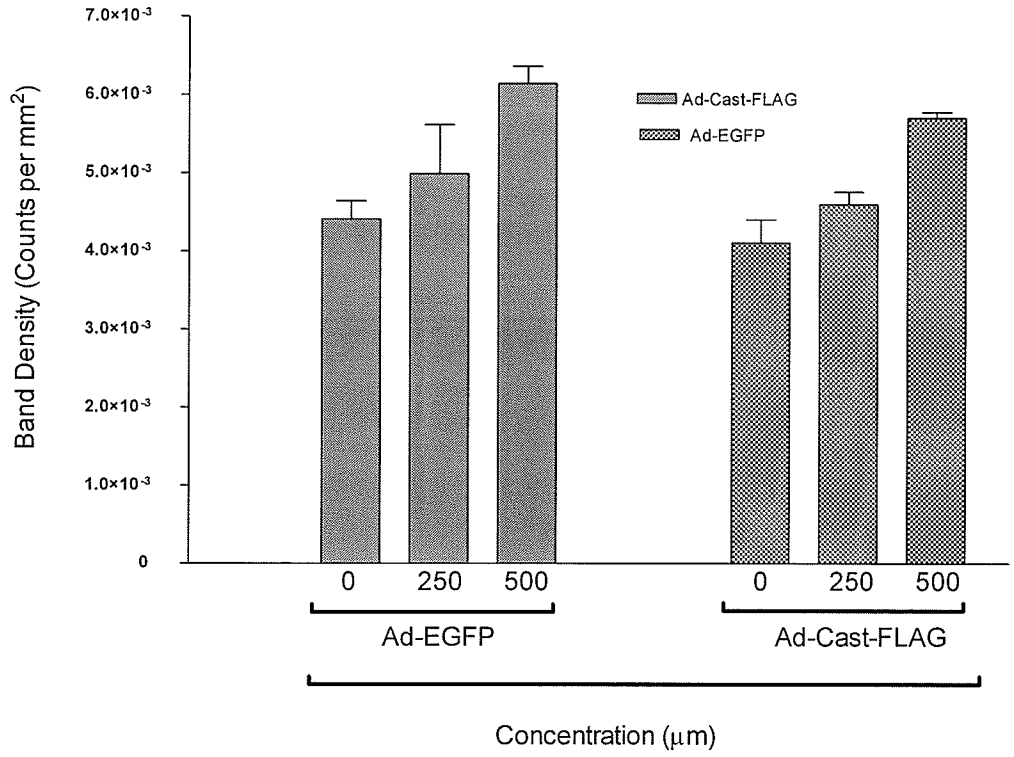

Figure 14b: Concentration-dependent increase in the level of expression of p53 in Ad-EGFP and Ad-Cast-FLAG infected VSMCs exposed to hydrogen peroxide.

The expression of p53 increased dose-dependently in both Ad-EGFP and Ad-Cast-FLAG infected VSMCs. There is not a significant increase in the resistance to hydrogen peroxide-induced apoptosis in Ad-Cast-FLAG infected VSMCs compared to the control. This bar graph represents three independent experiments.

H_2O_2 concentrations at or below 500 μM appeared not to induce significant caspase 3-specific SBP formation in control vascular smooth muscle cells and so further study of H_2O_2 effects were abandoned because these experimental conditions did not appear to offer an adequate model for testing involvement of calpain in caspase 3-mediated apoptosis. It was therefore decided to examine effects of staurosporine, which in other cells systems has been shown to activate caspase 3-mediated apoptotic cell death. As shown in Figure 15a, control vascular smooth muscle cells exposed to 1 μM staurosporine exhibited a small time-dependent increase in the formation of the caspase 3-mediated 120 kDa SBP. However, no obvious differences in the formation of this proteolytic fragment were observed in vascular smooth muscle cells infected with Ad-Cast-FLAG (Fig 15b). These data suggest that in vascular smooth muscle cells calpastatin induces little resistance to caspase 3-mediated cell death triggered by staurosporine or H_2O_2.

Despite the above results, we investigated the possibility that staurosporine-induced cell death (which was evident from visual identification of markedly increased incidence of rounded and detached cells) might result in the proteolytic modification of calpastatin given the fact that pan-caspase inhibition and calpain inhibition observed earlier arrested the production of its proteolytic fragments. As shown in Figure 16, exposure of vascular smooth muscle cells infected with Ad-Cast-FLAG to 1 μM staurosporine resulted in a time-dependent increase in the immuno-staining of all three (93, 84, 45 kDa) anti-FLAG reactive proteolytic fragments. These data, therefore, suggest that staurosporine-induced vascular smooth muscle cell death is associated with activation of an unidentified

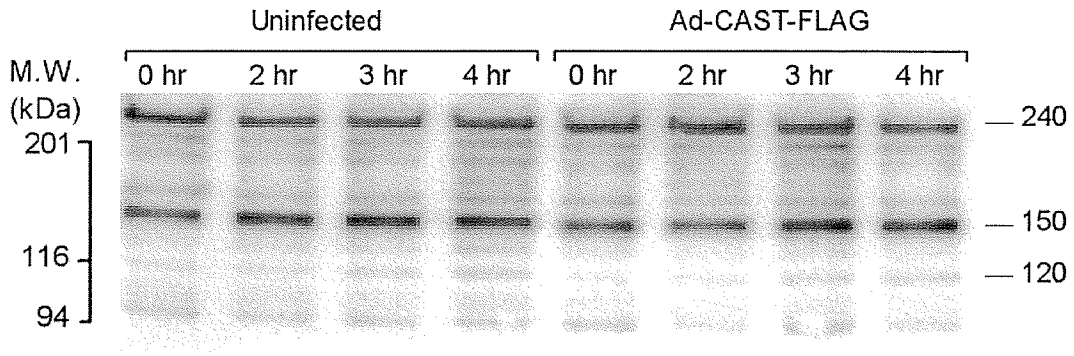

Figure 15a: Effect of calpain inhibition on the pattern of staurosporine-induced spectrin degradation.

This is the western blot of the 7.5% SDS-Polyacrylamide gel probed with spectrin antibody. Molecular weights have been shown on both sides of the blot VSMCs were exposed to 1μM staurosporine for 2, 3, 4 hours. There was a time-dependent increase in the level of expression of 120-kDa caspase 3 specific spectrin breakdown product (SBP) in both control and Ad-Cast-FLAG infected cells. No significant difference in the level of expression of the 120-kDa fragment was observed between control and Ad-Cast-FLAG infected cells.

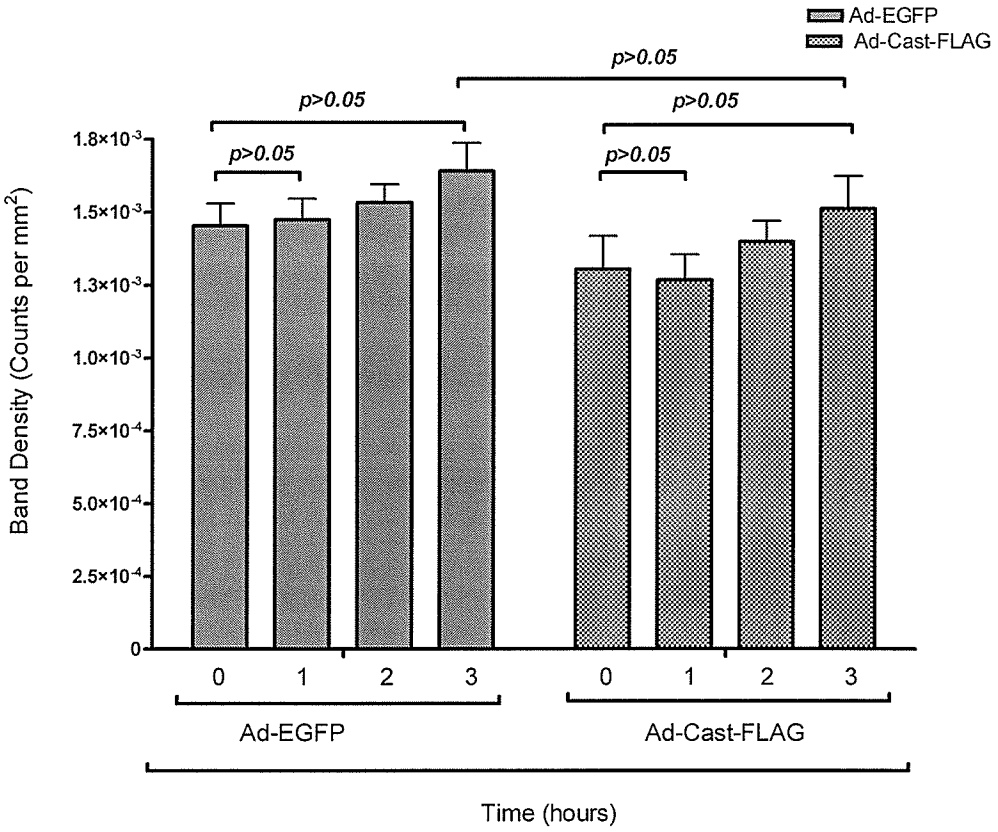

Figure 15b: Time-dependent increase in the level of expression of caspase-3 specific 120-kDa SBP in Ad-EGFP and Ad-Cast-FLAG infected VSMCs exposed to 1μM staurosporine.

The expression of caspase-3 specific 120-kDa SBP increased time-dependently in both Ad-EGFP and Ad-Cast-FLAG infected VSMCs. There was no significant increase in the resistance to staurosporine-induced apoptosis in Ad-Cast-FLAG infected VSMCs compared to the control. This bar graph represents three independent experiments

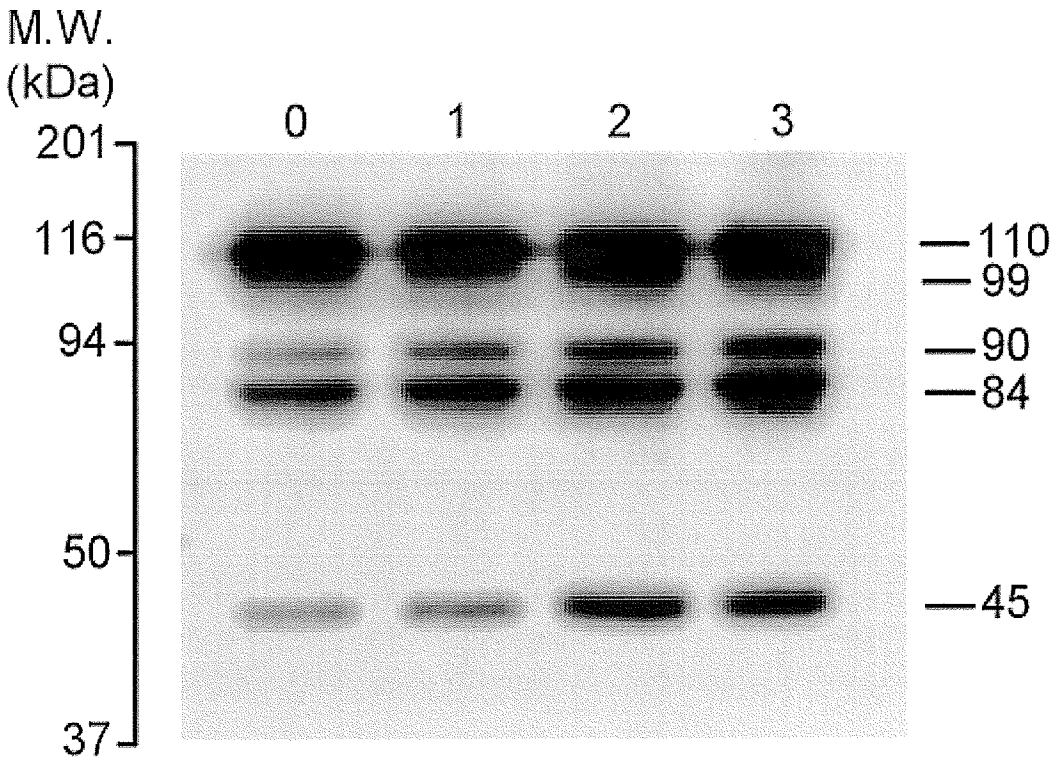

Figure 16: The effect of staurosporine induced apoptosis on fragmentation of calpastatin

This is the western blot of the 10% SDS-Polyacrylamide gel probed with anti-FLAG antibody. Molecular weights have been shown on both sides of the blot. As exposure time to staurosporine increased from 0 to 3 hours in Ad-Calp infected VSMCs, fragmentation of calpastatin intensified. A time dependent increase in expression of 93-, 84, 45 kDa fragments was evident.

protease which, from the data presented in Figure 15, may be a member of the caspase family of proteases.

Effects of Protease Inhibitors

To assess the role of calpain and caspases in the process of staurosporine-induced apoptosis, VSMCs were treated with 50 μM ALLN, ZVAD, FK-012 at the time of exposure to 1 μM staurosporine for four hours. As shown in Figure 17a, the expression of the 120 kDa caspase-3-specific band was markedly reduced in the pan-caspase inhibitor ZVAD treated cells. However in the case of ALLN (calpain inhibitor I) treated cells, over-expression of this band (120 kDa caspase-3-SBP) occurred. No significant difference was observed between FK-012 (inhibitor of caspase 8) treated cells and control (exposed only to 1 μM staurosporine with no drug treatment) cells (Fig 17b). As demonstrated in Figure 18, a marked over-expression of the tumor suppressor gene product, p53, was observed in ALLN treated cells. This finding suggests that ALLN treatment can predispose cells to apoptosis probably because it was inhibiting all forms of calpain including those that may have pro-life activity.

Figure 19, demonstrates the pattern of fragmentation of calpastatin in the above mentioned conditions after probing with anti-FLAG antibody. ALLN resulted in the appearance of a single peptide around 90 kDa whereas in all the other cases a discrete peptide doublet could be observed. Also 90-, 84-, 45-kDa fragments were markedly less expressed in ZVAD treated cells which suggests less degradation of calpastatin due to caspase inhibition.

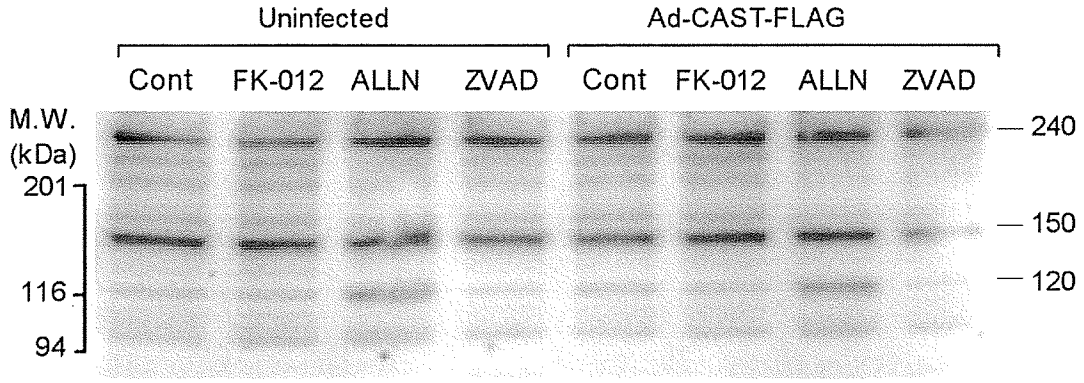

Figure 17 a: Effect of calpain/caspase inhibitors on the pattern of degradation of spectrin

This is the western blot of the 7.5% SDS-Polyacrylamide gel probed with spectrin antibody. Molecular weights have been shown on both sides of the blot. VSMCs (both uninfected and Ad-CAST-FLAG infected) were treated with staurosporine (1 μM) and a calpain/caspase inhibitor (50 μM) simultaneously for four hours. There was a marked over-expression of caspase 3-specific 120 kDa SBP in VSMCs treated with ALLN. The expression of caspase 3-specific 120 kDa SBP was markedly reduced in ZVAD treated cells.

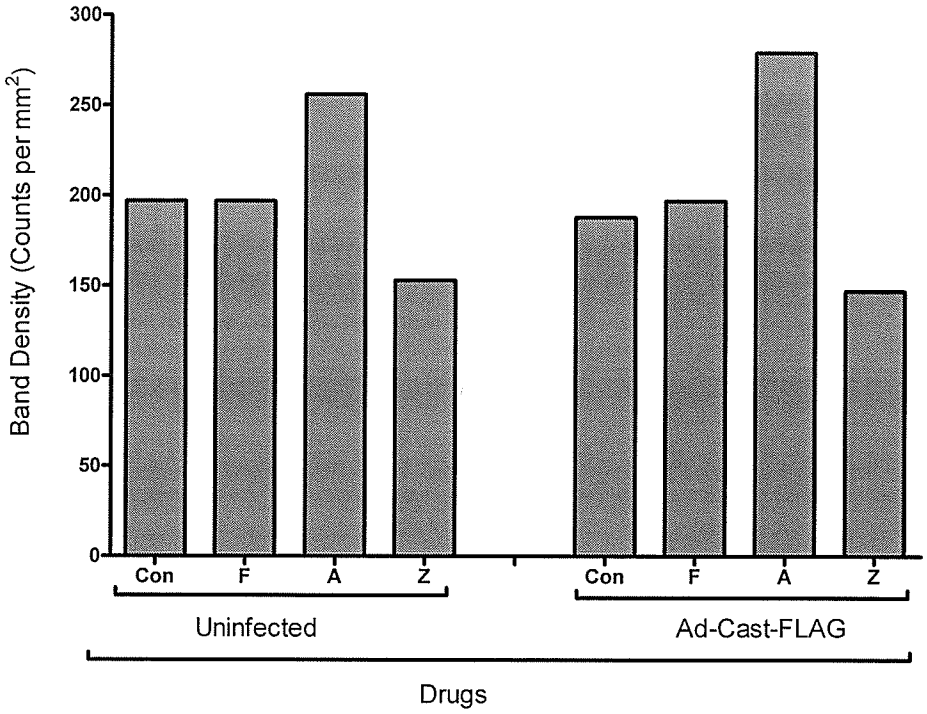

Figure 17 b: Effect of different caspase/calpain inhibitors on the level of expression of caspase-3 specific 120-kDa SBP in uninfected and Ad-Cast FLAG infected VSMCs

There was a significant increase in the level of expression of caspase-3 specific 120-kDa SBP in ALLN (A) treated VSMCs. There was a significant decrease in the level of expression of caspase-3 specific 120-kDa SBP in ZVAD (Z) treated VSMCs. There was no significant difference in the level of expression of caspase-3 specific 120-kDa SBP in FK-012 (F) treated VSMCs compared to the control.

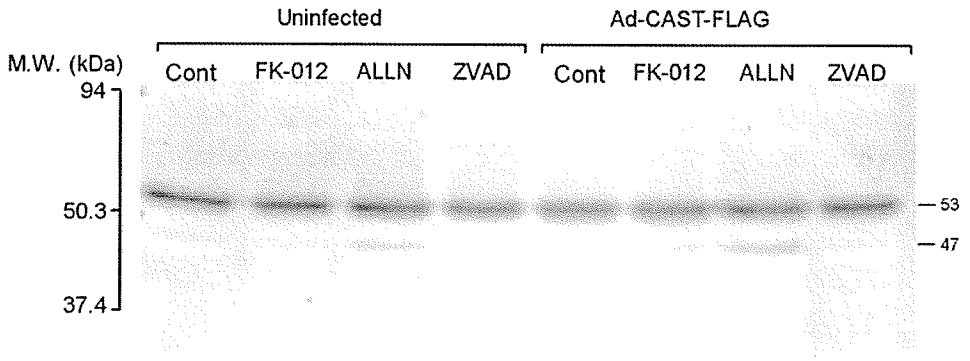

Figure 18: Effect of calpain/caspase inhibitors on the level of expression of p53

This is the western blot of the 10% SDS-Polyacrylamide gel probed with an anti-p53 antibody. Molecular weights have been shown on both sides of the blot. VSMCs (both uninfected and Ad-CAST-FLAG infected) were treated with staurosporine (1 μM) and one calpain/caspase inhibitor (50 μM) simultaneously for four hours. There was a marked over-expression of p53 in cells treated with ALLN.

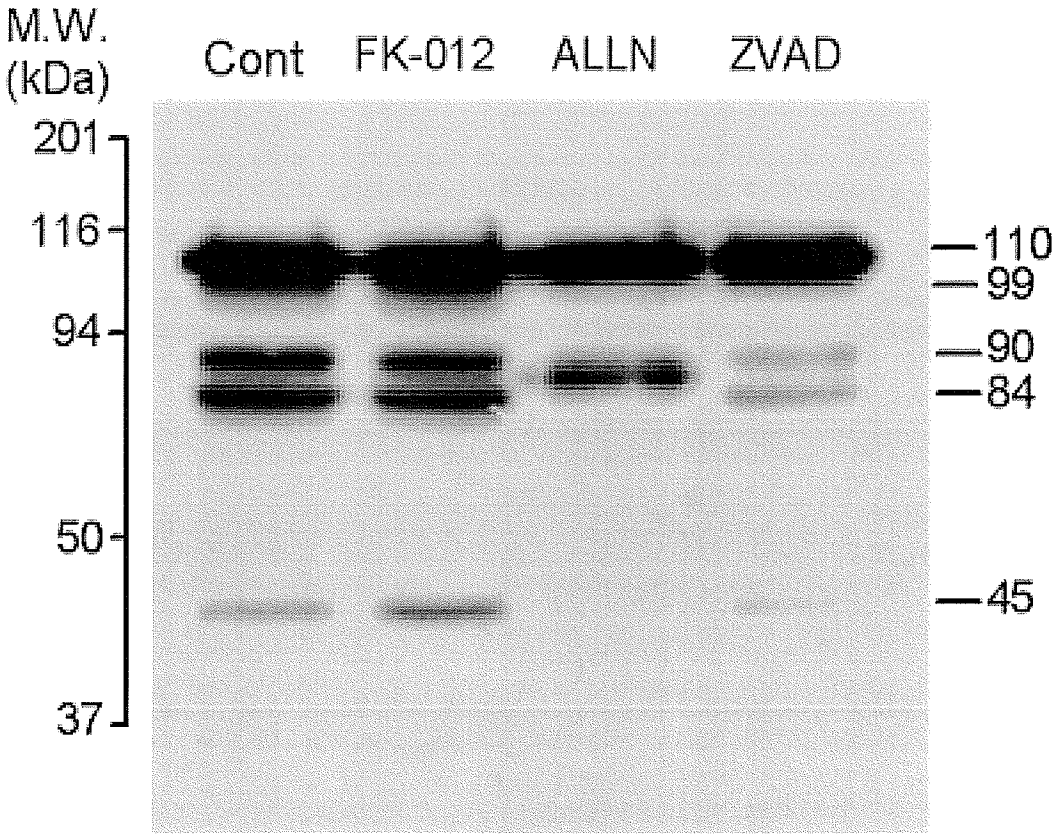

Figure 19: Effect of calpain/caspase inhibitors on the pattern of fragmentation of calpastatin

This is the western blot of the 10% SDS-Polyacrylamide gel probed with anti-FLAG antibody. Molecular weights are shown on both sides of the blot. VSMCs (both uninfected and Ad-CAST-FLAG infected) were treated with staurosporine (1μM) and a calpain/caspase inhibitor (50μM) simultaneously for four hours. There was a unique pattern of fragmentation of calpastatin in cells treated with ALLN, with a single band forming around 90 kDa. The level of expression of 90-, 84-, 45-kDa fragments were markedly reduced in ZVAD treated cells.

Apoptosis in Ad-Cast-FLAG infected HeLa Cells

One of the difficulties of studying involvement of caspase-dependent mechanisms in primary cell cultures derived from rabbit tissue is the lack of suitable antibodies capable of detecting apoptotic events using western blot approaches. This fact coupled with the apparent relative resistance of vascular smooth muscle cells to caspase 3-mediated staurosporine-induced cell death made it difficult to adequately test our hypothesis that calpain activation may be involved in caspase-3 activation during apoptosis. We were aware from the work of others in the Institute of Cardiovascular Sciences that HeLa cells could be readily shown to undergo caspase 3-mediated apoptotic cell death in response to staurosporine. Thus, it was decided to perform these studies by comparing the actions of staurosporine in HeLa cells infected with either Ad-EGFP (as a negative control) or Ad-Cast-FLAG. Figure 20a shows that in Ad-EGFP infected HeLa cells, 3 μM staurosporine induced a marked time-dependent increase in the production of the caspase 3-specific 120 kDa SBP. This proteolytic product was generated at the expense of the intact 240 kDa spectrin, as evident from the nearly complete fragmentation of this protein after three hours of exposure to staurosporine. This contrasts with the much reduced fragmentation of intact spectrin and attenuated formation of the caspase 3-specific 120 kDa SBP in HeLa cells infected with Ad-Cast-FLAG (Fig. 20b). To further investigate the possibility that calpastatin induced inhibition of calpain may limit activation of caspase 3 associated with staurosporine treatment, HeLa cell lysates were also probed on western blots with a monoclonal antibody that recognizes both intact and caspase 3-fragmented poly (ADP-ribose) polymerase (PARP). In Figure 21, intact PARP is shown as a 116 kDa immuno-reactive peptide which was cleaved by caspase 3 to an 85 kDa proteolytic fragment.

PARP is also known to be further processed to lower molecular weight fragments (50-60 kDa) by extensive fragmentation but our antibody was unable to detect those lower molecular weight fragments. As is the case for reduced SBP formation in Ad-Cast-FLAG infected HeLa cells, the effects of staurosporine upon formation of the 85 kDa caspase 3-specific proteolytic fragment of PARP was also attenuated (Figure 21). Figure 21 shows that one hour after staurosporine treatment substantial fragmentation of PARP occurred in Ad-EGFP infected HeLa cells but, in Ad-Cast-FLAG infected cells, production of the 85 kDa peptide was much reduced. After three hours, the immunostaining intensity of both intact and fragmented PARP was further reduced in Ad-EGFP infected HeLa cells whereas in Ad-Cast-FLAG infected cells, immunostaining intensity of the 85 kDa proteolytic fragment was preserved. Thus, these data support the hypothesis that calpain activation is associated with staurosporine-induced cell death (in HeLa cells) and that it was associated with mechanisms leading to activation of caspase 3. This possibility was supported by formation of a caspase-3-like proteolytic product of endogenous calpastatin (Wang et al., 1998) seen at around 85 kDa and reduced immunostaining of the 45 kDa band (Figure 22). Over-expressing hCast-FLAG in HeLa cells exposed to staurosporine had no obvious effect upon its fragmentation (Figure 22).

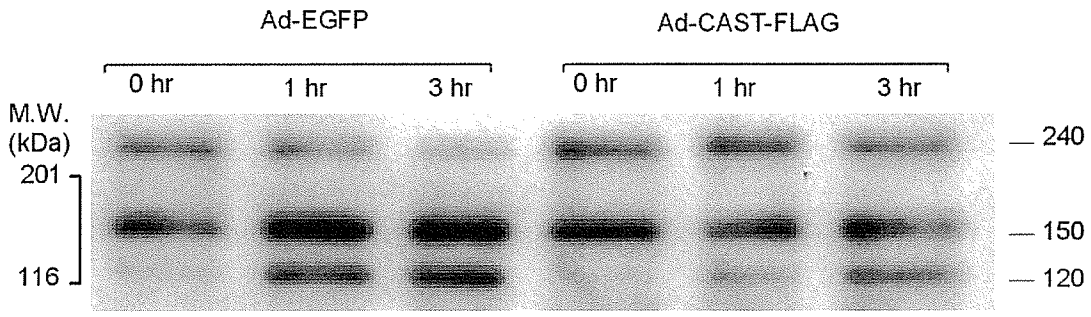

Figure 20 a: Effect of calpain inhibition on the pattern of staurosporine-induced spectrin degradation in Hela cells

This is the western blot of the 10% SDS-Polyacrylamide gel probed with spectrin antibody. Molecular weights have been shown on both sides of the blot. Reduced fragmentation of intact spectrin and attenuated formation of the caspase 3-specific 120 kDa SBP in HeLa cells infected with Ad-Cast-FLAG is quite clear in this image.

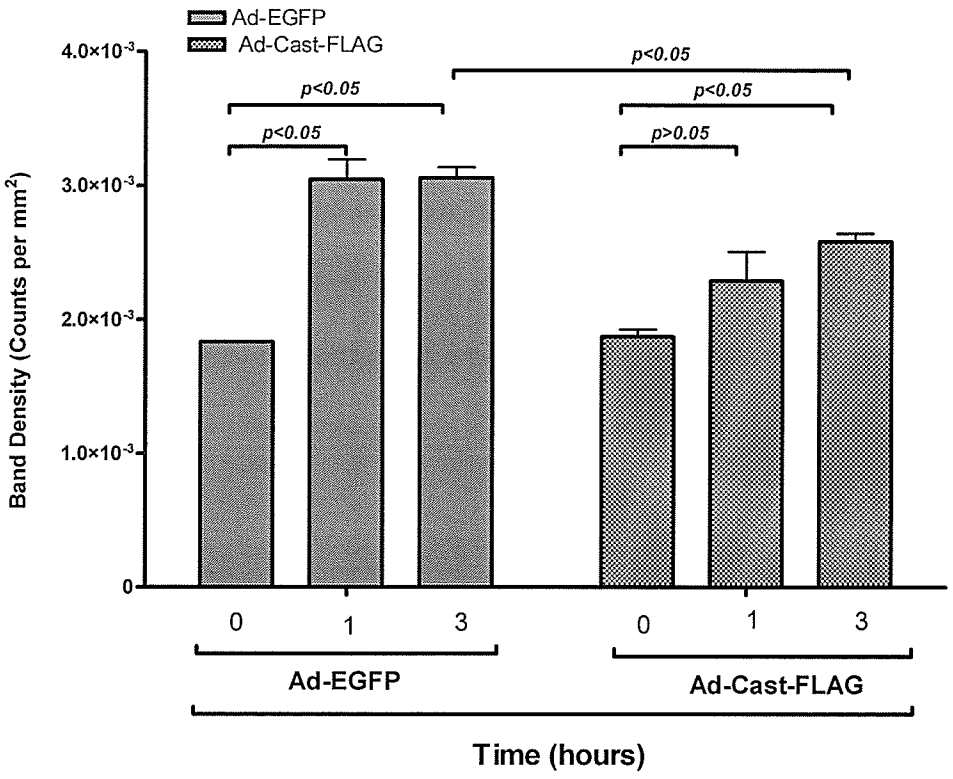

Figure 20 b: Time-dependent increase in the level of expression of caspase-3 specific 120-kDa SBP in Ad-EGFP and Ad-Cast-FLAG infected Hela cells exposed to 3μM staurosporine.

The expression of caspase-3 specific 120-kDa SBP increased time-dependently in both Ad-EGFP and Ad-Cast-FLAG infected Hela cells. There was a significant increase in the resistance to staurosporine-induced apoptosis in Ad-Cast-FLAG infected Hela cells compared to the control. This bar graph represents three independent experiments.

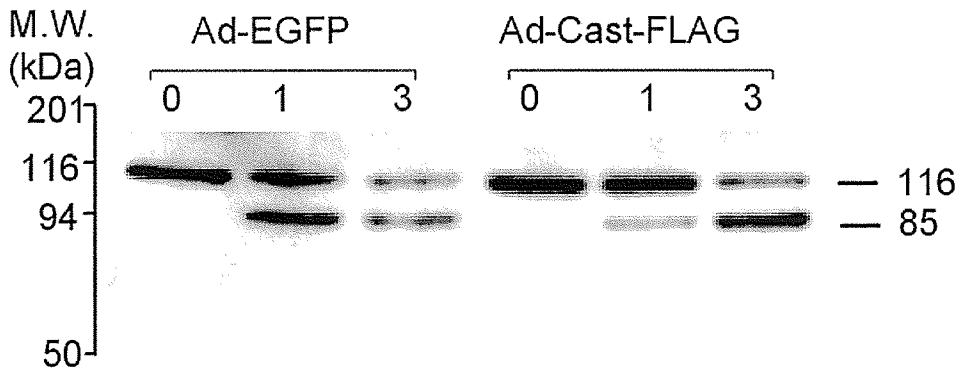

Figure 21: Effect of calpain inhibition on staurosporine-induced PARP degradation

This is the western blot of the 10% SDS-Polyacrylamide gel probed with an anti-PARP Antibody. Molecular weights have been shown on both sides of the blot. HeLa cells, both control (Ad-EGFP infected) and Ad-Cast-FLAG infected, were treated with 3μM staurosporine for 0, 1, 3 hours. After 1 hour exposure, production of the 85 kDa peptide was attenuated in Ad-Cast-FLAG infected cells compared to the control. After 3 hours exposure, both fragments were substantially cleaved in control cells.

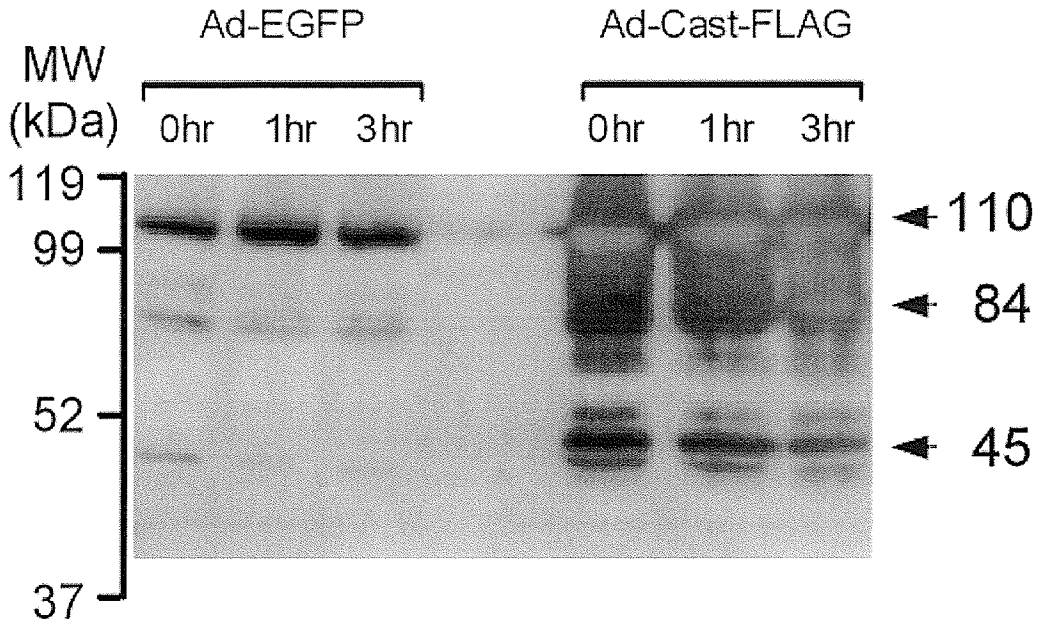

Figure 22: The effect of staurosporine induced apoptosis on fragmentation of HeLa cell calpastatin

This is the western blot of the 10% SDS-Polyacrylamide gel probed with anti-human calpastatin antibody. Molecular weights have been shown on both sides of the blot. As exposure time to staurosporine increased from 0 to 3 hours, increased fragmentation of calpastatin was observed through formation of additional peptide bands around 80 kDa and loss of immunostaining of peptides around 45 kDa.

DISCUSSION

Apoptosis is crucial for the proper functioning of the body's immunologic system, embryonic development and homeostasis in adult life (Boulares et al., 1999). Apoptotic cells have a characteristic morphology recognized by formation of apoptotic bodies, condensation of chromatin and membrane blebbing (Boulares et al., 1999). Compared to calpain, more research has been done about the role of caspases in apoptosis. Both intrinsic and extrinsic pathways of apoptosis lead to the activation of caspases. These two pathways ultimately converge on the activation of caspase 3, one of the so-called executioner caspases (Slee et al., 1999). More recently, the role of calpain in both caspase-dependent and caspase-independent pathways has been suggested. Calpain and caspases may not only share the same substrates (Burgess et al., 1999, Wang, 2000), but also calpain is capable of cleaving caspases (Lankiewicz et al., 2000, Nakagawa and Yuan, 2000). Many experiments done in the past used non-specific synthetic inhibitors of calpain in order to clarify its role in caspase-dependent apoptosis. The result of these experiments encouraged the researchers to consider modulating calpain activity as a therapeutic approach for a large variety of diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, cancers, and also cardiovascular and autoimmune pathologies (Thompson, 1995).

The aim of this study was to specifically evaluate calpain's role in the process of apoptosis induced in rabbit aortic vascular smooth muscle cells (VSMCs) and HeLa cells. In order to achieve our aim, calpain was selectively blocked through adenoviral delivery

of full-length human calpastatin containing a carboxy terminal antigenic FLAG tag (hCast-FLAG). Calpastatin is the only known specific endogenous inhibitor of calpain and has no inhibitory action on other proteases. By implementing such a strategy, we were going to test the hypothesis that calpain activation contributes to apoptosis. Specifically, we wanted to investigate if over-expressing hCast-FLAG in cultured cells could protect them from apoptotic cell death.

Previous fluorigenic assays by our lab of adenovirally expressed hCast-FLAG purified from VSMCs showed robust inhibition of *in vitro* calpain activity at orders of magnitude above that associated with endogenously expressed calpastatin. As shown in Figure 7, four days after infection was needed in order for VSMCs to highly express hCast-FLAG protein. This time was much longer than that required for expression in HeLa cells where high expression of hCast-FLAG was observed after only one day post-infection. It is probable that the slower rate of hCast-FLAG protein expression in VSMCs is due to their relative resistance to adenoviral infection in the absence of manipulating culture conditions.

To induce apoptosis, both H_2O_2 and staurosporine were employed. H_2O_2 is a common product of metabolism especially in phagocytic cells in regions of inflammation (Hyslop et al., 1995). In lower amounts, these agents can induce apoptosis (Choi et al., 2006) whereas at higher doses, necrosis will occur (Dypbukt et al., 1994,Lennon et al., 1991). H_2O_2 increases $[Ca^{2+}]_i$ in a biphasic manner, first through its release from intracellular sources and then via Ca^{2+} influx (Krippeit-Drews et al., 1999). In VSMCs, high

concentrations of H_2O_2 between 0.5 mM and 1 mM can cause apoptosis whereas lower concentrations (0.1 mM) results in cell cycle arrest in G_1 because of inhibition of cyclin-dependent kinase-2 (CDK2) activity (Deshpande et al., 2002). Also H_2O_2 increases the level of both p21 and p53 in treated VSMCs (Deshpande et al., 2002). In this study, concentrations of H_2O_2 up to a maximum of 0.5 mM were employed as higher concentrations were found in preliminary studies to produce significant cell lysis and loss of cytosolic proteins into the culture medium.

Staurosporine is a protein kinase inhibitor but the mechanism of staurosporine-induced apoptosis is still not clear. However, it has been suggested that the intrinsic pathway of apoptosis through the mitochondria is mainly responsible for induction of apoptosis by staurosporine (Tafani et al., 2001, Tafani et al., 2002). Both caspase-dependent and caspase-independent pathways have been considered responsible for staurosporine induced apoptosis (Belmokhtar et al., 2001, Zhang et al., 2004). In different cell types different mechanisms may be responsible for the apoptosis induced by staurosporine. For example, in human melanoma cell lines, staurosporine-induced apoptosis contains two phases. The first phase occurring shortly after exposure to staurosporine is caspase dependent. Broad caspase inhibitors and over-expression of Bcl-2 inhibited this phase but not the second (late) phase which is caspase-independent and is thought to be related to translocation of Apoptosis-Inducing Factor (AIF) from mitochondria into the nucleus (Zhang et al., 2004). In this study, staurosporine exposure was limited to time points of three hours and less.

The expression of a 120 kDa caspase-3-specific spectrin breakdown product (SBP) in western blots was considered as a primary marker of apoptosis in primary cultured rabbit aortic VSMCs. The lack of suitable antibodies for detecting apoptosis in rabbit tissues was a significant disadvantage when using rabbit aortic VSMCs. The parent 240-kDa alpha-spectrin protein is well known to be proteolytically sensitive. Proteolytic degradation by calpains and caspases leads to formation of 150 and 145-kDa SBPs, respectively (Nath et al., 1996) although sustained calpain-mediated proteolytic attack also leads to formation of this lower molecular weight 145 kDa SBP. However, a 120-kDa SBP is produced through caspase-3-specific proteolytic action and is, therefore, a useful marker event for the activation of caspase-dependent apoptosis (Wang et al., 1998). To provide an additional assessment, western blots were also probed for p53 levels, a tumor suppressor gene product that is known to be increased following treatment of cells with pro-apoptotic stimuli. p53 mediates apoptosis through transactivating expression of pro-apoptotic genes such as Bax gene. It is a negative regulator of cell proliferation either through arrest of the cell cycle in G_1 or, alternatively, apoptosis (Merino and Cordero-Campana, 1998).

Our results with VSMCs showed that they were quite resistant to both staurosporine- and H_2O_2-induced apoptotic cell death mediated by caspase-3. Both hydrogen peroxide and staurosporine caused an increased expression of the caspase 3-specific SBP. However, the extent of these increases was relatively modest compared to the extensive production observed in studies with other cell lines using similar experimental conditions. Also the difference between VSMCs infected with either Ad-EGFP or Ad-Cast-FLAG

was not significant. However, visual observation of cells exposed to either staurosporine or H_2O_2 revealed significant cell condensation and an obvious change in normal cellular morphology (not shown). These limited observations suggest that in VSMCs: (a) caspase 3-mediated apoptosis may not be the only pathway to cell death, and, (b) calpain activation does not have a significant effect on caspase-3-mediated apoptosis. In agreement with results from other studies (Deshpande et al., 2002), a concentration-dependent increase in p53 expression following treatment of VSMCs with staurosporine and H_2O_2 was detected. However, no significant difference was observed in cells infected with Ad-Cast-FLAG compared to the control cells.

To further test the hypothesis of an interaction between caspases and calpains during apoptosis, parallel studies were conducted with human cervical carcinoma (HeLa) cells. HeLa cells were selected because (a) we were informed of a strong caspase-3 response to staurosporine in these cells by other labs and (b) there are advantages of greater antibody availability when using human cells. In HeLa cells, a significant staurosporine-induced degradation of spectrin was noted with almost a complete disintegration of the 240-kDa parent protein and the strong formation of the 120 kDa caspase-3-specific SBP. Contrary to VSMCs, substantial resistance to spectrin fragmentation was observed in cells over-expressing hCast-FLAG. This finding was confirmed by comparing the pattern of PARP degradation in Ad-EGFP versus Ad-Cast-FLAG infected Hela cells (Figure 21).

Although the observations with HeLa cells supports the hypothesis that calpain activation feeds into the activation of caspase-3 during apoptotic cell death, the disparate

observations in VSMCs raises the possibility that these actions are cell type specific. One possible explanation is that differentiated cells like primary-cultured VSMCs are quite resistant to apoptosis so that calpastatin over-expression has little detectable impact upon this. Conversely, non-differentiated cells like HeLa cells were more susceptible to apoptosis and therefore inhibition of calpain activity through over-expression of calpastatin would have more dramatic and evident protective effect in these cells.

The above hypothesis is in agreement with previous studies showing that in fully differentiated adult cardiomyocytes, the differentiation-related suppression of the whole caspase-dependent apoptotic machinery is responsible for the protection of these cells against caspase-mediated cell death (Bahi et al., 2006). In other words, differentiation-related silencing of apoptotic genes may produce a switch from caspase-dependent to caspase-independent cell death, thus, accounting for the resistance of differentiated cardiomyocytes against apoptosis (Bahi et al., 2006). In the Bahi study (Bahi et al., 2006), pharmacological calpain inhibition did not have any significant effect on AIF cleavage in ischemic cardiomyocytes. Interestingly, EndoG, and not AIF, was considered responsible for cleavage of cardiac DNA (Bahi et al., 2006). Other studies have also shown that the intrinsic (mitochondrial) pathway of apoptosis is responsible for staurosporine-induced cell death and is blocked in differentiated and postnatal cardiomyocytes due to a lack of expression of apoptotic protease-activating factor-1 (Apaf-1) in these cells (Sanchis et al., 2003). Postmitotically differentiated cells such as mature cortical neurons (Yakovlev et al., 2001)(Ota et al., 2002), cardiomyocytes (Sanchis et al., 2003), and skeletal muscle cells (Burgess et al., 1999) express a

considerably lower level of Apaf-1 which can cause a significant reduction of apoptosome activity. Such a reduction in turn will lead to slower processing of procaspase-9 and, therefore, will provide a better chance for IAPs to bind and inactivate caspase-9 and block apoptosis (Wright et al., 2004). In contrast, IAPs appear to be ineffective in blocking apoptosis in mitotic cells like HeLa cells, HEK 293 cells, and fibroblasts due to increased expression of Apaf-1 and efficient apoptosome activity (Wright et al., 2004). All of these data suggests that neither calpains nor caspases have essential roles in apoptosis induced in differentiated cells. This result may explain why calpain inhibition in such cells cannot induce a significant resistance to apoptosis.

Several studies have suggested that calpain may contribute to the activation of caspase-dependent and caspase-independent cell death either through the conversion of Bcl2 family members (e.g. Bid to tBid) (Wood et al., 1998) or through activation of AIF (Polster et al., 2005). These actions are summarized in Figure 4. The results of the present study are consistent with this hypothesis, at least with respect to observations in HeLa cells. However, other results presented here suggest an additional level through which calpain may modulate the actions of caspases. Endogenous calpastatin fragmentation during apoptosis has been documented in previous studies (Wang et al., 1998). The mechanism responsible for this phenomenon is not yet clear. However, it has been shown that both calpain and several members of the caspase family can cleave calpastatin *in vitro*. According to our immunocytochemistry (Figure 10) and nuclei isolation studies (Figure 12), at low MOIs a single 110 kDa hCast-FLAG peptide was evident only in cytosolic fractions. However, infection at higher MOIs, led to the

reproducible formation of a series of lower molecular weight fragments. Also at higher levels of infection, we detected hCast-FLAG in the nuclei in agreement with previous observations with endogenous calpastatin (Nori et al., 1993). Furthermore, exposure to staurosporine led to a time-dependent increase in the degree of hCast-FLAG fragmentation in VSMCs. This finding is in agreement with the previously observed proteolytic susceptibility of calpastatin under similar apoptotic conditions (Wang et al., 1998). We noticed that treatment with a pan-caspase inhibitor (ZVAD) markedly inhibited both the fragmentation of hCast-FLAG and the production of the 120 kDa caspase-3 specific SBP. This finding suggests a crucial role for caspase activation in producing these effects. Interestingly ALLN, a strong synthetic calpain inhibitor, modified the typical pattern of calpastatin fragmentation and produced a unique hCast-FLAG fragment (85-90 kDa) and markedly increased production of a 120 kDa caspase-3 specific SBP. This finding suggests that ALLN can activate caspase 3 and cause apoptosis in VSMCs. ALLN has been reported to induce apoptosis in some cells (Akaike et al., 2003, Zhu et al., 2005) but cause protection against apoptosis in other cells (Debiasi et al., 1999, Kung et al., 2006). Such differences may be due to cell specifity. Nevertheless, our specific observations reported here are entirely novel. We hypothesize that ALLN blocks all forms of proteolytically activated calpain which can lead to inhibition of calpain-mediated cleavage and inactivation of caspase-3 in VSMCs. It is important to note that ALLN does not inhibit caspases.

Our results are in agreement with the following model of caspase/calpain/calpastatin mutual negative regulation shown in Figure 23. Phosphorylated calpastatin can bind to

and inhibit calpain both in the presence and absence of sub-micromolar Ca^{2+} concentrations (Averna et al., 2001, Averna et al., 2006, Melloni et al., 2006). Alternatively, calpain can cleave calpastatin and reduce this inhibition, possibly through the activation of separate calpain pools not bound to calpastatin. This is deemed feasible since not all calpain appears to be bound to calpastatin. The calpain inhibitory function of calpastatin depends on its phosphorylation status. Phosphorylation is an important mechanism for making calpastatin available for binding and inactivating calpain (Averna et al., 2006, Melloni et al., 2006). Calpain can cleave and inactivate caspase-3 (Wang et al., 1998) but caspase-3 is also capable of cleaving and disintegrating calpastatin (Wang et al., 1998). The latter actions may lead to the feed-forward activation of calpain through its dis-inhibition. Thus, in some cells, caspase-3 activation may help dis-inhibit calpain by proteolytically cleaving calpastatin. Indeed, the single site proteolysis of calpastatin by caspase-3 (Wang et al., 1998) is consistent with the production of a 85-90 kDa proteolytic fragment of Cast-FLAG and inhibition of its formation by ZVAD as shown in Figure 19. This proteolysis occurs within sub-domain C of intact calpastatin and loss of this structural integrity decreases the calpain-binding capability of the cleaved calpastatin. Also according to this model, we can predict that inhibition of calpain by ALLN can lead to dis-inhibition of caspase-3 (step D in Figure 23) which can exacerbate apoptosis as well as increase caspase-3-dependent fragmentation of calpastatin. This proposal is also consistent with the observed effects of calpain inhibition by calpain inhibitor II to induce caspase-dependent apoptosis in human acute lymphoblastic leukemia and non-Hodgkin's lymphoma cells (Zhu and Uckun, 2000). Thus, these observations collectively suggest that one of the functions of calpain is to regulate the activation of caspase-3.

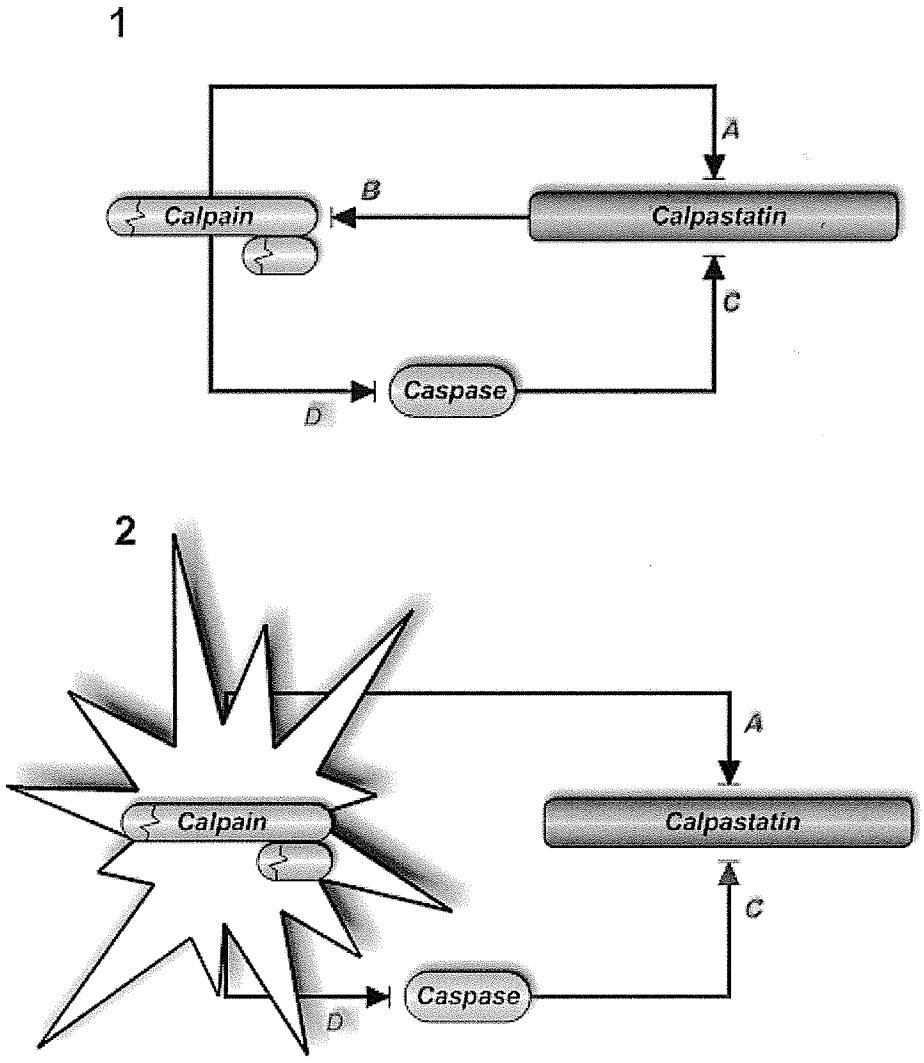

Figure 23. Proposed model of mutual negative regulation between Calpain, Calpastatin and Caspase 3.

In panel 1, Calpain inhibits calpastatin by proteolytic cleavage (at A) and calpastatin inhibits calpain (at B). Caspase 3 inhibits calpastatin (at C) by proteolytic cleavage and calpain also inhibits caspase 3 by proteolytic cleavage (at D). In panel 2, activation of caspase 3 (shown as red line) can inhibit calpastatin and lead to dis-inhibition of calpain (through loss of B). The prediction of this model is that caspase 3 activation can lead to feed-forward activation of calpain but also feed-back inhibition of caspase 3.

The mutual negative control of these three proteins might explain the pro-life function of calpain. Recent studies have shown an important role for calpain in promoting the Akt-dependent pro-survival pathways (Tan et al., 2006). Indeed, it has become increasingly clear that various pro-apoptotic stimuli can activate both pro-death and pro-life pathways with the final outcome being dependent upon the degree of activation down each arm. Interestingly, staurosporine-induced apoptosis in HeLa cells was not accompanied by fragmentation of endogenous calpastatin or hCast-FLAG. Overexpression of calpastatin in HeLa cells decreased caspase-mediated fragmentation of spectrin and PARP.

It seems that the model of mutual negative regulation of these three proteins is less important in HeLa cells as compared to VSMCs and calpain is more actively involved in promotion of apoptosis rather than inactivation of caspase-3. Such promotion of apoptosis by calpain can be done either through activation of a Bcl2 member or cleavage and, therefore, activation of AIF (Polster et al., 2005,Wood et al., 1998). Also calpastatin may not be cleaved markedly by caspase-3 in HeLa cells. However this hypothesis needs to be tested in future experiments.

In conclusion, calpain can play both pro-life and pro-death roles in the cells. Calpain's involvement in cell proliferation and its role in proteolytic cleavage and inactivation of p53 and caspases 7, 8, 9 are few examples of calpain's pro-life activity. However, cleavage and activation of caspase 12, AIF, and some Bcl-2 protein family members such as $Bclx_L$, Bid, and Bax clearly demonstrate calpain's active involvement in apoptosis. The final outcome of calpain activation in a cell depends on a variety of factors but it seems that the cell type and the nature of apoptotic stimuli are determining factors.

Apparently, in differentiated cells like VSMCs, apoptosis is not mediated by caspase-3 and this can be concluded from the pattern of degradation of spectrin. Furthermore, inhibition of calpain through over-expression of calpastatin has no significant effect on inhibition of caspase-3 mediated apoptosis. Therefore, it appears in differentiated cells such as VSMCs, calpain is not actively involved in apoptosis.

However, in non-differentiated cells like HeLa cells, apoptosis is actively mediated by caspase-3. Inhibition of calpain activity by over-expression of calpastatin induces a significant resistance to caspase-3 mediated apoptosis. Thus, calpain is actively involved in caspase-3 mediated apoptosis and its inhibition will lead to inhibition of caspase-3.

In VSMCs, ALLN induce caspase-3 mediated apoptosis most probably due to strong inhibition of calpain. Different results obtained when using ALLN compared to over-expression of calpastatin may be due to the presence of different functional pools of calpain.

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