FAS/FADD - INDUCED PRO-INFLAMMATORY RESPONSE IN VASCULAR SMOOTH MUSCLE CELLS

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Abstract

Fas/FADD-induced pro-inflammatory response in vascular smooth muscle cells

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Apoptosis of smooth muscle cells is a common feature of vascular lesions. Unlike necrosis, apoptosis is classically considered to be "silent," i.e., self-contained and non-inflammatory. I have found that signals initiated by Fas-associated death domain protein (FADD) overexpression in rat vascular smooth muscle cells in the carotid artery induce expression of monocyte-chemoattractant protein-1 (MCP-1) and Interleukin-8 (IL-8), and result in massive immigration of macrophages *in vivo*. These chemokines, and a specific subset of proinflammatory genes, are also upregulated in human vascular smooth muscle cells after treatment with Fas ligand (FasL) plus cycloheximide (Chx). I have determined that Fas/FADD-induced MCP-1 upregulation is regulated by increased synthesis and release of IL-1α and does not occur in cells derived from IL-1-receptor deficient mice. Inhibition of caspases and apoptosis only partly reduces gene induction in response to Fas/FADD activation. Calpain activation and cell death are the only known mechanisms through which IL-1α can be released. Calpain inhibitors effectively reduce Fas/FADD-mediated synthesis and release of IL-1α and consequently MCP-1 upregulation.
My data provides strong evidence for a non-apoptotic Fas/FADD-activated signaling pathway, which involves activation of caspases, calpain and IL-1α signaling, leading to the induction of a program of pro-inflammatory gene expression. An important pathophysiological consequence of this pathway could be that the secreted IL-1α acts on healthy neighboring cells to amplify the pro-inflammatory signal and prolong it beyond the life time of the cells initially stimulated by Fas/FADD.

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Chapter 1:

Fas/FADD-mediated activation of a specific program of inflammatory gene expression in vascular smooth muscle cells

Introduction

What is "apoptosis"?

"Apoptosis" refers to a sequence of cellular events, mediated by specific cell proteins, which leads to cell death. It has morphological features, including nuclear condensation and fragmentation, membrane blebbing and cell shrinkage, that distinguish it from "necrosis", which is characterized by cell membrane disruption, swelling and disintegration.

Many stimuli can affect apoptosis by feeding into one of two signaling pathways ("intrinsic" and "extrinsic") that result in the activation of members of the family of aspartic-specific cysteine proteases (caspases). In this thesis project, I focused mainly on the "extrinsic" pathway initiated by activation of the transmembrane receptor Fas (see below) in vascular SMCs. Binding of the soluble death ligand FasL to Fas induces Fas trimerization, followed by recruitment of the cytoplasmic adaptor molecule FADD [26], and other adaptor proteins. FADD then binds caspase 8 (Csp8), which becomes auto-proteolytically activated. The association of Fas/FADD and caspase 8, also called death-inducing signaling complex (DISC), subsequently activates the cascade of terminal effector caspases including caspases 3, 6 and 7 [25,126,129].

In cell types ("type II" cells) that do not express Csp8 abundantly, Fas-activated death signaling is transmitted largely through the "intrinsic pathway". In these cells, Csp8 does not efficiently activate Csp3 directly (as in "type I" cells), but instead facilitates (through cleavage) the binding of the pro-apoptotic proteins Bid and Bad to the anti-apoptotic mitochondrial membrane protein Bcl-2. The interaction of these two proteins, which are both Bcl-2 family members, causes changes in voltage-dependent mitochondrial channels, followed by the release of cytochrome C (reviewed [108]). Cytochrome C promotes interaction between the apoptotic protease APAF-1 and Csp9,

which then activates Csp3 and downstream effector caspases (reviewed in [18,59]). Overexpression of Bcl-2 or Bcl-X blocks apoptosis in type II cells, but is ineffective in type I cells. It has not been clearly established into which category vascular SMCs fall, and the pathway may depend on the apoptotic stimulus and the state of the cells.

Background

Signaling by death receptors.

Vascular SMCs express at least two transmembrane death receptors, Fas and TNF-R1 [49]. Soluble Fas ligand (FasL) is a weak inducer of SMC apoptosis unless the cells are "primed" [9,51]. Priming can be accomplished experimentally by including low concentrations of cycloheximide in the culture medium, or by adding mixtures of cytokines [51]. The nature of the priming is not clear, though in some cases, SMC sensitivity to FasL may be regulated by changes in the level of Fas expression on the cell surface [9]. TNF receptors use two well-established signaling pathways that operate in parallel. The anti-apoptotic (pro-inflammatory) pathway involves recruitment of TRADD then Traf2 then NIK and down the NF-κB pathway. The pro-apoptotic pathway involves FADD then Csp8 and down the apoptosis cascade. By contrast, Fas can activate apoptosis *via* recruitment of FADD then Csp8 (etc), but it does not have a well established anti-apoptotic pathway. Several studies have reported that Fas stimulation can result in activation of the transcription factor NF-κB. It has also been shown that overexpression of FADD [75,181] or Caspase 8 [23,75] can activate NF-κB in various cell lines, but the mechanism underlying this is not clear. The results of Wajant et al [181] suggest that activation of NF-κB occurs *via* a caspase-independent signaling pathway, because it can be activated by a mutant FADD that lacks the death effector domain (DED) that serves as the docking site for Csp8. Surprisingly, activation of NF-κB was also found to be mediated by Csp8 but without requirement for the caspase activity of Csp8. Hu et al [75] and Chaudhary et al. [23] reported that NF-κB is activated by overexpression of wild type Csp8, or a catalytic inactive form of Csp8, or even by the Csp8 DED domain alone. Another possible player with uncertain function is the Csp8

homologue FLIP/MRIT/CASPER. Wajant et al [181] found that overexpression of cFLIP inhibited Fas/FADD-initiated NF-κB activation, indicating that cFLIP can act as an inhibitor protein which must be downregulated (possibly by Chx) before NF-κB activation can occur. However, Hu et al [75] and Chaudhary et al [23] found that overexpression of cFLIP/Casper induces NF-κB activation and that this effect can be blocked by CrmA and caspase inhibitors. Although these studies do not present a clear picture of signaling from Fas to NF-κB, they do support the hypothesis that the biological function of Fas/FADD goes beyond their role in the apoptotic pathway. My findings further strengthen this notion.

Apoptosis and macrophages in vascular disease

Apoptosis plays a crucial role in development and homeostasis of the organism, including the sequelue of pathological processes. Apoptosis has been most thoroughly investigated in cells of hematopoietic (and epithelial) lineages, but also occurs, with some differences, in connective tissues. High rates of apoptosis are often observed in primary atherosclerotic lesions and in atherosclerotic vessels after angioplasty [50,64,137].

Although human atherosclerotic lesions are of great clinical interest, they are also histologically complex and have variable and uncertain histories and futures. Experimental animal models are better suited to determine possible causal relationships between apoptosis, lesion structure, and outcome. Balloon catheter injury of the rat carotid artery causes almost immediate death of many medial SMCs, at least in part *via* apoptosis. Perlman [137] reported that 70% of the SMCs were apoptotic at 1hr after balloon catheter injury. Similar kinetics were reported in a rabbit model [139] and pig model [105]. More surprising, perhaps, is the role of apoptosis in later evolution of the vascular lesion. Clowes et al. [29] observed that, despite continued proliferation of SMCs, especially at the luminal border of the neointima, the total number of neointimal SMCs is about the same at 2 and 12 weeks after balloon injury. The authors concluded that cell death must be occurring at a high rate to counterbalance this proliferation. Direct evaluation of apoptotic rates confirmed the presence of relatively large numbers of

apoptotic cells in the neointima [12,64,105]. However, the degree of apoptosis in response to vascular injury differs in different models. In a rabbit reinjury model, Kamenz [79] found no increase in apoptosis, while mouse models of atherosclerosis have revealed high rates of apoptosis [66,101].

In contrast to lymphatic cells, both connective tissue cells and the associated extracellular matrix play important structural roles. It would therefore make biological sense that apoptotic cell bodies would be removed / phagocytosed, as they might disturb the structural stability of the organ. Colocalization of macrophages with areas of apoptosis is often observed within atherosclerotic lesions, after angioplasty [64,87,88] and during embryonic development [72,133,196]. Macrophages are important cellular mediators in vascular disease, as scavengers and as sources of growth factors [150] and of matrix-degrading enzymes [96]. Kockx [87] reported that most apoptotic cells in atherosclerotic plaques were near the macrophage-rich necrotic core, and not seen in lesions without a macrophage-rich core. This observation is consistent with a recent report that identified increased numbers of macrophages, and decreased numbers of SMCs, at sites of plaque rupture in cases of sudden cardiac death, indicating a possible causal role for SMC apoptosis followed by macrophage invasion in these fatal events[89]. What is the causal relationship between apoptosis and macrophage accumulation in vascular lesions? Boyle et al. [15] demonstrated that macrophages can kill SMCs, which could suggest that the accumulation of macrophages is causing the SMC apoptosis. The converse relationship, however, seems more likely: that macrophages infiltrate the vessel wall to remove cell debris. But what does "attract" macrophages to the site of abundant apoptotic cell death. *In vitro*, macrophages can recognize apoptotic cells via vitronectin, thrombospondin, and phosphatidylserine receptors as well as through interaction with the plasma-membrane glycoprotein CD14 [43,156 35]. It seems conceivable that the increased numbers of macrophages observed in vascular lesions represent macrophages that, while passing through the tissue, were retained via interaction with apoptotic cells. Alternatively, it is possible that macrophages were actively recruited via cytokines produced as part of the SMC apoptotic program.

It is usually considered that apoptosis does not actively induce an inflammatory response [156] but some recent studies indicate that apoptosis can produce pro-inflammatory effects, and/or that apoptotic stimuli, including the Fas/Fas ligand system, can result in inflammation. Intraperitoneal inoculation of Fas-ligand expressing tumor cells induces processing and release of interleukin-1, causing a massive neutrophil infiltration *in vivo* [118]. Subcutaneous injection of agonistic anti-Fas monoclonal antibody triggers neoangiogenesis and local infiltration of inflammatory cells in a mouse model [10]. Expression of Fas-ligand on tumor cells, or pancreatic β-cell transplants, causes a strong, local inflammatory reaction. The authors speculate that Fas ligand expression induces apoptosis of infiltrating monocytes, which through the release of cytokines promote recruitment of neutrophils and further inflammatory responses [4,80,161].

In the first part of my thesis project, I present evidence from a system for regulating SMC apoptosis in the rat carotid artery, that SMCs, which undergo apoptosis, actively recruit macrophages through upregulation of a program of pro-inflammatory gene expression.

Fas/FADD-mediated apoptosis activates a specific program of inflammatory gene expression in vascular smooth muscle cells

In the Background section, I presented published evidence that increased apoptosis is a feature of atherosclerotic and restenotic lesions, both in humans and in animal models. Those studies establish a correlation between apoptosis and other characteristic changes in vascular lesions, including monocyte/macrophage accumulation, plaque rupture, *etc.* but do not distinguish whether apoptosis is the cause or the effect of any of those changes. In order to begin to address this question, I wanted to develop an experimental system that would allow me to induce SMC apoptosis *in vivo*, without exposing neighboring cells to effectors that could influence apoptosis or inflammation. In this report, I tested the hypothesis that macrophages and leukocytes are actively recruited into vascular lesions by apoptotic SMCs through induction of a specific program of SMC expression of pro-inflammatory genes. To accomplish this objective, I developed a system for initiating apoptosis directly in vascular SMCs *in vivo*, without exposing other

cells to any stimuli or apoptotic effectors, so that the initial responses were limited to, and could be evaluated in, the apoptotic cells

Results

Tet-regulated expression of FADD induces apoptosis of smooth muscle cells *in vitro*

I initiated apoptosis by over-expression of the "Fas associated death domain protein" (FADD) within the target SMCs. FADD is an adapter protein for the apoptotic signaling pathways triggered by Fas-ligand and TNF, and transient FADD overexpression is sufficient to cause apoptosis in mammalian cells [26]. As described in detail in the Methods section, I established tet-regulated FADD expression in an inbred Fischer rat SMC line (FRFADD cells) using a tetracycline-inhibited tTA regulatory system adapted after that of Shockett [165] such that FADD is expressed in the absence of tetracycline (tet) but not in the presence of tet (Fig.1.1). A nuclear-targeted myc-tagged β-galactosidase reporter is expressed from a second tet-regulated promoter. Vascular SMCs for transfection were derived from the inbred Fischer rat strain so that transfected cells could be reintroduced into syngeneic rats for the *in vivo* studies described below.

Expression of FADD after tet withdrawal resulted in apoptosis as evaluated in 3 ways. Morphology: In culture, removal of tet resulted in upregulation of FADD expression. FADD protein could be detected by Western blot within 24 hrs after tet removal (Fig.1.2). By three days after tet removal, the FRFADD cells displayed morphological alterations typical of apoptotic cells, including cell rounding, condensation, and detachment. The nuclei of the rounded FADD-expressing cells appeared condensed and fragmented as assessed by Hoechst 33342 DNA staining, and essentially no viable attached cells remained by 6 days (data not shown).
Cell proliferation and detachment: To determine the cumulative effect of FADD expression on a population of FRFADD cells, I determined the number of attached and floating cells at different times after plating in the presence of absence of tet. In the presence of tet, the FRFADD cells showed normal growth kinetics (Fig. 1.3). However, in the absence of tet, 80% to 90% of the cells had detached and were floating in the culture medium by 4 days, and essentially no viable attached cells remained by 6 days.

Phosphatidylserine exposure: To identify cells at an early stage in apoptosis, we used flow cytometry to determine the number of cells that stained with fluorescein-conjugated Annexin V (an indicator of phosphatidylserine exposure) but not with propidium iodide (a marker of loss of membrane barrier function common to apoptosis and necrosis). Annexin V-positive/PI negative cells were detected by 2 days after tet removal (Fig. 1.4).

Seeded FRFADD cells are incorporated into the vessel wall and exhibit tet-regulated FADD expression

In order to determine the consequences of SMC apoptosis *in vivo*, I "seeded" the FRFADD cells onto the surface of de-endothelialized Fischer rat carotid arteries (Fig. 1.5) [30]. The rats received tet in the drinking water for 2 weeks after seeding to keep FADD expression off. The seeded SMCs participated in forming a substantial and quiescent new layer of SMCs, the neointima (Fig.1.6). The effects of FADD expression were then determined by removing tet from the drinking water of half of the rats (Fig.1.6).

I first determined the location of the seeded cells and whether they exhibited tet-regulated expression of FADD and the co-expressed reporter gene (myc-tagged nuclear β-galactosidase) *in vivo*. Luminometer quantitation of the β-galactosidase content of carotid artery extracts showed that β-galactosidase activity peaked 7 days after removal of tet, with a greater than 1000 fold increase above the level in rats that continued to receive tet, then declined by more than 60% by day 15 (data not shown). I determined the location of the cells expressing the reporter by immunostaining for the myc tag. No positive staining was detectable in arteries of rats maintained on tet (Fig. 1.7). A small number of cells were positive by 4 days after tet withdrawal (not shown). By 7 days 70% to 80% of the neointimal cells were positive (Fig.1.7). By day 15, only a small fraction of the neointimal cells were still myc-tag positive (not shown). This pattern of tet-regulated reporter expression was confirmed for FADD protein expression by Western blot of tissue extracts (Fig. 1.8). The monoclonal anti-human FADD antibody recognized a specific FADD band (24 kD) in carotid extracts only after removal of tet, with a maximum at day 7 and a marked decline of expression at day 15. These data demonstrate

that FADD-transgene expression is efficiently regulated *in vivo*. The decline in reporter (and FADD) expression by 15 days is consistent with apoptosis of FADD-expressing cells (see below).

Expression of FADD causes apoptotic loss of neointimal SMCs.

To directly identify apoptotic SMCs, I evaluated sections of seeded arteries by TUNEL staining (data not shown). TUNEL-positive neointimal cells were found in carotid sections from rats at days 7 and 15 after removal of tet. The number of TUNEL-positive cells varied considerably between animals, ranging from 0.5% to 10% of the total number of neointimal cells per cross section. TUNEL-positive cells were almost never observed in the presence of tet. The use of TUNEL for quantitative evaluation of apoptosis is limited by the narrow window during which apoptotic cells can be detected. In order to obtain more reliable quantitative data, I directly determined the number of surviving FRFADD cells by measuring the amount of transgene DNA present per vessel segment (Fig. 1.9). In rats maintained on tet, the amount of seeded cell DNA did not change significantly. After removal of tet, it decreased by 55% at 7 days and by 85 % at 15 days, consistent with apoptotic loss of the seeded SMCs.

FADD-induced apoptosis *in vivo* results in leukocyte recruitment.

Despite the evidence that FADD overexpression resulted in massive apoptosis of seeded cells, the size of the neointima, and the total number of cells in it, decreased only slightly at 7 days (by 10%) and 15 days (by 20%) (data not shown). This suggested that apoptosis *in vivo* was accompanied by proliferation and/or immigration of other cells. I evaluated cell proliferation by immunostaining for incorporation of the thymidine analog BrdU released from a subcutaneous pellet implanted 24 hrs prior to sacrifice. The percentage of labeled cells was not significantly increased in seeded vessels 7 and 15 days after removal of tet, with 9% and 15 % (SEM 1% and 3%) versus 5% and 9% (SEM 1 and 1%) in the continued presence of tet. This suggested that additional/replacement cells, probably leukocytes, were entering the tissue from outside.

I evaluated macrophage immigration by immunostaining with the macrophage-specific monoclonal antibody ED-1. Very few macrophages were present within the seeded vessel if tet was present (Fig 1.10). By 7 days after tet withdrawal, massive macrophage infiltration was seen in the adventitial layer and to a smaller extent in the neointima (Fig.1.10). By 15 days, the neointima was filled with a large number of macrophages (Fig. 1.10). Only a few ED-1 positive cells were found in the medial layer at either time point. To quantitate the extent of macrophage invasion, I determined the area occupied by ED-1- positive cells as a percentage of total area of the neointima. Figure 1.11 shows that 7 days after tet removal, macrophages occupied approximately 6% of the neointima, compared to 1% in the control animals. By 15 days after tet removal, macrophages occupied 11% of the neointima.

In order to specifically evaluate neutrophil invasion, I immunostained with the neutrophil-specific monoclonal antibody RP-3. Virtually no neutrophils were detected in the presence of tet. By 7 and 15 days after tet removal, neutrophils could be found in the neointima of some vessels, but never in large numbers (data not shown).

FADD upregulates MCP-1 and IL-8 expression and secretion by SMC in culture and *in vivo*

The accumulation of macrophages in the neointima during the period of SMC apoptosis could result from "passive" retention of monocytes that would otherwise have passed through the tissue, or from active recruitment via release of leukocyte chemoattractants from the SMCs undergoing apoptosis. I used semi-quantitative RT-PCR to measure transcript levels for the macrophage chemoattractant MCP-1. In order to be certain of the source of the MCP-1, I first evaluated regulation of MCP-1 expression in cultured FRFADD cells. Figure 1.12 shows that MCP-1 transcript levels in FRFADD cells were elevated 60 fold by 4 days after tet removal ($p< 0.005$). Comparable analysis showed that expression of CINC, a rat member of the GRO/IL-8 family [200], is upregulated over 100 fold with similar kinetics ($p<0.001$)(data not shown). The upregulation of MCP-1 transcript level is dependent on expression of FADD and is not a side effect of the tTA transactivator. MCP-1 levels were not increased by tet withdrawal

in the parental cell line that expresses tTA but not the FADD transgene (data not shown). To confirm that induction of MCP-1 expression is directly correlated to overexpression of FADD and is not just an oddity of the specific FRFADD cell line that I was using, I transiently transfected cultures of FR344 SMCs with increasing amounts of an expression vector in which constitutive FADD expression is driven by the CMV promoter. MCP-1 transcript expression increased by 50 to 100 fold compared to mock transfected cells and was proportional to the amount of FADD vector transfected (data not shown).

Figure 1.13 shows that the conditioned medium from the FADD-expressing SMCs contains chemotactic activity for the THP-1 monocyte-like cell line. Addition of two different polyclonal MCP-1 antibodies (b and c), but not preimmune IgG (d) resulted in almost complete elimination of the induced chemotactic activity ($p < 0.005$), suggesting that MCP-1 accounts for most of the chemotactic activity. These data demonstrate that FADD expression in SMCs induces the synthesis and release of MCP-1 in biological active form and concentration.

FADD expression by SMCs also results in increased expression of MCP-1 and CINC/IL-8 *in vivo*. Figure 1.14 shows that MCP-1 and CINC/IL-8 transcript levels in carotid artery extracts increased 48 fold ($p< 0.01$) and 98 fold ($p< 0.05$) respectively at 7 days after withdrawal of tet. Transcript levels declined by 15 days after tet withdrawal, consistent with the apoptotic loss of the FADD-expressing cells.

Fas activation upregulates MCP-1 and IL-8 expression in normal human SMCs.

To determine whether MCP-1 expression could be induced by standard pro-apoptotic stimuli relevant to SMC apoptosis in atherosclerotic lesions [50] I treated low passage human arterial SMCs with either recombinant Fas ligand (FasL), or activating anti-Fas antibody, plus 1.5 μg/ml cycloheximide. This concentration of cycloheximide is below that resulting in complete inhibition of overall protein synthesis (about 10 μg/ml) and, by itself, had no detectable effect on SMCs. By 24 hrs after treatment, MCP-1 transcript levels were highly upregulated by both anti-Fas and FasL (data not shown). Release of IL-8 protein, as measured by ELISA, was also upregulated (data not shown).

Treatment with anti-Fas antibody or FasL alone, or cycloheximide alone, which did not induce apoptosis, did not increase transcript levels of either gene (data not shown).

Cultured SMCs are resistant to activation by FasL or anti-Fas antibodies alone. However, it has recently been reported that oligomerized and membrane-associated FasL can initiate apoptosis in other resistant cell types [76] and that factors other than cycloheximide, including IFN-γ, can sensitize SMCs to Fas activation [49]. In order to determine whether Fas activation can upregulate MCP-1 expression independent of any action of cycloheximide, I evaluated a form of recombinant soluble FasL (Oncogene Research Products, Cambridge, Massachusetts) with a FLAG epitope that permits oligomerization via an anti-FLAG antibody (Kodak, Rochester, New York). I found that this treatment did result in some apoptosis, and in a significant upregulation of MCP-1 expression in the absence of cycloheximide. These effects were increased by interferon-γ, but were much smaller than produced with the inclusion of cycloheximide (data not shown).

Fas activation of vascular SMCs affects transcript levels of a subset of genes involved in leukocyte recruitment and extracellular matrix remodeling.

I had initially chosen to evaluate expression of MCP-1 and IL-8 as possible mediators of the monocyte influx that accompanies FADD-induced SMC apoptosis *in vivo*. In order to determine how selective the changes in transcription were, and/or whether other genes might also be up- or down-regulated, I used a transcript array approach to survey changes in transcript levels for 4,000 known human genes. I found that the vast majority of expressed genes represented on the array was not affected (less than 2 fold change) by induction of apoptosis in cultured human SMCs. Figure 1.15 shows the 10 expressed genes whose transcript levels were upregulated by 8 fold or more at 24 hrs after initiating anti-Fas/cycloheximide treatment. In addition to MCP-1 and IL-8, transcripts for 5 other genes involved in inflammation were also upregulated: TNF-inducible protein TSG6, PAI 2, IL-6, GRO1 and IL-1α. None of the genes on the array filter were downregulated more than 2.5 fold in the apoptotic SMCs.

Discussion

Apoptosis is usually considered to be non-inflammatory.

It is usually stated that apoptosis is non-inflammatory [156]. This conclusion has been based largely on studies of apoptosis in hematopoietic cells, usually lymphocytes. These cell types play important regulatory roles but do not play significant structural roles in the tissues in which they are found. A rapid, silent, apoptotic death of these cells would not affect tissue structure. By contrast, in blood vessel walls, SMCs and the extracellular matrix that they secrete, constitute the structure of the artery wall, and programmed death of these cells may include adequate provisions for removing the cell bodies and remodeling the extracellular matrix. Apoptotic bodies and macrophages have often been observed in the same areas in vascular lesions [64,87,88] and during embryonic development [72,133,196]. However, poor kinetic resolution has made it impossible to evaluate a possible cause/effect relationships between apoptosis and other changes in the tissue. To do this, I used an experimental system, tet-regulated induction of FADD mediated apoptosis in seeded neointimal SMCs, that makes it possible to induce SMC apoptosis specifically in SMC in the artery wall *in vivo* in the absence of any mechanical or toxic insult. The properties and consequences of this regulated apoptosis could then be determined.

What are the consequences of SMC apoptosis *in vivo*?

In the tet-regulated FADD expression system, expression of FADD initiated SMC apoptosis as evaluated by all of the criteria that I applied. In culture, the SMCs showed nuclear condensation and fragmentation, and eventually detached from the dish. Unlike necrosis, the cells showed increased annexin V binding before membranes became permeable to PI. *In vivo*, I detected increased numbers of TUNEL-positive cells, and the DNA marker of the seeded cells became less abundant with time, consistent with apoptosis followed by degradation of seeded cell DNA. The most obvious consequence of the induced SMC apoptosis *in vivo* was a massive accumulation of macrophages [157]. I first observed macrophages in the adventitia at day 7. By day 15, the neointima was also filled with macrophages, covering about 11% of the total area of the neointima.

Neutrophils also appeared in the neointima, but in significantly lower numbers. As discussed in the Background section, apoptosis and macrophage accumulation are features of vascular injury and atherosclerotic lesion formation. Much of my focus to date has been to understand the mechanisms through which SMC apoptosis produces this macrophage accumulation.

How does apoptosis of SMCs result in macrophage accumulation? Either of two mechanisms could account for the accumulation of macrophages: active recruitment of monocytes/macrophages via release of chemokines, or "trapping" of monocytes/macrophages that were patrolling through the neointima and that recognized cell surface changes that mark apoptotic cells for phagocytosis (reviewed in [156]).

Upregulation of pro-inflammatory gene expression is part of the apoptotic program in vascular SMC.

To test the hypothesis that the macrophage accumulation resulted from active recruitment by the apoptotic cells, I determined expression levels for the major monocyte chemokine, MCP-1, and for CINC/IL-8, the rat member of the major pro-inflammatory neutrophil chemokine family. As detailed in the Results section, both MCP-1, and CINC/IL-8 were upregulated in SMCs in response to FADD overexpression in culture and *in vivo*. FADD expression also increased transcript and protein expression of MMP-9, a metalloproteinase implicated in tissue inflammation and repair (data not shown). To determine whether changes in gene expression also occur in untransfected SMCs stimulated by other apoptotic effectors, I evaluated cultured low passage human SMCs treated with FasL, or with an agonistic anti-Fas antibody. Consistent with the results in the FRFADD cells, induction of apoptosis in human SMCs resulted in a significant increase of MCP-1 and IL-8 transcription levels. Transcript array analysis also showed that, out of the 10 genes with the highest fold increase in transcript level ($\geq$ 8 fold), 7 are associated with inflammation and tissue remodeling. These genes were, in addition to IL-8 and MCP-1, the TNF-inducible protein 6 (TSG6), plasminogen activator inhibitor 2 (PAI 2), IL-6, GRO1 (another member of the IL-8 family) and IL-1α. TNF-inducible protein 6 (TSG6), and plasminogen activator inhibitor 2 (PAI-2), can act to inhibit

plasmin and/or downstream proteases that are part of the proteolytic cascade associated with inflammation. TSG-6 and PAI-2 expression are reported in inflammatory tissue and it has been suggested that they play roles in negative feedback regulation of inflammation [189]. However, the role of TSG-6, in particular, is complex, and TSG-6 expression also appears to promote SMC proliferation *in vivo* and in culture [197] and to function in organization of the extracellular matrix [93]. FADD expression also increased transcript and protein expression of MMP-9 (data not shown), a metalloproteinase implicated in tissue inflammation and repair [8,82,201], but this gene was not represented on the human transcript array and its expression by human SMCs was not determined. Expression levels of the vast majority of the approximately 4,000 known human genes represented on the filter array were not affected by induction of apoptosis and none of the genes was more than 2.5 fold downregulated in comparison to expression in control cells. This demonstrates that the pattern of transcript upregulation is specific and not just a consequence of general failure to regulate transcription during apoptosis.

Fas stimulation of IL-8 release has been described for astrocytes [153], colonic epithelial cells [1] and synoviocytes [163]. However, in those studies the authors emphasized that the enhanced production of IL-8 after Fas activation occurred independently of apoptosis. Abreu Martin et al [1] stated that although Fas antibody treatment in the absence of IFN -γ did not cause cell death, it greatly enhanced IL-8 secretion. Sekine et al [163]. showed that caspase inhibitors efficiently blocked apoptosis but did not affect anti-Fas induced IL-8 secretion. In astrocytes IL-8 seemed even to function as a survival factor and was predominantly released by cells that were apoptosis resistant [153]. In contrast to these reports, the data of my study suggests a closer relationship between induction of apoptosis and increased expression of IL-8 and MCP-1. 1) Incubation of human SMCs with anti-Fas antibody alone, or cycloheximide alone, was not sufficient to induce apoptosis, and did not increase MCP-1 or IL-8 transcript levels (data not shown). Only the combination resulted in cell death and only the combination upregulated MCP-1 and IL-8 expression. 2) FADD overexpression in rat vascular SMCs induced apoptosis that was accompanied by increased MCP-1 and IL-8 expression. FADD functions in the

apoptotic pathway to facilitate autoactivation of caspase 8 in response to Fas or TNF receptor stimulation.

Mechanisms of chemokine upregulation during SMC apoptosis.

The evidence above suggests that FADD and caspases are involved in activation of the pro-inflammatory program in SMCs. The possibility that FADD and caspases play roles in regulating cell functions in addition to death *per se*, is supported by the observation that FADD null mice do not survive embryonic development beyond day 11.5, and exhibit phenotypes that cannot be attributed directly to diminished apoptosis, including impaired cardiac development and lack of activation-induced T lymphocyte proliferation [198,202]. The phenotype of caspase 8 null mice [179] is similar. One mechanism through which apoptosis may affect transcript levels is suggested by the report that erythroblast differentiation is negatively regulated by cleavage of the transcription factor GATA 1 by caspases [33]. A second possibility is suggested by the fact that most of the genes that we found to be upregulated in response to anti-Fas/cycloheximide treatment are known to be transcriptionally regulated (at least in part) through NF-kappa B. These include: MCP-1 [138], IL-8 [67], Gro 1 [192], PAI 2 [102], IL-6 [39], IL-1 alpha [195]. Thus NF-kappa B activation might be a downstream event during Fas/FADD mediated caspase activation in SMCs.

'Slow' kinetics may be an important aspect of the gene expression program initiated by apoptosis in SMCs, and may be one of the factors that effectively restricts the program to certain cell types. It is obvious that a program that includes new gene transcription, translation, and protein secretion would not be possible if the time period from initial apoptotic stimulus to terminal apoptotic state was very short. Induction of apoptosis is relatively slow in the FADD-overexpressing rat SMC system. Although we detect FADD protein within 24 hours after transgene induction, annexin V positive cells and elevated cytokine RNA levels are not found before 48 hours, and increase together. Apoptosis of human SMCs treated with anti-Fas/cycloheximide is also relatively slow, with no morphological signs of apoptosis detectable until after 8 hours. In contrast, human SMCs treated with staurosporine, and lymphoid cells treated with anti-Fas,

became apoptotic within 1-2 hours (not shown). In neither of those cases did we detect MCP-1 transcript upregulation (data not shown). Cell types other than lymphocytes, including vascular SMCs, that are relatively resistant to apoptotic stimuli, may thus be more likely to survive long enough to display specific programs of transcript and protein upregulation.

Chemokines produced by apoptotic SMCs may play significant roles in vascular lesion development.

Macrophages and other leukocytes are important players in vascular disease. Macrophages are sources of cytokines and growth factors [150] and the integrity of fibrous plaques and their resistance to rupture may be critically influenced by the accumulation of macrophages, which represent rich sources of matrix-degrading enzymes [96]. We suggest that chemokines and other effectors produced by apoptotic SMCs may play significant roles in recruiting these leukocytes. The pro-inflammatory factors (including MCP-1 and IL-8 family members) that we identified as upregulated in apoptotic SMCs have been clearly implicated in establishing atherosclerotic lesions. MCP-1 is highly expressed in atherosclerotic plaques and after balloon catheter injury [130,199]. In two mouse models of atherosclerotic lesion formation, established by disrupting either the apolipoprotein E gene [14] or the LDL receptor gene [62], the size and macrophage content of the lesions were reduced when the MCP-1 receptor, CCR2, was also disrupted.

IL-8 is co-expressed with MCP-1 in atherosclerotic lesions and inflammatory cardiac tissue [86,162]. Fas ligation stimulates IL-8 secretion in various cell types [1,153,163]. It is usually considered to be a neutrophil chemokine, and its upregulation in our seeded vessels may account for the increased numbers of neutrophils in the apoptotic vessel wall. In addition, Il-8 may play an important synergistic role in monocyte trafficking. MCP-1 and IL-8 both activate leukocyte integrins to cause rolling monocytes to attach firmly, a necessary prelude to diapedesis through the endothelium and entry into the tissue [53]. In mice whose IL-8 receptor has been disrupted, macrophages do not accumulate in the vessel wall in an experimental model of atherosclerosis [13].

The data presented above suggests that programmed death of SMCs includes a specific and highly regulated program of SMC gene expression that facilitates removal of the cell body and remodeling of the extracellular matrix. The recruitment of macrophages by the induced production of MCP-1 and IL-8 may normally facilitate adaptive remodeling via growth factors, cytokines and proteases released from the macrophages, but may also contribute to the pathogenesis of vascular lesions when apoptosis is excessive.

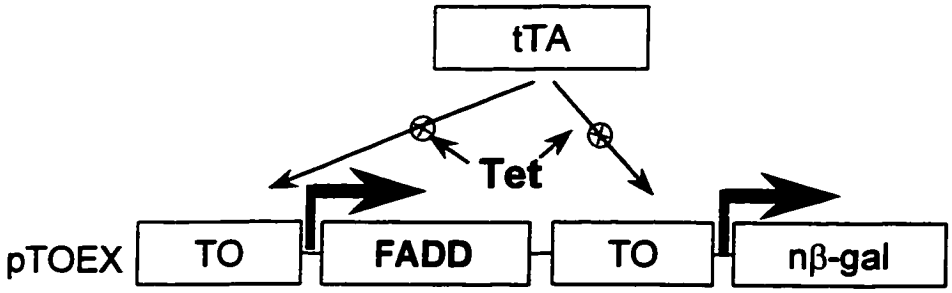

Figure 1.1: Tetracycline-regulated expression vector
Aortic SMCs from Fischer 344 rats were sequentially transfected with two expression vectors. The first plasmid contained the Tet transactivator protein (tTA) under the control of the Tet operator/promoter sequence. The second plasmid contained FADD as well as the reporter β-galactosidase, each under the control of separate copies of the tet operator/promoter.

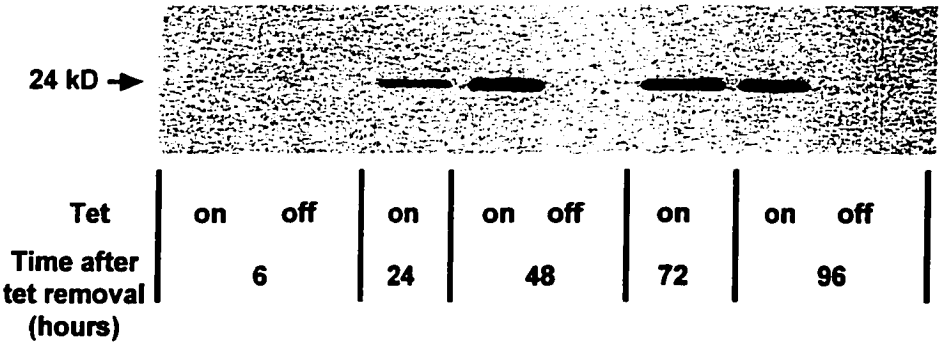

Figure 1.2: Induction of FADD protein expression after removal of tet
Cultured FRFADD cells incubated in the absence or presence of tet (for 6 hours and up to 96 hours) were extracted for protein determination and evaluated by SDS-PAGE followed by immunoblotting with anti-FADD monoclonal antibody. Extracts of cultured FRFADD cells incubated for 48 hours in the absence or presence of tet were used as positive and negative controls for detection of the 24kDa human FADD protein.

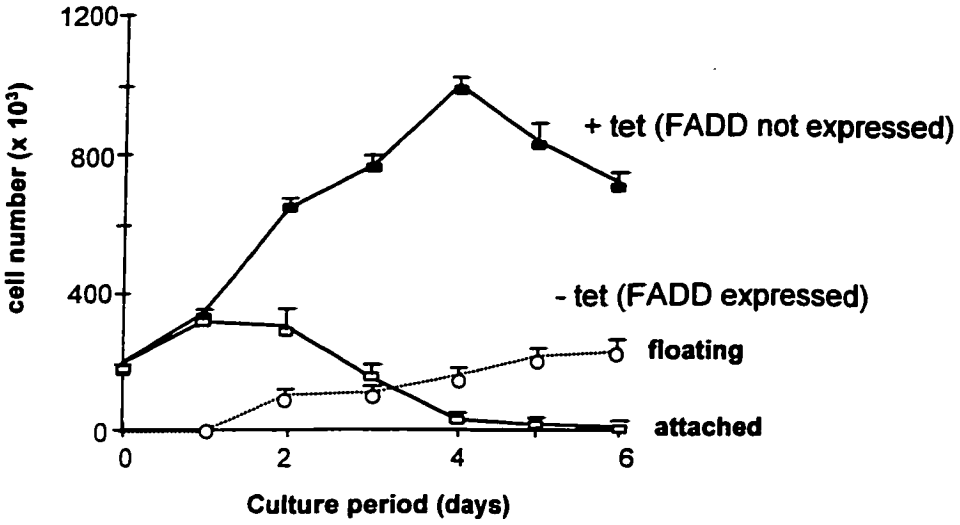

Figure 1.3: FADD expression results in detachment and loss of cultured smooth muscle cells FRFADD cells were plated in the presence (filled squares), or absence (open squares), of tet. At the indicated time points, detached cells (open circles) were collected from the culture medium and attached cells (squares) were trypsinized and cell numbers determined using a hemocytometer. No floating cells were detected in the presence of tet. Results are expressed as mean values ± SEM of triplicate cultures.

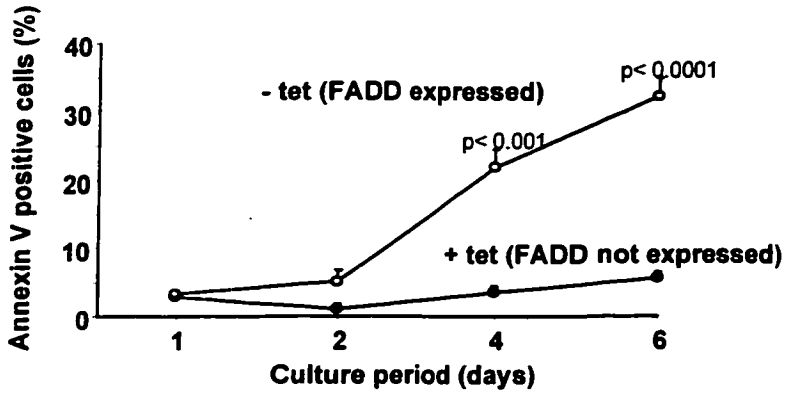

Figure 1.4: FADD expression by cultured smooth muscle cells results in early apoptotic changes determined by Annexin V binding. FRFADD smooth muscle cells were plated in the presence (filled circles), or absence (open circles), of tet. At the indicated time points, the cells were trypsinized, stained with FITC-conjugated Annexin V and propidium iodide (PI), and analyzed by flow cytometry. Early apoptotic cells, identified as cells that stain positive for Annexin V-FITC and negative for PI, are plotted as a percentage of total cells. Results are the mean ± SEM of determinations on quadruplicate cultures.

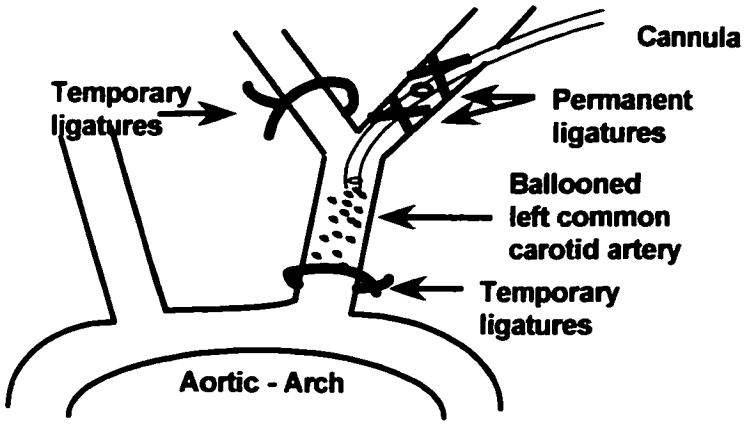

Figure 1.5: Seeding of rat carotid arteries
The distal carotid artery was temporarily isolated with ligatures placed around the carotid interna (at the bifurcation) and around the common carotid (10 mm proximal of the bifurcation). The common carotid was deendothelialized with a balloon catheter, which was introduced into the external carotid artery. Through a polyethylene tube a suspension of FRFADD SMCs was infused into the denuded area of the carotid artery. After 15 minutes the tubing was removed, the external carotid artery was tied off proximal to the incision hole, blood circulation was restored, and the wound was closed.

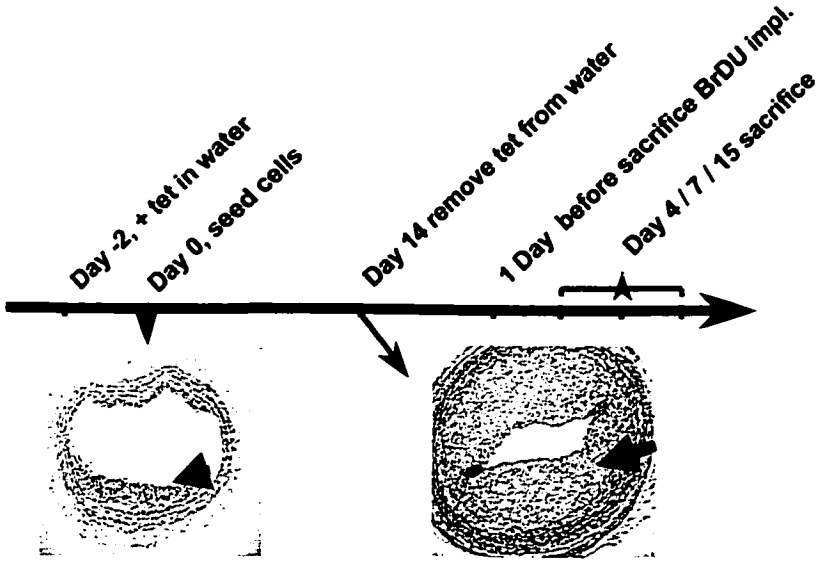

Figure 1.6: Seeding protocol
FRFADD cells were seeded into Fischer rat carotid arteries. Tet was included in the drinking water for 2 weeks to prevent FADD expression until a neointima had formed (blue arrow). After 2 weeks, tet was withdrawn from the drinking water of all except the control group and the rats were sacrificed after an additional 4, 7 and 14 days.

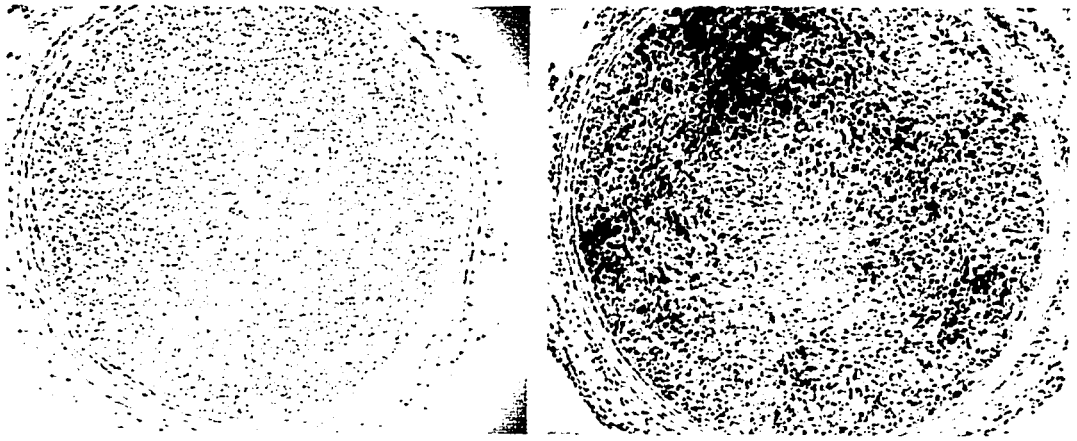

Figure 1.7: Induction and location of reporter expression in seeded neointimal SMCs after removal of tet

Expression of the myc-tagged nuclear-targeted beta-galactosidase reporter was detected by immunohistochemistry using the 9E10 monoclonal anti-myc antibody and visualized using nickel-intensified DAB (black nuclei). Other nuclei were counterstained with methyl green. Representative cross sections from control animals (with tet) (a) and from animals 7 days after tet withdrawal (b) are shown from a series of 5 animals for each time point and each group.

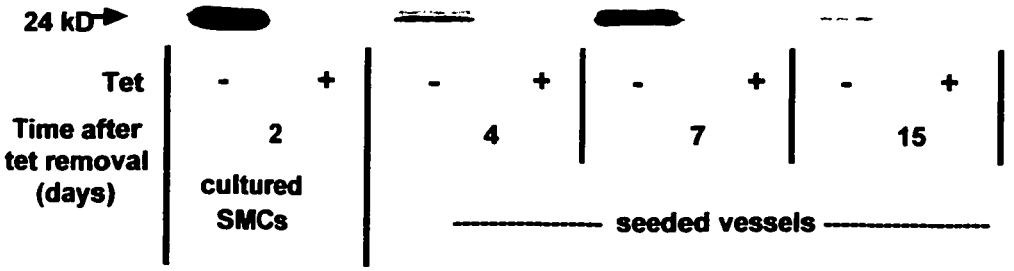

Figure 1.8: Induction of FADD protein expression after removal of tet

FRFADD cells were seeded into Fischer rat carotid arteries, and harvested at the indicated times after removal of tet. Cultured cells or vessel segments were extracted for protein determination and evaluated by SDS-PAGE followed by immunoblotting with anti-FADD monoclonal antibody. Extracts of cultured FRFADD cells incubated for 48 hours in the absence or presence of tet were used as positive and negative controls for detection of the 24kDa human FADD protein.

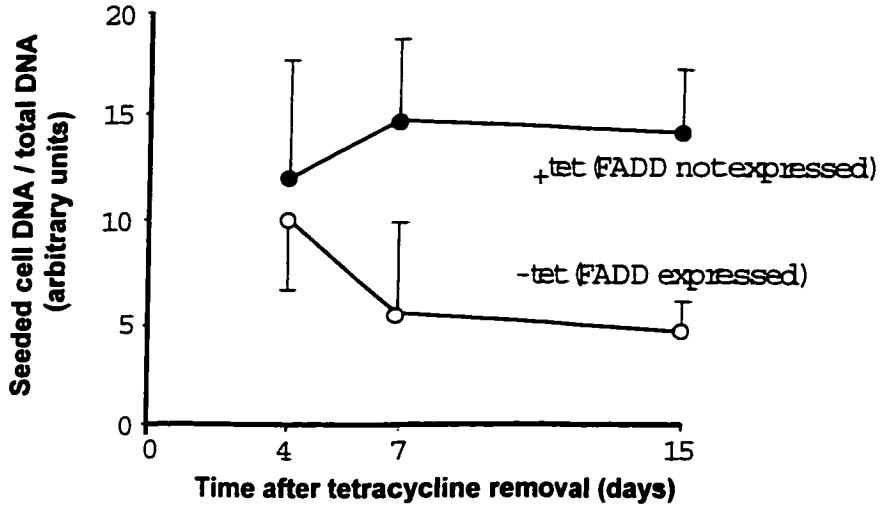

Figure 1.9: FADD expression results in loss of seeded SMCs from the neointima
FRFADD cells were seeded into the carotid artery of rats maintained on tet and allowed to form a neointima for 2 weeks. Tet was then removed (open circles) or continued (closed circles) for the additional period indicated on the abscissa. Extracts of seeded carotid artery segments (200 ng DNA per sample) were analyzed for FRFADD cell DNA content by competitive, semi-quantitative PCR for the neomycin resistance gene, which is only present in seeded cells. Results are expressed as mean $\pm$ SEM (n = 4 for 4 days, 6 and 5 for 7 and 15 days).

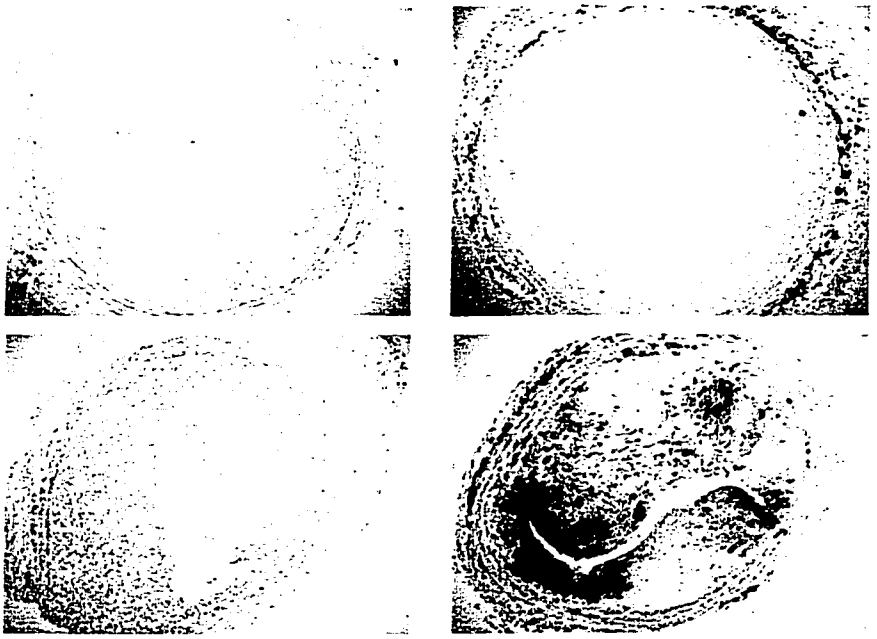

Figure 1.10: FADD expression results in recruitment of macrophages into the neointima
FRFADD cells were seeded into the carotid artery of rats maintained on tet in the drinking water for 2 weeks. Tet was then continued or removed, for an additional 7 days or 15 days. The carotid arteries were fixed and immunostained using a macrophage-specific monoclonal antibody (ED-1), and visualized using Vector red. Increasing numbers of macrophages invaded the adventitia and the seeded neointimas at 7 days and 15 days after removal of tet. In the presence of tet, only occasional macrophages were detected. n = 5 to 6 rats for each group, of which single representative micrographs are shown. Magnification x 100.

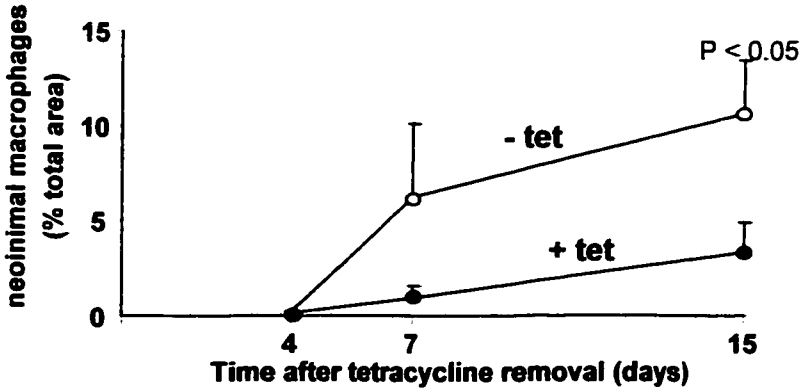

Figure 1.11: Quantitation of macrophage recruitment into the neointima after induction of FADD expression
To quantitate the extent of macrophage recruitment into the FRFADD-seeded carotids, the percentage of total neointimal area covered by ED-1 positive cells was determined using an image analysis program. Data is expressed as mean values ± SEM (n= 5 or 6 for each group) at various times after tet removal (open circles), or maintenance (closed circles).

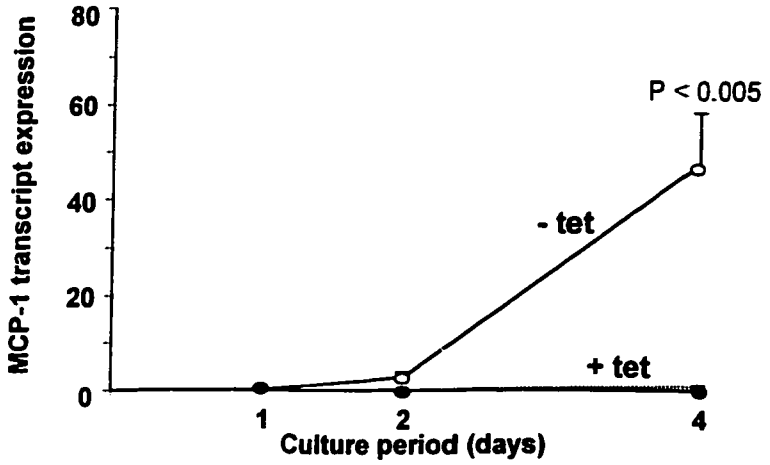

Figure 1.12: FADD expression results in upregulation of MCP-1 transcript levels *in vitro*
FRFADD cells were plated in the absence (open circles) or presence (closed circles) of tet, and RNA was extracted after 1, 2 and 4 days. MCP-1 transcript levels were determined by semi-quantitative RT-PCR, normalized to GAPDH levels, and expressed as mean +/- SEM.

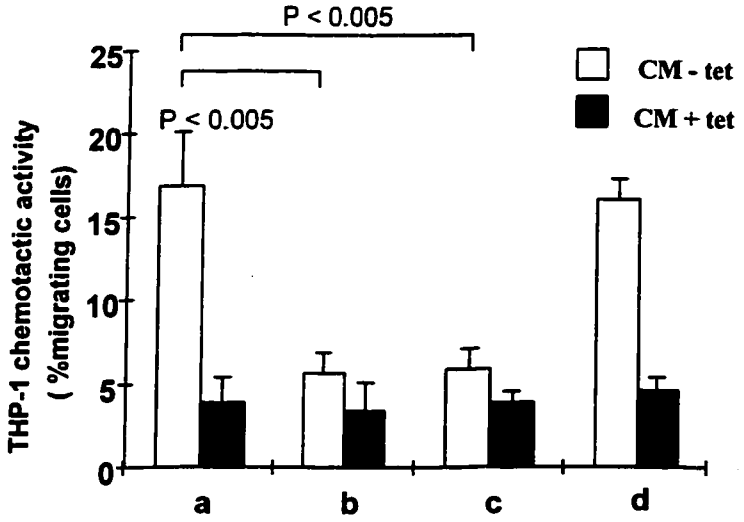

Figure 1.13: FADD expression results in secretion of active MCP-1
Conditioned media of FRFADD cells that were plated for 4 days without tet (open bars) or with tet (filled bars) was tested for monocyte chemotactic activity as described in Methods. Results are expressed as percentage of THP-1 cells (mean ± SEM) migrating during the course of the experiment. Conditioned media of FADD-expressing SMCs contain significantly higher levels of chemotactic activity as compared to cells maintained in tet (a). Addition of two different polyclonal antisera against MCP-1, R147 (b), and AAR12Z (c), neutralized this chemotactic effect almost completely. Preimmune IgG used as a negative control had no effect (d).

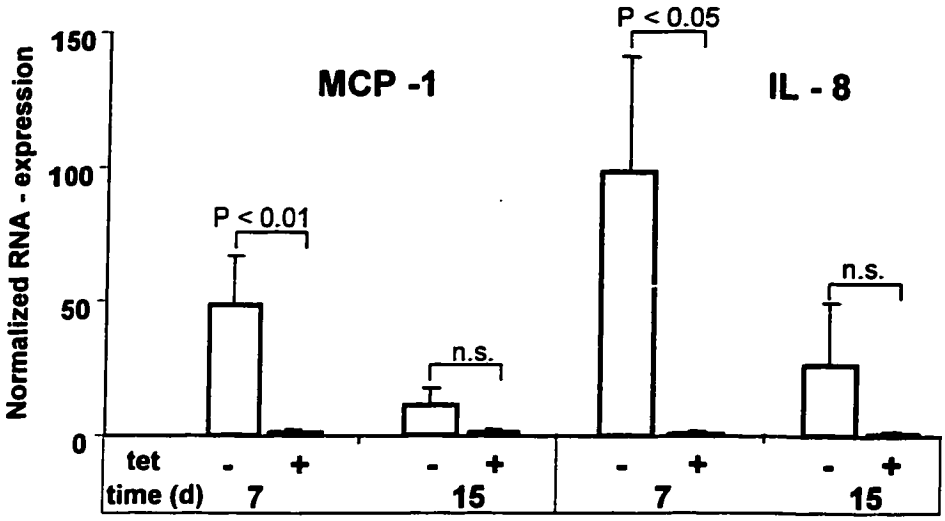

Figure 1.14: MCP-1 and IL-8 transcript expression is upregulated following FADD expression by FRFADD cells in rat carotid arteries *in vivo*

Expression of MCP-1 and IL-8 transcripts in seeded carotid arteries were determined by semi-quantitative RT-PCR and expressed as fold increase of transcript expression in the minus tetracycline animals (empty bars) compared to the plus tetracycline control animals (filled bars) at each time point (+ SEM). The absolute values of MCP-1 and IL-8 transcript levels in the plus tetracycline control arteries was low and unchanged at both time points (not shown). MCP-1 levels were normalized to β-actin levels. (n for each group = 4-7 animals)

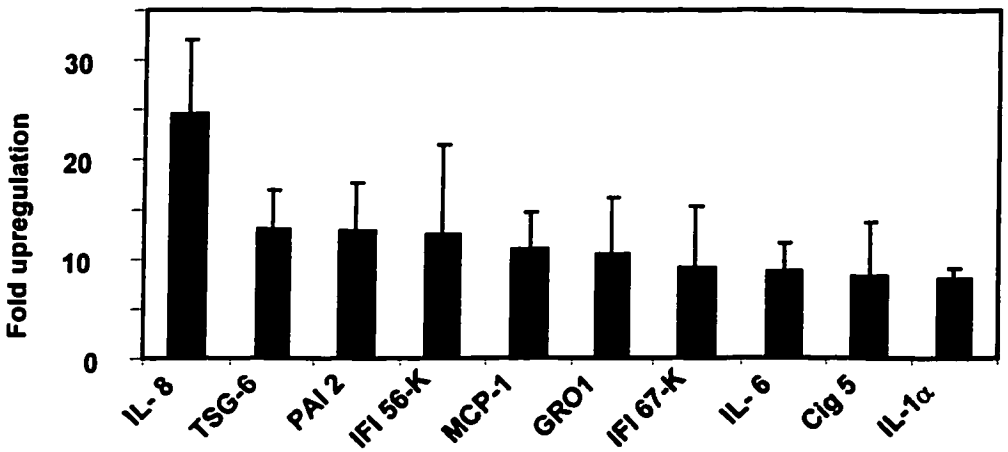

Figure 1.15: Induced expression of subset of genes in anti Fas antibody plus cycloheximide treated human SMCs

Human SMCs were incubated with Fas antibody plus c cycloheximide as described in figure 11. After 24 hours of treatment RNA was harvested and expression of 4000 known human genes was evaluated by array filter analysis. The 10 genes with the largest fold increase in transcript levels ($\geq$ 8 fold) as compared to non-treated control cells are listed in the table. Data are expressed as mean (n = 3).

Chapter 2

Mechanism of Fas/FADD mediated MCP-1 upregulation

Introduction

In the first part of my thesis I reported that induction of apoptosis through upregulation of FADD in rat vascular SMCs, causes accumulation of macrophages *in vivo*. FADD overexpression also triggered transcriptional upregulation of MCP-1 and IL-8 *in vitro* and *in vivo*. I confirmed the production and release of biologically significant amounts of MCP-1 and IL-8 proteins by chemotaxis and ELISA assays. Together these data suggested that macrophage recruitment after induction of apoptosis was at least in part mediated by FADD induced upregulation of proinflammatory chemokines.

Fas activation by FasL or agonistic anti-Fas antibody in cultured human SMCs also resulted in a dramatic increase of MCP-1 and IL-8 expression, excluding the possibility that cytokine upregulation in response to apoptotic stimuli may be simply an oddity of the FADD overexpressing SMC clone. In order to determine whether other genes might also be upregulated (or downregulated) that could either contribute to an inflammatory response or lead to other effects that had not been apparent in my initial evaluation of the *in vivo* response to FADD-induced apoptosis, I used a cDNA filter array (Research Genetics) to compare gene expression in cultured human SMCs before, and 24 hr after, treatment with FasL (in collaboration with Dr. Larry Adams). I found that expression of only 10 out of about 4,000 genes was increased by more than 8 fold, and that expression of none of the 4,000 genes was decreased by more than 2 fold. The 10 most highly upregulated genes consisted largely of genes that enhance or modulate inflammation, including MCP-1, IL-8, the IL-8 family member GRO1, and IL-1α. This indicates that changes in gene expression in apoptotic SMCs represent a coordinated response rather than a general collapse of normal regulatory mechanisms during death. I hypothesized that gene induction as a response to pro-apoptotic stimuli is a specific feature of connective tissue cells, which are relatively resistant to apoptosis, in contrast to

lymphocytes that undergo apoptosis very rapidly but do not exhibit upregulation of proinflammatory genes.

In this second part of my thesis, I further investigated the mechanisms of Fas/FADD mediated induction of MCP-1 and focused specifically on the role of interleukin 1 (IL-1) signaling. IL-1 is one of the most potent inflammatory cytokines and a well-known inducer of MCP-1 and IL-8 expression [182]. Its involvement in the development of vascular diseases has been well documented in a number of *in vivo* and *in vitro* studies, which I will discuss in more detail in the background section. One isoform of IL-1, IL-1β is activated and released through cleavage by caspase 1/ IL-1β converting enzyme (ICE), which connects this inflammation inducing cytokine with the apoptosis signaling pathway [22,123,177]. Confirming this notion, Miwa et al [118] demonstrated that the neutrophil inflammatory response to FasL presented by tumor cells depends in part on IL-1β production, because the inflammatory response was substantially reduced in an IL-1α/β knockout mouse.

My working hypothesis is that Fas/FADD induced upregulation of expression of MCP-1 (and other genes in the pro-inflammatory program) is mediated through IL-1 signaling. A potential physiological consequence of Fas/FADD-induced release of IL-1α, would be that it serves to upregulate MCP-1 expression by neighboring SMCs as well as by the apoptotic SMCs. This could extend the period of MCP-1 production beyond the death of their apoptotic neighbors.

Background

Interleukin 1α and Interleukin 1β

Interleukin 1 is a major mediator in the pathogenesis of acute and chronic inflammatory events, regulating multiple functions such as proliferation, differentiation and activation of various cell types. The cytokine consists as two isoforms, interleukin 1 alpha (IL-1 α) and interleukin 1 beta (IL-1 β). Both are synthesized as 31-35 kD pro-molecules and share 25% amino acid homology across the entire precursor structure and 22% amino acid homology over their mature form [107]. Although, many cell types have

surface IL-1 receptors and IL-1 protein can be detected in plasma and other fluids during inflammatory diseases, IL-1 is not considered a secretory protein, because it does not contain a hydrophobic leader sequence and is not released via the classic secretory pathways [151].

IL-1β, is not fully active in its proform and is usually released after being processed into the 17-kD mature form by caspase 1/ IL-1β converting enzyme (ICE) [22,123,177]. The importance of ICE in the cleavage and release of IL-1β was demonstrated by the ability of ICE-specific inhibitors to prevent secretion of the cytokine from activated macrophages [117]. In addition, monocytes and macrophages from ICE-deficient mice do not secrete IL-1β in response to LPS [91,95]. ICE is synthesized as a 45kD precursor and autocatalytically processed to from an active homodimeric enzyme of 10- and 20kD [177]. ICE was the first identified caspase, but, although it can trigger apoptosis through activation of caspase 3, its major physiological role seems to be the cleavage of IL-1β.

The 31-kD proform of IL-1α is fully biologically active as a precursor and is mainly released during cell death, where it can be subsequently cleaved into its mature form by extracellular proteases [71,84,123]. In intact cells, IL-1α is cleaved into its mature form and released through activation of the calcium-dependent proteases calpain 1 (μ-calpain) and calpain 2 (m-calpain) (see below) [81,85,151,172]. After stimulation of cells with the calcium ionophore ionomycin, TNF or lipopolysaccaride (LPS), the 17-kD mature form of the protein is only detectable in the media and not in the cell lysate, suggesting that production, processing and release of the cytokine are closely linked. Analysis of IL-1α secretion in cell lines expressing either its precursor form or mature form revealed that the mature form is the preferential format of release [166]. Whereas transiently transfected cells secrete only 2% of the precursor IL-1α following incubation with the calcium ionophore ionomycin or LPS, cells expressing mature IL-1α essentially secrete all of the protein into the medium [166]. The requirement for stimuli such as LPS or ionomycin in order to induce release of the cytokine suggests that presence of mature IL-

1α protein by itself is not a sufficient signal for secretion and that secretion is modulated by additional factors.

Pathophysiological roles of IL-1 have been described in inflammation, bacterial and viral infection, activation of the immune system and activation of the hypothalamic-pituitary -endocrine system [36]. In contrast to IL-1β , IL-1α is not commonly found in the circulation or in body fluids except during severe diseases, in which case it may have been released in larger amounts by dying cells. It seems therefore that IL-1β functions as a systemic, hormone-like mediator intended to be released from cells, whereas IL-1α is a mediator of locally defined inflammatory events. The biological function of IL-1 has been extensively studied using IL-1 protein and - receptor knockout mice. IL-1β deficient mice exhibited reduced acute phase response to sterile tissue damage induced by turpentine administration. Interestingly, IL-1β -/- mice were highly susceptible to infection with Lisetria monocytogens and not protected from LPS induced toxicity [92,94]. Data regarding IL-1α deficient mice are limited to one report, which showed that in contrast to IL-1β deficient mice, these animals were not resistant to turpentine induced inflammation [73]. The phenotype of IL-1 receptor I (IL-1RI) deficient mice was similar to that of IL-1β knockout mice. The animals exhibited no obvious developmental abnormalities and appeared healthy and fertile. IL-1 receptor I (IL-1RI) deficient mice did not develop an acute phase response and fever after turpentine administration and were not protected from LPS-induced toxicity [92,94]. Other studies showed that IL-1RI knock-out mice are at least partially protected in inflammation models such as renal ischemia/reperfusion, pancreatitis and allergen challenge [17,34,65].

Interleukin receptors and Interleukin receptor-antagonist

Two receptors for IL-1 have been identified. The type I receptor (IL-1 RI), which is the signal-transducing receptor has no intrinsic enzymatic activity. After IL-1 binds to IL-1RI, the cytoplasmic domain of the receptor interacts with the IL-1 receptor associated protein (IL-1R-AcP) and a series of other proteins, including the serine/threonine

interleukin-receptor associated kinases IRAK and MyD88 (which is also involved in Toll-like receptor (TLR) signaling) in order to fully activate NF-κB [63,127,146]. IRAK and MyD88, which both consist of an N-terminal death domain (DD) and a C-terminal protein kinase domain, induce NF-κB activation when overexpressed, whereas their DD-lacking dominant negative (DN) mutants block IL-1-induced signaling [2,3,19,113,127,146,187]. The current model for IL-1 mediated NF-κB activation involves sequentially: IL-1RI, IL-1RacP, MyD88, IRAK, TRAF6 and NF-κB inducing kinase (NIK).

The interleukin 1 type II receptor (IL-1 RII) acts as a decoy, which binds IL-1 but does not transduce a signal. Therefore, IL-1 RII serves as an 'antagonist' of IL-1 activity (reviewed by [135]). Depending on species and cell type, the two receptors show differential binding characteristics for IL-1α and IL-1β. Typically IL-1α is reported to bind preferentially to IL-1 RI ($Kd_{IL-1\alpha}$ = 50pM vs $Kd_{IL-1\beta}$= 1nM), while IL-1β has a higher affinity for IL-1 RII ($Kd_{IL-1\alpha}$ = 1.5 μM vs $Kd_{IL-1\beta}$= 2nM) [27,37,78].

Besides IL-1RII, the second regulator of IL-1 activity is a protein called IL-1 receptor antagonist (IL-1ra) [40]. It resembles IL-1α and IL-1β in its amino acid sequence, three-dimensional folding pattern and gene structure [132]. IL-1ra inhibits IL-1 activity through occupying the IL-1RI (with a higher binding affinity than IL-1α and IL-1β), without triggering a signaling response [5,60,90]. Several forms of IL-1ra have been reported. One form is secreted by activated monocytes and macrophages and possibly constitutively released by hepatocytes [40,46,47]. Other forms, which are lacking the signal peptide sequence are retained within the cytoplasm of intact cells, e.g. epithelial cells form which IL-1ra was originally identified [40,68,106,128].

The role of IL-1 in the development of vascular diseases

Most investigations of the expression and biological function of IL-1 has focused on monocytes and macrophages. However, a growing number of reports within the last few years have implied that IL-1 also regulates the active participation of other cell types, such as vascular SMCs and endothelial cells, in local immune and inflammatory

processes.The major cells of the blood vessel wall, endothelial cells, SMCs and fibroblasts, produce IL-1α and IL-1β, when stimulated with e.g. TNF or IL-1 itself [97,98,185]. In atherosclerotic lesions, IL-1 is secreted by foam cells (macrophages), endothelial cells and SMCs [150]. Balloon angioplasty of rat carotid arteries induces rapid upregulation (within 6 hours) of IL-1β, IL-1RI, IL-1RII and IL-1ra (note: IL-1α expression was not evaluated) [184]. Furthermore, immunohistochemical staining of vein-coronary bypass grafts identified IL-1α accumulation only in sclerotic grafts, but not in non-occluded vessels [16].

What are the effects of IL-1 production and release within the vessel wall? In cultured endothelial cells, IL-1 induces transendothelial passage of neutrophils through gap junctions [122]. IL-1 also acts on endothelial cells and SMCs to upregulate expression of chemokines including IL-8 and MCP-1, which further facilitate accumulation and activation of neutophils and macrophages [182]. IL-1 seems to function as an indirect mitogen for SMCs, through upregulation of PDGF and its respective receptors [144]. Several *in vivo* studies have confirmed the important role of IL-1 in the development vascular lesions. Immunohistochemical staining of stenotic arterial grafts revealed colocalization of IL-1α with proliferating SMCs as well as accumulation of macrophages [16]. Chronic administration of IL-1α or IL-1β through a cytokine-absorbed cotton mesh, which was wrapped around the exterior of the vessel wall, caused the development of neointimal stenosis even without preceding injury to the endothelial layer [45]. Increased shear stress after contralateral ligation of the common carotid artery induced a 7 fold smaller neointimal area in IL-1RI deficient mice as compared to wild-type mice[145].

Further understanding of the role of IL-1 in vascular pathology has been obtained through investigations on the expression and behavior of its physiological antagonist, the IL-1ra. Increased levels IL-1ra were detected in human atherosclerotic carotid artery plaques, after balloon angioplasty and in serum of patients with stable angina pectoris [48,58]. Overexpression of IL-1ra in transplanted rat hearts protected the myocardium from ischemia-reperfusion injury, by attenuation of the inflammation-induced damage, including apoptosis [173]. Interestingly, IL-1ra deficient mice developed lethal arterial

inflammation at sites of high shear stress in the major arteries [131]. However, the genetic background seems to play an important role in the development of IL-1ra deficient mice. Whereas arterial inflammation was characteristic for IL-1ra deficient MF1 albino mice, IL-1ra deficient Balb/c mice spontaneously developed a chronic inflammatory arthropathy that resembled rheumatoid arthritis [74]. Together, these studies highlight the important role of IL-1 mediated inflammation in the development of vascular pathology.

Calpains

Calpains are calcium-dependent cystein proteases that are widely expressed as both ubiquitous (calpain 1 and calpain 2) and tissue specific isoforms. Calpain 1 and calpain 2 are heterodimers consisting of a specific 80 kD catalytic subunit and a common 28 kD regulatory subunit (reviewed by [121]). Calpains are involved in the regulation of cell migration and adhesion by participating in the turnover of cytoskeletal proteins such as focal adhesion kinase, talin and the cytoplasmic tail of β1 and β3 integrin. [20,31,142,158,168].

Alterations in intracellular calcium homeostasis have been implicated both in promoting or inhibiting apoptosis in many cell types. An overload of calcium by excitotoxicity induces both necrotic and apoptotic cell death [111]. Chelation of extracellular calcium protects against apoptosis induced by glucocorticoids in thymocytes, but leads to apoptosis in other cell types such as in cultured neuronal cells [169]. Calcium ionophores have been shown to inhibit apoptosis in interleukin-3-dependent hemopoietic cells and in cyclosporine treated renal tubular cells [24,149]. Considering that calpains are activated in response to increased calcium levels it is not surprising that a growing body of evidence has emerged, implicating functional connections between calpains and caspase activation. Common substrates of calpains and caspases have been identified such as fodrin, calpastatin, actin, PARP and TAU [110,141,180,183]. Furthermore several reports demonstrated calpain-mediated cleavage of caspase-3, caspase-7, caspase-8 and caspase-9 [11,28,152,190]. Squier et al [170] found that apoptosis induced by protein

synthesis-inhibition in neutrophils required calpain activity, whereas Fas mediated apoptosis was unaffected by calpain inhibitors. Moreover, in this study depletion of calpastatin, which is the natural calpain-inhibitor, was sufficient to cause apoptosis of neutrophils. In HL-60 cells the pro-apoptotic protein Bax was cleaved by calpains during drug-induced apoptosis [191], suggesting calpains may be involved in modulating cell death by acting selectively on cellular substrates. However, although the calpain inhibitor calpeptin effectively blocked both drug-induced Bax cleavage and calpain activation, it did not prevent PARP cleavage or cell death. Even though most of the data listed above suggest a pro-apoptotic or at least apoptosis-supporting role, the role of calpains in the regulation of apoptosis remains controversial and seems dependent on cell type and stimulus. There are also a number of reports demonstrating an opposite function for calpains. An inhibitory role of calpain in apoptosis has been shown in limb-girdle muscular dystrophy type 2A disease, which results from calpain 3 deficiency [7]. Biopsies of limb-girdle muscular dystrophy type 2A, revealed myonuclear apoptosis, which was correlated with altered subcellular distribution of Iκ-B and NF-κB. While overexpression of Iκ-B prevented nuclear translocation of NF-κB and led to apoptosis of primary myogenic satellite cells, activation of calpain 3 caused Iκ-B degradation, indicating that calpain 3 may control Iκ-B turnover and thus indirectly regulate NF-κB -dependent expression of survival genes [7]. Calcium ionophore treatment in neuroblastoma cells induced rapid calpain activation and cleavage of caspase-9, generating a truncated caspase-9 form that was unable to activate caspase-3 in cell lysates [28]. In addition calpains were found to prevent activation of procaspase-9 through degradation of the apoptotic protease-activating factor-1 (APAF-1), suggesting that calpains may act as negative regulators of apoptosis by effectively inactivating upstream caspases [147].

Although the role of calpain in IL-1α processing and release is well documented, it still remains unclear, how calpain is activated under physiological circumstances. *In vitro* calpain 1 shows proteolytic activity at calcium concentrations of 5-50 μm, whereas calpain 2 requires concentrations of 200-1000 μM [114,124]. However, both calcium levels are at least 10 to 50 times higher than present in the cytosol, suggesting that calpain

activity might be regulated by additional mechanisms. Calpains undergo calcium-dependent autolysis, a multi-step self-proteolytic event, resulting in increased enzyme activity and sensitivity to calcium [55]. Autolysis results in conversion of the 80-kD catalytic subunit to 76-kD via a short-lived 78-kD intermediate product, followed by the conversion of the 30-kD regulatory subunit to an 18-kD form. Upon longer incubations in the presence of calcium, autolysis progresses to complete degradation of the proteinase into smaller, inactive fragments [134]. Yet, these findings are based on *in vitro* studies and required unphysiologically high calcium concentrations. More and more evidence has emerged, indicating that the non-autolyzed form of calpain is also active under physiological conditions [41,120]. Another important mechanism of calpain activation may be through membrane association. Under normal conditions calpains are not membrane bound. However, after increasing the cytosolic calcium concentration a significant amount of non-autolyzed 80-kD calpain was detected at the plasma membrane, which was found to be active on its preferred substrates [174]. Therefore, membrane-binding of physiologically active non-autolyzed calpain may simply sequester the enzyme away from its natural, cytosolic inhibitor calpastatin. It has been proposed, that membrane phospolipids, such as phosphatidyl inositol, may be key factors for membrane-association of calpains as well as reduction of the calcium requirement for autolysis [140,155]. Interestingly, EGF triggers calpain 2 downstream of extracellular signal-related kinase (ERK)/mitogen-activated protein kinase signaling and not phospholipase C signaling, which mobilizes intracellular calcium suggesting a novel mechanism of activation [54].

Recent studies provided evidence that calpains may be involved in the development of vascular diseases. Vascular SMCs contain large amounts of calpain 1 and 2 and their counter-part calpastatin [109]. Treatment with calpain inhibitors or antisense-oligonucleotide against m-calpain inhibited serum-stimulated proliferation of human vascular SMCs in a dose-dependent fashion [6]. Calpain inhibitors also effectively inhibited spreading of SMCs onto fibrinogen [136]. Degradation of collagen by active matrix metalloproteinases is a classic feature of extracellular matrix remodeling in atherosclerotic and restenotic lesions. Carragher et al. [21] demonstrated that collagen

fragments rapidly induce initiation of calpain-mediated cleavage of $pp125^{FAK}$, paxillin and talin, resulting in focal adhesion disassembly and cell rounding.

My data presented here raise the interesting possibility that calpains regulate the induction of a pro-inflammatory gene program in response to apoptotic stimuli in vascular SMCs.

Results

Fas-induced MCP-1 upregulation is mediated by IL-1α

Fas stimulation, as well as FADD expression, resulted in the upregulation of MCP-1 and a limited number of other pro-inflammatory genes. The increase in transcription of this specific subset of genes could be "directly" induced by Fas/FADD, for example through activation of transcription factors, such as NF-kappa B (NF-κB). Alternatively, Fas/FADD expression of pro-inflammatory genes could be induced "indirectly" through activation and/or release of a pro-inflammatory cytokine.

IL-1 appeared to be the ideal candidate for the latter hypothesis, because it is a potent inducer of MCP-1 and IL-8 expression and IL-1β maturation and release is controlled by ICE/caspase-1 activity. In order to test whether IL-1 is a significant component of the pathway(s) leading from Fas to MCP-1 upregulation, SMCs were co-incubated with FasL/Chx plus soluble IL-1 receptor antagonist (IL-1ra), which occupies IL-1 receptors but has no intrinsic activity. IL-1ra reduced the extent of upregulation of MCP-1 at 24 hrs after Fas stimulation by >90% (Fig. 2.1B), supporting the notion that IL-1 plays a crucial role in Fas/FADD-induced MCP-1 upregulation. This was further corroborated by evaluating MCP-1 RNA levels in response to FasL/Chx in IL-1RI-deficient embryonic fibroblasts (Fig 2.2). The extent of FasL/Chx induced cell death in wildtype and IL-1RI deficient cells was very similar (data not shown). However, MCP-1 transcription was increased only in wild-type cells and did not occur in IL-RI knock-out cells. Treatment

with FasL in the absence of Chx also caused MCP-1 expression only in wildtype cells, although to a lesser extent.

In most inflammatory conditions, the predominant form of IL-1 is thought to be IL-1β, which is secreted mainly by leukocytes. Using ELISA, IL-1β was not detected in conditioned medium of FasL/Chx-treated human SMCs (data not shown). However, SMCs did secrete significant amounts of IL-1α in response to Fas activation (Fig 2.3A). Incubation with either Chx or FasL alone did not cause measurable IL-1α release into the medium (data not shown). Confirming that IL-1α release might act as an intermediary effector of MCP-1 upregulation, co-incubation with a neutralizing antibody specific for IL-1α (which does not recognize IL-1β) reduced FasL/Chx-induced MCP-1 upregulation almost as effectively as the addition of IL-1ra (Fig 2.1B).

Increased expression and synthesis of IL-1α in response to FasL/Chx treatment

To determine whether Fas activation stimulates the release of pre-stored IL-1α or whether transcriptional upregulation and induction of cytokine synthesis proceeds its secretion, I determined IL-1α RNA levels by PCR Taqman analysis as well as IL-1α protein concentration in total cell lysates by ELISA. IL-1α RNA expression was low in untreated and Chx treated SMCs but increased dramatically (by 290 fold) after incubation with FasL/Chx (Fig 2.4A). Co-incubation with IL-1ra or a neutralising anti-IL-1α antibody reduced elevated IL-1α RNA levels by more than 90% (Fig. 2.4B), indicating that Fas-induced IL-1α upregulation is to a large extent regulated by an autocrine/paracrine effect of IL-1α. This notion was supported by ELISA analysis of IL-1α protein content in control and FasL/Chx treated SMCs. This assay detects both precursor and mature forms of the cytokine. IL-1α protein content was very low (5 pg/ml) in untreated and FasL- treated SMCs, but increased by 30 fold in response to FasL/Chx treatment (Fig 2.5A). The addition of IL-1ra also reduced IL-1α protein content in FasL/Chx treated cells, although to a smaller degree than expected, considering its dramatic effect on IL-1α transcription (Fig 2.5B). It is possible that even a small upregulation of IL-1α RNA expression results in a substantial increase in IL-1α protein

content and that IL-1α transcription may be more tightly regulated via an auto-/paracrine effect than protein synthesis and release of the cytokine. Interestingly, while treatment with FasL alone did not have a measurable effect on the SMCs, Chx treatment stimulated IL-1α transcription (Fig. 2.4A) and protein synthesis (Fig. 2.5A) to a small extent, but, as mentioned earlier, did not cause secretion of the cytokine (data not shown). Therefore, the combination of FasL plus Chx must trigger one or several pathways that not only stimulate IL-1α production but also regulate its release and thus its biological activity.

Fas activates IL-1α signalling and MCP-1 upregulation via caspase-dependent and a caspase-independent pathway

How does FasL/Chx regulate IL-1α synthesis and release? In contrast to IL-1β, the regulatory mechanisms of IL-1α production, activation and secretion are still relatively unclear and controversial. As mentioned earlier, neither IL-1α nor IL-1β contain a secretory sequence. While IL-1β is released after cleavage into its activated/mature form by ICE/caspase 1, IL-1α , which is not a substrate for ICE/caspase1, is released in response to necrotic and apoptotic cell death through a yet unknown mechanism(s). It seemed possible that Fas ligation stimulates increased release of IL-1α through activation of caspases and apoptosis. In order to determine the role of caspase activation on Fas-induced IL-1α signalling and MCP-1 upregulation, I incubated human SMCs with FasL/Chx with or without the addition of either a pan-caspase inhibitor IDUN1529 (IDUN) [194] or inhibitors of caspase 8 (Z-IETD-FMK), caspase 1 (Z-YVAD-FMK) or caspase 3 (Z-DEVD-FMK) (all in 50μM final concentration).

The potency of the caspase inhibitors was established by determining the number of surviving SMCs that were still attached after 24hr FasL/Chx treatment. The number of viable, attached cells decreased by only 45% after incubation with FasL/Chx alone (Fig. 2.6A), which confirms that SMCs are relatively resistant to apoptotic stimuli, as compared to other cell types such as lymphocytes, which die within a few hours, (Fig. 2.6B). As expected, all caspase inhibitors reduced FasL/Chx mediated cell death by 70 to

98%, with IDUN being most potent and the caspase 3 inhibitor the least effective (Fig. 2.6B). Caspase inhibitors also effectively diminished IL-1α release (Fig. 2.3B), which corresponded well with their anti-apoptotic effects. This suggests that most of the measured IL-1α in the medium was released by cells in response to caspase activation and consequential apoptosis. In addition, IL-1α release appears to be at least in part regulated via an autocrine/paracrine mechanism, because blocking of IL-1 signalling by IL-1ra also decreased cytokine concentration in the medium by 50% (Fig. 2.3B) (without reducing cell death).

Surprisingly, caspase inhibitors only partially reduced Fas-triggered MCP-1 upregulation with a 55% decrease after addition of IDUN and the caspase 8 inhibitor and an even smaller effect in response to caspase 1 and caspase 3 inhibitors (22% and 7% reduction respectively) (Fig. 2.1B). Considering that inhibition of IL-1α signalling via IL-1ra and anti-IL-1α Ab almost completely eliminated MCP-1 upregulation and that release of IL-1α was effectively reduced by caspase inhibitors, one would have expected a much stronger effect of caspase inhibitors on Fas induced MCP-1 upregulation. This suggests that the relatively low levels of IL-1α in the presence of caspase inhibitors are sufficient to upregulate MCP-1 transcription. But what causes this release of IL-1α? There are two likely explanations: 1) Caspase activation is not completely blocked by caspase inhibitors and is still able to trigger some secretion of the cyotkine; or 2) IL-1α release in response to Fas stimulation may be controlled by additional, caspase-independent regulatory mechanisms.

The concentrations of IL-1α in whole cell lysates and in conditioned medium were below the detection limit of Western Blot analysis, which requires concentrations in the ng/ml- range. To obtain information about the intracellular localization of IL-1α, as well as its behaviour in response to Fas activation with and without the addition of caspase inhibitiors, I evaluated cultured SMCs by immunostaining with an anti- IL-1α antibody. FasL/Chx-treated cells that were still attached to the culture plate, consistently displayed a strong nuclear staining pattern for IL-1α, which was not observed in untreated SMCs (Fig. 2.7A). The notion of nuclear translocation of IL-1α in response to

Fas activation was confirmed by ELISA analysis of nuclear protein extracts. The amount of IL-1α in 10 μg nuclear protein from FasL/Chx treated SMCs was 60 times higher than in untreated cells (Fig. 2.7B). Chx treatment alone also induced a very weak nuclear IL-1α staining (Fig. 2.6A). Caspase inhibition with IDUN had only a small reducing effect on the strong Fas-induced IL-1α nuclear staining, providing further evidence for a caspase-independent regulatory mechanism of Fas-induced IL-1α signalling. Inclusion of either IL-1ra (Fig 2.7A) and/or an anti-IL-1α antibody (not shown) resulted in a strong reduction of nuclear staining intensity in response to FasL/Chx, which implies that translocation into the nucleus requires release and receptor-binding of the cytokine.

FasL/Chx induces IL-1α signalling and MCP-1 upregulation in part through calpains

Besides cell death, activation of calpains is the only known mechanism of IL-1α cleavage/maturation and release [81,85,151,172]. To determine the involvement of calpain activation in Fas-induced IL-1α production and release, as well as MCP-1 upregulation, SMCs were co-incubated with FasL/Chx and either a synthetic calpain inhibitor, MDL 28170, or a calpastatin-like peptide, CS-P. These inhibit both calpain 1 and calpain 2 activities.

Calpain inhibitors effectively decreased IL-1α release in a dose-dependent manner and were as potent as the pan-caspase inhibitor IDUN (Fig. 2.8). In certain cell types, and in response to certain stimuli, calpain activation has been reported to be pro-apoptotic and inhibition of calpains was found to prevent apoptosis [152,170,190]. However, in this study, calpain inhibitors did not reduce Fas-induced cell death, excluding the possibility that calpain inhibitors reduce IL-1α release through an anti-apoptotic property (data not shown). This data also indicates, that apoptotic cell death by itself, is not sufficient to induce the release of IL-1α. In addition, inhibition of calpains alone did not cause any cell loss and was therefore not pro-apoptotic. Calpain inhibition (with MDL 28170) was more effective than IDUN treatment in reducing Fas stimulated IL-1α RNA

expression (Fig. 2.4B), and IL-1α protein synthesis (Fig. 2.5B), as well as in preventing nuclear translocation of IL-1α (Fig. 2.7A). Consistent with this and the decrease in IL-1α release described above, calpain inhibitors also reduced FasL/Chx - triggered MCP-1 upregulation in a dose-dependent fashion and were more effective than caspase inhibitors (Fig. 2.9).

Together, these data demonstrate that calpains function as important regulatory factors of Fas-induced IL-1α activation and the consequent upregulation of pro-inflammatory genes. Furthermore, the divergent effects of caspase - and calpain inhibitors on IL-1α expression and nuclear translocation strongly suggest the Fas signalling to IL-1α is in part transduced via a caspase-independent, calpain-dependent signalling pathway.

FADD induces MCP-1 expression in an IL-1dependent fashion

The adaptor protein FADD binds directly to Fas during activation of the apoptosis signalling cascade. FADD overexpression in rat SMCs induces apoptosis as well as MCP-1 and IL-8 upregulation *in vivo* and *in vitro*. Since FasL/Chx treatment of human SMCs also triggers MCP-1 and IL-8 expression, I hypothesized that Fas transduces its pro-inflammatory signal via FADD. If this would be the only pathway, FADD-induced gene expression would also depend on IL-1α signaling, which would be activated via a caspase-dependent as well as a caspase-independent pathway.

To further investigate the role of FADD in the transduction pathway from Fas to MCP-1 upregulation, I used a retroviral expression system to express FADD and a dominant-negative form of FADD (FADD DN) that lacks the death effector domain (DED) and can not recruit caspase 8 or induce apoptosis. The retroviral vector contains an IRES (internal ribosome entry site) downstream of the reading frame of the test gene that permits transcription of the EGFP expression marker. The infection efficiency ranged consistently between 40%-50% as evaluated by the percentage of cells that were EGFP positive (data not shown). Expression of FADD and FADD DN was confirmed by Western Blot analysis (data not shown).

Cells were harvested 60 hours after infection with the transgene. During this time period, the number of FADD or FADD DN expressing cells did not change in comparison to control SMCs that were expressing the empty vector (Fig. 10). Although this suggests that FADD overexpression did not cause cell death, I can not rule out the possibility that FADD induced apoptosis was masked by a compensatory increase in SMC proliferation. At later time points (by 3 and 4 days after infection), substantial cell death occurred only in FADD overexpressing cells. This is consistent with my findings in FADD overexpressing rat SMCs, in which measurable cell death did not occur until 72 hours after induction of transgene expression, and again documents the great tolerance of SMCs towards apoptotic stimuli. Similar to our results obtained in rat SMCs, FADD overexpression in human SMCs resulted in a substantial upregulation of MCP-1, which did not require the addition of Chx (Fig. 2.11A). Increased MCP-1 expression was substantially reduced by IL-1ra and a neutralizing anti-IL-1α antibody, supporting the hypothesis that IL-1α is a crucial mediator in FasL/Chx and FADD-induced gene expression (Fig. 2.11B). Consistent with this, FADD overexpression stimulated IL-1α transcription (Fig. 2.12A) and release (Fig. 2.13A) and induced nuclear translocation of the cytokine (Fig. 2.14). Analogous to FasL/Chx treated cells, FADD-activated IL-1α signalling was in part regulated via an autocrine/paracrine mechanism, because the addition of IL-1ra partly inhibited expression and release of the cytokine (Fig. 2.12B and Fig. 2.13B). Expression of FADD DN did not upregulate IL-1α or MCP-1, which excludes the possibility that FADD signals in a retrograde fashion via Fas and/or the TNF pathway through binding of TRADD.

The role of caspase- and calpain activity in FADD-induced IL-1α signalling and MCP-1 upregulation

To further support my hypothesis that Fas-induced gene expression is mediated via FADD, it was important to establish that both FasL/Chx- and FADD-activated IL-1 signalling and MCP-1 upregulation are at least in part mediated via the same pathways. FADD-overexpressing SMCs were treated with either a caspase- (IDUN) or a calpain inhibitor (MDL 28170). Like in FasL/Chx treated cells, inhibition of caspase activity in

FADD-overexpressing cells efficiently reduced IL-1α upregulation (by 60%) (Fig. 2.12B) and IL-1α release (by 60%) (Fig. 2.13B). Calpain inhibition with MDL 28170 was even more effective than IDUN in both reducing FADD-triggered IL-1α RNA expression (by 70%) (Fig. 2.12B) and IL-1α release (by 80%) (Fig. 2.13B). However, in contrast to Fas-stimulated SMCs, upregulation of MCP-1 in FADD-overexpressing cells was completely unaffected by caspase inhibition. Incubation with MDL 28170 caused a dose-dependent decrease in MCP-1 RNA levels and was almost as effective as inhibition of IL-1α signalling, suggesting that calpain activity plays a dominant role in FADD-induced MCP-1-expression.

These data support the hypothesis that Fas/FADD-induced gene expression is mediated through activation of caspase-dependent (yet apoptosis-independent) and calpain-dependent pathways, which involve activation of IL-1α synthesis and release, and result in further expansion and amplification of the signal.

Discussion

Fas induced upregulation of MCP-1 is mediated by IL-1α signaling

The second part of my thesis was aimed at obtaining a better understanding of the regulatory mechanisms underlying Fas/FADD induced MCP-1 upregulation. I concentrated on MCP-1 for 2 reasons: 1) MCP-1 is one of the most highly upregulated genes in response to Fas stimulation and 2) the production and release of MCP-1, which is the major macrophage chemoattractant, can best explain the predominant phenotype (macrophage accumulation) in our animal model of regulated induction of apoptosis via FADD overexpression [157], as well as the findings of others, demonstrating co-localization of apoptotic SMCs and macrophages in diseased or injured vessels [64,87,88].

Several studies have reported that Fas stimulation and FADD-overexpression can result in activation of the transcription factor NF-κB [75,181], but the mechanism underlying this is not clear. Fas/FADD-induced NF-κB activation would be a reasonable explanation for my findings, since most of the pro-inflammatory genes that were highly

expressed in response to Fas stimulation are known to be transcriptionally regulated by NF-κB. Alternatively, Fas/FADD could stimulate gene-expression through activation and/or release of a mediator, such as a pro-inflammatory cytokine. This would have the physiological advantage that the signal could spread from potentially dying cells to unaffected neighboring cells, which would also extend the period of gene induction beyond apoptosis of the "initiating' cells.

Pursuing this latter hypothesis, I chose to focus on IL-1 as a possible mediator of Fas- induced upregulation of pro-inflammatory genes for several reasons. IL-1 is a major pro-inflammatory chemokine and, as I discussed in the background section, plays a crucial role in the development of vascular diseases. Using gene array analysis, I have shown that IL-1α was one of the genes with the greatest increase in transcript level in response to FasL/Chx treatment [157]. In addition, IL-1 can induce MCP-1, IL-8, Gro-1, IL-6, TSG-6 and PAI2 expression, which were all among the ten most highly upregulated genes after Fas stimulation and are known to be involved in regulation of inflammation and tissue remodeling [77,100,116,171,182]. Miwa et al [118] demonstrated that the neutrophil inflammatory response to FasL presented by tumor cells depends in part on IL-1β release, because inflammation was substantially reduced in IL-1α/β knockout mice.

One possible mechanism through which Fas could upregulate MCP-1 expression in human SMCs, would be via activation of ICE/caspase1 or an ICE-like protease, resulting in cleavage of pro-IL-1β and secretion of mature IL-1β [182]. Human SMCs treated with FasL/Chx for 24 hr did not secrete IL-1β, but they did release significant amounts of IL-1α as determined by isoform-specific ELISA assays. Using 3 different approaches, I could show that IL-1α release is a significant component of the pathway(s) leading from Fas to chemokine upregulation. (1) Addition of IL-1 receptor antagonist (IL-1ra), which blocks both IL-1 receptors [5], inhibited most of the MCP-1 upregulation in response to FasL/Chx. (2) A neutralizing antibody that is specific for IL-1α (i.e. does not recognize IL-1β) was almost as effective, supporting a role for IL-1α but not IL-1β. (3) Fas activation by either incubation with FasL or FasL/Chx failed to induce MCP-1 expression in mouse embryonic fibroblasts derived from IL-1RI-deficient animals, but

caused increase in MCP-1 RNA levels in wildtype cells. The results, obtained by comparing IL-1RI-deficient and wildtype embryonic fibroblasts, are important because they emphasize a critical role for IL-1 signaling in Fas- mediated gene induction. In addition, they provide evidence that the pro-inflammatory response to Fas stimulation is not an exclusive feature of SMCs, but instead also occurs in other connective tissue cell types. MCP-1 upregulation in wildtype mouse embryonic fibroblasts in response to treatment with FasL alone shows that the addition of Chx is not required for all cell types, yet enhances the magnitude of Fas mediated effects. Together, these data provide strong evidence that Fas stimulates MCP-1 expression through increased release of IL-1α.

Mechanisms controlling IL-1α signaling in response to Fas stimulation

IL-1β seems to function more as a systemic, hormone-like mediator during acute and chronic inflammatory diseases, whereas IL-1α seems to primarily control locally defined inflammatory events [36]. Vascular pathologies involving SMC apoptosis, such as atherosclerotic plaques and restenotic lesions, consist of locally circumscribed inflammations, which would be typically mediated by IL-1α signaling. How do SMCs preferentially activate IL-1α– over IL-1β in response to Fas-stimulation? SMCs do not store IL-1α or IL-1β under normal conditions. Using gene array analysis, I found that transcript levels of both IL-1 isoforms were equally low in unstimulated, low passage human SMCs (data not shown). Fas-stimulation, however, causes exclusively increased expression of IL-1α but not IL-1β, suggesting that transcription of both cytokines is differently regulated. Schoenbeck et al. [159] showed that in contrast to endothelial cells, human vascular SMCs were only able to process IL-1α protein but not IL-1β into its mature form.

But why would it be advantageous for SMCs to selectively use IL-1α as a mediator of Fas-induced gene expression? IL-1α is biologically active in its mature and its pro-form, which may be an advantage over IL-1β, because even unprocessed IL-1α released from dead cells would be able to propagate the pro-inflammatory signal to neighboring cells. Furthermore, slow progression from Fas stimulation to apoptotic cell

death appears to be crucial for Fas/FADD-induced gene upregulation [157]. As described earlier, activation and release of IL-1β are preceded by cleavage into its mature form through ICE/caspase 1, which would be activated by caspase 8 in response to Fas-stimulation. Active caspase 8, however, would also induce activation of caspase 3, which is usually followed by rapid execution of the apoptotic death program. Thus, it seems possible that the time period from IL-1β activation to actual cell death would not be sufficient for new gene transcription, translation, and protein secretion.

How does Fas/FADD induce IL-1α signaling in human SMCs? Relatively little is known about the regulatory mechanisms of IL-1α signaling. Due to its prevalence in immune cells and its important role in a large variety of inflammatory diseases, research on IL-1 has mainly focused on IL-1β. Under normal culture conditions human SMCs express minimal IL-1α protein and most of the studies on the biologic activities of IL-1α have been performed using the recombinant, 17-kD mature form of IL-1α. To obtain a better understanding of Fas/FADD-induced activation of IL-1α, I focused on four potential mechanisms controlling IL-1α signaling: IL-1α transcription and protein synthesis, IL-1α release and nuclear translocation.

IL-1α RNA expression in SMCs:

IL-1α RNA levels in unstimulated SMCs were very low and increased dramatically in response to FasL/Chx treatment. This increase could be almost completely eliminated by co-incubation with IL-1α inhibitors. As both inhibitors, IL-1ra and anti-IL-1α Ab, only block extracellular, released IL-1α, it appears that IL-1α expression is predominantly regulated in an autocrine/paracrine fashion via the release of the cytokine. However, considering the very small amounts of IL-1α protein in untreated human SMCs, it seems more likely that IL-1α release enhances and prolongs its own transcriptional upregulation, which was originally initiated by additional factors.

FasL treatment alone had no detectable effect on human SMCs and the addition of Chx was necessary for FasL to induce cell death and gene upregulation. While protein synthesis inhibitors predominantly prevent translation, they have been also found to

either superinduce gene expression, when co-incubated with cytokines [38,56] or induce transcriptional upregulation of certain genes by themselves [69,115,160].

The mechanisms of this seemingly paradox response to inhibition of translation are unclear and controversial. Turner et al. [178] found that the Chx-induced increase in IL-1α and IL-1β mRNA in human monocytes was entirely attributed to prolonged half-lives of the transcripts, rather than transcriptional upregulation. Other reports confirmed this data and demonstrated that transcripts with increased expression levels in response to protein synthesis inhibition contained a potential mRNA destabilizing AU-rich sequence within their 3' untranslated region. [38,52,69,148,164]. Another model proposes the existence of labile proteins, which regulate transcript degradation and whose synthesis is blocked by protein synthesis inhibitors [38,143]. I found that Chx treatment alone caused a small increase in IL-1α RNA and protein levels in human SMCs. It is possible that the addition of FasL triggers the release of Chx-induced IL-1α protein, which then through autocrine/paracrine effects further drives IL-1α upregulation and consequently MCP-1 expression. However, two lines of evidence suggest that other factor besides Chx initiate IL-1α upregulation in response to FasL/Chx treatment: 1) FasL treatment alone induced MCP-1 upregulation in mouse embryonic fibroblasts. 2) FADD-overexpressing cells exhibited increased IL-1α expression without the addition of Chx, indicating that IL-1α upregulation may be initiated via Fas/FADD. Apoptosis of vascular SMCs *in vivo* occurs most commonly in environments that contain high cytokine concentrations, such as atherosclerotic plaques and restenotic lesions. It is conceivable that under these circumstances, SMCs may already contain significant amounts of IL-1α protein and therefore be "pre-conditioned" to respond to Fas-activation.

IL-1α protein content in SMCs:

Pro-IL-1α remains in the cytosol after translation without accumulating in any specific organelle. I found that unstimulated human SMCs contained only minimal IL-1α. Consistent with IL-1α RNA expression, incubation with Chx, alone and to a much larger extent with FasL/Chx, resulted in an increase in intracellular IL-1α, as determined

by ELISA analysis of whole cell lysates. Interestingly, IL-1 protein levels were not as dramatically reduced by co-incubation with IL-1ra, as compared to Fas-induced transcriptional upregulation of IL-1α and MCP-1. Besides transcription and translation, the intracellular content of IL-1α protein is determined by the stability of the protein and by its release. It seems reasonable to assume that any factor that would reduce IL-1α release could consequently cause accumulation of intracellular cytokine. The notion of "constipated" cells is supported by my findings using caspase- and calpain inhibitors in FasL/Chx treated cells. The inhibitors substantially reduced Fas-induced IL-1α release and consequently IL-1α–, as well as MCP-1 transcription, but had almost no effect on IL-1α protein content. Some investigators have considered that intracellular pro-IL-1α regulates important physiological functions, such as cell differentiation, migration and senescence in certain cell types [70,104,112]. However, my data indicate that Fas-induced gene expression in SMCs is predominantly regulated by IL-1α release and that intracellular IL-1α does not play a crucial role in this system.

Release of IL-1α

Binding of extracellular IL-1α to the IL-1RI appeared to be the key factor for Fas/FADD induced IL-1α– and MCP-1 upregulation. As mentioned in the Background section, IL-1α does not contain a secretory sequence and is not released via the classic secretory pathways [151]. Transient transfection of 3T3 cells with plasmids encoding either pro-IL-1α or mature 17-kD IL-1α, did not result in the release of IL-1α into the medium, suggesting that IL-1α is not released without a stimulus [166 176]. FasL/Chx treatment and FADD-overexpression caused release of IL-1α into the medium. Therefore, one can assume that Fas/FADD stimulates not only IL-1α synthesis, but also IL-1α release. So far, there are only two mechanisms of IL-1α release known: cell death, during which pro- and mature IL-1α can be released [71,84,123] and calpain activation, which is preceded by the cleavage of the cytokine into its mature form [81,85,151,172]. IL-1α release in response to Fas/FADD stimulation was regulated by caspase- and calpain-activation, which will be further discussed in the sections below.

Nuclear translocation of IL-1α

FasL/Chx treatment and FADD-overexpression resulted in translocation of IL-1α into the nucleus, which was demonstrated in 2 ways: by immunostaining with a specific anti-IL-1α Ab and by ELISA analysis of nuclear protein extracts. Several published studies have described nuclear localization of IL-1α, however, how and in what form IL-1α reaches the nucleus is still a mystery. Initially, Mizel et al. [119] reported that radiolabelled recombinant 17-kD IL-1α was rapidly internalized after binding to its receptors and was found to be associated with the nucleus after two to three hours. Others have demonstrated, that nuclear IL-1α was still bound to IL-1RI, which contains a typical nuclear import sequence, and that only the IL-1α /IL-RI complex but not mature IL-1α alone could bind to immobilized DNA [32,61,186]. A nuclear localization sequence is also present on pro-IL-1α but not mature IL-1α [188]. Using specific anti-pro-IL-1α antibodies and transfecting endothelial cells with a plasmid containing the first 115 amino acids of pro-IL-1α, which included the nuclear localization sequence, it appeared that pro-IL-1α could also localize to the nucleus [103]. Nevertheless, it is still controversial discussed whether internalization and nuclear localization of IL-1α are required to transduce IL-1α signals [103,112,167].

As I have shown by immunostaining, Fas-induced nuclear localization of IL-1α was markedly reduced in response to co-incubation with either IL-1ra, anti-IL-1αAb (not shown) or a calpain inhibitor, whereas IL-1α content in total cell lysates (as shown by ELISA) did not change dramatically after these treatments. This would suggest that IL-1α synthesis is not sufficient to trigger IL-1α translocation, but requires the release and receptor-binding of the cytokine. Additional studies comparing IL-1α concentrations in total cell lysates and in nuclear protein extracts after Fas/FADD stimulation with or without inhibitors need to be done to further confirm this hypothesis. It is noteworthy that nuclear translocation of IL-1α in response to FADD-overexpression was not limited to EGFP-positive and therefore presumably FADD-expressing cells. Conversely, not all EGFP-positive cells displayed a strong nuclear signal for IL-1α, indicating that the signal

that causes IL-1α migration to the nucleus can freely spread and is not limited to the confines of its source, which again speaks more in favor for released IL-1α being translocated to the nucleus. However, human SMCs treated with recombinant mature IL-1α did not exhibit positive nuclear staining at 2-3 hours after incubation with the cytokine, although cells responded to IL-1α within several minutes, as shown by complete degradation of Iκ-B (data not shown). SMCs displayed a strong nuclear staining pattern in every cell 24 hours after treatment, suggesting that either IL-1α translocation is very slow or that additional processes besides IL-1α binding to the receptor may have to occur in order to facilitate IL-1α translocation. The latter hypothesis could be explained by the recent finding of ATP-dependent association of the chaperone-type HSP74 family member Mortalin with the IL-1R receptor [154].

The significance of nuclear translocation of IL-1α in regards to Fas/FADD mediated MCP-1 upregulation is unclear. However, the responses of MCP-1 upregulation and nuclear translocation to all the treatment conditions were very similar, so that evaluation of nuclear translocation was at least useful as an additional tool to monitor the effects of Fas/FADD stimulation in SMCs.

The role of caspases in IL-1α signaling and MCP-1 upregulation

The release of IL-1α appeared to be the key factor for upregulation of IL-1α and MCP-1 in response to FasL/Chx treatment and FADD-overexpression. How does Fas/FADD stimulation result in IL-1α release? A causal relationship between apoptosis and IL-1α secretion/release has been described previously, although the mechanisms responsible for this finding were not defined [71]. FasL-expressing tumor cells *in vivo* caused caspase-dependent but ICE/caspase1-independent release of IL-1β, followed by neutrophil infiltration [118]. It seems therefore possible that analogous to IL-1β, IL-1α cleavage and subsequent release may be also facilitated by active caspases. Alternatively, IL-1α release could simply be a consequence of progressive membrane disintegration in the course of apoptotic cell death.

To test the hypothesis that caspase activation or apoptosis play a critical role in Fas-induced IL-1α release and consequent MCP-1 upregulation, I co-incubated FasL/Chx treated human SMCs with either a pan-caspase inhibitor (IDUN 1529) or inhibitors of caspase 8, caspase 1 and caspase 3. Although, "specific" caspase inhibitors preferentially bind and block activation of one or two distinct caspases, it is important to keep in mind that they are not completely specific [175,194]. Therefore, I considered the different caspase inhibitors more as a broad spectrum of tools to identify effects of caspases in MCP-1 induction rather than to unambiguously determine the role of a specific caspase. Caspase inhibitors prevented FasL/Chx-stimulated cell death and also efficiently blocked IL-1α release, suggesting that caspase activation /apoptosis may be important regulatory mechanisms in Fas/FADD induced IL-1α secretion/release. However, the potent inhibitory effect on cell death and IL-1α release did not correlate with considerably weaker effects of caspase inhibitors on IL-1α– and MCP-1 gene expression. Since upregulation of both cytokines seemed to depend on the binding of extracellular IL-1α to its receptor, it seems reasonable to conclude that the amount of IL-1α, which is released even in the absence of cell death in the presence of caspase inhibitors is sufficient to upregulate MCP-1 expression. The low levels of IL-1α released in the presence of caspase inhibitors may result from either incomplete caspase inhibition or the involvement of an additional, caspase-independent pathway. Nevertheless, these data indicate that IL-1α is secreted even in the absence of cell death. This concept of a cell death independent mechanism, leading to the release of IL-1α is further supported by three findings:1) Nuclear localization of IL-1α in FasL/Chx treated cells was almost completely unaffected by caspase inhibition with IDUN; 2) FADD-overexpression induced release of IL-1α (and MCP-1 upregulation) prior to driving a detectable number of cells to undergo apoptosis; and 3) MCP-1 upregulation in FADD-overexpressing cells was completely unaffected by IDUN.

Despite the fact that FADD-overexpressing SMCs did not undergo apoptosis, caspase inhibition with IDUN caused a 50% decrease in IL-1α transcription and release in those cells. This supports the intriguing hypothesis that caspases may be involved in

the regulation of additional cell functions, besides activation of apoptotic cell death. Although, as stated earlier, results obtained with "specific" caspase inhibitors require cautious interpretation, the discrepancy between the effects of IDUN and caspase8 inhibitors on the one side and caspase1 and caspase3 inhibitors on the other were remarkable. While all inhibitors effectively reduced IL-1α release, caspase3 and caspase1 inhibitors had almost no effect on FasL/Chx induced MCP-1 upregulation, which decreased by more than 50% in response to IDUN and caspase8 inhibition. Since IDUN also strongly inhibits caspase8 activity [194], these data suggest a potential non-apoptosis related role for caspase8 in Fas-mediated gene induction. The following preliminary data support this hypothesis: overexpression of full-length caspase8 in human SMCs also induces IL-1α release and MCP-1 upregulation, prior to or without, the induction of apoptosis. Expression of a deletion mutant of caspase8, missing the catalytic domain, did not cause comparable effects. In contrast to wildtype cells, caspase8-deficient embryonic fibroblasts lacked increased MCP-1 expression in response to FasL/Chx.

The role of calpains in IL-1α signaling and MCP-1 upregulation

The data obtained by using caspase inhibitors indicated that Fas/FADD-induced IL-1α release and consequent MCP-1 upregulation, may be regulated in part via a caspase / apoptosis independent pathway. Besides cell death, the only other known mechanism of IL-1α release involves the calcium-dependent cystein proteases calpain 1 and calpain 2 [81,85,151,172]. However, apart from the well-documented fact that calpains cleave pro-IL-1α into its 17kD-mature form, the process of calpain-mediated IL-1α release is still unclear. To test, whether calpains are involved in Fas/FADD-mediated IL-1α release, I used 2 different calpain inhibitors, a pharmacological compound (MDL 28170) and a peptide-based inhibitor, encoded by exon 1B of human calpastatin (CS-P). Both calpain inhibitors reduced Fas/FADD-triggered IL-1α release in a dose-dependent manner and were as effective as IDUN. Calpain inhibitors were even more effective than IDUN in reducing IL-1α and MCP-1 transcript upregulation, indicating that IL-1α-

mediated gene induction in response to Fas/FADD stimulation is at least in part transduced via calpain activity.

As discussed in the Background section, calpain activation *in vitro* requires non-physiologically high calcium concentrations. The mechanisms responsible for calpain activation *in vivo* are still largely unknown [114,124]. Calpains have been associated with apoptosis, particularly in neuronal cells and lymphocytes, as both death-preventing and death-inducing factors [11,28,152,190]. Calpain inhibitors did not affect the rate of cell death in response to FasL/Chx treatment, suggesting that calpains do not directly modulate apoptosis in human SMCs. It seems reasonable to assume that calpains are activated by caspases, e.g. through cleavage of their endogenous inhibitor calpastatin [183]. However, two lines of evidence argue against the notion that caspases are the only activators of calpains: 1) Calpain inhibitors were significantly more effective than a caspase inhibitor (IDUN) in reducing Fas-activated IL-1α RNA expression and 2) MCP-1 upregulation in FADD-overexpressing cells was significantly inhibited by a calpain inhibitor (MDL 28170), whereas IDUN was completely ineffective. One focus of my future studies will be to determine the mechanisms underlying Fas/FADD mediated calpain activation. To do this, it will be necessary to establish a method, which allows me to reliably monitor calpain activity levels in human SMCs.

Conclusions

Based on the presented results, I propose the following sequence in Fas/FADD induced gene expression: Fas stimulation causes the recruitment of FADD, which induces, through yet unknown pathways, IL-1α RNA transcription and calpain activation. Calpains mediate maturation and release of IL-1α, which further promotes and enhances IL-1α– and consequently MCP-1 RNA upregulation. In a second wave, Fas/FADD-activated caspases (caspase8?) further stimulate IL-1α release, possibly through an increase in calpain activity after degradation of calpastatin. In the final phase, SMCs undergo apoptosis resulting in the release of accumulated pro-IL-1α, which ensures the

propagation and continuation of the signal beyond the life time of the cells initially stimulated by Fas/FADD. Although, the data presented here does not fill all the gaps of this hypothetical scenario, it provides intriguing information about novel interactions and additional functions of pathways, that were commonly thought to be involved only in the regulation of life or death.

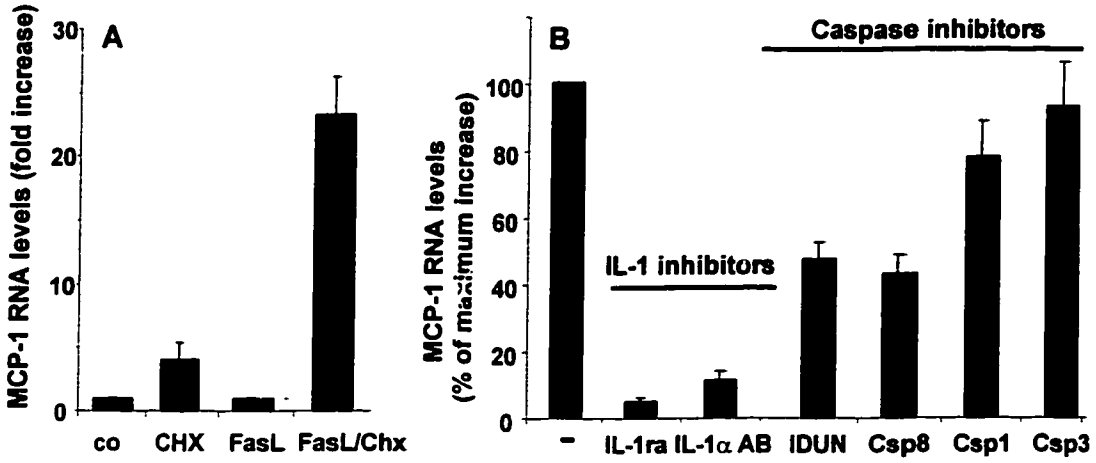

Figure 2.1: MCP-1 upregulation in response to FasL/Chx is mediated by IL-1α
A: Human SMCs were incubated 24 h with soluble Fas ligand (FasL) with or without cycloheximide (Chx) or cycloheximide alone. MCP-1 transcript levels were determined by Taqman realtime PCR and normalized to 18s ribosomal RNA levels. Data are expressed as fold increase of MCP-1 expression compared to untreated cells (co). B: Human SMCs were treated with FasL/Chx and analyzed as in A, but were co-incubated with or without either IL-1 inhibitors {IL-1 receptor antagonist (IL-1ra) or anti-IL-1α antibody (IL-1αAB)} or caspase inhibitors {a pan caspase inhibitor (IDUN), or inhibitors that are more specific for caspase 8 (Csp8), caspase 1 (Csp1) or caspase 3 (Csp3)}. Data are expressed as percentage of maximum MCP-1 RNA levels in FasL/Chx (-) stimulated cultures without inhibitors.

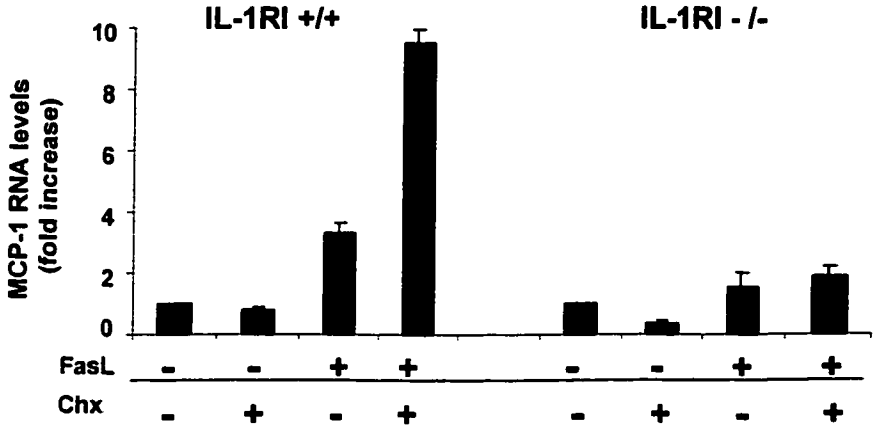

Figure 2.2: IL-1 receptor deficient cells do not upregulate MCP-1 expression in response to Fas stimulation
Primary embryonic fibroblasts of IL-1 receptor deficient (IL-1RI -/-) and wildtype (IL-1RI +/+) mice were either untreated or stimulated with Chx, FasL or FasL plus Chx for 24 h. MCP-1 RNA levels were determined using RT-PCR Taqman. Data are expressed as fold increase of MCP-1 expression compared to untreated.

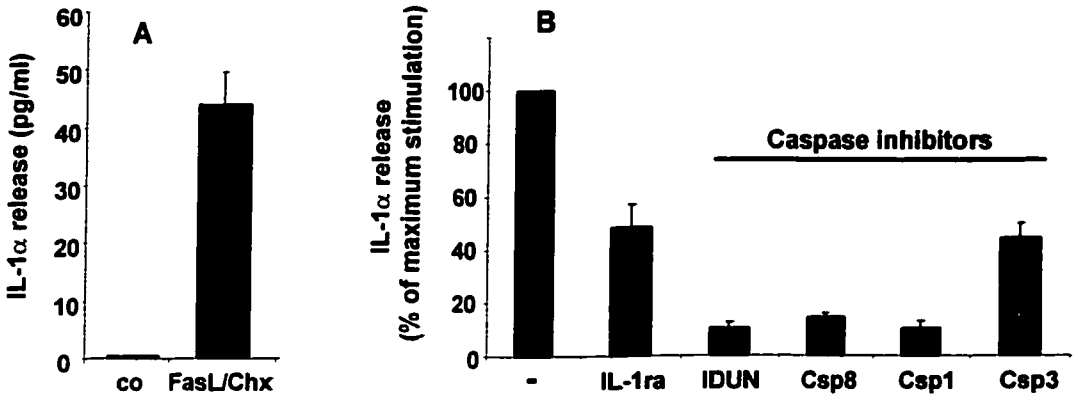

Figure 2.3: Caspase inhibitors cause decrease in FasL/Chx induced release of IL-1α
A: IL-1α protein concentration was determined by ELISA in conditioned medium of untreated human SMCs (co) and after FasL/Chx treatment for 24 h. B: Human SMCs were treated with FasL/Chx with or without the addition of either IL-1ra or caspase inhibitors. Data are expressed as percentage of IL-1α release in FasL/Chx treated SMCs without inhibitor.

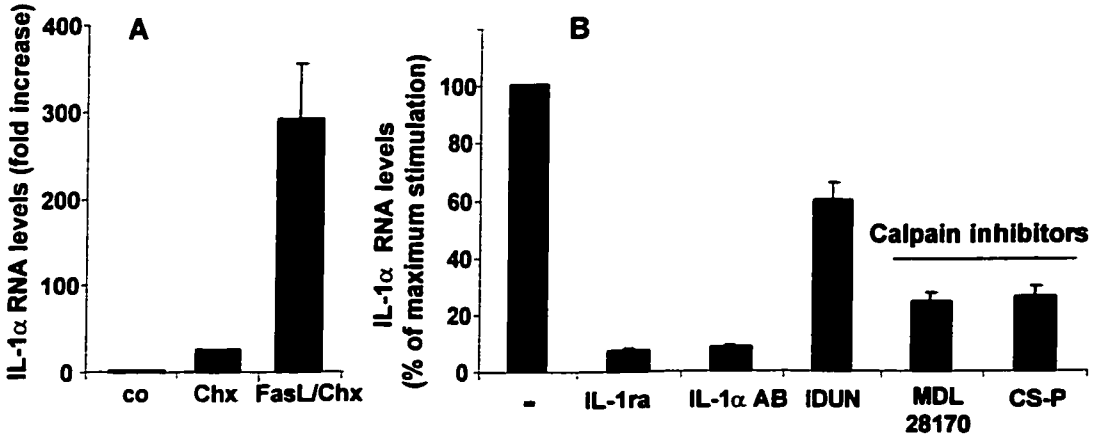

Figure 2.4: FasL/Chx induces IL-1α upregulation in IL-1α and calpain dependent fashion
A: Human SMCs were incubated for 24 h with FasL/Chx or Chx alone. IL-1α transcript levels were determined by Taqman realtime PCR and normalized to 18s ribosomal RNA levels. Data are expressed as fold increase of IL-1α expression compared to untreated SMCs (co). B: Human SMCs were treated with FasL/Chx and co-incubated with or without either IL-1 inhibitors (IL-1ra or anti-IL-1α antibody) or a pan caspase inhibitor (IDUN 1529), or calpain inhibitors (either MDL 28170 (20μM) or a calpastatin-like peptide CS-P (50μM)). Data are expressed as percentage of IL-1a RNA levels in FasL/Chx stimulated cultures without inhibitors (-).

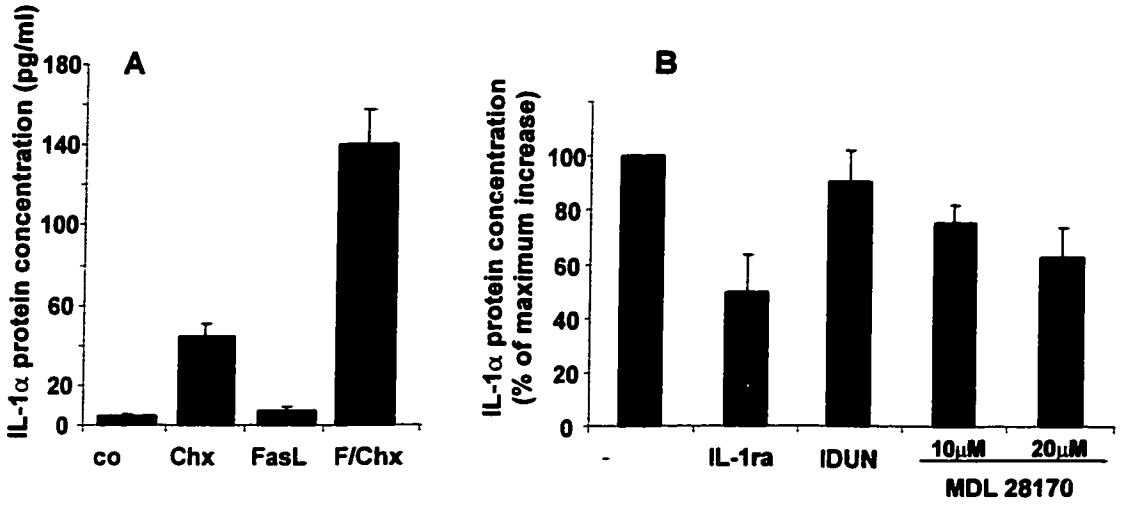

Figure 2.5: Increased intracellular IL-1α protein concentration in response to FasL/Chx is not significantly reduced by caspase- or calpain inhibition
A: IL-1α protein concentration in equal amounts of total cell lysate (5μg) in untreated human SMCs (co) and after FasL/Chx treated SMCs (24 hrs) was determined by ELISA. B: Human SMCs were treated with FasL/Chx with or without the addition of either IL-1ra, a pan-caspase inhibitor (IDUN) or a calpain inhibitor (MDL 28170). Data are expressed as percentage of IL-1α content in FasL/Chx treated cultures without inhibitor.

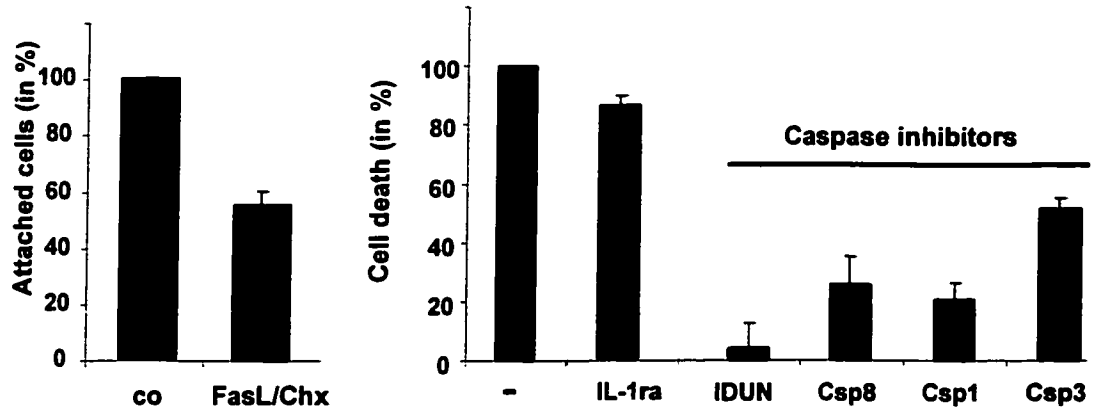

Figure 2.6: Caspase inhibitors reduce effectively FasL/Chx induced cell death
A: Human SMCs cells treated with FasL/Chx for 24 hours. Cells that remained attached were washed, trypsinized and cell numbers determined using a coulter counter. Data are expressed as percentage of attached untreated control cells. B: FasL/Chx treated cells were co-incubated with IL-1 receptor antagonist (IL-1ra), or caspase inhibitors (IDUN, or caspase 8, caspase 1 and caspase 3 inhibitor). Attached SMCs were analyzed by coulter counter. Data are expressed as percentage of cell death in response to FasL/Chx alone.

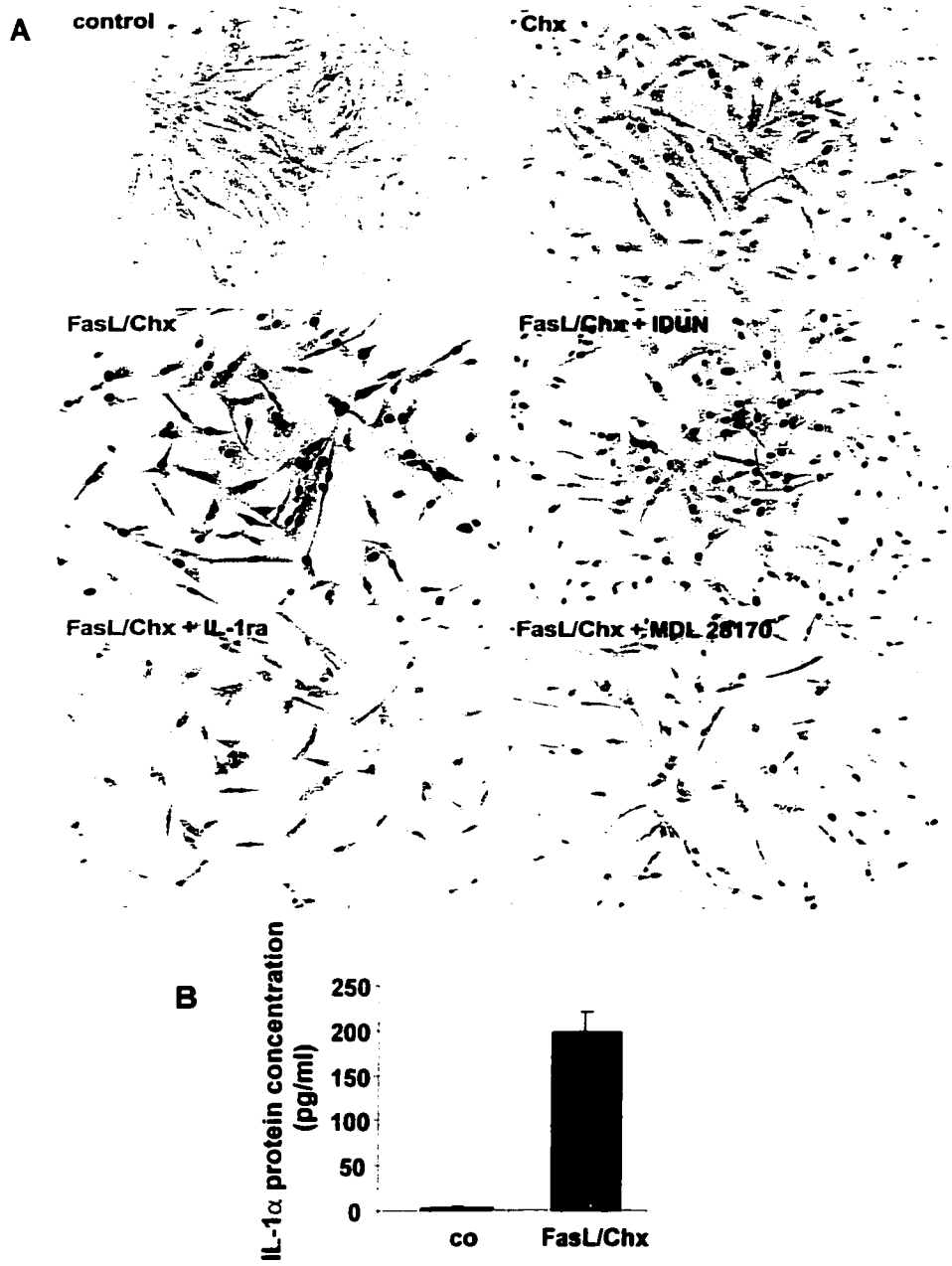

Figure 2.7: Nuclear translocation of IL-1α in response to FasL/Chx occurs independent of caspase activation

A: Human SMCs were either untreated (control) or incubated with FasL/Chx with or without caspase inhibitor (IDUN), IL-1ra or a calpain inhibitor (MDL 28170; 20mM). The cells were fixed, immunostained with an IL-1a specific antibody and visualized using a rhodamine conjugated secondary antibody. Magnification 10x. B: IL-1a concentration in nuclear extracts of untreated and FasL/Chx treated cells were determined by ELISA.

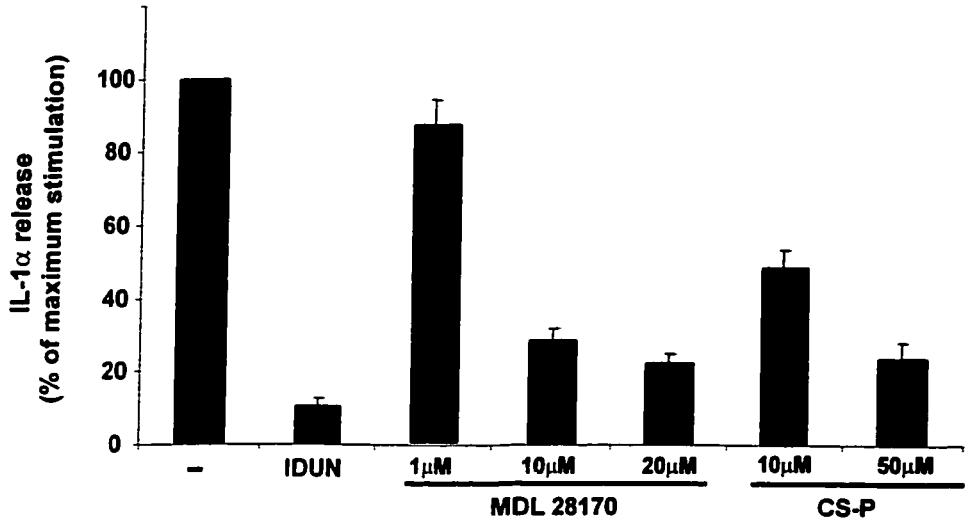

Figure 2.8: Calpain inhibitors reduce in FasL/Chx stimulated release of IL-1α
Human SMCs were co-incubated for 24 hr with FasL/Chx plus calpain inhibitors (MDL 28170 and CS-P) in the indicated concentrations. IL-1α concentration in conditioned medium was determined by ELISA. Data are expressed as percentage of IL-1α release in FasL/Chx treated SMCs without inhibitor.

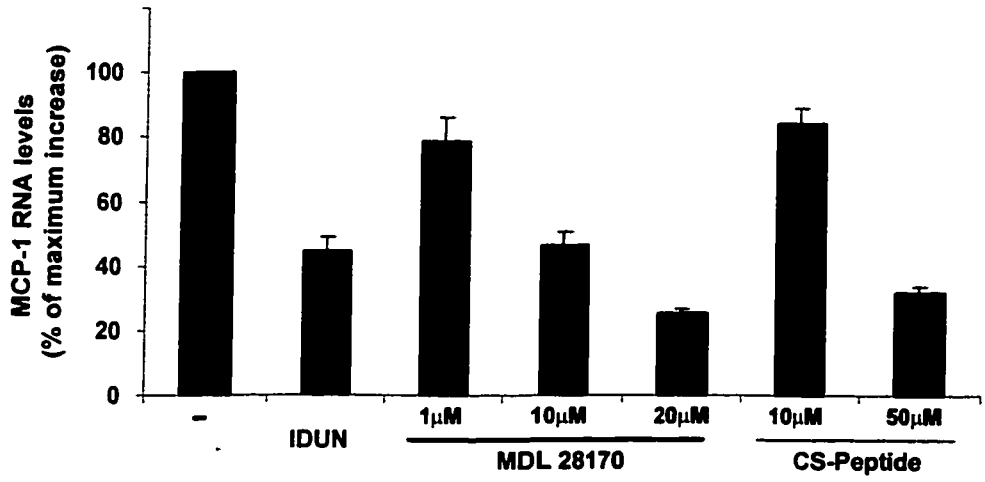

Figure 2.9: Fas-induced MCP-1 upregulation is in part regulated by calpain activity
Human SMCs were treated for 24 hr with FasL/Chx and co-incubated with calpain inhibitors (MDL 28170 and CS-P). MCP-1 transcript levels were determined by Taqman realtime PCR. Data are expressed as percentage of transcript levels in stimulated cultures without inhibitors.

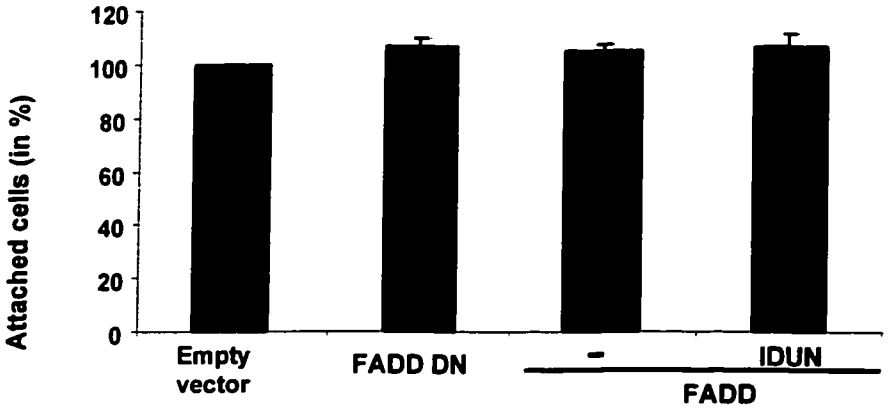

Figure 2.10: FADD-overexpression in human SMCs does not induce cell death during the observation period

Human SMCs were either infected with a retroviral vector containing human FADD cDNA, or a dominant-negative acting deletion mutant of FADD, which lacks the death effector domain (FADD DN) or the empty vector. A subset of FADD expressing cells was treated with the caspase inhibitor IDUN. Sixty hrs after infection cultures were trypsinized and cell numbers determined by coulter counter. Data are expressed as percentage of attached cells that were infected with the empty vector.

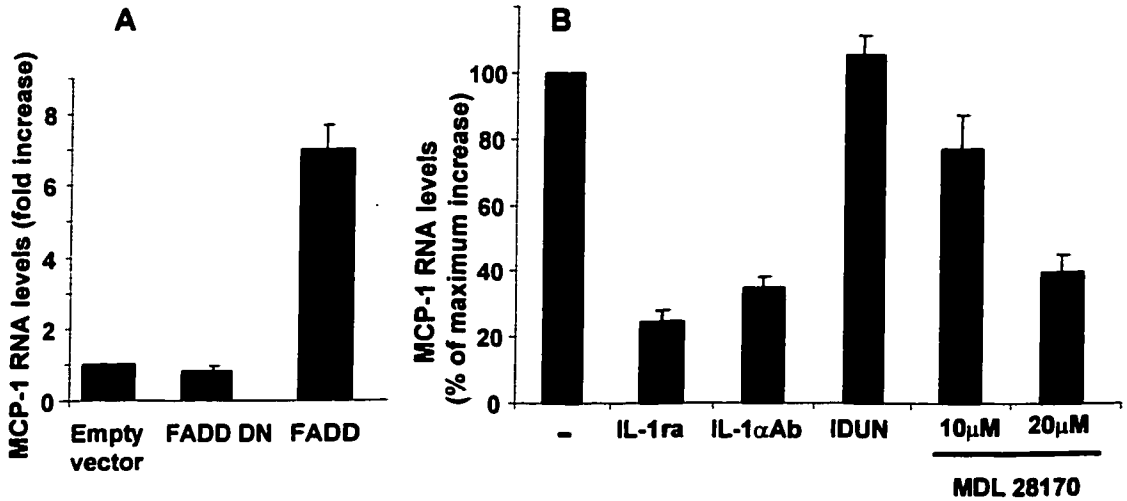

Figure 2.11: FADD-induced upregulation of MCP-1 is IL-1α and calpain dependent but caspase independent

A: Human SMCs infected with either the empty retroviral vector, or FADD or dominant-negative acting FADD (FADD DN) were harvested for RNA extraction 60hrs after infection. MCP-1 transcript levels, normalized to 18s, were determined by Taqman realtime PCR. Data are expressed as fold increase of MCP-1 expression compared to cultures infected with the empty vector. B: FADD expressing SMCs were treated with inhibitors of either IL-1a (IL-1ra and IL-1aAB), caspase (IDUN) or calpain (MDL 28170). RNA harvest and MCP-1 RNA levels analysis were performed as in A. Data are expressed as percentage of transcript levels in FADD expressing SMCs without inhibitors.

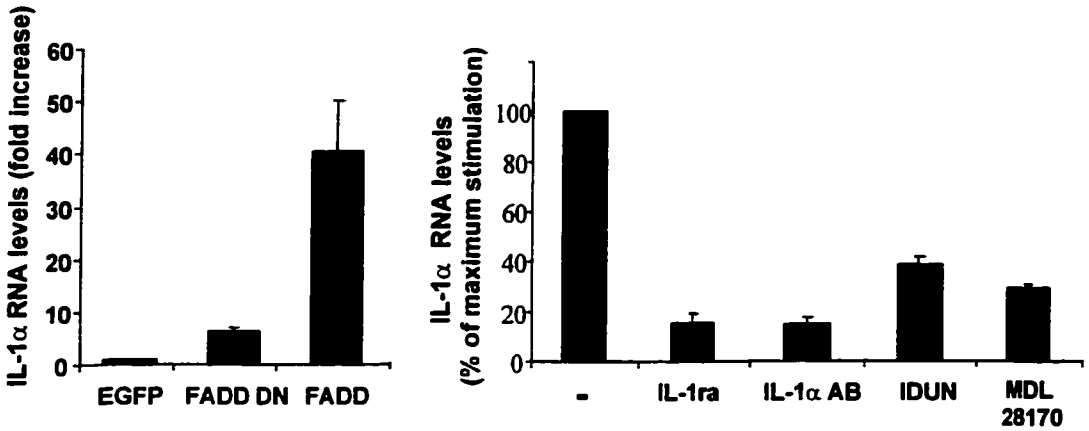

Figure 2.12: FADD-induced upregulation of IL-1α RNA is mediated in an IL-1α , caspase- and calpain dependent pathway

A: IL-1α RNA levels in human SMCs were determined 60 hr after retroviral infection with either the empty vector, FADD DN or FADD. Data are expressed as fold increase of MCP-1 expression compared to cultures infected with the empty vector. B: FADD-expressing SMCs were incubated with IL-1α-, caspase- (IDUN) or calpain- (MDL28170) inhibitors. MCP-1 RNA transcript levels were determined at 60 hr. A. Data are expressed as percentage of transcript levels in FADD expressing SMCs without inhibitors.

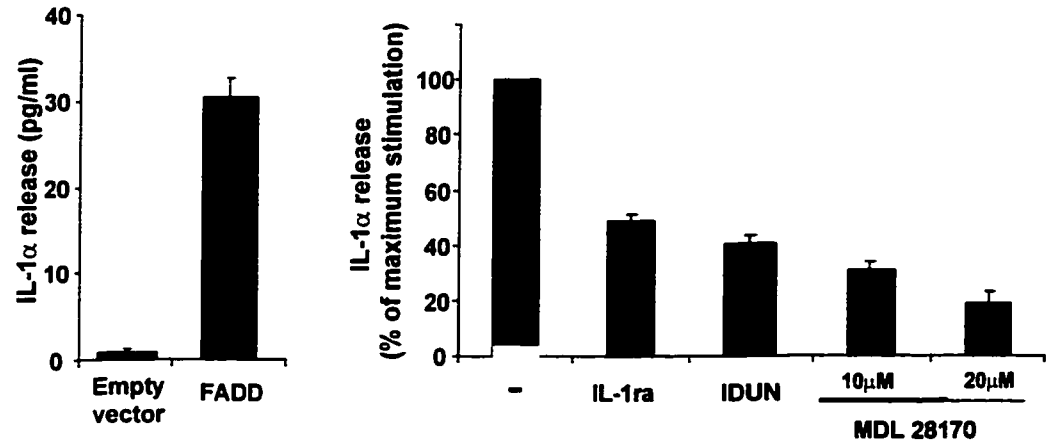

Figure 2.13: FADD induced IL-1α release is in part mediated by IL-1, caspases and calpain

A: Human SMCs were infected with either FADD or the empty vector. After 36 hr medium was changed to 1% FBS containing DMEM.. IL-1α protein concentration was determined by ELISA after 24 hr.

B: FADD expressing SMCs were treated with IL--ra, a caspase- (IDUN) or a calpain inhibitor (MDL 28170). IL-1α concentration of released IL-1α was determined as in A. Data are expressed as percentage of IL-1α release in FADD expressing SMCs without inhibitor.

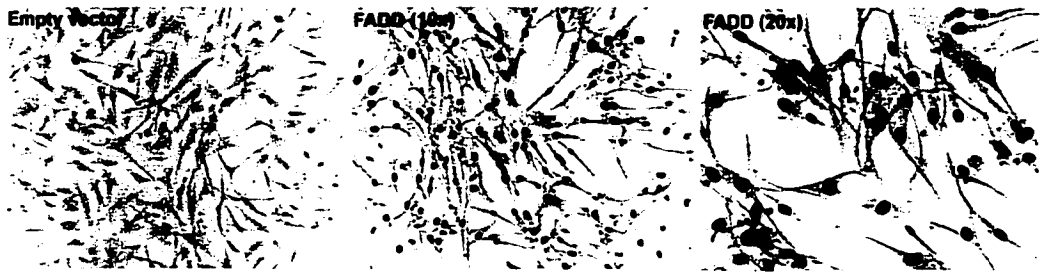

Figure 2.14: Nuclear translocation of IL-1α in response to FasL/Chx occurs independent of caspase activation

Human SMCs were either transfected with FADD or the empty vector. The cells were fixed, immunostained with an IL-1a specific antibody and visualized using a rhodamine conjugated secondary antibody. Magnification 10x and 20x.

METHODS

Establishing a rat SMC line with tetracycline-regulated expression of FADD (FRFADD).

Aortic SMCs were isolated from Fischer 344 rats (Simonson Lab., Gilroy, CA) and cultured as described by Clowes et al [30]. SMCs expressing FADD under control of the Tet operator/promoter regulatory system [57] were obtained by two sequential gene transfections. Fischer 344 SMCs were first co-transfected with a plasmid encoding the Tet transactivator protein (tTA) under the control of the Tet operator/promoter sequence [165] and a plasmid conferring antibiotic resistance (pSV2NEO) as a selectable marker. SMC clones that survived G418 selection were screened for tightly regulated expression of tTA by performing transient transfections with plasmids containing a reporter gene, luciferase, under control of the Tet operator/promoter. The SMC clone showing the best tetracycline-regulated expression of luciferase was used for the next round of transfection. This SMC clone was co-transfected with a plasmid containing human FADD [26] under control of the Tet operator/promoter along with a hygromycin resistance plasmid. Hygromycin-resistant SMC clones were tested for tetracycline-regulated expression of FADD by western blots (see below), and the clone showing the highest expression in the absence of tet with the lowest expression in the presence of tet was used for subsequent experiments. The plasmid containing human FADD under the control of the Tet operator/promoter also contained a second Tet operator/promoter regulating expression of a reporter protein: β-galactosidase modified with a nuclear localization signal at the N-terminus and six repeats of the myc epitope tag at the C-terminus. Co-expression of this marker gene facilitated the identification of the seeded cells (see below).

Cell culture

FRFADD SMCs were cultured in DMEM with 10% calf serum in the presence of 1 μg/ml tetracycline. For induction of transgene expression, cells were plated in DMEM supplemented with 1% calf serum without the addition of tetracycline. Low passage cultures of human SMCs were generously provided by Dr. R. Ross, University of

Washington, Seattle Washington. IL-1R WT and knock-out primary embryonic fibroblasts were a generous gift from Dr. Jaques Peschon, Immunex, Seattle Washington. Experiments were performed between passage 6 and 10, with cells grown in DMEM with 10% fetal calf serum for 36 hours prior to initiation of experiments. Cells were induced to undergo apoptosis by replacing the medium with fresh medium containing 0-1 % fetal calf serum, 2 μg/ml CD 95 monoclonal antibody (CH 11) (Coulter Corporation) or 100 ng/ml recombinant FasL, and 0.05-1.5 μg/ml cycloheximide. Recombinant human soluble FasL, generously provided by Dr. Peter Kiener (Bristol-Myers Squibb, Pharmaceutical Research Institute, Princeton, NJ), was expressed as fusion protein consisting of the extracellular domain of human FasL [83].

To inhibit caspases, we added 50 μM of the caspase inhibitor IDUN 1529 (IDUN Pharmaceuticals, Inc., La Jolla, CA) [194], or 50 μM of the caspase1 Z-YVAD-FMK, the caspase3 inhibitor Z-DEVD-FMK (both from Calbiochem, La Jolla, CA) or the caspase8 Z-IETD-FMK (Enzyme Systems Products, Livermore, CA). Calpains were inhibited by using either the synthetic calpain inhibitor MDL 28170 or the calpastatin-like peptide CS Peptide (both from Calbiochem, La Jolla, CA).

ELISA assay

Concentrations of IL-1α in culture supernatants were determined by ELISA using a human IL-1α ELISA development system (DuoSet, R & D Systems, Minneapolis, MN) according to the vendor's instructions.

Flow cytometric analysis

Cell viability and surface exposure of phosphatidylserine were analyzed by flow cytometry of 10,000 cells using a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Cells were washed and incubated with FITC-conjugated Annexin V (R&D Systems) to evaluate phosphatidylserine exposure, plus propidium iodide (R&D systems) to evaluate membrane permeability. Only cells that stained positive for Annexin V and negative for propidium iodide were considered to be apoptotic.

In vivo studies

To establish a carotid neointima containing FRFADD cells, the cell seeding procedure was performed according to the method of Clowes et al [30]. Rats received tet (1mg/ml) plus sucrose (2.5mg/ml) with the drinking water for 4 to 7 days prior to seeding to bring circulating tet levels up to an inhibitory concentration. Male Fischer 344 rats (250 – 300g) were anesthetized by intraperitoneal injection of xylazine (Anased; Lloyd Laboratories) 4.6 mg/ kg body weight, and ketamine (Ketaset; Aveco Co Inc) 70 mg/kg body weight and Azepromazine (Fermenta, Kansas City) 0.1 mg/kg body weight. The left common carotid artery was surgically exposed. The distal carotid artery was temporarily isolated with ligatures placed around the internal carotid (at the bifurcation) and around the common carotid (10 mm proximal of the bifurcation). To de-endothelialize the carotid, a 2 French balloon embolectomy catheter (Baxter Healthcare Co) was introduced into the external carotid artery and passed into the common carotid artery. A suspension of trypsinized FRFADD SMCs (100,000 in 40μl of culture medium with 5% calf serum) was then infused into the denuded area by means of a polyethylene tube. After 15 minutes the tubing was removed and the external carotid artery was tied off proximal to the incision hole. Blood circulation was restored, and the wound was closed. The rats were provided with tet (2.5mg/ml) in the drinking water for the subsequent weeks while a quiescent neointima developed. To permit determination of cell proliferation rate, BrdU pellets (50 mg per animal) (Boehringer Mannheim) were implanted subcutaneously 24 hours before the animals were sacrificed. At various times after withdrawing tet, rats were killed by intravenous injection of sodium pentobarbital (Anthony Products Co) 10 ml / kg body weight. The carotid artery was dissected and adhering connective tissue was removed.

Western Blot Analysis

Cultured cells were washed and solubilized in lysis buffer (10mM potassium phosphate pH 7.8, 0.2% Triton X-100, 1mmol/L phenylmethylsulfonyl fluoride, 10μg/ml). Seeded carotid artery segments were homogenized in 1 ml of lysis buffer. The protein extracts were centrifuged to remove particulate material then concentrated by ultrafiltration at 4

deg using Centricon 30 concentrators (Amicon). Aliquots containing 10μg protein (BCA protein assay kit, Rockford, IL) were reduced with 0.7% BME and loaded on 12% SDS-PAGE gels and transferred to nitrocellulose. The primary antibody directed against human FADD (Transduction Laboratories) was used at 1:250 dilution. A 1:1000 dilution of anti-rabbit IgG horseradish peroxidase conjugate (Amersham) was used as the secondary antibody and the bands visualized using enhanced chemiluminesence (NEN Life Sciences, Boston MA)

Immunohistochemistry and Immunocytochemistry

Carotid arteries seeded with FRFADD SMCs were fixed with methyl Carnoy's fixative for 12 hours at room temperature. The tissue was paraffin-embedded and sectioned (6 μm). The dehydrated tissue sections were incubated with the following different primary antibodies: 9E10 monoclonal antibody for the myc-tag [42], ED-1 (Serotec Inc., Raleigh, NC) for macrophages, and RP-3 (generous gift from Dr. Rick Johnson, University of Washington, Seattle Washington) for neutrophils. The sections were then incubated with biotinylated secondary antibodies followed by enzyme-conjugated (horseradish peroxidase or alkaline phosphatase) avidin-biotin complexes (ABC Standard or Elite, Vector Laboratories, Burlingame, CA). Finally, the sections were incubated in substrate; diaminobenzidine for horseradish peroxidase or Vector Red (Vector Laboratories, Burlingame, CA) for alkaline phosphatase, and nuclear counterstained with hematoxylin or methyl-green.

Plated human SMCs were washed with cold phosphate-buffered saline and fixed with 2% paraformaldehyde. Cells were incubated with a monoclonal anti-human IL-1α antibody (R&D Systems, Minneapolis, MN) in blocking buffer for 1 hour, followed by incubation with a rhodamine-labelled anti-mouse secondary antibody (Dako Corp., Carpinteria, CA).

DNA isolation and semiquantitative, competitive PCR

To monitor the loss of seeded FRFADD SMCs after removal of tet, we determined the concentration of the neomycin resistance gene, which is only present in seeded FRFADD cells, in 200ng total DNA by semiquantitative, competitive PCR [193]. For this assay, we

developed a competitive template which consists of a plasmid containing the neo resistance DNA modified by site-directed mutagenesis to alter a single base in a position between the PCR primer sites. This mutation introduced a novel restriction enzyme site (Hind III) within the 730 bp PCR-amplified product. The relative amount of neo DNA in the carotid arteries was measured by determining how much competitive template DNA must be added to each carotid DNA sample to produce equal amounts of wild type and mutant, HindIII cleaved, PCR products. DNA from comparable segments of the seeded carotid arteries was isolated using the QIAamp Tissue Kit (Qiagen Inc, Valencia, CA) according to manufacturer's protocol. Primer sequences used for PCR amplification were: 5'-GGTGGA GAGGCTAT TCG GCTA TGACTG-3' and 5' – GTCAAGAAGGCGATAGAAGG CGATGCG (product size: 730 bp). Each sample was analyzed three times.

RNA Isolation and semiquantitative RT-PCR

Total RNA from cultured cells or seeded carotid arteries was isolated using TRIZOL reagent (Life Technologies, INC, Grand Island, NY). RNA was converted to cDNA by standard methods using reverse transcriptase (Life Technologies, Gaithersburg, MD) and random primers (Life Technologies, Gaithersburg, MD). The cDNA was amplified under nonsaturating PCR conditions using specific primers. Primer sequences (sense primers are indicated first) are as follows: rat GAPDH, 5'-CCTCTGGAAAGCTGTGGCGT-3' 3' and 5'-TTGGAGGCCATGTAGGCCAT-3' (product size: 430 bp); rat beta-actin, 5'-TAAAACGCAGCTCAGTA ACAGTCCG-3' and 5'- CGTTGACATCCGT AAA GACCTCTA-3' (product size: 279 bp); rat MCP-1, 5'-TATGCAGGTCTCTG TCACGC-3' and 5'- AGTGCT TGAGGTGGTTGTGG-3' (product size: 414 bp); CINC, 5'- GCCAATGAGCTG CGCTGTCAATGC-3' and 5' – CTTGGGGACACCTTTTAG CATCTT – 3 (product size: 340 bp). Human GAPDH 5'– CGGAGTCAACGGATTTGGT CGTAT – 3' and 5'– AGCCTTCT CCATGGTGGTGAAGAC –3' (product size: 324 bp); HUMAN MCP-1 5' – CAA ACTGAAGCTCGCACTCTCGCC – 3' and 5' – ATTCTTGGGTTGTGGAGTGAG TGTTCA – 3' (product size: 354 bp); human Il-8 5'- ATGACTTCCAAGCTGGC

CGTG –3' and 5' – CTTCTCCACAACCCTCTGCAC – 3' (product size: 289 bp). To determine relative transcript levels in the cultured cells or seeded carotid arteries, cDNA samples were serially diluted and PCR amplification performed with radiolabeled nucleotides ^{125}. PCR products were separated by PAGE and band intensity was measured using phosphorimager analysis. Care was taken to ensure that all PCR products fell within the range of exponential amplification. Results from different PCR runs cannot be combined directly because it is not possible to adequately standardize all of the variables. Therefore, some samples were included in all of the PCR runs for a given set of primers in order to provide a reference for combining results from different runs. Independent amplification of a control gene, such as GAPDH (for cultured cells) and beta-actin (for seeded carotid arteries) was done to correct for differences in efficiency of RNA isolation and reverse transcription. The final, normalized, results were calculated by dividing the relative transcript levels of the test genes by relative amount of the GAPDH or beta-actin transcripts.

For Taqman™ quantitative PCR [99], MCP-1, IL-1α and 18S primers and Taqman™ probes were obtained from PE Biosystems (Foster City, California, USA). PCR was carried out in triplicate in a PE 5700 with standard cycling parameters. Threshold (C_T) values were calculated by the GeneAmp 5700 SDS Detector software.

Analysis of chemotactic activity

The ability of FRFADD cell conditioned media to induce monocyte/macrophage chemotaxis was assessed by minor modification of a fluorescence-based assay using 96-well chemotaxis chambers containing polycarbonate filters with 8 μm pores (ChemoTX, Neuro Probe Inc., Gaithersburg, MD) [44]. In brief, THP-1 cells (5x 10^6 / ml), a human monocyte/macrophage cell line, were incubated for 30 min at 37^{O}C in HEPES-buffered RPMI- 0.1% BSA and calcein AM (10μm) (Molecular Probes, Eugene, OR), then washed twice and resuspended at 4x 10^6 / ml in HEPES-buffered RPMI- 0.1% BSA for the assay. The chamber wells were filled with 29 μl of culture supernatant, the filter applied, and THP-1 cells (25 μl containing 1x 10^5 cells) were placed directly onto the

filter sites. All determinations were performed in quadruplicate for each experiment. The chambers were incubated for 2h (37^{O}C and 5% CO_2). To assess the contribution of MCP-1 to the measured monocyte/macrophage chemoattractant activity in culture supernatants, the test media were preincubated for 30 minutes at 37^{O}C with two different rabbit anti-rat/mouse MCP-1 antibodies R147 (a generous gift from Dr. Jeffrey S. Warren, University of Michigan Medical School, Ann Arbor, MI) and AAR12Z (Serotec Ltd, Oxford England) or pre-immune rabbit IgG as a negative control. At the end of the incubation period, non-migrating cells on the origin (top) side of the filter were removed by washing and aspirating with excess RPMI-BSA and gentle wiping with a tissue. To determine the percentage of cells that had migrated into the bottom chamber during the course of the experiment, the chemotaxis chamber was placed in a multi-well fluorescent plate reader (Cyto Fluor II, PerSeptive Biosystems, Framingham, MA) and fluorescence determined in the bottom-read position (excitation: 485 nm; emission: 530 nm). The data are reported as the percentage of THP-1 cells (mean ± SD) that migrated into bottom chamber during the course of the experiment.

Array probing and analysis

The gf211 "Named Genes" array (Research Genetics, Huntsville, AL) containing probes for 4,048 human genes was used to survey and quantify transcript expression in human SMCs. Labeling, hybridization, washes and data analysis was performed as specified by the manufacturer. Briefly, equivalent amounts of total RNA from each treatment condition were used as template for oligo dT-primed synthesis of ^{33}P-labeled first strand cDNA. Equivalent total cpm of column-purified labeled probe was added to the hybridization reactions and allowed to mix overnight in a roller bottle hybridization chamber. Arrays from the same lot were used for each experiment. After washing, the blots were exposed to Phosphorimager screens (Molecular Dynamics, Sunnyvale, CA) and scanned on a Storm Phosphorimager at 50 micron pixel size (Molecular Dynamics, Sunnyvale, CA). Scans were imported into the array data analysis program Pathways (Research Genetics, Huntsville, Al) and sample expression was quantified. All scans were normalized to the total genomic "landing lights" hybridization levels. Care was

taken to use equivalent total probe activities and to choose phosphorimager scans of similar maximum strength and background levels for all comparisons.

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Curriculum Vitae

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Publications:

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