Fas and Fas-Associated Death Domain Protein Regulate Monocyte Chemoattractant Protein-1 Expression by Human Smooth Muscle Cells Through Caspase- and Calpain-Dependent Release of Interleukin-1α

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Fas and Fas-Associated Death Domain Protein Regulate Monocyte Chemoattractant Protein-1 Expression by Human Smooth Muscle Cells Through Caspase- and Calpain-Dependent Release of Interleukin-1α

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Abstract—We previously reported that treatment of human vascular smooth muscle cells (SMCs) with proapoptotic stimuli, including Fas ligand plus cycloheximide (FasL/Chx), or overexpression of Fas-associated death domain protein (FADD) result in increased expression of monocyte chemoattractant protein-1 (MCP-1) and other proinflammatory genes. In this study, we demonstrate that Fas/FADD-induced MCP-1 upregulation is driven by an autocrine/paracrine signaling loop in which interleukin (IL)-1α synthesis and release are activated through caspase- and calpain-dependent processes. Untreated SMCs contain very little IL-1α protein or transcript. Both were increased greatly in response to Fas/FADD activation, primarily through an autocrine/paracrine pathway in which secreted IL-1α stimulated additional IL-1α synthesis and release. Caspase 8 (Csp8) activity increased in response to FasL/Chx treatment, and Csp8 inhibitors markedly reduced IL-1α release and MCP-1 upregulation. In contrast, Csp8 activity was not significantly increased in response to FADD overexpression and caspase inhibitors did not effect FADD-induced MCP-1 upregulation. Both FasL/Chx treatment and FADD overexpression increased the activity of calpains. Calpain inhibitors reduced IL-1α release and MCP-1 upregulation in both FADD-overexpressing SMCs and FasL/Chx-treated SMCs without blocking Csp8 activity. This indicates that calpains are not required for activation of caspases and that caspase activation is not sufficient for IL-1α release and MCP-1 upregulation. These data suggest that calpains play a dominant role in Fas/FADD-induced IL-1α release and MCP-1 upregulation and that caspase activation may function to amplify the effects of calpain activation. **(*Circ Res*. 2003;93:515-522.)**

Key Words: apoptosis ■ interleukin ■ inflammation ■ calpain ■ monocyte chemoattractant protein-1

High rates of apoptosis are often observed in atherosclerotic lesions and in blood vessels after angioplasty.[1,2] Although apoptosis is generally regarded as a noninflammatory event, we previously reported that induction of rat vascular smooth muscle cell (SMC) apoptosis, via regulated overexpression of Fas-associated death domain protein (FADD), causes local accumulation of macrophages and triggers transcriptional upregulation of monocyte chemoattractant protein-1 (MCP-1) in vitro and in vivo.[3] A specific subset of proinflammatory genes, including MCP-1, is upregulated in cultured human SMCs after treatment with Fas ligand plus cycloheximide (FasL/Chx).[3] Upregulation of these proinflammatory genes could explain the macrophage accumulation observed in our rat model of regulated induction of apoptosis as well as the reports that apoptotic SMCs often colocalize with macrophages in diseased or injured vessels.[2,4] We hypothesized that the pattern of gene induction in response to proapoptotic stimuli is characteristic of vascular SMCs and other connective tissue cells, which are relatively resistant to apoptosis. This would distinguish the response of connective tissue cells from that of lymphocytes and epithelial cells, which undergo apoptosis very rapidly and without significant upregulation of proinflammatory genes.

Most of the proinflammatory genes that are highly expressed in response to Fas stimulation are known to be regulated by the transcription factor nuclear factor (NF)-κB. NF-κB can be activated by overexpression of FADD[5,6] or caspase 8 (Csp8)[6] in various cell lines, but the mechanism underlying this activation has not been determined. Based on our previously published data,[3] we hypothesize that Fas and FADD activate NF-κB and consequent gene upregulation indirectly via activation or release of a cytokine. Because inhibition of interleukin (IL)-1α markedly decreased MCP-1 upregulation in FasL/Chx-treated SMCs,[3] we speculated that IL-1α could play this role. IL-1α is a proinflammatory chemokine whose involvement in the pathogenesis of vascu-

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lar diseases has been implicated by several studies in vitro and in vivo.[7,8] IL-1 is a potent activator of NF-κB signaling and has been reported to induce expression of MCP-1, IL-8, GRO-1, IL-6, TSG-6, and PAI2, which are among the 10 genes that we reported to be most highly upregulated after FasL/Chx stimulation of human SMCs.[9,10] In the present report, we additionally investigate the mechanisms of Fas/FADD-mediated induction of MCP-1, focusing specifically on the role of IL-1α signaling. Our findings demonstrate that Fas/FADD-induced expression of MCP-1 is predominantly mediated through increased synthesis and release of IL-1α. This increase in IL-1α release can occur independent of apoptosis per se and is regulated by both caspase- and calpain-dependent pathways.

Materials and Methods

Reagents and Cells

The following antibodies and reagents were used: anti-IL-1α antibody (5 μg/mL) and IL-1 receptor antagonist (100 ng/mL) (R&D System, Minneapolis, Minn), IDUN1529 (IDUN Pharmaceuticals, La Jolla, Calif), Z-IETD-FMK (Enzyme Systems, Livermore, Calif), MDL28170, and calpastatin-like peptide (CS-P) (Calbiochem, San Diego, Calif). Human SMC cultures (passages 7 through 9) were provided by Dr E. Raines (University of Washington, Seattle, Wash) and used between passages 7 and 10. SMCs were cultured in DMEM plus 10% FCS for 48 hours, followed by a 12-hour incubation in DMEM plus 1% FCS before initiation of experiments. SMCs were induced to undergo apoptosis by treatment with 100 ng/mL recombinant human FasL[11] (kindly provided by Dr Peter Kiener, Bristol-Myers Squibb, Princeton, NJ) plus 1.5 μg/mL cycloheximide. Inhibitors of IL-1α, caspases, and calpains were added 1 hour before treatment with FasL/Chx.

Retroviral Vector Infection

The retroviral expression vector pBM-IRES-EGFP, in which the reporter EGFP is expressed from an internal ribosome entry site downstream of the transgene cloning site, was generously provided by G. Nolan (Stanford University, Stanford, Calif). The cDNAs of FADD[12] and FADD DN[12] were cloned into the *Eco*RI/*Bam*HI cloning site and verified by sequencing. CrmA was cloned into the retroviral expression vector pBM-IRES-Puro (provided by Dr. E. Raines). High-titer retrovirus preparations were obtained using Phoenix amphotropic packaging cell line (American Type Culture Collection, Manassas, Va) as previously described.[13] For infection, 6×10^4 human SMCs were plated into 12-well plates 24 hours before infection and incubated with 3 to 5 mL virus stock for 12 to 18 hours in the presence of 5 μg/mL polybrene. Infection efficiency, determined by analyzing expression of EGFP by flow cytometry, was 45% to 50%. After retroviral infection, cells were cultured for an additional 18 hours in DMEM plus 10% FCS, followed by a 24-hour incubation in 1% FCS. CrmA-expressing SMCs were enriched by puromycin selection (9 μg/mL for 48 hours). FADD-overexpressing cells were treated with calpain and caspase inhibitors immediately after removal of the retrovirus-containing medium until cell harvest.

TaqMan (Real-Time Quantitative RT-PCR) Analysis

Total RNA from cultured cells was isolated using TRIZOL reagent (Life Technologies). RNA was reverse transcribed by standard methods using RT (Life Technologies). For TaqMan real-time PCR, MCP-1, IL-1α, and 18S primers and TaqMan probe were purchased from PE Biosystems. An Applied Biosystems Prism 5700 Sequence Detection System (Applied Biosystems) was used with the default thermal cycling program (95°C for 10 minutes followed by 40 cycles of 95°C, 20 seconds, 60°C, 1 minute). Reactions were performed in triplicate and normalized by dividing the relative transcript levels of the gene of interest by relative level of 18S RNA transcript.

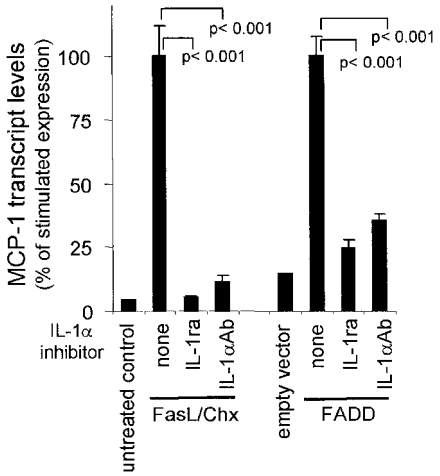

Figure 1. Inhibition of IL-1α signaling decreases Fas/FADD-induced MCP-1 upregulation. Cultured human SMCs were treated with FasL (100 ng/mL) plus Chx (1.5 μg/mL) for 24 hours or infected with FADD plasmid 60 hours before measurement. Inhibitors were included from 1 hour before addition of FasL/Chx and immediately after infection with the FADD plasmid. IL-1ra was used at 100 ng/mL, and the neutralizing anti–IL-1α antibody (IL-1αAb) at 5 μg/mL. MCP-1 transcript levels were determined by TaqMan real-time RT-PCR and expressed as percentage (mean±SEM from at least 3 experiments, each in triplicate) of levels in FasL/Chx-stimulated or FADD-overexpressing cells in the absence of inhibitors.

Immunocytochemistry and ELISA for IL-1α Protein

Cells were fixed with 2% paraformaldehyde and incubated with 5 μg/mL monoclonal anti-IL-1α antibody (R&D System) in blocking buffer for 1 hour, followed by incubation with rhodamine-conjugated rabbit anti-mouse secondary antibody (DAKO, A/S, Denmark). Concentrations of IL-1α in culture supernatants, cell lysates, and nuclear protein extracts were determined by ELISA using a human IL-1α–specific ELISA development system (DuoSet, R&D Systems) according to the vendor's instructions. Conditioned medium was harvested 24 hours after medium change (DMEM plus 0.5% to 1% FCS). Proteins were extracted from whole cells using lysis buffer (1% Triton, 10 mmol/L Tris, 1.0 μg/mL aprotinin, 10 μg/mL leupeptin, 0.2 mmol/L phenylmethylsulfonyl fluoride), and protein concentration was determined using the MicroBCA assay (Pierce). Nuclear extracts were prepared as described by Dignam et al,[14] and protein content was determined using the MicroBCA assay.

Csp8 Activity Assay

Cell extracts were prepared as described above. Protein extracts were added to 50 μL of ×2 caspase cleavage buffer (40 mmol/L PIPES, pH 7.2, 200 mmol/L NaCl, 20% sucrose, 0.2% CHAPS, 20 mmol/L DTT) containing the Csp8 substrate 40 μmol/L AC-IETD-AMC and incubated for 120 minutes at 37°C. To determine specific activity, fluorescence was measured using a Packard FluoroCount plate reader (Packard Instruments) with an excitation wavelength of 360 nmol/L and an emission wavelength of 460 nmol/L.

Results

Role of IL-1α in FasL/Chx- and FADD-Induced MCP-1 Upregulation

As previously reported,[3] treatment of human SMCs with FasL/Chx (24 hours) resulted in increased expression of MCP-1, and this upregulation was almost completely blocked by coincubation with either recombinant IL-1 receptor antagonist (IL-1ra) or a neutralizing anti–IL-1α antibody (Figure

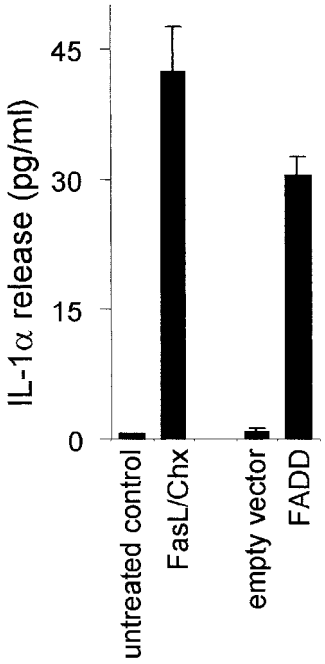

Figure 2. Release of IL-1α in response to Fas/FADD stimulation. Cultured human SMCs were treated with apoptotic stimuli as in Figure 1. IL-1α levels in the culture medium at the end of the treatment period were determined by IL-1α–specific ELISA, as described in Materials and Methods. Data are expressed as picogram of IL-1α protein per milliliter of conditioned medium (mean±SEM from at least 3 experiments, each in triplicate).

1). An early event in Fas activation of the apoptosis signaling cascade is the interaction of Fas with the adaptor protein FADD. Overexpression of FADD using a retroviral vector was sufficient to upregulate MCP-1 transcript expression in human SMCs (Figure 1). Similar to FasL/Chx-treated SMCs, MCP-1 upregulation in response to FADD overexpression was significantly reduced by including IL-1α inhibitors in the culture medium (Figure 1). MCP-1 transcription was not increased by overexpression of a dominant-negative form of FADD that lacks the death effector domain that serves as the docking site for Csp8 (data not shown). This argues against the possibility that FADD induces transcription in human SMCs via retrograde activation of Fas or tumor necrosis factor signaling, eg, through recruitment of adaptor proteins such as Dax[15] or TRADD.[5]

In most inflammatory conditions, the predominant form of IL-1 is thought to be IL-1β, which is secreted largely by leukocytes. Neither FasL/Chx-treated nor FADD-overexpressing SMCs released detectable amounts of IL-1β, as evaluated by an IL-1β–specific ELISA (data not shown). However, SMCs did secrete significant amounts of IL-1α in response to these treatments (Figure 2).

Regulation of IL-1α Expression

To determine whether Fas/FADD activation stimulates the release of IL-1α from a presynthesized store or whether de novo IL-1α synthesis precedes the increased secretion, we determined levels of IL-1α transcript and intracellular protein. IL-1α transcript levels were low in untreated SMCs but increased dramatically after incubation with FasL/Chx and in response to FADD overexpression (Figure 3A). Coincubation with IL-1ra or with a neutralizing anti-IL-1α antibody reduced IL-1α transcript levels by more than 85% (Figure 3A), indicating that Fas/FADD-induced IL-1α upregulation is mediated or amplified, to a large extent, via an autocrine/paracrine pathway. As required for such an autostimulatory pathway, exogenous IL-1α was capable of upregulating IL-1α transcript expression (Figure 3A).

To evaluate intracellular IL-1α protein levels, we used an ELISA that detects both the precursor and mature forms of the cytokine. Untreated SMCs contained very small amounts of IL-1α protein. FasL/Chx incubation and FADD overexpression increased intracellular levels of IL-1α protein by more than 30- and 10-fold, respectively (Figure 3B). The concentration of Chx that we used (1.5 μg/mL) only partially blocked overall protein synthesis, as measured by radiolabeled amino acid incorporation (80% inhibition, data not shown). The net increase in IL-1α protein expression in response to FasL/Chx treatment presumably reflects the net effect of decreased efficiency of overall protein synthesis and greatly increased transcript levels for this specific protein.

To determine the subcellular localization of the cell-associated IL-1α protein, we immunostained cultured human SMCs using an antibody against IL-1α (Figures 4A through 4F). Untreated SMCs did not show detectable immunostaining (Figures 4A and 4D), consistent with the very low content of IL-1α protein detectable by ELISA in whole cell extracts (Figure 3B). FasL/Chx treatment and FADD overexpression, however, resulted in a strong nuclear signal for IL-1α (Figures 4B and 4E), which was confirmed by ELISA, showing a 60-fold increase in IL-1α protein in nuclear

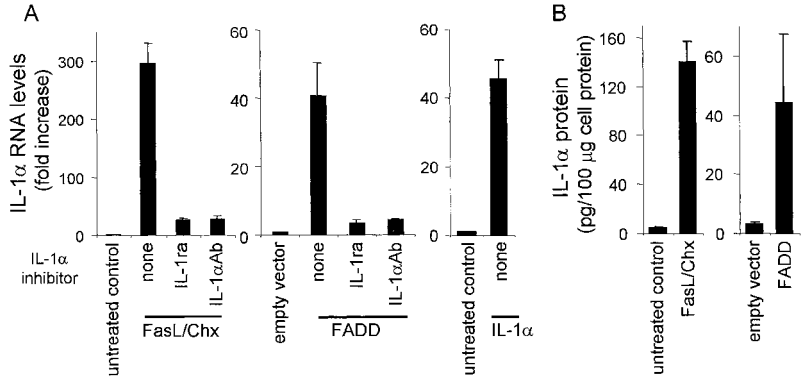

Figure 3. Increased IL-1α transcript and intracellular protein in response to Fas/FADD activation. Cultured human SMCs were treated with apoptotic stimuli and inhibitors as in Figure 1. A, IL-1α transcript levels were determined by TaqMan real-time RT-PCR. Data are expressed as fold increase over level in untreated control SMCs (mean±SEM from at least 3 experiments, each in triplicate). B, IL-1α protein levels in cell lysates were determined by IL-1α–specific ELISA. Results are expressed as picogram of IL-1α protein per 100 μg of total cell protein (mean±SEM from at least 3 experiments).

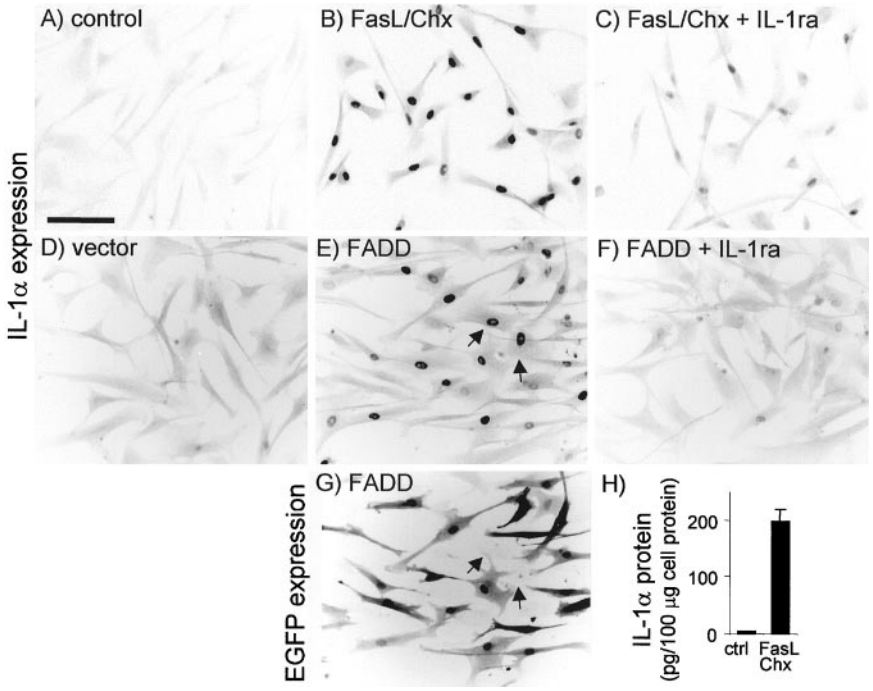

Figure 4. Nuclear accumulation of IL-1α in response to Fas/FADD stimulation. Cultured human SMCs were treated with apoptotic stimuli and inhibitors as in Figure 1. A through F, Cells were fixed, immunostained with an anti-IL-1α antibody, and examined by fluorescent microscopy. Scale bar=50 μm. G, The same field of FADD plasmid–infected cells shown in panel E is evaluated for the infection reporter EGFP. Arrows in E and G indicate cells that are positive for nuclear IL-1α protein but negative for EGFP expression. H, To quantitate nuclear content of IL-1α protein, nuclear protein extracts were evaluated by IL-1α–specific ELISA. Results are expressed as picogram of IL-1α protein per 100 μg nuclear protein (mean±SEM, n=3).

extracts (Figure 4H). Coincubation with IL-1ra (Figures 4C and 4F) or with an anti-IL-1α antibody (not shown) almost completely abolished FasL/Chx- and FADD-induced nuclear staining, suggesting that nuclear accumulation of IL-1α requires release and receptor binding of the cytokine. Figure 4G shows that after retroviral infection, nuclear accumulation of IL-1α was not limited to FADD-expressing cells (marked by coexpression of EGFP). This is a graphic demonstration that a proapoptotic stimulus in a subset of SMCs can result in activation of neighboring cells. Taken together, these findings suggest that Fas/FADD initiate synthesis and release of IL-1α, which in turn causes upregulation of proinflammatory genes, including IL-1α itself, in both the initially stimulated SMCs and in neighboring cells.

Fas and FADD Activate IL-1α Signaling via Caspase-Dependent and Caspase-Independent Pathways

Cell death and caspase activation have been implicated in IL-1α processing or release in other systems.[16,17] Figure 5 shows that the pan-caspase inhibitor IDUN1529 reduced FasL/Chx- and FADD-induced IL-1α release, with a maximum inhibition of 90% and 60%, respectively. Similar results were obtained by inhibition of Csp8 using Z-IETD-FMK or overexpression of the cowpox virus protein CrmA (data not shown).

As previously published, IDUN1529 and Z-IETD-FMK essentially completely blocked apoptotic death in response to FasL/Chx treatment but inhibited MCP-1 upregulation by only ≈50% (Figures 6A and 6B).[3] CrmA overexpression was as effective as IDUN1529 in inhibiting FasL/Chx-induced Csp8 activity and apoptosis (Figures 6A and 6C) but inhibited MCP-1 upregulation by only ≈35% (Figure 6B). FADD-overexpressing SMCs did not exhibit a significant increase in Csp8 activity or show overt signs of apoptotic cell death during the time period studied (60 hours) (Figures 6C and 6A), and MCP-1 upregulation in response to FADD overexpression was completely unaffected by the presence of the caspase inhibitor IDUN1529 (Figure 6B). This indicates that IL-1α release and MCP-1 upregulation in response to proapoptotic stimuli can be augmented by, but do not require, caspase activation and can be dissociated from loss of cell integrity or other changes that occur during the terminal stages of apoptotic cell death.

Calpain Activation Plays a Significant Role in Fas/FADD-Induced IL-1α Signaling and MCP-1 Upregulation

Other than caspase activation and cell death, the only known mechanism regulating IL-1α release involves the activation of calpains, a family of calcium-dependent cysteine pro-

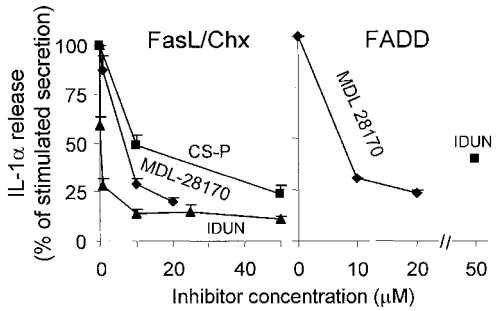

Figure 5. Fas and FADD activate IL-1α release via caspase- and calpain-dependent pathways. Cultured human SMCs were treated with apoptotic stimuli and different concentrations of the pan-caspase inhibitor IDUN1529 or the calpain inhibitors MDL28170 and CS-P as in Figure 1. IL-1α concentration in conditioned medium was determined by IL-1α–specific ELISA. Data are expressed as the percentage of IL-1α released from FasL/Chx- or FADD-activated SMCs in the absence of inhibitors (mean±SEM from at least 3 experiments, each in triplicate).

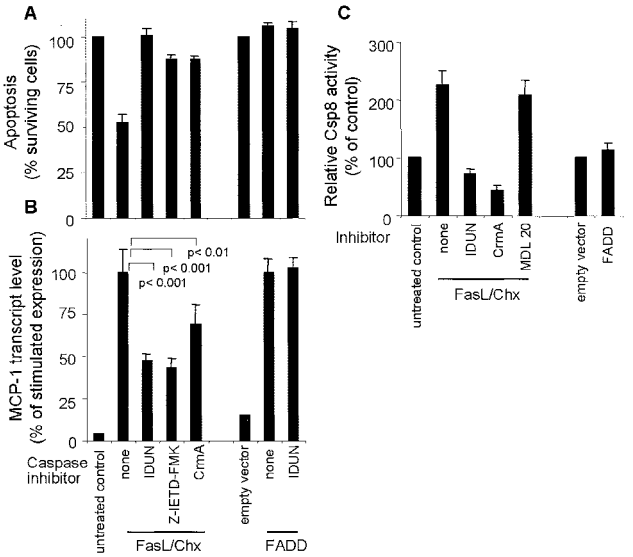

Figure 6. Caspase inhibitors block apoptosis and decrease MCP-1 upregulation in response to FasL/Chx but not FADD overexpression. Cultured human SMCs were treated with apoptotic stimuli and inhibitors as in Figure 1. The pan-caspase inhibitor IDUN1529 and the caspase-8 inhibitor Z-IETD-FMK were used at 50 μmol/L, and the calpain inhibitor MDL28170 was used at 20 μmol/L. CrmA-expressing SMCs were enriched by puromycin selection after infection with the CrmA/Puro vector. A, Apoptosis was evaluated by determining the numbers (mean±SEM from at least 3 experiments) of surviving attached cells as a percentage of the numbers in untreated control cultures. B, MCP-1 transcript levels were determined by TaqMan real-time RT-PCR and normalized to 18S ribosomal RNA levels. Data are expressed as a percentage of the MCP-1 RNA level in FasL/Chx-stimulated or FADD-overexpressing cells in the absence of inhibitors (mean±SEM from at least 3 experiments, each in triplicate). C, Csp8 activity in whole-cell extracts was determined using the AC-IETD-AMC fluorogenic substrate. Data are expressed as relative Csp8 activity as a percentage of activity in untreated control cells (mean±SEM from at least 3 experiments).

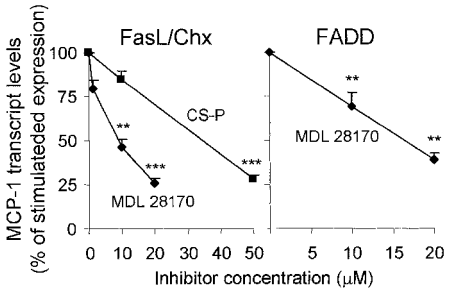

Figure 8. Calpain inhibitors block Fas/FADD-induced MCP-1 upregulation. Cultured human SMCs were treated with apoptotic stimuli and the calpain inhibitors MDL28170 and CS-P at the concentrations indicated. MCP-1 transcript levels were determined by TaqMan real-time RT-PCR. Data are expressed as percentage of the of MCP-1 RNA level in FasL/Chx-stimulated or FADD-overexpressing cells in the absence of inhibitors (mean±SEM from at least 3 experiments, each in triplicate). $^{**}P \leq 0.015$; $^{***}P < 0.001$.

teases.[18,19] To evaluate regulation of calpain activity in SMC response to apoptotic stimuli, we used BOC-LM-CMAC, a calpain-specific fluorescent substrate.[20] Figure 7 shows that both FasL/Chx treatment and FADD overexpression resulted in a 2-fold increase in calpain activity. To evaluate the importance of this calpain activation in IL-1α signaling and MCP-1 upregulation, we incubated SMCs with either the synthetic calpain inhibitor MDL28170 or a peptide (CS-P) encoded by exon 1B of the endogenous calpain inhibitor calpastatin. The calpain inhibitors reduced FasL/Chx-induced IL-1α release as effectively as did caspase inhibitors and were more potent than caspase inhibitors in inhibiting release from FADD-overexpressing SMCs (Figure 5). Consistent with the inhibition of IL-1α release, calpain inhibitors blocked FasL/Chx- and FADD-triggered MCP-1 upregulation in a dose-dependent fashion (Figure 8). Calpain activation has been reported to be involved in regulation of apoptosis in other cell types.[21–23] However, calpain inhibitors did not induce SMC death (assayed by LDH release, data not shown) and did not reduce Csp8 activation by FasL/Chx (Figure 6C), which argues against the possibility that calpain inhibitors block IL-1α release and gene upregulation through inhibition of apoptotic cell death. Because calpain inhibitors did not affect Csp8 activation in FasL/Chx-treated SMCs, it seems that caspase activation is not sufficient to induce IL-1α release and MCP-1 upregulation and that calpain activation has a central role in the regulatory pathways from Fas/FADD to IL-1α release and gene induction. However, caspases do augment these path-

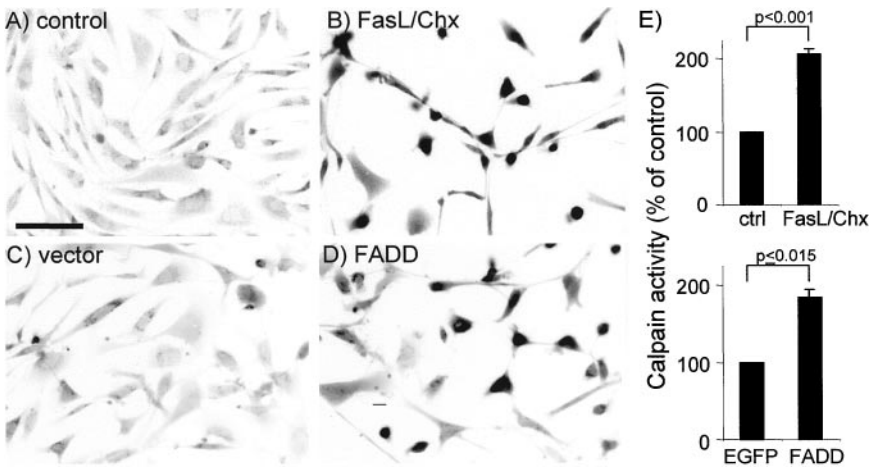

Figure 7. Calpain activation in response to Fas/FADD stimulation. Cultured SMCs that were treated with FasL/Chx (24 hours) or were expressing FADD (60 hours) were loaded with 10 μmol/L of the calpain substrate Boc-LM-CMAC (Molecular Probes)[20] that becomes fluorescent after cleavage by calpain. After 40 minutes, the cells were washed with PBS and observed by fluorescent microscopy using a Hamamatsu C5810 digital camera on a Nikon Optiphot-2 microscope. Scale bar=50 μm. Images were pseudo-colored to grayscale and inverted so that darkness correlates with increased fluorescence intensity and calpain activity. The intensity of fluorescent signal from the reaction product was quantitated using Image Quant (Molecular Dynamics) and is shown in the bar graphs as mean±SEM of increase over signal from untreated cultures (n=3 to 4).

ways, as evidenced by the observation that the combination of MDL and IDUN was more effective than either alone in inhibiting MCP-1 upregulation in response to FasL/Chx (74% inhibition by MDL28170, 54% inhibition by IDUN, 85% inhibition by both; $P<0.01$; data not shown).

Discussion

Proinflammatory gene expression (including MCP-1) is greatly upregulated in vascular SMCs in response to FasL/Chx treatment and FADD overexpression[3] (Figure 1). The results presented above confirm that this upregulation is predominantly dependent on signaling through IL-1α as an intermediary factor. IL-1α and IL-1β use the same receptors but differ considerably in cell source and in mechanisms of regulation. In general, IL-1β is produced by inflammatory cells and functions as a systemic mediator in acute and chronic inflammatory diseases. IL-1α is produced by connective tissue cells, including vascular SMCs, and is usually associated with locally confined inflammatory conditions[24] and other conditions that increase cell stress.[25,26] Vascular diseases, including atherosclerotic plaques and restenotic lesions, have characteristics of locally circumscribed inflammation, which typically involves IL-1α signaling. Human vascular SMCs are able to process and release IL-1α but not IL-1β,[27] consistent with our finding that Fas and FADD activation of SMCs results in release of IL-1α and not IL-1β (Figure 2).

What regulates IL-1α production and function in response to proapoptotic stimuli? We first considered the possibility that FasL/Chx and FADD induce the processing or secretion of an intracellular store of presynthesized IL-1α protein. Apoptosis of vascular SMCs in vivo often occurs in environments that contain high cytokine concentrations.[1,2,28] Under these circumstances, it is possible that SMCs may contain significant amounts of IL-1α protein and are thus preconditioned to release the cytokine in response to Fas and FADD activation. However, unstimulated cultured SMCs contain only small amounts of IL-1α protein (Figure 3B) and low levels of IL-1α transcript (Figure 3A). IL-1α transcript and protein expression increase after stimulation by FasL/Chx or FADD, and this increase is largely blocked by coincubation with IL-1ra or anti-IL-1α antibody (Figures 3A and 3B). Because IL-1α expression by SMCs is itself stimulated by IL-1α (Figure 3A), it seems that secreted IL-1α functions in a positive-feedback autocrine/paracrine loop that amplifies an initial IL-1α stimulus over the ensuing 24 hours. Although it is possible that this autocrine/paracrine loop is initiated by release of the small amount of presynthesized IL-1α, preliminary data suggest that the initial upregulation reflects a rapid but brief direct upregulation of IL-1α (and MCP-1 expression) by Fas/FADD that does not depend on prior IL-1α release. In the present report we focused on evaluating the regulation of the late/amplification phase of IL-1α synthesis and release.

Our immunohistochemical studies showed that Fas and FADD stimulation resulted in accumulation of IL-1α protein within the nucleus (Figures 4A through 4F). Nuclear accumulation was dependent on release of IL-1α itself, because it was efficiently inhibited by agents that blocked the activity of extracellular IL-1α. Several reports have described nuclear localization of IL-1α, but how and in what form IL-1α reaches the nucleus are still unknown, and the role of nuclear localization in IL-1–dependent signal transduction is controversial.[29–31] Nuclear localization of the amino-terminal pro-piece of IL-1α has been found to induce apoptosis in tumor cell lines but not in normal cells.[32] We found that inhibition of secreted IL-1α using IL-1ra completely blocked Fas/FADD-induced nuclear accumulation of IL-1α in human SMCs but had no inhibitory effect on cell death (data not shown). Although the significance of nuclear IL-1α is not clear, evaluation of IL-1α nuclear translocation was a useful marker to demonstrate that IL-1α activation effectively spread from FADD-overexpressing SMCs to neighboring cells that were not expressing the transgene (Figures 4E and 4G).

Like IL-1β, IL-1α is synthesized as a larger pro-form, does not contain a secretory signal sequence, and is not released via classic secretory pathways.[33] Cultured cells transiently transfected with plasmids encoding either pro-IL-1α or mature 17-kDa IL-1α do not release the cytokine into the medium, suggesting that IL-1α is not released without a stimulus.[25,34] Our data indicate that release of IL-1α in response to FasL/Chx stimulation and FADD overexpression does not merely reflect the disruption of the cell membrane that follows terminal apoptosis, because it occurs relatively early after stimulation and can still occur even when terminal apoptosis is not observed (Figure 6). In contrast to IL-1β activation/release, which is known to be regulated through activation of caspase 1/ICE, which then cleaves the precursor to the mature form of IL-1β, IL-1α is not a direct substrate for this caspase.[35] However, previous reports suggested that other caspases play important, although undefined, roles in IL-1α release.[16,17] Our data confirm a role for caspases in IL-1α production and release in response to FasL/Chx-treated human SMCs, because IL-1α release was greatly reduced by caspase inhibitors (Figure 5). In contrast, IL-1α release in FADD-overexpressing cells was not accompanied by a significant increase in Csp8 activation, and caspase inhibition was much less effective in reducing IL-1α production or release.

Other than cell death and caspase-activated processes, the only known mechanism of IL-1α release involves calpain-mediated cleavage of pro-IL-1α into its 17-kD mature form. Calpains are calcium-dependent cysteine proteases that are widely expressed as both ubiquitous and tissue-specific isoforms.[18,19,33] Calpains have been associated with apoptosis, particularly in neuronal cells and lymphocytes, as both death-preventing and death-inducing factors,[21,22,36] but the mechanisms of calpain activation in cells are still largely unclear. In vitro, calpains undergo calcium-dependent autolysis, a multistep self-proteolytic event, resulting in increased enzyme activity and sensitivity to calcium.[37] It has been proposed that in vivo membrane phospholipids, such as phosphatidylinositol, may regulate both the membrane association of calpain and the calcium requirements for autolytic processing.[38,39] We found that FasL/Chx and FADD treatment increased calpain activity in SMCs (Figure 7). Because Fas and FADD treatment of SMCs activate phosphatidylinositol-3 kinase (authors' unpublished observation, 2003), it is possible that the elevated levels of PIP3 increase membrane association and proteolytic activation of

calpains. Caspase-mediated cleavage and inactivation of the endogenous calpain inhibitor calpastatin has also been proposed as a mechanism for calpain activation, suggesting a possible interconnection between calpain and caspase pathways.[40]

FasL/Chx- and FADD-triggered IL-1α production (Figure 5) and MCP-1 upregulation (Figure 8) were more effectively inhibited by the calpain inhibitors MDL28170 and CS-P than by caspase inhibitors (compare Figures 5, 6, and 8). Calpain inhibitors neither induced cell death by themselves (data not shown) nor prevented Csp8 activation in response to FasL/Chx treatment (Figure 6C), suggesting that calpains are not acting by modulating Fas/FADD-induced apoptotic death of SMCs. Because calpain inhibitors suppressed IL-1α release and MCP-1 upregulation in FasL/Chx-treated SMCs without blocking Csp8 activity, it seems that caspase activation is not sufficient for IL-1α release and MCP-1 upregulation. Caspases seem to play an even smaller role in SMC response to overexpression of FADD. FADD overexpression did not result in marked Csp8 activation during the period in which MCP-1 expression was upregulated, and caspase inhibitors did not block MCP-1 upregulation. These data suggest that calpains play a more important role in Fas/FADD-induced IL-1α release and MCP-1 upregulation than do caspases and that caspase activation may function to amplify the effects of calpain activation. In contrast to IL-1β, IL-1α can be released and biologically active in both precursor and cleaved mature forms.[16] We used a variety of approaches to determine which forms are released by SMCs in response to Fas/FADD stimulation and activation of caspases and calpains. However, fibroblasts and smooth muscle cells release less IL-1 than do monocyte and macrophages, where most of the work on IL-1 processing has been done, and our results were inconclusive because of the very low levels of IL-1α protein (data not shown).

The results presented above provide insights into the proinflammatory signaling pathways initiated by Fas and FADD and support the hypothesis that these proteins play important roles in the regulation of processes other than terminal apoptotic cell death. We demonstrate that Fas/FADD-induced MCP-1 upregulation is driven by an autocrine/paracrine signaling loop in which IL-1α synthesis and release are activated through caspase- and calpain-dependent processes. IL-1α activation could be of physiological significance in vivo as a mechanism for spreading of Fas/FADD-induced proinflammatory signals to healthy neighboring cells. Our findings suggest the possibility that calpains may represent a therapeutic target to prevent or treat vascular inflammatory disease.

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