Macrophages but not smooth muscle cells undergo z-VAD-fmk-induced nonapoptotic cell death depending on receptor interacting protein 1 (RIP1) expression: implications for the stabilization of macrophage-rich atherosclerotic plaques

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Abbreviations: Boc-D, t-butyloxycarbonyl-Asp(O-methyl)-fluoromethylketone; LC3, microtubule associated protein light chain 3; mTOR, mammalian target of rapamycin; RIP1, receptor interacting protein 1; SMC, smooth muscle cell; TNFα, tumor necrosis factor-α; TNF-R, TNF-receptor; z-FA-fmk, benzyloxy carbonyl-Phe-Ala-fluoromethylketone; z-VAD-fmk, benzyloxy carbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone

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Abstract

Several lines of evidence suggest that macrophages play a key role in atherosclerotic plaque destabilization and rupture. Selective removal of macrophages from plaques via pharmacological therapy could therefore represent a promising approach to stabilize ‘vulnerable’, rupture-prone lesions. Yet, how macrophages can be eliminated from plaques without influencing other cell types, including smooth muscle cells (SMCs), is unknown. In the present study, we report that benzoyloxycarbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk), a caspase inhibitor with broad specificity, induces nonapoptotic cell death of J774A.1 and RAW264.7 macrophages, but not of SMCs. Cell death was characterized by bulk degradation of long-lived proteins, processing of microtubule associated protein light chain 3, and cytoplasmic vacuolization, which are all markers of autophagy. However, also necrosis occurred and the number of necrotic cells rapidly increased during z-VAD-fmk treatment. Primary mouse peritoneal macrophages were resistant to z-VAD-fmk mediated cell death, but unlike SMCs they underwent z-VAD-fmk mediated necrosis after pre-treatment with IFN-γ. Further evidence indicated that the expression level of receptor interacting protein 1 (RIP1) mediates the sensitivity to z-VAD-fmk. Importantly, upon z-VAD-fmk treatment J774A.1 macrophages overexpressed and secreted several chemokines and cytokines, including tumor necrosis factor-α (TNFα). The combination of z-VAD-fmk and TNFα, but not TNFα alone, induced SMCs necrosis via a mechanism that required RIP1 expression. These results suggest that z-VAD-fmk, despite its selective cell death inducing capacity, would be detrimental for the stability of atherosclerotic plaques due to enlargement of the necrotic core, stimulation of inflammatory responses and indirect induction of SMC death.
Introduction

Monocyte recruitment to the arterial wall is an early event in atherogenesis (Lusis, 2000). Resident monocytes accumulate lipids and are rapidly transformed into macrophage-derived foam cells. Lesional macrophages contribute to apoptosis of smooth muscle cells (SMCs) via Fas/Fas-L interactions, production of reactive oxygen species and the release of proinflammatory cytokines (Boyle, 2005). Moreover, secretion of metalloproteinases by macrophages results in breakdown of interstitial collagen fibers and thinning of the fibrous cap (Newby, 2005). In line with these findings, it is generally assumed that macrophages play a key role in atherosclerotic plaque destabilization and rupture (Boyle, 2005; Hansson, 2005). Given the knowledge that atherosclerosis is an inflammatory disease, it has been proposed that powerful immunosuppressant or anti-inflammatory agents might offer attractive therapeutic opportunities for the prevention of acute coronary syndromes (Hansson, 2005). However, an increased incidence of cardiovascular events in patients treated with a cyclooxygenase-2 inhibitor indicate the need for a cautious approach to the use of this type of anti-inflammatory compounds in cardiovascular disease (Bresalier et al., 2005). Removal of macrophages from plaques via pharmacological therapy represents a promising alternative approach to stabilize ‘vulnerable’, rupture-prone lesions. Yet, the mechanisms whereby macrophages can be eliminated from plaques without influencing other cell types, including SMCs, are unknown. Systemic therapy with statins reduced but did not eliminate macrophages from atherosclerotic plaques (Crisby et al., 2001). Recently, Yu et al. (2004) demonstrated that the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk) induces nonapoptotic death of monocytes and macrophages. Caspase inhibitors such as z-VAD-fmk are widely used both in vitro and in vivo to block cytokine release and initiation of apoptosis (Ekert et al., 1999; Nicholson, 2000). It is plausible that these compounds do not stimulate nonapoptotic death in all mammalian cell types. In the present study, we tested the hypothesis that z-VAD-fmk induces cell death of macrophages, but not of SMCs. The potential of z-VAD-fmk in the treatment of rupture-prone atherosclerotic plaques is discussed.
Methods

Cell culture. The murine macrophage cell line J774A.1 was grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 20 U/ml polymyxin B and 10% fetal bovine serum. Alternatively, peritoneal macrophages were isolated 5 days after injection of 2 ml Brewer’s thioglycolate medium (Sigma-Aldrich, St. Louis, MO) into the peritoneal cavity of C56BL/6 mice as reported previously (McCarron et al., 1984). Primary macrophages were added to culture flasks and allowed to adhere for 2 hours at 37°C. Non-adherent cells were removed by 3 washes of warm medium. Adherent cells were >99% macrophages as assessed by immunocytochemical detection of the macrophage marker F4/80 (anti-F4/80, clone Cl:A3-1; Serotec, Oxford, UK). Smooth muscle cells were isolated from mouse or rabbit aorta by collagenase type 2 (Worthington, Lakewood, NJ) and elastase (Sigma-Aldrich) digestion (60-90 minutes at 37°C) at 300 U/ml and 5 U/ml final concentration, respectively, and cultured in Ham F10 medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. C2C12 myoblasts were grown in DMEM containing 10% fetal bovine serum and antibiotics. z-VAD-fmk, Boc-D-fmk (t-butyloxycarbonyl-Asp(O-methyl)-fluoromethylketone) and z-FA-fmk (benzyloxycarbonyl-Phe-Ala-fluoromethylketone) (Alexis, Lausen, Switzerland) were stored at −20°C as a 44.1 mM stock solution in DMSO. Only fresh dilutions of the peptide inhibitor stocks were used in serum-containing culture medium. Evaluation of cell viability was based on the incorporation of the supravital dye neutral red by viable cells (Lowik et al., 1993). To examine de novo protein synthesis, cells were pulse-labeled for 1 hour at 37°C with 5 µCi Pro-mix L-[35S] in vitro cell labeling mix (Amersham Biosciences, Uppsala, Sweden) in cysteine/methionine free DMEM (Invitrogen). After homogenization of cells in hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100), labeled proteins were precipitated with 10% trichloroacetic acid, resuspended in 0.2 N NaOH and measured by liquid scintillation counting. Degradation of long-lived proteins was determined in serum-containing medium according to a method previously reported (Furuta et al., 2004). For DNA fragmentation assays, cultured cells (10^6) were lysed in 0.5 ml hypotonic lysis buffer supplemented with 250
µg proteinase K. Lysates were incubated for 1 hour at 50°C, then supplemented with 5 µl volumes of DNase-free RNase A (2 mg/ml) and incubated for an additional hour at 37°C. The samples were precipitated overnight with 1/10 volume of 3 M sodium acetate and one volume of isopropanol. DNA pellets were air-dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). After electrophoresis in 2% agarose, DNA laddering was visualized under UV light by staining the agarose gel with ethidium bromide. To detect initiation of necrosis, adherent cells were trypsinized (0.05% trypsin for SMCs, 0.25% trypsin for J774A.1 macrophages) and collected by centrifugation. Cells were resuspended in PBS, labeled with propidium iodide (1 µg/ml final concentration) and immediately analyzed on a FACSort flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using Cell Quest software (BD Biosciences). Alternatively, Hoechst 33258 (Sigma-Aldrich) was prepared in distilled water at 1 mg/ml and added to the culture medium at a final concentration of 10 µg/ml. After 30 min at 37°C, Hoechst labeling was examined with a fluorescence microscope (Olympus, Tokyo, Japan) using UV light excitation. Secretion of IL1β, TNFα and MIP-1α was determined via Quantikine colorimetric sandwich ELISA (R&D Systems, Minneapolis, MN).

**Plasmid construction and transient transfection.** The mouse receptor interacting protein (mRIP) coding sequence was excised from plasmid pEF1-mRIP (BCCM/LMBP plasmid collection, Ghent, Belgium) as a BamHI/XbaI fragment and cloned in the similarly opened vector pEGFP-N3 (Clontech Laboratories, Palo Alto, CA), obtaining pCMV-mRIP as a final expression plasmid. A control plasmid without mRIP insert was constructed by digesting pEGFP-N3 with EcoRI/NotI, followed by blunting of vector ends with Klenow polymerase and self ligation. SMCs (10^6 cells) were transiently transfected with 5 µg purified plasmid DNA via Nucleofector technology (program U-25) using the Human AoSMC Nucleofector kit (Amaxa GmbH, Koeln, Germany).

**Western blot analysis.** Cultured cells were lysed in an appropriate volume of Laemmli sample buffer (BioRad, Richmond, CA). Cell lysates were then heat-denatured for 4 minutes in boiling water and loaded on a SDS polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA).
according to standard procedures. Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% non-fat dry milk (BioRad) for 1 hour. After blocking, membranes were probed overnight at 4°C with primary antibodies in antibody dilution buffer (TBS-T containing 1% non-fat dry milk), followed by 1 hour incubation with secondary antibody at room temperature. Antibody detection was accomplished with SuperSignal West Pico or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

The following primary antibodies were used: mouse monoclonal anti-RIP1 (clone 38) and anti-caspase-3 (clone 19) from BD Transduction Laboratories (Lexington, KY), mouse monoclonal anti-β-actin (clone AC-15) from Sigma-Aldrich and rabbit polyclonal anti-cleaved caspase-3 from Cell Signaling Technology (Beverly, MA). Rat anti-LC3 polyclonal antibody raised against the synthetic peptide H2N-PSDRPFKQRRSFADC-CONH2 was prepared by Eurogentec (Liege, Belgium). Peroxidase-conjugated secondary antibodies were purchased from DakoCytomation (Glostrup, Denmark).

Electron microscopy. Samples were fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 hours, then rinsed (3x10 minutes) in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% saccharose and postfixed in 1% OsO4 solution for 1 hour. After dehydration in an ethanol gradient (70% ethanol [20 minutes], 96% ethanol [20 minutes], 100% ethanol [2x20 minutes]), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope at 80 kV.

Microarray analysis. Total RNA was prepared from cultured cells using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). All RNA samples were treated with RNase-free DNase I. RNA quality was verified on an Agilent 2100 Bioanalyser using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA). Samples were then analyzed by the Microarray Facility of the Flanders Interuniversity Institute for Biotechnology (VIB, Leuven, Belgium) using the Whole Mouse Genome Oligo Microarray Kit (Agilent
Technologies) representing over 41,000 mouse genes and transcripts. To define differential gene induction, we used a 5-fold threshold value.

**Real time quantitative RT-PCR.** Relative abundance of mRNA species was assessed using the 5′ fluorogenic nuclease assay (TaqMan) on an ABIPrism 7700 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers and fluorogenic probes (Table 1) were designed using Primer Express software (Applied Biosystems). Probes were 5′-FAM (reporter) and 3′-TAMRA (quencher) labeled. Quantitative RT-PCR was performed in duplicate in 25 µl reaction volumes consisting of 1× Master Mix and 1× Multiscribe and RNase inhibitor Mix (TaqMan One Step PCR Master Mix Reagents Kit, Applied Biosystems). PCR cycling parameters were: reverse transcription at 48°C for 30 minutes, inactivation of RT at 95°C for 10 minutes, followed by 40 cycles consisting of incubations at 95°C for 15 seconds and 60°C for 1 minute. Relative expression of mRNA species was calculated using the comparative C\textsubscript{T} method. All data were controlled for quantity of RNA input by performing measurements on the endogenous reference gene \(\beta\)-actin (Taqman \(\beta\)-actin detection reagent, Applied Biosystems).

**Statistical analysis.** Values are presented as mean ± S.E.M. Probability levels less than 0.05 were considered as statistically significant.
Results

z-VAD-fmk induces nonapoptotic cell death in macrophages, but not in smooth muscle cells. Mouse J774A.1 macrophages and vascular smooth muscle cells (SMCs) isolated from mouse or rabbit aorta were treated in vitro with the broad spectrum caspase inhibitor z-VAD-fmk. In contrast to SMCs, viability of J774A.1 cells treated with z-VAD-fmk rapidly decreased (Fig. 1A). Similar results were obtained with z-VAD-fmk treated RAW264.7 macrophages (data not shown), suggesting a cell type specific initiation of cell death. Caspase family inhibitor Boc-D-fmk, but not cysteine protease inhibitor z-FA-fmk induced J774A.1 macrophage cell death at the same concentration as z-VAD-fmk (not shown). Besides resistance against z-VAD-fmk induced cell death, SMCs showed normal cell proliferation in the presence of z-VAD-fmk. Resistance against z-VAD-fmk induced cell death was also observed in the mouse myoblast cell line C2C12. Dose-response experiments showed that J774A.1 macrophage cell death could be detected within 24 hours if cells were treated with ≥30 µM z-VAD-fmk (Fig. 1B). However, pretreatment of J774A.1 cells with IFN-γ increased the sensitivity for z-VAD-fmk considerably (Fig. 1A-B). Nonetheless, IFN-γ primed SMCs remained insensitive to z-VAD-fmk induced cell death (Fig. 1A). Primary mouse peritoneal macrophages were resistant to z-VAD-fmk, but underwent z-VAD-fmk induced cell death after pre-treatment with IFN-γ (Fig. 2A). Concentration-response studies showed that 100 µM z-VAD-fmk was required to obtain a significant number of dead cells within 24 hours (Fig. 2B). Therefore, we chose 100 µM z-VAD-fmk for all further experiments. This concentration has also been used previously by Yu et al. (2004) to induce z-VAD-fmk mediated cell death in macrophages.

The type of J774A.1 macrophage death after z-VAD-fmk treatment was not characterized by activation of caspase-3 and internucleosomal DNA fragmentation typical of apoptosis, albeit levels of procaspase-3 in macrophages progressively decreased during treatment (Fig. 3A). However, bulk degradation of long-lived proteins occurred (Fig. 3B), which is a hallmark of autophagy (Mizushima, 2004), and such degradation could be blocked...
by the autophagy inhibitor 3-methyladenine (Fig. 3B). Furthermore, z-VAD-fmk-treated J774A.1 macrophages showed conversion of the 18-kDa protein microtubule-associated protein 1 LC3 (LC3-I) into the 16-kDa protein LC3-II (Fig. 3C), which is considered a reliable marker of autophagosome formation (Mizushima, 2004). Transmission electron microscopy (TEM) of J774A.1 cells treated with z-VAD-fmk for 8 hours revealed an intact nonpyknotic nucleus and numerous vacuoles in the cytoplasm characteristic of autophagy (Fig. 4B-C) as opposed to untreated cells showing normal cell morphology (Fig. 4A). Some cells, however, were necrotic (Fig. 4D). Necrotic cells were characterized by cellular swelling, presence of a non-electron dense cytoplasm, rupture of the plasma membrane and formation of necrotic debris. Labeling of z-VAD-fmk treated cells with propidium iodide or Hoechst confirmed initiation of necrosis and indicated that the number of necrotic cells rapidly increased with time (Fig. 3D). Pretreatment of J774A.1 cells with IFN-γ followed by exposure to z-VAD-fmk did not trigger autophagy but readily induced necrotic cell death (Fig. 5).

**Receptor interacting protein 1 mediates z-VAD-fmk induced cell death.** Because receptor interacting protein 1 (RIP1) is involved in the regulation of nonapoptotic cell death (Holler et al., 2000; Yu et al., 2004), expression of this protein was examined in macrophages and SMCs. Western blots showed that RIP1 was less abundantly expressed in SMCs and mouse peritoneal macrophages versus J774A.1 macrophages (Fig. 6A). IFN-γ strongly upregulated RIP1 expression (4-5 fold after 16 hours) in macrophages, but not or only weakly (1.5-2 fold after 16 hours) in SMCs (Fig. 6B). RIP1 forms a complex with heat-shock protein HSP-90 and disruption of HSP-90 function by the HSP-90 inhibitor geldanamycin causes RIP1 degradation (Lewis et al., 2000). Accordingly, RIP1 protein concentrations progressively diminished in macrophages treated with geldanamycin (Fig. 6C). These RIP1 deficient cells were more resistant to z-VAD-fmk-induced cell death as compared to control cells. On the other hand, transient transfection of SMCs with plasmid DNA encoding RIP1 substantially enhanced z-VAD-fmk-mediated cell death (Fig. 6D).
**z-VAD-fmk induced macrophage death triggers inflammatory responses.** To examine potentially important downstream pathways linked to z-VAD-fmk induced cell death, a full genome microarray representing over 41,000 mouse genes or transcripts was probed with cDNA isolated from z-VAD-fmk-treated versus DMSO-treated J774A.1 macrophages. After analysis of the hybridization data, 38 genes with a >5-fold differential expression level could be identified (see supplemental data). Of these, 35 genes were upregulated in z-VAD-fmk treated cells and 3 genes downregulated. Upregulation of 2 cytokines (IL1β and TNFα) and 5 chemokines (Groβ, MIP-1α, MIP-1β, MCP-3 and IP-10) was confirmed by real time RT-PCR (Table 2). ELISA experiments revealed that the release of IL1β, TNFα and MIP-1α protein in the culture medium was significantly increased after z-VAD-fmk treatment (Fig. 7).

**z-VAD-fmk combined with TNFα induces necrosis of smooth muscle cells.** Because RIP1 is a crucial component of the TNF-signalling pathway (Hsu et al., 1996), the effect of TNFα on SMC viability in the presence of z-VAD-fmk was investigated in more detail. z-VAD-fmk combined with TNFα, but not TNFα or z-VAD-fmk alone, induced SMC death (Fig. 1A and 8A). Degradation of RIP1 by geldanamycin protected SMCs against TNFα/z-VAD-fmk induced cell death (Fig. 8B). Western blotting demonstrated that RIP1 protein levels in SMCs were not significantly altered after stimulation with TNFα (Fig. 8C). However, treatment of SMCs with TNFα plus z-VAD-fmk resulted in a substantial loss of RIP1 protein (Fig. 8C). TNFα plus z-VAD-fmk did not induce either apoptosis (absence of procaspase-3 activation and oligonucleosomal DNA fragmentation [Fig 8D]) or autophagy (absence of enhanced degradation of long-lived proteins [Fig. 8E]), but stimulated necrosis, as shown by staining with propidium iodide (Fig. 8F). Dose-response experiments indicated that a concentration of at least 30 µM z-VAD-fmk was required to induce TNFα/z-VAD-fmk mediated necrosis. Examination by TEM confirmed that TNFα plus z-VAD-fmk treated cells did not develop ultrastructural alterations typical of apoptosis or autophagy such as chromatin condensation, cytoplasmic fragmentation and increased vacuolization (not shown).
Discussion

Although caspase inhibitors have yet to reach the clinic, preclinical studies are compelling and the plethora of diseases in which they might have beneficial effects makes their therapeutic potential enormous (Nicholson, 2000). Recent evidence suggests that caspase inhibitors not only arrest apoptosis, but also have the unanticipated effect of promoting nonapoptotic cell death (Yu et al., 2004). In the present study, we report that benzyloxy carbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone (z-VAD-fmk), a caspase inhibitor with broadspectrum activity, induces nonapoptotic death of J774A.1 and RAW264.7 macrophages, but not of smooth muscle cells (SMCs). The broadspectrum caspase inhibitor Boc-D-fmk produced a similar effect. Primary mouse macrophages underwent z-VAD-fmk mediated cell death after pre-treatment with IFN-γ. Since macrophages favor atherosclerotic plaque destabilization and rupture, these findings might be an interesting starting point to develop new therapeutics for coronary artery syndromes.

At least two types of nonapoptotic death can be distinguished: autophagic death, also known as type II programmed cell death, and necrosis. Autophagy is a normal physiological process involved in routine turnover of cell constituents that protects cells against various conditions of stress. From this perspective, autophagy is in the first place a survival and not a cell death pathway (Lockshin and Zakeri, 2004; Edinger and Thompson, 2004). Necrosis, on the other hand, is a disorderly mode of cell death characterized by loss of plasma membrane integrity and leakage of cell content. Macrophages undergoing z-VAD-fmk-induced cell death revealed bulk degradation of long-lived proteins, processing of microtubule associated protein light chain 3 (LC3), and cytoplasmic vacuolization, which are all markers of autophagy (Mizushima, 2004). Because many viral pathogens have caspase inhibitors, z-VAD-fmk could mimick a viral infection so that some cell types including macrophages may be forced to initiate the autophagic pathway as a “fail-safe” mechanism of nonapoptotic cell death. However, also necrosis occurred and the number of necrotic cells rapidly increased during z-VAD-fmk treatment. We cannot rule out the possibility that necrosis represents a terminal stage of autophagy (Bursch, 2001). This implies that z-VAD-fmk initiates autophagic...
death which in turn evolves to secondary necrosis, similar to what happens with apoptotic cells if they are not phagocytized by neighbouring cells. One can also assume that necrosis is indirectly induced. Indeed, z-VAD-fmk treated macrophages secrete several cytokines in large amounts prior to undergoing cell death, as discussed below. These cytokines in combination with z-VAD-fmk may induce necrotic death rather than autophagy. In line with this theory, pre-treatment of J774A.1 macrophages with IFN-γ readily induced necrosis without the occurrence of autophagy.

Chloro/fluoromethyl ketone peptide inhibitors including z-VAD-fmk are believed to be broad spectrum caspase inhibitors, but they can also potently bind and inhibit cysteine proteases of the cathepsin family at concentrations that are commonly used to demonstrate an involvement of caspases (Schotte et al., 1999). This finding questions the interpretation of the results. Indeed, autophagy might become more prominent following z-VAD-fmk treatment of macrophages as lysosomal degradation of their contents is disrupted. However, z-FA-fmk, an inhibitor of cysteine proteases such as cathepsin B, did not induce J774A.1 macrophage death. Recent evidence suggests that mammalian cells possess a low constitutive level of active caspase-8 that carries out cellular regulatory processes (Chun et al., 2002). Downregulation of caspase-8 protein spontaneously induces features of autophagy via a mechanism that requires the autophagy specific genes Atg7 and beclin 1 (Yu et al., 2004). Because z-VAD-fmk is a potent inhibitor of caspase-8, it likely exerted its cell death effect through the inhibition of caspase-8. Interestingly, receptor interacting protein 1 (RIP1), which is involved in the regulation of both apoptotic and nonapoptotic cell death (Lin et al., 1999; Holler et al., 2000; Yu et al., 2004), is a well known substrate of caspase-8 (Lin et al., 1999). It is therefore plausible that cleavage of RIP1 by caspase-8 inhibits nonapoptotic death. In this study, we demonstrate that RIP1 is less abundantly expressed in SMCs as compared to J774A.1 macrophages, which may explain the different sensitivity to z-VAD-fmk between both cell types. Primary peritoneal macrophages were insensitive to z-VAD-fmk, but unlike SMCs, pretreatment peritoneal macrophages with IFN-γ increased the sensitivity to z-VAD-fmk induced cell death considerably due to upregulation of RIP1 expression. Moreover,
selective degradation of RIP1 in J774A.1 macrophages by geldanamycin protected cells against z-VAD-fmk induced death, whereas overexpression of RIP1 in smooth muscle cells stimulated z-VAD-fmk mediated cell death. Overall, these findings strongly suggest that RIP1 is an important effector molecule of z-VAD-fmk induced cell death.

The molecular pathway underlying z-VAD-fmk induced cell death downstream of RIP1 remains unclear. Recently, it has been demonstrated that c-Jun N-terminal kinase (JNK) and JNK-activating kinase MAP kinase kinase 7 (MKK7) as well as protein synthesis in general are essential for z-VAD-fmk induced cell death (Yu et al., 2004). To identify gene products that might respond to z-VAD-fmk treatment, we performed microarray studies with cDNA isolated from macrophages incubated with z-VAD-fmk. Among the 38 genes that were differentially expressed (>5-fold), we found a strong upregulation of several chemokines (Groβ, MIP-1α, MIP-1β, MCP-3 and IP-10) and cytokines (IL1β and TNFα). Increased secretion of IL1β, TNFα and MIP-1α protein by z-VAD-fmk treated macrophages was confirmed by ELISA. These findings confirm previous results showing that necrotic cells, in contrast to apoptotic cells, can retain the opportunity to synthesize inflammatory proteins in an attempt to provide “danger” signals for the surrounding cells and immune competent cells (Saelens et al., 2005). Of note, maturation of IL1β requires caspase-1 and caspase-4 (the human homologue of murine caspase-11), which are both blocked by z-VAD-fmk. It is therefore likely that the increased levels of IL1β in the macrophage culture medium after z-VAD-fmk treatment represent unprocessed (inactive) protein. Interestingly, z-VAD-fmk combined with TNFα, but not z-VAD-fmk or TNFα alone, induced cell death of SMCs. Several studies have shown that blockade of caspases in some cell lines sensitizes them to TNFα-mediated cytotoxicity. The authors refer to this type of cell death as necrosis (Vercammen et al., 1998; Holler et al., 2000), apoptosis (Cowburn et al., 2005), ‘cell death with apoptotic-like and necrotic-like features’ (Liu et al., 2003), ‘nonapoptotic’ cell death (Khwaja and Tatton, 1999) or a ‘transitional stage between apoptosis and necrosis’ (Luschen et al., 2000). Such descriptions underline the complicated nature of the phenomenon but, at
the same time, dictate the necessity to use an adequate set of tools for the registration of cell death. Our results indicate that TNFα combined with z-VAD-fmk did not induce either apoptosis (absence of procaspase-3 activation and oligonucleosomal DNA fragmentation) or autophagy (absence of enhanced degradation of long-lived proteins and cytoplasmic vacuolization). However, the combination stimulated necrosis as shown by staining with propidium iodide. Although the underlying mechanism is unknown, RIP1 seems to be an important effector molecule because downregulation of RIP1 expression by geldanamycin protected SMCs against z-VAD-fmk plus TNFα-induced cell death. Western blots demonstrated that RIP1 in SMCs was not upregulated in response to TNFα. This finding suggests that the expression level of RIP1 to induce TNFα/z-VAD-fmk mediated cell death in SMCs is less critical as compared to z-VAD-fmk induced cell death. However, Holler et al. (2000) reported that the kinase activity of RIP1 is essential for TNFα-induced necrosis. Since RIP1 is a crucial component of the TNF-receptor (TNF-R) signalling complex (Meylan and Tschopp, 2005), it is tempting to speculate that recruitment of RIP1 to TNF-R after TNFα-stimulation under nonapoptotic conditions (e.g. caspase blockade) induces formation of a "necrosome-complex", analogous to the formation of an apoptosome during TNFα-induced apoptosis, in which the activation of RIP1 is a crucial event. Further research is needed to unravel the downstream signaling events and RIP1 substrates.

In summary, z-VAD-fmk treatment can induce nonapoptotic death of (IFN-γ primed) macrophages, but leaves smooth muscle cells unaffected. This difference in sensitivity to z-VAD-fmk is largely based on differential RIP1 expression. Although removal of macrophages may contribute to stabilization of atherosclerotic plaques, administration of z-VAD-fmk has several adverse effects. First, z-VAD-fmk induces macrophage necrosis which contributes to enlargement of the necrotic core of advanced lesions. Secondly, z-VAD-fmk treated macrophages release large amounts of chemokines and cytokines in an early stage of the cell death process which inevitably would lead to inflammation and plaque progression. Third, secretion of TNFα by macrophages during z-VAD-fmk treatment sensitizes smooth
muscle cells to undergo z-VAD-fmk dependent necrosis. Loss of smooth muscle cells is detrimental for plaque stability as it results in decreased synthesis of interstitial collagen fibers and thinning of the fibrous cap. Development of chemical compounds that selectively induce a pure autophagic type of cell death without necrosis and synthesis of inflammatory molecules might bypass the undesired adverse effects of z-VAD-fmk treatment.
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References


Footnotes

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**Fig. 1.** Effect of z-VAD-fmk on the viability of J774A.1 macrophages and smooth muscle cells (SMC). (A) J774A.1 cells and SMCs were exposed to 100 µM z-VAD-fmk in serum-containing medium for up to 24 hours (upper panel). In some experiments, cells were exposed to 100 U/ml IFN-γ for 16 hours prior to z-VAD-fmk treatment (lower panel). Initiation of cell death was examined by neutral red viability assays. **p<0.01 vs untreated cells, ++p<0.01 vs without IFN-γ pretreatment (ANOVA, followed by Dunnett test). (B) Viability of J774A.1 macrophages after exposure to various concentrations of z-VAD-fmk for 24 hours with or without pre-treatment with IFN-γ (100 U/ml, 16 hours). Results represent the mean±SEM of 3 independent experiments. **p<0.01 vs control (ANOVA, followed by Dunnett test).

**Fig. 2.** Effect of z-VAD-fmk on the viability of primary mouse peritoneal macrophages. (A) Cells were exposed to 100 µM z-VAD-fmk in serum-containing medium for up to 24 hours. In some experiments, cells were exposed to 100 U/ml IFN-γ for 16 hours prior to z-VAD-fmk treatment. Initiation of cell death was examined by neutral red viability assays. *p<0.05, **p<0.01 vs untreated cells (ANOVA, followed by Dunnett test). (B) Viability of IFN-γ primed (100 U/ml, 16 hours) peritoneal macrophages after exposure to z-VAD-fmk (10^{-6}-10^{-4} M) for 24 hours. Results represent the mean±SEM of 3 independent experiments. *p<0.05, **p<0.01 vs control (ANOVA, followed by Dunnett test).

**Fig. 3.** Characterization of z-VAD-fmk induced cell death in J774A.1 macrophages. Cells were treated with 100 µM z-VAD-fmk for up to 24 hours. (A) To detect apoptosis, cleavage of procaspase-3 (procasp-3) and internucleosomal DNA fragmentation was analyzed using Western blotting (upper panel) and agarose gel electrophoresis (lower panel), respectively. J774A.1 cells treated with cycloheximide (CHX) for 16 hours served as a positive control. (B) Degradation of long-lived proteins as a biochemical marker for autophagy was studied by
treating cells with medium containing DMSO (control) or medium supplemented with z-VAD-fmk in the presence or absence of the autophagy inhibitor 3-methyladenine (3-MA). **p<0.01 vs control (unpaired Student’s t test). (C) Western blot analysis of microtubule associated protein light chain 3 (LC3) processing was performed as an alternative method to detect autophagy. (D) Necrosis was examined by treating cells with DMSO (control) or z-VAD-fmk for 24 hours followed by Hoechst labeling and fluorescence microscopy (left panels). Scale bar=100 µm. Alternatively, z-VAD-fmk treated cells were labeled with propidium iodide and analyzed by flow cytometry (right panel).

**Fig. 4.** Ultrastructural features of J774A.1 macrophages treated with 100 µM z-VAD-fmk for 8 hours. Panel A shows an untreated control with normal cell morphology. Treatment of macrophages with z-VAD-fmk induced cell death evocating autophagy, as shown in panel B and C, and is characterized by extensive vacuolization. Besides autophagy, necrotic cells were present (panel D). Results are representative of 2 independent experiments. N indicates nucleus. Scale bar=3 µm.

**Fig. 5.** Characterization of cell death in J774A.1 macrophages treated with IFN-γ (100 U/ml) for 16 hours, followed by exposure to 100 µM z-VAD-fmk. Autophagic cell death was evaluated by assaying degradation of long-lived proteins 6 hours after z-VAD-fmk treatment (A) or by performing Western blots for LC3 processing (B). In the latter experiments, z-VAD-fmk treated J774A.1 cells without IFN-γ pretreatment were used as a positive control. Necrosis was examined by PI labeling and flow cytometry (C).

**Fig. 6.** Effect of receptor interacting protein 1 (RIP1) expression on z-VAD-fmk induced cell death. (A) Comparison of RIP1 expression levels in J774A.1 cells versus smooth muscle cells (SMC) or mouse peritoneal macrophages via Western blotting. The results indicate that RIP1 is less abundantly expressed in SMCs and peritoneal macrophages as compared to J774A.1 cells. β-actin served as a loading control. (B) Western blot detection of RIP1 in
J774A.1 macrophages, peritoneal macrophages and SMCs treated with IFN-γ for 0-16 hours. (C) Pretreatment of macrophages with geldanamycin (1 µg/ml, 8 hours) reduces z-VAD-fmk induced cell death. Western blotting indicated a prominent decline in RIP1 protein levels after geldanamycin treatment. Cells were then treated with 100 µM z-VAD-fmk in the presence of geldanamycin for 24 hours. Cell viability was determined and compared with that of cells treated with z-VAD-fmk in the absence of geldanamycin. *** p<0.001 vs z-VAD-fmk treated cells (unpaired Student’s t test). (D) Overexpression of RIP1 sensitizes smooth muscle cells to z-VAD-fmk induced cell death. Smooth muscle cells were transiently transfected with pCMV-mRIP encoding RIP1 or with a control plasmid. Elevated levels of RIP1 protein could be demonstrated via Western blotting 16 hours after transfection. RIP1 transfected cells showed reduced cell viability as compared to control cells when they were treated with 100 µM z-VAD-fmk for 16 hours immediately after transfection. Bar graphs represent mean±SEM of 3 independent experiments. *** p<0.001 vs control (unpaired Student’s t test).

Fig. 7. Release of inflammatory proteins after initiation of z-VAD-fmk induced macrophage death. Macrophages were treated with 100 µM z-VAD-fmk or DMSO for 2-16 hours. The release of IL1β (A), TNFα (B) and MIP-1α (C) in the culture supernatant was determined via a quantitative sandwich enzyme immunoassay. Results represent the mean±SEM of 3 independent experiments. * p<0.05, ** p<0.01, *** p<0.001 vs DMSO treated control (unpaired Student’s t test).

Fig. 8. Effect of TNFα on smooth muscle cell (SMC) viability in the presence of z-VAD-fmk. (A) Viability of SMCs after exposure to TNFα (10 ng/ml) or TNFα (10 ng/ml) combined with 100 µM z-VAD-fmk for up to 24 hours. ** p<0.01 vs untreated cells (ANOVA, followed by Dunnett test). (B) Pretreatment with geldanamycin protects SMCs against TNFα/z-VAD-fmk induced cell death. SMCs were pretreated with 1 µg/ml geldanamycin for 8 hours, followed by incubation with a mixture of TNFα (10 ng/ml) and z-VAD-fmk (100 µM) in the presence of
geldanamycin for 24 hours. Western blots showed downregulation of RIP1 protein after geldanamycin treatment. Cell viability was determined and compared with that of cells treated with TNFα plus z-VAD-fmk in the absence of geldanamycin. ***p<0.001 vs geldanamycin nontreated cells (unpaired Student’s t test). (C) Analysis of RIP1 protein expression in SMCs by Western blotting after treatment with TNFα (10 ng/ml) or TNFα (10 ng/ml) combined with z-VAD-fmk (100 µM) for 0-24 hours. (D-F) Characterization of SMC death after incubation with TNFα and z-VAD-fmk. (D) Cleavage of procaspase-3 (procasp-3) and internucleosomal DNA fragmentation typical of apoptosis during treatment (0-24 hours) could not be detected. SMCs treated with TNFα (10 ng/ml) and cycloheximide (20 µg/ml) for 8 hours served as a positive control. (E) In contrast to SMCs that underwent amino acid deprivation in Earle’s Balanced Salt Solution (EBSS) for 6 hours, SMCs that were treated with TNFα and z-VAD-fmk did not show enhanced degradation of long-lived proteins typical of autophagy. **p<0.01 vs control (ANOVA, followed by Dunnett test). (F) However, SMCs could be labeled with propidium iodide after incubation with TNFα plus z-VAD-fmk. Bar graphs represent the mean±SEM of 3 independent experiments.
### TABLE 1

PCR primers and fluorogenic probes used in real time quantitative RT-PCR

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<th>Gene</th>
<th>Forward and reverse primer</th>
<th>Probe</th>
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<td>5’-TCAACCAACAAAGTGATATTCTCCATGAGCTTTGTAC-3’</td>
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<td>5’-AGCTGCTCCTCCACTTGGTGTTTGCTA-3’</td>
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<td></td>
<td>5’-CACCACCTAGTTGTTGTCTTTGAGA-3’</td>
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<tr>
<td>Cxcl2/Grozβ</td>
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<td>5’-CGCCCAGACAGAGTCATAGCCACTCTCTC-3’</td>
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<td></td>
<td>5’-TCTTTGGATGATTTTCTGAACCA-3’</td>
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<tr>
<td>Ccl3/MIP-1α</td>
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TABLE 2

Real time RT-PCR confirmation of mRNA cytokine and chemokine upregulation identified via microarray screening in J774A.1 macrophages treated with 100 µM z-VAD-fmk for 4 hours versus DMSO treated controls

Values are mean ± S.E.M.

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<th>Accession N°</th>
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<td>Cxcl10/IP-10</td>
<td>chemokine (C-X-C motif) ligand 10</td>
<td>6±1</td>
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**Figure 1**

(A) Viability with and without IFN-γ pretreatment following treatment with z-VAD-fmk (100 µM) for 4, 16, and 24 hours. J774A.1 and SMC cell lines are shown.

(B) Viability of J774A.1 cells untreated or treated with IFN-γ over a range of z-VAD-fmk concentrations (log M).

**Legend:**
- J774A.1
- SMC
- ** and ++ indicate significant differences compared to control.
Figure 2

A

B

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3
Figure 4
Figure 5

A

Degradation long-lived proteins, %

<table>
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<th>0%</th>
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<tr>
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B

Positive Control

LC3-I  LC3-II

C

Propidium iodide

Control  z-VAD-fmk  8 h  4 h  2 h

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6
Figure 7

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Figure 8