Caspase-3 Activation Triggers Extracellular Cathepsin L Release and Endorepellin Proteolysis*

Jean-François Calhier1, Isabelle Siros1,2, Patrick Laplante3, Stéphanie Lepage1, Marc-André Raymond1, Nathalie Brassard3, Alexandre Prat5, Renato V. Iozzo6, Alexey V. Pshezhetsky7, and Marie-Josée Hébert1,3

From the 1Research Centre, Centre Hospitalier Universitaire de Montréal (CHUM) and Montreal Cancer Institute and 2Neuroimmunology Research Laboratory, Research Centre, CHUM, Université de Montréal, Montreal, Quebec H2L 4M1, Canada, 3Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, and 4Department of Medical Genetics, Centre Hospitalier Universitaire Sainte-Justine, Université de Montréal, Montreal, Quebec H3T 1C5, Canada

Proteolysis of extracellular matrix components and the production of cryptic bioactive factors play key roles in vascular remodeling. We showed previously that extracellular matrix proteolysis is triggered by the apoptosis of endothelial cells (EC), resulting in the release of an anti-apoptotic C-terminal fragment of endorepellin (LG3). Here, we characterize the endorepellin-cleaving proteases released by apoptotic EC using a multifaceted proteomics strategy. Cathepsin L (CathL), a cysteine protease known to be associated with cardiovascular disease progression in animal models and humans, was isolated from medium conditioned by apoptotic (CathL), a cysteine protease known to be associated with car-

process, as most clinical risk factors of atherosclerosis (such as hypertension (1, 2), hyperglycemia (3, 4), oxidized low density lipoproteins (LDLs) (5, 6) and oxidative stress (7)) induce EC apoptosis. Interventions aimed at preventing EC apoptosis in animal models of transplant vasculopathy, an immune-mediated form of atherosclerosis, prevent neointima formation, indicating that EC apoptosis is an important pro-atherosclerotic trigger (8–13). During vascular remodeling, EC injury and apoptosis are followed by migration of α-smooth muscle actin-positive cells (smooth muscle cells (SMC) and myofibroblasts) that accumulate within the intima through a state of resistance to apoptosis largely dependent on Bcl-xL overexpression (14–16).

Recent evidence from our group and others suggests that apoptotic EC favor neointima formation through the release of paracrine mediators, which in turn, increase Bcl-xL expression and inhibit the apoptosis of vascular SMC and fibroblasts (17–20). The production of biologically active mediators by apoptotic EC is at least partially dependent on pericellular proteolysis, leading to basement membrane and extracellular matrix (ECM) degradation with the release of cryptic anti-apoptotic factors (18–20). A C-terminal fragment of endorepellin (perlecain domain V) released in association with EC apoptosis was found to heighten Bcl-xL expression in SMC and fibroblasts (18–20). Perlecain is a basement membrane modular proteoglycan composed of five structural domains (21). The C-terminal domain, also called endorepellin, comprises three laminin-like globular (LG1–LG3) modules interspaced by four epidermal growth factor-like repeats. The C-terminal LG3 motif mediates most of the anti-apoptotic and fibrogenic activity of endorepellin (18–20, 22). LG3 levels were recently observed to be increased in patients with chronic renal allograft rejection (23), further supporting the contention that EC apoptosis and ECM proteolysis play key roles in vascular remodeling.

In the last two decades a convincing body of evidence has demonstrated that the ECM is an extremely dynamic structure of crucial importance for the regulation of cell adhesion, migration, survival, and differentiation. Proteolysis of basement membrane and/or ECM components (such as collagen IV, XV, XVIII, and perlcan) and liberation of split products with new morphogenetic protein-1; LC, liquid chromatography; MS/MS, tandem mass spectroscopy.

Apoptosis of endothelial cells (EC) is increasingly recognized as an important component of the “response to injury”
functions (such as tumstatin, arrestin, endostatin, and endorepellin LG3 motif) have central roles in angiogenesis, wound-healing, tissue-remodeling, and atherosclerosis (24).

We reported previously that LG3 production by apoptotic EC occurs downstream of caspase activation (18, 20). Yet endorepellin does not harbor caspase-cleavage sites (25). This suggested that caspase activation leads to the release/activation of an as yet uncharacterized protease(s) which could then cleave endorepellin and release LG3. Hence, we used a multifaceted proteomics strategy to characterize the endorepellin-cleaving proteases released by apoptotic EC. Here, we describe the importance of cathepsin L (CathL), a lysosomal endoprotease associated with the development of atherosclerotic diseases in animal models and humans, as a key enzyme responsible for LG3 production by apoptotic EC.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human umbilical vein endothelial cells (HUVEC, Clonetics), WI-38 human fibroblasts (ATCC), and A7R5 vascular SMC (ATCC) were cultured as described elsewhere (18, 20). Caspase inhibitors (ZVAD-FMK, DEVD-FMK, and LEHD-FMK) were purchased from R&D Systems. The CathL inhibitors ZFF-FMK and ZFA-FMK were from Calbiochem and MP Biomedicals, respectively. t-PA-STOP was obtained from American Diagnostica, Inc. Human recombinant endorepellin was purified as documented previously (22). Tissue plasminogen activator (t-PA) protein levels were measured with commercially available enzyme-linked immunosorbent assay kits (MedSystems) according to the supplier’s protocol. Human CathL, recombinant t-PA, and human $\alpha_2$-macroglobulin, a bone morphogenetic protein-1 (BMP-1) inhibitor, were obtained from Sigma-Aldrich, Roche, and R&D Systems, respectively.

Conditioned Media—Serum-free media conditioned by apoptotic or non-apoptotic EC were generated as described previously (18–20). In brief, to generate medium conditioned by non-apoptotic EC (SSC-no-apo), confluent HUVEC were exposed to the pan-caspase inhibitor ZVAD-FMK (100 $\mu$M) for 2 h, washed, and then serum-starved for 4 h in RPMI 1640 (Invitrogen), whereas in medium conditioned by apoptotic EC (SSC-apo), HUVEC were instead exposed to DMSO for 2 h, washed, and then serum-starved for 4 h. For CathL inhibition, HUVEC were pretreated with ZFF-FMK (15 $\mu$M), ZFA-FMK (100 $\mu$M) or DMSO (control), washed, and serum-starved for 4 h. To inhibit t-PA proteolytic activity, HUVEC were exposed to t-PA-STOP (1.23 nmol/ml) for 4 h during serum starvation. Conditioned media were collected and stored at $-20 \, ^\circ\text{C}$. Mitomycin C (MMC, Sigma-Aldrich) served as another pro-apoptotic stimulus (26). In brief, confluent HUVEC were grown to confluence in endothelial cell basal medium (Clonetics) and exposed to MMC at 0.01 mg/ml or vehicle for 24 h, and the conditioned media were harvested. To generate medium conditioned by autophagic EC, HUVEC grown to confluence in endothelial cell basal medium were exposed to rapamycin (Wyeth) (1 $\mu$g/ml) or vehicle for 4 h, and the conditioned media were harvested. For BMP-1/Tolloid-like metalloprotease inhibition, HUVEC were incubated with $\alpha_2$-macroglobulin (27 nm) or vehicle (phosphate-buffered saline/glycerol) during serum starvation for 4 h.

Characterization of the Proteases Produced by Apoptotic and Non-apoptotic EC—To characterize the complete set of proteases secreted by apoptotic EC downstream of caspase activation, we tested two comparative proteomic approaches: SDS-PAGE-LC-MS/MS and two-dimensional LC-MS/MS (27). SSC-apo and SSC-no-apo were centrifuged to eliminate cell debris and apoptotic bodies (28). Proteins in supernatants were concentrated, denatured, and digested with trypsin. Tryptic peptides were fractionated by weak anion exchange high performance liquid chromatography and analyzed by LC-MS/MS or resolved by SDS-PAGE, digested “in gel,” and analyzed by LC-MS/MS. In addition, to specifically characterize proteins with anti-apoptotic activity released during EC apoptosis, we conducted a functional analysis of SSC-apo, where the medium was fractionated with fast protein liquid chromatography and tested for anti-apoptotic activity on SMC. The biologically active fraction was resolved by SDS-PAGE, and bands present only in the bioactive fraction were sequenced with LC-MS/MS.

Immunoblotting and In Vitro Digestion Assay—For all experiments, equal volumes of all conditioned media were concentrated by centrifugation through a 10-kDa Vivascience concentrator according to the manufacturer’s specifications (Vivascience). For in vitro digestion assays, recombinant endorepellin (148.5 ng) (22) was incubated with t-PA (51 ng) or CathL (37.8 ng) at 37 °C for 48 h in RPMI 1640 (total volume of 40 $\mu$L). A fixed volume for all conditions was loaded onto the gel. Proteins were separated on 12% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Western blotting was performed as described elsewhere (17, 20), and the membranes were probed with a polyclonal rabbit antibody against the LG3 fragment of perlecan (17, 20) or monoclonal anti-CathL antibody (BD Transduction Laboratories). As a loading control, proteins from the same digestion sample were loaded on a gel and stained with Coomassie Blue.

Detection of Intracellular CathL Activity—CathL activity in EC was measured with the CathL detect kits (Calbiochem) as specified by the supplier. The subcellular localization of active CathL, defined by the presence of the fluorescent product, was evaluated by confocal microscopy (Leica S5 confocal microscope).

Fluorescence Microscopy for the Assessment of Apoptosis and Necrosis—Fluorescence microscopy of unfixed/unpermeabilized adherent cells stained with Hoechst 33342 (1 $\mu$g/ml) and propidium iodide (5 $\mu$g/ml) was undertaken as described in our previous work (18–20).

Subcellular Localization of Proteases during Apoptosis—HUVEC were grown on glass-bottom Petri dishes (Mat-Tek Corp.) coated with 1% gelatin and serum-starved for 4 h. The cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X-100. They were then blocked and stained with anti-t-PA antibody (American Diagnostica Inc.) and a biotinylated anti-mouse antibody (Dako) followed by a streptavidin-Alexa-488 antibody (Invitrogen). Nuclei were stained with To-Pro3/DNA (Molecular Probes) for confocal microscopy.

Evaluation of Lysosomal Destabilization—Lysosomal permeabilization was assessed by fluorescence microscopy with
Cathespin L Release Downstream of Caspase-3 Activation

TABLE 1
List of proteases found exclusively in SSC-apo or in the bioactive fraction
Sources were UniProtKR, Stanford Micro Array, and Swiss-Prot Databases. Letters in parentheses indicate the residues that are not present in the peptide but are adjacent in the protein sequence.

| Protein name  | Type of LC-MS/MS approach | # of peptides identified | Peptide sequence |
|---------------|---------------------------|-------------------------|-----------------|
| ADAM 17 Cathespin L | Functional Two-dimensional LC SDS-PAGE | 2 | (R)ADPDPMNTCCK (L) |
| ADAMTS 4 | Functional Two-dimensional LC SDS-PAGE | 2 | (R)QVRQPQAPQHSTCILRA (5) |
| SPUVE t-PA | Two-dimensional LC SDS-PAGE | 1 | (R)YSFVPRPSFPRTPTQDNLR (R) |
| t-PA | Two-dimensional LC SDS-PAGE | 1 | (R)LYGMNEEGWR (R) |
| | | | (R)KVFQEPLFYEAPR (S) |
| | | | (R)ADPDPMNTCCK (L) |
| | | | (R)YSFVPRPSFPRTPTQDNLR (R) |

acridine orange (Calbiochem) staining (5 μg/ml for 15 min at 37 °C) 26).

Caspase-3 Silencing—HUVEC were grown on gelatin 1% until 50% confluence. The cells were then transfected with Oligofectamine (Invitrogen) employing ON-TARGETplus SMARTpool caspase-3 small interfering (si)RNA (Dharmacon RNAi Technologies, Thermo Scientific) or control siRNAs at a final concentration of 100 nM annealed oligo. After 72 h the cells were serum-starved for 4 h. To assess caspase-3 silencing, protein extracts from HUVEC were harvested and immunoblotted for caspase-3 (Abcam).

Statistical Analysis—The results are expressed as the mean ± S.E. The data were analyzed by Student’s t test (with Bonferroni corrections when appropriate) or analysis of variance. p < 0.05 was deemed significant for all tests.

RESULTS

Characterization of Proteases Released during Apoptosis of EC—Using a multifaceted proteomic strategy, five proteases were identified in SSC-apo and were not found in SSC-no-apo (Table 1). CathL and t-PA were observed to have putative cleavage activity for endorepellin of potential relevance to LG3 production, according to computer simulation (ACD Protein Manager software) (Fig. 1A). To confirm this activity, human recombinant endorepellin was incubated in the presence of CathL or t-PA, and fragmentation was evaluated by immunoblotting with an antibody specific for the C-terminal LG3 motif (22). CathL and t-PA were observed to have putative cleavage activity for endorepellin of potential relevance to LG3 production, according to computer simulation (ACD Protein Manager software) (Fig. 1A).

Because we reported previously that in normal non-apoptotic cell systems, BMP-1/Tolloid-like metalloprotease is a key protease implicated in endorepellin cleavage (25), we also tested the possibility that BMP-1 could participate in apoptosis-induced LG3 production. α2-Macroglobulin, a BMP-1 inhibitor (29), did not modulate LG3 release, suggesting that BMP-1 is not involved in our system (Fig. 1C).

CathL Plays a Central Role in the Generation of LG3 by Apoptotic EC—Having confirmed that CathL and t-PA cleave recombinant endorepellin, we then investigated whether inhibiting their activity during the development of EC apoptosis decreases production of the anti-apoptotic LG3 fragment. HUVEC were exposed to ZFF-FMK (15 μM) and ZFA-FMK (100 μM), two irreversible and cell-permeable CathL inhibitors, or to vehicle for 2 h and serum-starved for 4 h. 5 ml of medium conditioned by HUVEC in the presence of ZFF-FMK, ZFA-FMK, or vehicle were concentrated, the same volumes were loaded, and proteins were separated by SDS-PAGE and membranes probed for LG3. The amount of proteins released by HUVEC during serum starvation was not altered by CathL inhibition, as evaluated by Ponceau red staining (Fig. 2A). Yet both inhibitors significantly reduced LG3 release (Fig. 2A).

Also, to evaluate whether inhibition of CathL activity during conditioning altered the anti-apoptotic activity of conditioned medium, the development of apoptosis was estimated in SMC and fibroblasts exposed to these conditioned media. Consistent with our previous results, serum starvation of SMC and fibroblasts for 24 h induced a significant apoptotic response (18–20). Apoptosis of SMC and fibroblasts was inhibited by medium conditioned by apoptotic HUVEC (SSC Control) (Fig. 2, B and C). Inhibition of CathL activity in HUVEC during conditioning significantly decreased the anti-apoptotic activity of conditioned medium on SMC and fibroblasts (Fig. 2, B and C).

We then evaluated whether inhibiting CathL activity in HUVEC during serum starvation could reduce LG3 release through an indirect inhibitory effect on EC apoptosis. CathL inhibition in HUVEC during serum starvation did not inhibit EC apoptosis nor did it modulate necrosis, whereas the pan-caspase inhibitor, ZVAD-FMK, significantly reduced apoptosis compared with the control (Fig. 2D).

Increased levels of the active form of CathL (25 kDa) were found in 5 ml of SSC control compared with an equal volume of SSC produced by HUVEC in which development of apoptosis was blocked by pan-caspase inhibition (ZVAD-FMK) (Fig. 3A). Collectively, these results indicate that EC apoptosis leads to increased LG3 production at least in part through CathL-dependent proteolysis.

Increased CathL levels were also found in medium conditioned by apoptotic HUVEC exposed to the DNA-damaging agent MMC compared with an equal volume of medium conditioned by non-apoptotic HUVEC (Fig. 3A) (18). Because serum starvation may also activate autophagy, a non-apoptotic form of programmed cell death, we tested the possibility that autophagy induction per se could activate CathL release. HUVEC were exposed to rapamycin (1 μg/ml for 4 h), a pharmacological inducer of autophagy acting through inhibition of the mammalian target of rapamycin (mTOR) (30). HUVEC exposed to rapamycin developed an autophagic response, as
assessed by acridine orange staining (data not shown) in the absence of apoptosis. However, CathL levels were not increased in medium conditioned by autophagic EC. Because necrosis can result in the release of proteases (31), we questioned whether EC necrosis also enhanced CathL release. HUVEC were heated for 30 min at 65 °C, a method that induced primary EC necrosis in previous work (26). Active CathL levels were not increased in medium conditioned by necrotic HUVEC compared with an equal volume of medium conditioned by normal HUVEC (data not shown). These results indicate that CathL is translocated extracellularly during EC apoptosis and plays a key role in pericellular proteolysis and LG3 release but not in the regulation of EC apoptosis per se.

Because proteases may directly activate receptors and signaling pathways of importance for inhibition of apoptosis, we evaluated whether CathL released by apoptotic EC exerted direct anti-apoptotic activity on target cells (32). SMC were exposed to SSC-apo concomitantly with ZFF-FMK or vehicle. Inhibition of CathL proteolytic activity in SMC exposed to SSC failed to blunt the anti-apoptotic response of SMC (Fig. 3B). Of note, ZFF-FMK concentrations used on SMC proved to be effective in inhibiting endorepellin cleavage during apoptosis of EC and in suppressing the anti-apoptotic activity of SSC. These results suggest that the proteolytic activity of CathL is required for the production of anti-apoptotic ECM fragments during EC apoptosis but not for the direct activation of anti-apoptotic pathways in SMC.

Inhibition of t-PA activity in serum-starved EC did not prevent the development of apoptosis but attenuated LG3 production (data not shown and Fig. 4A). However, no differences were apparent in the levels of t-PA present in medium conditioned by apoptotic HUVEC (SSC control) compared with medium conditioned by non-apoptotic HUVEC (SSC ZVAD-FMK), as

---

**FIGURE 1. Identification of proteases released by apoptotic EC.** A, schematic representation of the C-terminal fragment of perlecan with putative cleavage sites for t-PA and CathL, EGF, epidermal growth factor. B, immunoblot showing increased LG3 levels after digestion of recombinant endorepellin (148.5 ng) with either CathL (37.8 ng) or t-PA (51 ng) for 48 h in RPMI 1640. Coomassie Blue staining is used as a protein-loading control. The blots are representative of three independent experiments. C, immunoblotting for the LG3 fragment in media conditioned by HUVEC serum-starved for 4 h in the presence of α₂-macroglobulin (SSC α₂-MG), a BMP-1 inhibitor, or vehicle (SSC Control). Ponceau red staining was used as a protein-loading control. The blots are representative of two separate experiments.
evaluated by enzyme-linked immunosorbent assay (Fig. 4B), indicating that t-PA in soluble form is not increased in SSC-apo. However, confocal microscopy with t-PA immunostaining demonstrated t-PA translocation toward the periphery of apoptotic cells and bodies (Fig. 4, C2–3 and C5). Pan-caspase inhibition with ZVAD-FMK during serum starvation prevented t-PA translocation (Fig. 4 C3). The data suggest that during EC apoptosis, t-PA translocates at the cell periphery but is not released in soluble form by EC and plays a lesser role in endorepellin cleavage.

Mechanisms of CathL Translocation during EC Apoptosis—CathL activity was tracked in serum-starved EC with fluorescence-based CathL activity detection kits and confocal microscopy. In HUVEC exposed to normal medium, CathL activity was found exclusively in intracellular organelles (Fig. 5A). Extra-lysosomal CathL activity was found in HUVEC serum-starved for 4 h (Fig. 5A). HUVEC serum-starved for up to 4 h showed a progressive and statistically significant increase in cells with lysosomal destabilization, as assessed by acridine orange staining (Fig. 5B). Inhibition of caspase activation with ZVAD-FMK in serum-starved HUVEC significantly decreased the percentage of cells with lysosomal destabilization compared with serum-starved HUVEC (Fig. 5C). ZFF-FMK and ZFA-FMK significantly albeit incompletely reduced lysosomal destabilization in serum-starved HUVEC by 8 and 30%, respectively (data not shown). These results suggest that lysosomal permeabilization occurs downstream of caspase activation. Extra-lysosomal CathL release would then further enhance lysosomal destabilization and facilitate CathL translocation outside the lysosomal compartment.

To define the key caspase responsible for CathL translocation, serum-free media were conditioned on HUVEC in which caspase-3 or caspase-9 activation had been inhibited with DEVD-FMK and LEHD-FMK, respectively. ZVAD-FMK served as a pan-caspase control inhibitor. Equal volumes (5 ml) of conditioned media were concentrated and loaded onto the gel. As expected, all inhibitors (ZVAD-FMK, DEVD-FMK, and LEHD-FMK) reduced
Caspase-3 activation in serum-starved HUVEC, as evaluated by immunoblotting, whereas only ZVAD-FMK and LEHD-FMK blocked caspase-9 activation (data not shown). Yet all inhibitors prevented extracellular CathL release and suppressed LG3 production (Fig. 6A, left panel, and B) These findings indicate that the downstream effector caspase-3 is likely an important regulator of CathL release. To further confirm the importance of caspase-3 in CathL translocation, siRNAs were used to silence caspase-3 expression in HUVEC (Fig. 6C). Caspase-3 silencing inhibited EC apoptosis (Fig. 6D). When equal volumes of media conditioned by either caspase-3-silenced serum-starved HUVEC or controls were harvested and compared, decreased CathL and LG3 levels were found in media conditioned by caspase-3-silenced HUVEC (Fig. 6, A, middle panel, and B). Decreased CathL levels were also found in caspase-3-silenced HUVEC exposed to MMC for 24 h (Fig. 6A, right panel). Collectively, these results demonstrate that caspase-3 activation in apoptotic EC triggers CathL activation and release, which in turn degrades endorepellin and releases LG3.

**DISCUSSION**

ECM remodeling is a central feature of various repair processes. Vascular repair, either normal or pathological, as seen in atherosclerosis, transplant vasculopathy, and immune-mediated vasculitides, involves substantial remodeling of the vasculature. Matrix metalloproteases are classical regulators of vascular remodeling (33, 34), but recent results demonstrate that serine and cysteine proteases also play key roles in the initiation and progression of atherosclerotic lesions (35–37). Increased CathL, -B, and -S levels have been reported within EC, SMC, and macrophages in animal models and human atheromatous disease during neointima formation and in abdominal aortic aneurysms (24, 37–39). Recent studies in LDL receptor-deficient and apolipoprotein E-null mice revealed that CathS, -K, and -L gene deficiencies significantly reduced the development of atherosclerosis (40–42).

A mounting body of evidence suggests that ECM proteolysis leads to the release of cryptic, bioactive ECM fragments or embedded growth factors, which in turn may dramatically alter angiogenesis, platelet activation, recruitment of mononuclear leukocytes and SMC and myofibroblast differentiation (21). Recent work by our group and others demonstrated that EC apoptosis represents a previously unrecognized pathway contributing to ECM proteolysis (5, 6, 18, 19, 43, 44). We showed that in EC, apoptosis triggers endorepellin proteolysis and LG3 release, which interacts with α2β1 integrins on EC, platelets, and fibroblasts, and triggers angiostatic, thrombogenic, and anti-apoptotic responses (20, 45–47).

In the present work we aimed at characterizing the proteases released by apoptotic EC and responsible for endorepellin cleavage. Three complementary MS/MS strategies were adopted: two approaches were comparative (i.e. medium conditioned by apoptotic HUVEC compared with medium conditioned by non-apoptotic HUVEC), and the third aimed at identifying proteases present in the functionally active anti-apoptotic fraction of SSC-apo. None of the proteomic strategies employed here identified BMP-1/Tolloid-like metalloprotease (a protease we recognized previously as a key enzyme implicated in endorepellin cleavage in non-apoptotic cell culture) (25) in medium conditioned by apoptotic EC. Also, we showed that inhibiting BMP-1 activity in HUVEC during conditioning did not modulate LG3 production. These results raised the possibility that proteolytic mechanisms involved in endorepellin processing in normal live cells differ from the pathways activated during apoptosis.

Two proteases isolated in medium conditioned by apoptotic EC, CathL, and t-PA had putative cleaving activity for endorepellin when analyzed with ACD Protein Manager Proteo-
Because various proteases are synthesized as inactive zymogens and most cathepsins function within a narrow pH optimum, characterization of a protease through MS/MS approaches does not necessarily indicate proteolytic activity. Hence, putative endorepellin cleaving activity was first confirmed by showing that recombinant endorepellin exposed to either CathL or t-PA in vitro was cleaved to generate a 23-kDa fragment recognized by an antibody specific for the C-terminal 40 amino acids of endorepellin. When equal volumes of media conditioned by either apoptotic or non-apoptotic EC were concentrated and compared, Western blotting confirmed the higher levels of active CathL in medium conditioned by apoptotic EC.

We then asked whether CathL release by HUVEC was specific to apoptotic cell death or associated with various forms of cell death. We showed that HUVEC develop classical features of apoptosis upon 4 h of serum starvation. Yet autophagic cell death may also be initiated by nutrient deprivation and develops through the suppression of mTOR; for review, see Ref. 48. Autophagy is an evolutionarily conserved catabolic process that mediates the degradation of cytoplasmic components and may in certain situations assume a cell-killing function (48). Yet, we found that pharmacological stimulation of autophagy with the mTOR inhibitor rapamycin did not stimulate CathL release. These results suggested that apoptosis but not autophagy played a central role in regulating CathL release. In support of this contention, we showed that another pro-apoptotic stimulus, MMC, also increased the release of the active CathL form. Necrotic cell death has been associated with the lysosomal destabilization and liberation of various proteases (48, 49). However, two levels of evidence indicate that necrosis does not participate

**FIGURE 4.** T-PA translocates to the cell periphery in association with caspase activation. **A,** immunoblotting for the LG3 fragment in media conditioned by HUVEC serum-starved for 4 h in the presence of t-PA-STOP or vehicle. Ponceau red staining is used as a protein-loading control. Each blot is representative of two separate experiments. **B,** protein levels of soluble t-PA in media conditioned by HUVEC pretreated with DMSO or ZVAD-FMK for 2 h and serum-starved for 4 h (n = 3 per condition). **C,** t-PA immuno-staining in HUVEC exposed to normal medium (C1) or serum-free medium (C2 to C5) for 4 h. Isotype control staining in HUVEC serum-starved for 4 h (C6), t-PA staining in HUVEC pretreated with DMSO (C7) or ZVAD-FMK (C8) and serum-starved for 4 h. White arrows, apoptotic cells; green t-PA; blue, To-Pro3/DNA. Scale = 50 μm.

**FIGURE 5.** Caspase activation leads to lysosomal permeabilization and release of active CathL. **A,** confocal microscopy with Acrysil-Violet CathL substrate in HUVEC exposed to normal medium (left panel) or serum-free medium (right panel) for 4 h. The white arrow points to extra-lysosomal translocation of the cleaved substrate. **B,** percentage of HUVEC with lysosomal destabilization, as evaluated by acridine orange staining, after exposure to normal medium (white bars) or serum-free medium (black bars) for 2–4 h (*, p < 0.0001 versus normal medium; n = 14 per condition). **C,** percentage of cells with lysosomal destabilization in HUVEC pretreated with either ZVAD-FMK or vehicle (Control) for 2 h followed by 4 h of serum-starvation (*, p < 0.001 versus ZVAD-FMK; n = 10 per condition).
in CathL release and endorepellin processing in our system. First, serum starvation for 4 h in HUVEC increased apoptosis but not necrosis. Also, induction of primary necrosis in HUVEC did not increase the release of the active 25-kDa form of CathL. Collectively, these data support a key role for apoptosis in CathL translocation and release.

Measurements of t-PA levels by enzyme-linked immunosorbent assay did not confirm increased levels of soluble t-PA in medium conditioned by apoptotic EC. Caspase-dependent peripheral t-PA translocation was found in apoptotic cells and bodies. Collectively, these observations suggest that CathL but not t-PA plays a predominant role in endorepellin cleavage in association with apoptosis.

Activation of various cathepsins during apoptosis has been reported to occur downstream or upstream of caspase activation or to replace caspase activity on key substrates in certain systems (49, 50). Hence, we investigated whether CathL inhibitors prevented LG3 release through an anti-apoptotic effect on serum-starved EC or through inhibition of CathL-dependent endorepellin cleavage or both. Inhibitors of CathL activation failed to modulate the apoptosis of serum-starved EC but largely prevented LG3 release, demonstrating that in this system CathL activity is crucial for endorepellin processing but not for the regulation of EC apoptosis. Also, caspase inhibition prevented lysosomal permeabilization, indicating that extra-lysosomal CathL translocation is not an early apoptotic event and occurs downstream of caspase activation. CathL inhibitors partially inhibited lysosomal destabilization, suggesting that CathL translocation may act as a positive feedback loop to further increase lysosomal permeability once the caspases have been activated.

We also tested the possibility that CathL could exert direct anti-apoptotic activity on target cells (i.e. SMC). Various proteases have been implicated in apoptosis inhibition through interactions with protease-activated receptors (51, 52). Yet, blocking CathL activity in SMC exposed to medium conditioned by apoptotic EC did not inhibit the anti-apoptotic response of SMC. These findings suggest that proteases released by apoptotic EC trigger an anti-apoptotic response in target cells largely through ECM proteolysis and the production of bioactive cryptic ECM fragments, such as LG3. CathL released from hemangiendothelioma cells (53) has been recently shown to cleave collagen XVIII, another ECM component, releasing endostatin and further highlighting the ECM-degrading capacity of CathL. Biochemical inhibition and caspase-3 silencing in serum-starved EC confirmed that caspase-3 activation plays a central role in CathL release. We also showed that silencing caspase-3 expression in EC exposed to another pro-apoptotic stimulus (DNA damage with MMC) blocked the release of active CathL.

**FIGURE 6. Caspase-3 activation is central to extracellular release of active CathL.** A, left panel, immunoblotting showing active (25 kDa) CathL levels in media conditioned by HUVEC pretreated with ZVAD-FMK, LEHD-FMK, DEVD-FMK, or vehicle for 2 h followed by serum starvation for 4 h (respectively, SSC ZVAD-FMK, SSC LEHD-FMK, SSC DEVD-FMK, and SSC Control). Middle panel, immunoblotting showing active (25 kDa) CathL levels in media conditioned by HUVEC treated with siRNA for caspase-3 or control followed by serum starvation for 4 h. Right panel, immunoblotting showing active (25 kDa) CathL levels in media conditioned by HUVEC treated with siRNA for caspase-3 (MMC siRNA Casp-3) or control (MMC siRNA Control) followed by MMC treatment for 24 h. For all experiments 5 ml of conditioned medium were concentrated and compared. Ponceau red staining is used as a protein-loading control. Blots are representative of two separate experiments. B, immunoblotting for LG3 under the same conditions as in A. Blots are representative of three separate experiments. C, immunoblotting for caspase-3 in HUVEC treated with caspase-3 siRNA or control siRNA and exposed to serum starvation for 4 h. Tubulin is shown as a loading control. Blots are representative of five separate experiments. D, percentage of apoptotic cells in HUVEC exposed for 4 h to normal medium, serum-free medium, and HUVEC treated with either siRNA for caspase-3 or control followed by serum starvation for 4 h (*, p < 0.00001 versus SS siRNA control; n = 9 per condition).
Cathepsin L Release Downstream of Caspase-3 Activation

These results demonstrate a novel role for caspase-3 in regulating protein trafficking and export.

Pro-inflammatory mediators, such as interleukin-1β, tumor necrosis factor-α, and interferon-γ, and growth factors, such as basic fibroblast growth factor and vascular endothelial growth factor, are known to increase the expression and secretion of cathepsins by SMC, EC, and macrophages (37). Our data indicate that caspase-3 activation during apoptosis of EC is a novel mechanism for the initiation of CathL release, which in turn plays a central role in endorepellin proteolysis and LG3 production, a cryptic activator of platelets (37, 54, 55).

In conclusion, EC apoptosis is a novel pathway contributing to CathL release and ECM proteolysis. Activated caspase-3 is the key effector controlling CathL release, which in turn initiates endorepellin degradation and LG3 production, a cryptic ECM fragment of potential importance in human vascular diseases.

Acknowledgments—We thank the J. L. Lévesque Foundation for renewed support. We also thank Ovid Da Silva for editing this manuscript.

REFERENCES

1. Hamet, P., and deBlois, D. (2001) Can J. Cardiol. 17, Suppl. A, 26–28
2. Vogt, C. J., and Schmid-Schönbein, G. W. (2001) Microcirculation 8, 129–139
3. Baumgartner-Parzer, S. M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A., and Waldhauß, W. (1995) Diabetes 44, 1323–1327
4. Du, X. L., Sui, G. Z., Stockklauser-Farber, K., Weiss, J., Zink, S., Schwipbert, B., Wu, Q. X., Tschope, D., and Rosen, P. (1998) Diabetologia 41, 249–256
5. Dimmelser, S., Haendeler, J., Galle, J., and Zeiher, A. M. (1997) Circulation 95, 1760–1763
6. Sata, M., and Walsh, K. (1998) J. Clin. Investig. 102, 1682–1689
7. Heermeier, K., Schneider, R., Heinloth, A., Wanner, C., Dimmeler, S., and Galle, J. (1999) Kidney Int. 56, 1310–1312
8. Shimizu, A., Yamada, K., Sachs, D. H., and Colvin, R. B. (2002) Kidney Int. 61, 1867–1879
9. Shimizu, A., Yamada, K., Sachs, M. S., Sachs, D. H., and Colvin, R. B. (2000) J. Am. Soc. Nephrol. 11, 2371–2380
10. Shimizu, A., Yamada, K., Sachs, D. H., and Colvin, R. B. (2000) Kidney Int. 58, 2546–2558
11. Ishii, S., Sawada, T., Kubota, K., Fuchinoue, S., Teraoka, S., and Shimizu, A. (2005) Kidney Int. 67, 321–332
12. Choy, J. C., Cruz, R. P., Kerjner, A., Geisbrecht, J., Sawchuk, T., Fraser, S. A., Hudig, D., Bleackley, R. C., Jirik, F. R., McManus, B. M., and Granville, D. J. (2005) Am. J. Transplant. 5, 499–499
13. Choy, J. C., Kerjner, A., Wong, B. W., McManus, B. M., and Granville, D. J. (2004) Am. J. Pathol. 165, 127–133
14. Pollman, M., Hall, J., Mann, Z., Zhang, L., and Gibbons, G. (1998) Nat. Med. 4, 222–227
15. Suzuki, J., Isobe, M., Morishita, R., Nishikawa, T., Amano, J., and Kaneda, Y. (2000) Cardiovasc. Res. 45, 783–787
16. Sata, M., and Walsh, K. (1998) J. Am. Soc. Nephrol. 9, 1–31
17. Raymond, M. A., Vigneault, N., Lutgens, E., Lutgens, S. P., Faber, B. C., Heeneman, S., Gijbels, M. M., de Vos, W. V., Vermeulen, S. A., Haas, J. L., and Cleutjens, J. P. (2003) FASEB J. 17, 515–517
18. Raymond, M. A., Mollica, L., Vigneault, N., Desormeaux, A., Chan, J. S., Filep, J. G., and Hebert, M. J. (2003) J. Biol. Chem. 28, 4238–4249
19. Raymond, M. A., Labelle, A., Abe, J., Iozzo, R. V., and Hebert, M. J. (2006) J. Biol. Chem. 281, 30383–30392
20. Lutgens, E., Lutgens, S. P., Faber, B. C., Heeneman, S., Gijbels, M. M., de Vos, W. V., Vermeulen, S. A., Haas, J. L., and Cleutjens, J. P. (2003) FASEB J. 17, 515–517
21. Raymond, M. A., Mollica, L., Vigneault, N., Desormeaux, A., Chan, J. S., Filep, J. G., and Hebert, M. J. (2003) FASEB J. 17, 515–517
22. Raymond, M. A., Mollica, L., Vigneault, N., Desormeaux, A., Chan, J. S., Filep, J. G., and Hebert, M. J. (2003) FASEB J. 17, 515–517
23. Raymond, M. A., Mollica, L., Vigneault, N., Desormeaux, A., Chan, J. S., Filep, J. G., and Hebert, M. J. (2003) FASEB J. 17, 515–517
24. Raymond, M. A., Mollica, L., Vigneault, N., Desormeaux, A., Chan, J. S., Filep, J. G., and Hebert, M. J. (2003) FASEB J. 17, 515–517
25. Raymond, M. A., Mollica, L., Vigneault, N., Desormeaux, A., Chan, J. S., Filep, J. G., and Hebert, M. J. (2003) FASEB J. 17, 515–517
(2004) J. Cell Biol. 166, 97–109
46. Bix, G., Iozzo, R. A., Woodall, B., Burrows, M., McQuillan, A., Campbell, S., Fields, G. B., and Iozzo, R. V. (2007) Blood 109, 3745–3748
47. Woodall, B. P., Nystrom, A., Iozzo, R. A., Eble, J. A., Niland, S., Krieg, T., Eckes, B., Pozzi, A., and Iozzo, R. V. (2008) J. Biol. Chem. 283, 2335–2343
48. Degterev, A., and Yuan, J. (2008) Nat. Rev. Mol. Cell Biol. 9, 378–390
49. Leist, M., and Jaattela, M. (2001) Nat. Rev. Mol. Cell Biol. 2, 589–598
50. Jaattela, M., and Tschopp, J. (2003) Nat. Immunol. 4, 416–423
51. Sambrano, G. R., Huang, W., Faruqi, T., Mahrus, S., Craik, C., and Coughlin, S. R. (2000) J. Biol. Chem. 275, 6819–6823
52. Ramachandran, R., Sadofsky, L. R., Xiao, Y., Botham, A., Cowen, M., Morice, A. H., and Compton, S. J. (2007) Am. J. Physiol. Lung Cell. Mol. Physiol. 292, L788–L798
53. Felbor, U., Dreier, L., Bryant, R. A., Ploegh, H. L., Olsen, B. R., and Mothes, W. (2000) EMBO J. 19, 1187–1194
54. Gacko, M., and Glowinski, S. (1998) Clin. Chem. Lab. Med. 36, 449–452
55. Gacko, M., and Glowinski, S. (1998) Clin. Chim. Acta 271, 171–177