Autologous Apoptotic Cell Engulfment Stimulates Chemokine Secretion by Vascular Smooth Muscle Cells

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Apoptosis of vascular smooth muscle cells (VSMCs) occurs *in vivo* under both physiological and pathological settings. The clearance of apoptotic cells may be accomplished in part by the surrounding normal VSMCs. However, the fate of internalized apoptotic cells, the rate of intracellular degradation, and the consequences of these processes to VSMC biology are unknown. Electron microscopy and confocal fluorescence imaging showed that rat VSMCs effectively bound and internalized autologous apoptotic VSMCs *in vitro*. Within 2 hours, the internalized apoptotic cells were delivered to lysosomes, and the majority of these internalized cells and their proteins were efficiently degraded by 24 hours. After degradation was completed, the phagocytic VSMCs remained viable with normal rates of proliferation. Clearance of apoptotic cells by VSMCs did not induce the release of vascular wall matrix proteases but was associated with a 1.6-fold increase in transforming growth factor- β 1 release. Interestingly, clearance of apoptotic cells stimulated VSMCs to secrete monocyte-chemoattractant protein-1 and cytokine-induced neutrophil chemoattractant. The coordinated release of transforming growth factor- β 1 and chemokines suggests that autologous apoptotic cell clearance stimulates VSMCs to release molecules that specifically recruit professional phagocytes while simultaneously dampening the inflammatory response and preventing vascular injury. (*Am J Pathol* 2005, 167:345–353)

Apoptosis of vascular smooth muscle cells (VSMCs) has been documented *in vivo* during physiological ves-

sel wall remodeling after birth as well as in pathological settings such as atherosclerosis and hypertension.^{1–4} Moreover, it has been well documented that VSMCs from a variety of species, including humans, undergo apoptosis on removal of serum-surviving factors or on exposure to cytokines and oxidants.^{5–8} The induction of vascular smooth muscle apoptosis necessitates effective mechanisms for removal of the apoptotic cells. The efficient removal of apoptotic cells by phagocytic cells, which is facilitated by the expression of multiple receptors that recognize apoptotic cells, is a critical process because it completes apoptosis and protects tissues and organs from pathological consequences and/or inflammation.^{9–13} Whereas the majority of the studies on apoptotic cell removal have been focused on professional phagocytic cells such as macrophages, the participation of the VSMCs and the consequences in clearing their own apoptotic cells is not well understood at present.

By electron microscopy, VSMCs in the vascular wall and in culture have been found to phagocytize necrotic cells as well as yeast and latex beads.¹⁴ Subsequently, it was shown that rat VSMCs internalize apoptotic cells derived from homologous cells in part by the recognition of the exposed phosphatidylserine on the apoptotic cell outer membrane.¹⁵ In addition, VSMCs internalize doxorubicin-coated mast cells.¹⁶ Although these three studies establish that VSMCs are capable of phagocytosis, whether VSMCs can handle a phagocytic load comparable with professional phagocytes has not been determined. Also, the physiological and pathological responses of VSMCs after apoptotic cell engulfment and degradation are not known at present.

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It is entirely possible that VSMCs participating in the process of apoptotic cell clearance are activated to release factors that would promote uncontrolled proliferation, remodeling of the extracellular matrix, or release of inflammatory mediators. VSMC hyperplasia and release of proteases are critical determinants that promote deleterious vascular wall remodeling in pathological conditions such as atherosclerosis.^{17–19} Furthermore, VSMCs are able to release inflammatory mediators that could recruit macrophages and leukocytes in the vascular wall, which contribute to the development and progression of atherosclerotic lesions.^{17–19}

Therefore, we investigated the fate, rate, and consequences of autologous apoptotic cell clearance by cultured VSMCs. The data revealed an asynchronous binding and engulfment of apoptotic cells by VSMCs that was completed within 24 hours. Delivery to lysosomes and complete degradation of the engulfed cells was completed within the next 24 hours without any significant effect on cellular morphology, viability, and rate of proliferation. Removal of apoptotic cells did not result in the secretion of proteases, and it was accompanied by an increase in transforming growth factor (TGF)- β 1, which modulates inflammatory responses by phagocytes and mammary epithelial cells after apoptotic cell clearance.^{11,15,18} Apoptotic cells also stimulated VSMCs to release two chemokines: monocyte/macrophage chemokine molecules monocyte-chemoattractant protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1). The recruitment of phagocytic cells in the presence of a modulator of inflammation may further facilitate apoptotic cell removal while minimizing inflammation and potentially injury to the vascular wall.

Materials and Methods

Induction of Apoptosis

Primary aortic smooth muscle cells were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion as described previously.²⁰ Cells were cultured in Dulbecco's medium supplemented with 10% bovine fetal serum. To induce apoptosis, the cells were treated with 1) a mixture of 250 U/ml tumor necrosis factor- α (TNF- α), 600 U/ml interleukin (IL)-1 β , 300 U/ml interferon (IFN)- γ , and 5 μ g/ml lipopolysaccharide for 72 hours; 2) 8 μ mol/L camptothecin; or 3) 20 ng/ml Fas-ligand for 24 hours in Dulbecco's medium supplemented with 5% bovine fetal serum. The apoptotic and nonapoptotic dead cells were distinguished using flow cytometry analysis with double labeling for annexin V conjugated with fluorescein isothiocyanate (FITC) and with red-fluorescent propidium iodide. Detection of annexin V-FITC/propidium iodide labeling was performed using 488-nm excitation and 530/30-nm bandpass filter for FITC detection and a 585/45-nm filter for PI labeling. At least 10,000 events were collected and analyzed ungated using the Quadrant Statistic in Cell Quest program (Becton-Dickinson, San Jose, CA). Cell labeling for flow cytometry was per-

formed using the Vybrant apoptotic assay kit per manufacturer's instructions (Molecular Probes, Eugene, OR).

Phagocytosis Assay

After induction of cell death, cells present in the culture medium were recovered and washed four times by centrifugation (600 $\times$ g) and re-suspension in 40 ml of phosphate-buffered saline (PBS), followed by final re-suspension in Dulbecco's modified Eagle's medium/2.5% fetal bovine serum before co-incubation with naïve smooth muscle cells. Apoptotic cells were then added at a ratio of 2:1 or 3:1 to naïve nonstimulated cells and allowed to bind for 2 hours at 37°C. After 2 hours of co-incubation, dead cells that were not attached or internalized were removed by extensive washing (five washes with gentle swirling after each addition of Dulbecco's modified Eagle's medium/2.5% fetal bovine serum medium). The cells were then incubated in fresh medium supplemented with 2.5% bovine fetal serum and harvested at various time points. The harvested cells were washed extensively with ice-cold PBS and processed for immunological, electron microscopic, and biochemical analyses. To examine the contribution of lysosome acidification in the degradation of apoptotic cells, the cells were pre-treated with chloroquine for 2 hours followed by extensive washing before the addition of the apoptotic cells. In addition, cells were challenged with 25 mmol/L NH₄Cl to alter the lysosomal pH during the co-incubation with apoptotic cells, and degradation of tyrosine-nitrated protein was followed over time by immunocytochemistry (data not shown).

Immunocytochemical Staining for Confocal Microscopy

Cells were co-incubated with apoptotic cells labeled with annexin V conjugated with FITC for 2 hours. The cells were washed several times, and fresh medium was added for an additional 2-hour incubation. After acetone/methanol fixation, the cells were blocked with bovine serum albumin and incubated with the monoclonal anti- α -actin antibody conjugated with Cy3 (2 μ g/ml mouse IgG2a; Sigma, St. Louis, MO) and 4-6-diamidino-2-phenyl-indole dihydrochloride. The cells were scanned with a Leica DM IRE2 HC fluor-TCS 1-B-UV microscope coupled to a Leica TCS SP2 spectral confocal system/UV. The scan head contains an AOBBS, acoustical-optical tunable filter, for simultaneous control of up to six laser lines. The three fluorochromes were sequentially scanned with a green HeNe (543 nm/1.2mW), the 488-nm line of a blue argon (458 nm/5mV 488 nm/20mW; 514 nm/20mW), and blue diode (405 nm/20mW) laser. This system allows pixel-by-pixel quantification, registration, and averaging of multiple images and superimposition of fluorescence images.

Electron Microscopy

Cells were co-incubated for 2 hours with apoptotic cells, then washed several times, and incubated in fresh medium supplemented with 2.5% serum for an additional 2 hours. Cells were postfixed with 2% osmium tetroxide and *en bloc* stained with saturated aqueous uranyl acetate. Cells were dehydrated through graded alcohol and embedded in EPON. Sections (70 nm thick) were cut on Leica Ultracut FCS and mounted on 200 mesh copper grid. Sample was stained with 7% uranyl acetate in 50% ethanol, washed, and counterstained with bismuth subnitrite. Grids were observed in JEOL Jem 1010, and digital images were captured using AMT for 12 hours aided by a Hamamatsu CCD camera.

Immunoelectron Microscopy

Immunoelectron microscopy was performed on cells fixed in 4% paraformaldehyde plus 0.2% glutaraldehyde, dehydrated in graded alcohol, and embedded in LR White. Sections (90 nm thick) were picked up on nickel grids. Grids were incubated with blocking buffer (1% ovalbumin fraction V plus 0.2% cold water fish skin gelatin) for 1 hour. Grids were then incubated with polyclonal anti-p53 antibody overnight. Grids were rinsed and stained with gold-labeled anti-rabbit for 2 hours at room temperature. Excess gold was washed off, and grids were stained in 2% aqueous uranyl acetate and observed in electron microscope as mentioned.

Detection of Inducible Nitric Oxide Synthase (iNOS)

iNOS protein expression and cellular localization was investigated as described previously.²¹ Cells were lysed and centrifuged at $14,000 \times g$. Extracted proteins were quantified by the Bradford assay. Proteins (50 μ g) were separated on 10% polyacrylamide gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were incubated with 125 ng/ml mouse monoclonal anti-iNOS antibody (Transduction Laboratories, San Jose, CA). After washing with PBS-0.05% Tween 20, the membranes were incubated with a goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate (BioRad) at 1:2500 dilution for 1 hour at room temperature. The proteins were detected by enhanced chemiluminescence. For immunocytochemistry, cells were fixed with a mixture of cold acetone/methanol. Nonspecific binding was blocked by incubation for 1 hour with 10% bovine serum albumin and normal goat serum in PBS plus 0.3% Triton X-100 at room temperature. The cells were then incubated with an anti-iNOS monoclonal antibody (500 ng/ml; Transduction Laboratories) and with affinity-purified anti-nitrotyrosine antibody 432²¹ overnight at 4°C. The cells were washed three times with PBS plus 0.3% Triton X-100 and incubated with Alexa Fluor 488 goat anti-mouse IgG antibody

(Molecular Probes) at 1:500 and with a Cy3-conjugated affinity-purified anti-rabbit IgG (Molecular Probes) at 1:200 dilution for 1 hour. The nucleus was stained with DAPI. Fluorescence images were obtained using an Olympus IX70 inverted microscope.

Cell Viability and Proliferation

Cells were seeded at 1.5×10^5 cells/ml, and after 2 days in culture, they were co-incubated with apoptotic cells as described above. Cells were assayed for apoptosis/necrosis using the Vybrant Apoptosis Assay (Molecular Probes). Control cells, camptothecin-treated cells, and VSMCs 48 hours after the completion of phagocytosis were incubated with annexin V-FITC and propidium iodide and analyzed by flow cytometry. Cell viability was also determined by trypan blue (0.4%) exclusion after 24, 48, and 72 hours after completion of phagocytosis.

For the cell proliferation assay, cells were washed and fixed for immunocytochemistry as described above. After blocking, the cells were incubated overnight at 4°C with an anti-Ki 67 monoclonal antibody (1 μ g/ml; Pharmingen, Inc., San Jose, CA). The cells were then washed with PBS plus 0.3% Triton X-100 and incubated with Alexa fluor488 goat anti-mouse IgG antibody (Molecular Probes) at 1:500 dilution for 1 hour at room temperature. Several random fields were then examined, and cells positive for anti-Ki 67 were counted. The results are expressed as the percentage $\pm$ SD of positive cells in the total cells counted. Cell proliferation after phagocytosis was further assayed by harvesting control and phagocytic cells 48 hours after co-culture with the apoptotic cells. After cell lysis, protein content of each condition was assayed by the bicinchoninic acid method (BCA Protein Assay; Pierce, Rockford, IL).

Elastin Degradation

The elastase activity was evaluated as described before^{22,23} with minor changes. Insoluble [¹⁴C]elastin (bovine ligamentum nuchae; Elastin Products, St. Louis, MO) was prepared by suspending 0.2 g of elastin powder in 9 ml of 0.15 mol/L sodium borate buffer, pH 9.0, for 18 hours with continuous stirring. [¹⁴C]formaldehyde (750 μ Ci) was added to the elastin suspension followed by the addition of three boluses of 100 μ l of freshly prepared solution of NaCNBH₃ (200 mmol/L stock). The reaction mixture was continuously stirred for an additional 18 hours at 4°C. [¹⁴C]elastin was centrifuged $10,000 \times g$ for 30 minutes and washed many times by re-suspending in cold water until the activity of the supernatant was at minimum level of 150 cpm/100 μ l and a specific activity of 322 cpm/ μ g [¹⁴C]elastin was achieved. After cells were co-incubated for 2 hours with apoptotic cells and washed several times, 250 μ g of [¹⁴C]elastin was added to the cells and incubated at 37°C for 24, 48, and 72 hours. As a positive control, cells were stimulated with 500 U/ml IFN- γ plus 10 ng/ml IL-1 β for 24, 48, and 72 hours. After each incubation time, culture media were

collected and centrifuged at $16,000 \times g$ for 30 minutes, and 200 μ l of the supernatant was transferred into 5 ml of ScintiSafe 30% (Fisher Scientific, Pittsburgh, PA) for scintillation counting of digested [14 C]elastin. The results are presented as mean $\pm$ SD and were performed in 12 separate wells and counted in triplicate.

TGF β 1 and Cytokine Production

Established enzyme-linked immunosorbent assays (ELISAs) were used to evaluate the production of TGF β 1, CINC-1 (the rat equivalent of GRO/IL-8), and MCP-1 in the conditioned cell media from control cells, camptothecin-treated cells, and cells after 48 hours of incubation with apoptotic cells. The ELISAs were performed according to the recommendations of the manufacturer (TGF β 1 and CINC-1; R&D Systems, Inc., Minneapolis, MN) (MCP-1; Biosource International, Camarillo, CA).

Figure 1. Phagocytosis of autologous apoptotic cells by VSMCs. **a:** Representative EM image of an apoptotic cell generated from VSMCs treated with LPS, TNF- α , IL-1 β , and IFN- γ . Note the nuclear fragment (n) containing condensed chromatin (arrows). **b to f:** EM images of VSMCs were incubated with apoptotic cells for 2 hours (*, apoptotic cell; arrowheads, lysosomes containing apoptotic cell fragments). Bars = 2 μ m in each frame. **f:** Magnified image of inset in **e** showing immunogold labeling with anti-p53 (arrows), confirming that these were apoptotic cell fragments.

Figure 2. Phagocytosis of apoptotic cells by VSMCs. **a to h:** Sequence of confocal optical sections showing apoptotic cells engulfed by a naive VSMC in culture after 2 hours of co-incubation. The cytoskeleton of the VSMC was labeled with actin-conjugated with Cy3 (red), the nucleus was stained with DAPI (blue), and the apoptotic cell was labeled with FITC-conjugated annexin V (green) before internalization. Bar = 10 μ m. **i:** xz projection of the image stack, showing phagocytosed apoptotic cells within the cell (arrowhead).

Results

Binding/Phagocytosis of Apoptotic Cells

Exposure of rat aortic VSMCs to a mixture of cytokines plus LPS resulted in cell death, with the majority of the dead cells present in the culture supernatant after 72 hours. The detached cells had rounded appearance and contained nuclear fragments with condensed chromatin (Figure 1a). By flow cytometry, $88.6 \pm 8.1\%$ ($n = 6$ independent determinations) of the dead cells were positive for FITC-annexin V, consistent with exposure of phosphatidylserine to the outer membrane leaflet. Similar results were obtained after treatment of cells with camptothecin or Fas-ligand (data not shown), well-known inducers of VSMC apoptosis.

Uptake of apoptotic cells was examined by electron microscopy. As shown in Figure 1, b to d, apoptotic cells were tightly associated with the surface of VSMCs, and with time, the apoptotic bodies were completely internalized. Consistent with previous data,¹⁵ 332 ± 90 ($n = 4$) apoptotic cells/100 VSMCs either were engaged or were in various phases of phagocytosis within the first 2 hours of co-incubation. The initial large phagosome containing a single apoptotic cell was eventually broken down into multiple smaller vesicles. The material in the lumen of these vesicles was enriched for p53, which has been shown to mediate apoptosis in rat VSMCs,⁵ as determined by immunogold electron microscopy (EM; Figure 1, e and f).

We also examined apoptotic cell uptake by confocal fluorescence microscopy. Apoptotic cells labeled with FITC-conjugated annexin V were harvested and washed extensively to remove cellular debris and then co-incubated with naive nonstimulated VSMCs. As shown in Figure 2, the apoptotic cells were internalized by VSMCs.

Degradation of Apoptotic Cell by VSMCs

Previously, it was shown that exposure of rat VSMCs to the same mixture of cytokines plus LPS that induced

Figure 3. Apoptotic cells contain iNOS. Apoptotic cells were generated by exposure of rat VSMCs to LPS, TNF- α , IL-1 β , and IFN- γ and then fixed and stained with anti-iNOS antibody (**a**) and DAPI (**b**). **c** and **d**: Merged fluorescence and phase contrast images, respectively. Bar = 2 μ m.

apoptosis also induces iNOS and induces nitration of tyrosine residues in proteins.²¹ We found that apoptotic cells were enriched for iNOS (Figure 3). Expression of iNOS also accompanies induction of apoptosis after treatment of cells with camptothecin or Fas-ligand (not shown). Because iNOS was not expressed by cultured,

Figure 5. Time course of lysosomal iNOS degradation. **a**: VSMCs were incubated for 2 hours at 37°C with apoptotic cells and then further incubated for varying amounts of time 37°C with or without pretreatment with chloroquine. The cells were then harvested, and iNOS expression was determined by immunoblot. GAPDH was used as a protein loading control. **b**: Densitometric analysis of three independent experiments (mean $\pm$ SD). Before incubation with apoptotic cells, VSMCs did not express detectable levels (nd) of iNOS. Chloroquine significantly inhibited iNOS degradation, indicating lysosomal degradation of iNOS. * P < 0.05, analysis of variance with Tukey post hoc analysis.

naïve VSMCs (Figures 4 and 5), we used iNOS as a marker to study the time course of apoptotic cell degradation.

The kinetics of apoptotic cell degradation are shown in Figures 4 and 5. We assayed for both iNOS and tyrosine-nitrated proteins formed as a result of nitric oxide production by iNOS.²¹ To avoid a potential confounding contribution of naïve cell proliferation, these

Figure 4. Degradation of iNOS and nitrated proteins after apoptotic cell phagocytosis. VSMCs were either untreated (**a**, **f**, **k**, and **p**) or incubated with apoptotic cells for 2 hours, followed by further incubation of 2 hours (**b**, **g**, **i**, and **q**), 4 hours (**c**, **h**, **m**, and **r**), 24 hours (**d**, **i**, **n**, and **s**), or 48 hours (**e**, **j**, **o**, and **t**) at 37°C. The cells were then fixed and stained with anti-iNOS (**a** to **e**), anti-nitrotyrosine (NTyr; **f** to **j**), and DAPI (**k** to **o**). Merged images are shown in **p** to **t**. At early incubation times, VSMC lysosomes contained both iNOS and nitrated proteins (arrowheads), however, by 24 to 48 hours, most of this material had been completely degraded. Bar = 10 μ m.

Table 1. Effect of Phagocytosis on Vascular Smooth Muscle Properties

	Viability*	Proliferation [†] /protein [‡]	Protease release [§]
Naïve cells	2.3 ± 1.1	31 ± 3	bd
	5.1 ± 0.6	1.95 ± 0.06	
Cells after phagocytosis	2.6 ± 0.7	26 ± 4	bd
	6.3 ± 0.6	2.13 ± 0.03	
Cytokine/camptothecin treated	7.5 ± 0.6	nd	257 ± 72
	22.7 ± 1.7 [¶]	1.66 ± 0.03	(n = 12)

*Cell death determined by flow cytometry. Top number indicates the percentage ± SD of propidium iodide-labeled cells (n = 6), and bottom number indicates percentage ± SD of annexin V-positive cells (n = 6). The data were obtained 48 hours after incubation with apoptotic cells using cells treated with camptothecin for 48 hours as a positive control.

[†]Proliferation was determined by the expression of the ki67 antigen and by measuring protein in the adhered cells 48 hours after incubation with apoptotic cells. The number represents the percentage ± SD of cells stained positive for ki67 antigen (n = 5).

[‡]Protein is expressed as mg/ml, and the values represent the mean value ± SD of six independent determinations.

[§]Release of proteases was evaluated by the degradation of elastin as described in detail in Materials and Methods.

[¶]Significance at P < 0.05 using ANOVA and Tukey's test for post hoc pairwise multiple comparison.

bd, below level of detection; nd, not determined.

experiments were performed using VSMC treated with salicylic acid to inhibit cell proliferation.²⁴ Both iNOS and nitrotyrosine immunoreactivities diminished over time and were virtually absent 48 hours after engulfment of apoptotic cells. Degradation of iNOS was also confirmed by immunoblot (Figure 5). Moreover, pretreatment of naïve VSMC with chloroquine, which prevents lysosome acidification and inhibits lysosome protease function,²⁵ slowed the rate of iNOS degradation. Collectively, these data support the notion that engulfed apoptotic cells were effectively degraded by VSMC lysosomes.

VSMCs Responses to Autologous Phagocytosis of Apoptotic Cells

On completion of apoptotic cell degradation, the cells remain viable as determined by flow cytometry for both necrotic and apoptotic cells (Table 1). The cells that had completed the process of phagocytosis proliferate at a normal rate evaluated by the expression of Ki67 antigen and by measuring protein concentration (Table 1). Seventy-two hours after completion of phagocytosis, 26 ± 4% of cells were Ki67 positive compared with 31 ± 3% of control cells (n = 5).

Moreover, 24 hours after completion of phagocytosis, the cells did not show appreciable release of elastolytic proteases, similar to naïve control cells. By comparison, cytokine-stimulated cells that were not exposed to apoptotic cells degraded 257 ± 72 ng of elastin per 10⁵ cells per 24 hours (n = 12), consistent with a previous report.²² This suggests that internalization of apoptotic cells did not activate breakdown of the extracellular matrix by VSMCs.

Previous studies have shown that macrophages and epithelial cells secrete the anti-inflammatory molecule TGFβ1 after phagocytosis of apoptotic cells.^{26–28} Therefore, it was determined whether VSMCs might exhibit a similar response. The data in Figure 6 show that VSMCs challenged with apoptotic cells secrete 1.6-fold more TGFβ1 in the conditioned media compared with naïve cells. The engulfment and phagocytosis of apoptotic cells also induced the release of two chemokines:

MCP-1, a monocyte/macrophage attractant, and CINC-1, a rat member of the GRO/IL-8 family (Figure 6). A 2.7- and 1.6-fold increase in the levels of MCP-1 and CINC-1, respectively, was measured in conditioned media from cells incubated for 48 hours with apoptotic cells compared with naïve cells. Note that secretion of these chemokines was not associated with expression of pro-inflammatory molecules such as iNOS by the VSMCs on completion of the apoptotic cell removal (Figures 4 and 5).

We confirmed that the increase in MCP-1 release was not derived from the camptothecin-treated apoptotic cells, because the levels of MCP-1 in the conditioned media of camptothecin-treated cells (273 ± 72 pg/mg cell protein) were comparable with naïve cells. However, camptothecin treatment did stimulate VSMCs to secrete CINC-1 (92 ± 15 pg/mg protein, P < 0.05 versus nonstimulated control cells). It is unlikely that the responses were due to the apoptotic cells themselves, because the apoptotic cells were extensively washed before incubation with VSMCs (described in detail in Materials and Methods), and the chemokine secretion was measured 48 hours after challenge with the apoptotic cells, a time at which apoptotic cells had been cleared from the culture medium, internalized, and degraded. Taken together, the results suggest that

Figure 6. TGFβ1 and cytokine production by VSMCs. TGFβ1, MCP-1, and CINC-1 release into the culture medium by control and cells incubated for 48 hours with apoptotic cells. The levels of TGFβ1, MCP-1, and CINC-1 were assayed by ELISA and normalized to total cell protein (n = 6). The values represent the mean ± SD. The values for TGFβ1, MCP-1, and CINC-1 in the cells incubated with apoptotic cells were significantly different from controls (*P < 0.05, Student's t-test).

autologous apoptotic cells provide a stimulus that causes VSMCs to simultaneously secrete anti-inflammatory hormones and phagocyte chemoattractants.

Discussion

Apoptotic cell removal and degradation of the internalized material is the final phase of apoptosis.^{9–13} Thus, effective and rapid removal of apoptotic cells completes the apoptotic process and avoids pathological consequences.^{9–13} Herein, we provided evidence that naïve VSMCs effectively cleared autologous apoptotic cells. We also found that apoptotic cell clearance stimulated VSMCs to simultaneously secrete two chemokines, MCP-1 and CINC-1, and an anti-inflammatory hormone, TGF β 1. Whereas previous studies have shown that macrophages and epithelial cells secrete TGF β 1 in response to phagocytosis of apoptotic cells,^{26–28} this is the first documentation that the same process stimulates secretion of MCP-1 and CINC-1 by VSMCs. These data suggest that VSMCs participating in the process of removing apoptotic cells signal to specifically recruit phagocytes into vessel wall but not other inflammatory cells. Consistent with the absence of pro-inflammatory mediators during this process, VSMCs internalizing and degrading apoptotic cells did not express iNOS and did not release elastolytic proteins.

Inappropriate signaling and accumulation of large numbers of macrophages has been demonstrated during the progressive changes of lesion development in atherosclerosis.^{29–33} The production of chemokines has been attributed to cellular injury and even to VSMC apoptosis in the vascular wall.³⁴ Moreover, effective blockade of chemokine release has been shown to diminish atherosclerotic burden in different mouse models of the disease.^{35,36} However, chemokines in conjunction with TGF β 1 may provide a mechanism to recruit phagocytic cells without the associated damage that might otherwise occur during inflammation. This has the potential to maximize the benefit of recruiting phagocytic cells to efficiently remove apoptotic cells while minimizing the potential risk associated with this recruitment. Consistent with this, previous studies have shown that apoptotic cell removal by professional phagocytes and mammary epithelial cells is associated with the release of TGF β 1, suppression of pro-inflammatory cytokines, and release of other growth hormones such as vascular endothelial growth factor (VEGF).^{25–28}

Whereas the removal of apoptotic cells from the vascular wall is essential in preventing activation of thrombin and increased risk for thrombosis,^{17,18} the persistent accumulation of phagocytes could predispose the tissue to adverse injury. The possibility of vascular wall injury could be exaggerated by considering that the presence of oxidized LDL within the vascular wall during lesion development was shown to delay the removal of apoptotic cells^{37–39} and potentially prolong the resident time of phagocytic cells. This underscores the potential importance of TGF β 1 secretion by VSMCs as a means to limit

excessive recruitment of phagocytic cells to sites of vascular remodeling or injury.

Our findings that VSMCs participate in apoptotic cell clearance are supported by previous studies. Garfield et al¹⁴ used electron microscopy to show that VSMCs phagocytize necrotic cells, yeasts, and 0.3- μ m latex spheres. Moreover, Bennett et al¹⁵ demonstrated that VSMCs in part bind phosphatidylserine exposed in the outer leaflet to engage and engulf autologous apoptotic cells. We have expanded on these studies by demonstrating that VSMCs effectively degrade engulfed apoptotic cells primarily by incorporation into lysosomes and complete the process without suffering deleterious effects. Apoptotic cell processing was asynchronous, and at early times, VSMCs had both surface-bound and internalized fractions. The lack of synchronization in the engagement and internalization of the apoptotic cells may relate to the different size of the apoptotic cells as well as possible variations in the expression of the recognition domains for phosphatidylserine or other factor(s) of naïve VSMCs.¹⁵ Based on immunohistochemical and biochemical analysis (Figures 4 and 5), lysosomal proteases degraded the contents of the internalized apoptotic cells. This included oxidatively modified, tyrosine-nitrated proteins present in the apoptotic cells, suggesting that lysosomal hydrolysis was not hindered by the presence of these modified proteins.

The clearance of apoptotic cells by VSMCs under the conditions studied in this work did not influence cell viability, the rate of cell proliferation, or the secretion of elastolytic molecules. These observations are significant because loss of VSMCs can translate into loss of a clearance mechanism, which in turn, could result in the buildup of apoptotic cells and cell fragments in the vascular wall. Moreover, a decline in viable VSMCs has been shown to result in decreased collagen production that predisposes plaques to rupture, which can contribute to acute myocardial infarction, a common and significant adverse consequence of atherosclerosis.^{17–19} Furthermore, excessive accumulation of VSMCs along with phagocytes and T lymphocytes is believed to play a critical role in the progression of atherosclerosis.^{1–3,17–19} Our data indicate that the process of removing apoptotic cells does not contribute to signaling events that lead to VSMC hyperplasia. Atherosclerotic changes within the vascular wall also include extensive remodeling of the extracellular matrix, and VSMCs are known to release enzymes capable of degrading elastin and collagen, the prominent constituents of the extracellular matrix within the vascular wall.²² However, the process of removing autologous apoptotic cells does not appear to contribute to the secretion of elastolytic molecules (Table 1).

Overall, the studies herein for the first time reveal that undisturbed smooth muscle cells are able to successfully process and completely degrade internalized apoptotic cells and that this process was accompanied by stimulated hormone and chemokine secretion. Completion of the process did not influence cellular viability, morphology, and rate of proliferation or induce the release of enzymes that can reconstruct the vascular wall matrix. The removal of apoptotic cells was associated with a

coordinate release of TGF β 1, which has the capacity to locally suppress the expression of pro-inflammatory molecules, and chemokines, which have the potential to recruit phagocytic cells to sites at which apoptotic VSMCs are located. Whereas the successful removal and degradation of apoptotic cells underscores a critical role for VSMCs in vessel wall remodeling, the release of chemokines indicates a previously unrecognized molecular event in the recruitment of phagocytes within the vascular wall. Whether this recruitment could produce adverse effects in the vascular intima *in vivo* remains to be determined. Nonetheless, our results suggest that coordinating phagocytes and VSMCs to clear apoptotic cells is likely to be a critical step that determines whether vessel wall alterations lead to a healthy or pathological phenotype.

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