Granzyme B Induces Smooth Muscle Cell Apoptosis in the Absence of Perforin

Involvement of Extracellular Matrix Degradation


Objective—T cell–induced cytotoxicity, of which granzyne B is a key mediator, is believed to contribute to the pathogenesis of inflammatory vascular diseases. In this report, we investigate the mechanism of granzyne B–induced smooth muscle cell (SMC) death.

Methods and Results—The addition of purified granzyne B alone to cultured SMCs caused a significant reduction in cell viability. Chromatin condensation, phosphatidylycerine externalization, and membrane blebbing were observed, indicating that the mechanism of granzyne B–induced SMC death was through apoptosis. Activated splenocytes from perforin-knockout mice induced SMC death through a granzyne B–mediated pathway. Inhibition of the proteolytic activities of caspases and granzyne B prevented granzyne B–induced SMC death, whereas attenuation of granzyne B internalization with mannose-6-phosphate (M6P) did not. Further, granzyne B induced the cleavage of several SMC extracellular proteins, including fibronectin, and reduced focal adhesion kinase phosphorylation.

Conclusions—These results indicate that granzyne B can induce apoptosis of SMCs in the absence of perforin by cleaving extracellular proteins, such as fibronectin. (Arterioscler Thromb Vasc Biol. 2004;24:2245-2250.)

Key Words: granzyne B ▪ perforin ▪ smooth muscle cell ▪ apoptosis ▪ extracellular

The granule exocytosis pathway is one of the main mechanisms through which T cells kill pathogenic cells. Granzyne B is a cytotoxic serine protease contained within cytotoxic T cell granules that induces apoptosis of target cells. On activation, granzyne B is exocytosed from T cells and is endocytosed through a receptor-dependent mechanism into intact vesicles of target cells. In the presence of perforin, granzyne B is released into the cytoplasm of target cells, where it induces apoptosis through the indirect or direct activation of caspases. Because granzyne B requires perforin to be delivered to the cytosol of target cells, induction of cell death by this serine protease is believed to require perforin.

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Vascular cell death induced by activated T cells contributes to the pathogenesis of several inflammatory vascular diseases. Cytotoxic T cells are observed in Takayasu arteritis and giant cell arteritis, and cytotoxic granule components derived from these lymphocytes localize to medial smooth muscle cells (SMC) in these diseases, suggesting that granule-mediated SMC death may contribute to vascular damage. Similar localization of T cell cytotoxic granule components to medial SMCs is observed in aortic aneurysms, suggesting that this type of SMC death may contribute to weakening of the vessel wall and eventual rupture of the aorta. Both granzyne B and perforin are also abundant in allograft arteries, whereupon perforin-containing T cells localize to the immediate subendothelial space and granzyne B localizes to terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive macrophages and SMCs in both the intima and media. In allograft arteries, there is significant medial SMC death that is mediated by cytotoxic T cells. We have recently shown, using a rat heterotopic heart transplant model, that immune-mediated medial degradation and medial cell apoptosis is associated with severe defects in SMC constriction of allograft arteries. Finally, granule-mediated SMC death may also play a role in the pathogenesis of atherosclerosis as SMC apoptosis contributes to plaque destabilization and rupture, which is the leading cause of myocardial infarction. Granzyne B is observed in and around TUNEL-positive SMCs in both the intima and media of atherosclerotic arteries, suggesting that the granule pathway may be mediating SMC death in this disease.
Although T cell-induced SMC death contributes to a number of vascular pathologies and affects vasomotor function in allograft arteries, the mechanisms through which this event is mediated has not been investigated. Because immunohistological experiments have implicated the granule pathway in the induction of SMC death during autoimmunity, vascularized organ transplant rejection, and atherosclerosis, we have investigated the mechanisms through which granzyme B induces SMC death in vitro. Surprisingly, granzyme B can induce SMC death in the absence of perforin. We provide evidence indicating that the mechanism through which granzyme B induces SMC apoptosis in the absence of perforin is mediated by the proteolysis of extracellular proteins.

Materials and Methods

Reagents
Please refer to online supplementary data available at http://atvb.ahajournals.org for details.

Quantitation of Cell Viability
Human coronary artery SMCs and HeLa cells were grown to 70% confluence in 96-well plates, maintained in medium +0.2% fetal calf serum for 48 hours, and treated with either granzyme B or granzyme B plus Ad5 (10 to 100 pfu/cell). In experiments using inhibitors and mannose-6-phosphate (M6P), SMCs were incubated with z-VAD.fmk (100 μmol/L), z-AAD.cmk (25 μmol/L), or soluble M6P (20 mmol/L) for 30 minutes before the addition of granzyme B. Cell viability was quantitated with an MTS assay as per the manufacturer’s instructions (Promega, Madison, Wis).

Assessment of Apoptosis
Chromatin condensation was assessed by staining SMC nuclei with the DNA dye Hoechst 33342 and phosphatidylserine outer membrane localization determined by staining unfixed cells with Alexa 488-labeled Annexin V. Please see online supplementary data for details.

Silver Staining
Please refer to supplementary data for details.

Splenocyte Killing Assays
Splenocyte killing assays were performed using calcein-loaded Fisher rat aortic SMCs or concanavalin A (Con A)-treated rat splenocytes as targets, and Con A-activated C57BL/6 (wild-type) or perforin-knockout splenocytes as effectors. Calcein release was used to quantitate SMC death. The following formula was used to calculate percent specific calcein release:

\[
\text{% specific calcein release} = \left( \frac{\text{calcein release in sample} - \text{spontaneous release}}{\text{total calcein release} - \text{spontaneous release}} \right) \times 100
\]

Please refer to supplementary data for details.

Cell Lysis and Western Blot
SMCs were grown to 70% confluence on 60 mm tissue culture (TC) dishes, maintained in SmBM +0.2% fetal calf serum for 48 hours, and treated with granzyme B (75 nM). After 24 hours, cells were lysed and Western blot analysis was performed as described previously.10

Granzyme B Uptake Assay
See supplementary data for details.

Assessment of Proteolysis of Extracellular Proteins
SMCs were grown to 70% confluence in a 24-well TC plate and maintained in SmBM +0.2% fetal calf serum for 48 hours. SMCs were then washed 3 times with cold PBS, and lysed with 0.25 mol/L NH4OH for 30 minutes. Biotinylation of the extracellular proteins was performed using a biotinylation kit as per the manufacturer’s instructions (Pierce Chemicals). At the indicated time points, samples of the supernatant were collected. At 24 hours after treatment, supernatant was collected, and total extracellular proteins collected in SDS lysis buffer and Western blot performed to assess the protein profile.

Statistics
An ANOVA was performed to determine significance between granzyme B-treated and control groups in the dose response experiments. In all other experiments, a Student t test was performed. P value (α-error) of <0.05 was considered significant.

Results

Granzyme B Alone Induces Apoptosis of SMCs
Granzyme B alone reduced SMC viability (Figure 1A). There was a significant decrease in cell viability beginning at 37.5
nM, and this reached a minimum of 56.5±5.9% of control at 150 nM. The addition of Ad5, which mimics the granzyme B-delivery actions of perforin in vitro through its endosomolytic properties, did not affect the granzyme B-induced decrease in SMC viability, indicating that the addition of an endosomolytic agent may not potentiate granzyme B-induced cytotoxicity toward SMCs. By transfecting SMCs with Ad5 containing a GFP vector and assessing GFP-positive SMCs, we have ensured that Ad5 was capable of transfecting ~50% of SMCs, indicating that Ad5 is an effective protein delivery agent in SMCs (data not shown). As a control, HeLa cells were also treated with granzyme B and consistent with previous reports granzyme B and Ad5 acted in concert to induce a dramatic and significant reduction in HeLa cell viability (Figure IA, available online at http://atvb.ahajournals.org). Importantly, granzyme B alone did not affect HeLa cell viability, suggesting that perforin-independent granzyme B-induced cell death is cell-type specific.

To ensure that the cytotoxicity of purified granzyme B toward SMCs was not a result of perforin contamination, silver staining and Western blotting for perforin was performed on purified granzyme B preparations. These preparations contained a single band at 32 kDa, which is the molecular weight of granzyme B (Figure IB and IC). The absence of other bands in either assay indicates that perforin (molecular weight ~66 kDa) was not present in the preparations.

Assessment of SMC morphology after 24 hours of treatment with granzyme B (75 nM) indicated that this protease induced morphological alterations such as cell rounding and surface detachment (Figure IIA, available online at http://atvb.ahajournals.org). Granzyme B did not have any effect on HeLa cells in the absence of an endosomolytic agent. Because the observed morphological changes in granzyme B-treated SMCs are indicative of apoptosis, SMCs were stained with Hoechst 33342 and Annexin V to visualize chromatin condensation and the externalization of phosphatidylserine, respectively, to further characterize apoptosis. Granzyme B induced marked chromatin and nuclear condensation (Figure 1B), as well as phosphatidylserine outer membrane localization in several SMCs (Figure 1B), indicating that granzyme B was inducing SMC apoptosis. Quantitation of Annexin V-positive cells 24 hours after treatment with granzyme B revealed a significant increase in SMC apoptosis (19.0±2.6% in granzyme B-treated SMCs as compared with 10.3±0.7% in untreated SMCs, P<0.04).

To assess the timing of granzyme B-induced SMC apoptosis, we temporally characterized the morphological changes induced by this protease. Assessment of dark and light field photomicrographs of SMCs labeled with Alexa 488-conjugated Annexin V indicated that SMCs displaying Annexin V-positivity were also undergoing morphological changes of apoptosis (Figure IIB). Granzyme B-induced SMC apoptosis was a slow event. Morphological changes did not begin until at least 16 hours after treatment and were not abundant until 20 hours after treatment. At 24 hours and 48 hours after treatment, several SMCs were undergoing cell rounding, surface detachment, and membrane blebbing (Figure IIC).

**Splenocytes Induce SMC Death Through a Perforin-Independent Granzyme B-Mediated Pathway**

Limited SMC death was induced by activated splenocytes at time points at or before 24 hours, and splenocyte-induced SMC death was not optimal until 48 hours. At this time point, splenocytes induced SMC death in a dose-dependent manner, reaching a maximum of 41.4±3.0% specific calcein release at an effector:target (E:T) of 25:1 (Figure IIIA, available online at http://atvb.ahajournals.org). There was no SMC death induced by inactivated splenocytes or the supernatant from activated splenocytes at 48 hours, indicating that SMC death was mediated by activated T cells and was not caused by the passive release of granule components at 48 hours (data not shown). In addition, Con A-activated mouse splenocytes induced cell death of isolated rat splenocytes by 6 hours, indicating that the slow time course of SMC death is not a result of the assay system but represents a reduced sensitivity of SMCs to immune-mediated cell death as compared with certain other cell types (Figure IIIB). Also, activated syngeneic splenocytes did not induce cell death.

SMCs were incubated with wild-type or perforin-knockout Con A-activated splenocytes at an E:T of 12:1 in the presence or absence of z-AAD.cmk (a synthetic granzyme B inhibitor) to determine the contribution of granzyme B to splenocyte-induced SMC death in the absence of perforin. We have previously shown that z-AAD.cmk does not inhibit apoptosis induced by caspases10 and this inhibitor does not prevent TRAIL-induced apoptosis (data not shown), indicating that z-AAD.cmk does not block receptor-mediated apoptosis. There was a reduction in SMC death induced by perforin-knockout splenocytes as compared with wild-type splenocytes, indicating that SMCs are sensitive to cell death induced by a perforin pathway. However, the addition of z-AAD.cmk to perforin-knockout splenocytes further reduced SMC death, indicating that activated T cells can kill SMCs through a perforin-independent granzyme B-mediated pathway (Figure 1C). Finally, because we have shown that purified granzyme B alone induces SMC apoptosis, the calcein release measured in these experiments represents membrane damage that occurs during late-stage apoptosis.

**Granzyme B–Induced SMC Apoptosis Is Dependent on Caspase and Granzyme B Proteolytic Activity**

Granzyme B treatment resulted in the processing of procaspase-3 to its p17 fragment, suggesting that the caspase cascade was being activated in SMCs (Figure IV, available online at http://atvb.ahajournals.org). z-VAD.fmk completely inhibited the granzyme B-mediated loss in cell viability, indicating that caspase activation is required for perforin-independent SMC death (Figure 2). SMCs were also treated with the granzyme B inhibitor z-AAD.cmk (25 μmol/L) for 30 minutes before the addition of granzyme B to assess the contribution of granzyme B proteolysis to SMC death. Similar to z-VAD.fmk, z-AAD.cmk completely inhibited the reduction in SMC viability (Figure 2).
Granzyme B Acts Extracellularly to Induce SMC Apoptosis

Although granzyme B-induced SMC death was not apparent until 16 hours after treatment, GrB-488 was associated with SMCs as early as 2 hours, and this interaction was more pronounced at 24 hours (Figure V, available online at http://atvb.ahajournals.org). The pattern of GrB-488 localization with SMCs appeared to be very punctate and perinuclear, which is similar to that observed in other cell types and is suggestive of its localization to intact vesicles. To inhibit granzyme B uptake, SMCs were incubated with 20 mmol/L soluble mannose-6-phosphate (M6P) before treatment with GrB-488. M6P has been shown to inhibit granzyme B internalization in other cell types. At 2 hours and 24 hours, M6P attenuated GrB-488 association with SMCs, indicating that M6P can attenuate granzyme B internalization into SMCs (Figure V).

Because soluble M6P attenuates granzyme B uptake into SMCs, these cells were incubated with 20 mmol/L soluble M6P for 30 minutes before the addition of granzyme B. At 24 hours after treatment, MTS assay was performed. Soluble M6P did not inhibit SMC death, indicating that granzyme B internalization is not required for SMC death and that granzyme B is acting extracellularly to induce cell death (Figure 3).

Granzyme B Acts Extracellularly to Induce SMC Apoptosis

Granzyme B Induces the Cleavage of Multiple Extracellular Proteins

Treatment of SMC matrix with granzyme B induced the cleavage of a number of extracellular proteins (Figure 4). In the collected insoluble proteins, 4 protein bands between ~50 to 70 kDa and ~236 kDa disappeared 24 hours after treatment with granzyme B and cleavage fragments of ~25 to 39 kDa were evident in the matrix at this same time point. Further, at least 6 proteins and/or cleavage fragments ranging in molecular weight from ~29 to 148 kDa were eluted into the supernatant as early as 2 hours after granzyme B treatment. To ensure that the SMC extracellular protein preparations used were devoid of intracellular proteins, Western blotting for β-actin was performed on the collected supernatant and extracellular proteins. β-actin was apparent in SMC lysates (positive control) but was absent from matrix and supernatant preparations.

To identify extracellular proteins that are cleaved by granzyme B, we performed Western blots for fibronectin, collagen, and vitronectin on lysates from untreated and granzyme B-treated SMCs. In SMCs treated with granzyme B for 24 hours, there was a reduction in the total amount of fibronectin in lysates collected from SMCs. In the supernatants of granzyme B-treated SMCs at 24 hours, a fibronectin cleavage product was detected (Figure 4). No cleavage of collagen IV or vitronectin was observed (Figure VIA, available online at http://atvb.ahajournals.org). Therefore, granzyme B induces the cleavage of fibronectin in SMC extracellular matrices but does not affect collagen IV or vitronectin.

To determine whether granzyme B was inducing SMC apoptosis through the generation of soluble pro-apoptotic fragments from extracellular proteins or through the disruption of established cell–matrix interactions, we collected the supernatant from granzyme B-treated SMC extracellular proteins (which contain granzyme B-generated cleavage products) and inhibited granzyme B activity with the addition of z-AAD.cmk before the addition of these fragments to SMCs for 24 hours. The granzyme B-generated SMC extracellular protein fragments were not capable of inducing SMC death (Figure VIB). Because these results suggest that granzyme B is disrupting cell–matrix interactions, we evaluated the integrity of extracellular matrix-derived intracellular signals by assessing focal adhesion kinase (FAK) phosphorylation by Western blot analysis. In adherent cell types, FAK is activated by phosphorylation in response to cellular interactions with extracellular components and this kinase regulates survival signals. Disruption of FAK activation through elimination of extracellular-derived signals can induce apoptosis. Treatment of SMCs with granzyme B for 24 hours reduced
FAK phosphorylation but did not alter the levels of total FAK protein (Figure 4). These results indicate that granzyme B is inducing SMC death by disrupting extracellular SMC signals through the proteolysis of extracellular proteins.

Discussion

In the current study, we demonstrate that granzyme B can induce SMC apoptosis in the absence of perforin. Perforin-independent granzyme B-induced SMC death was dependent on granzyme B and caspase proteolytic activity, and did not require granzyme B internalization. Further, granzyme B induced the cleavage of multiple extracellular proteins, including fibronectin, and disrupted extracellular SMC interactions. These data suggest that the mechanism of perforin-independent granzyme B-induced cell death involves the proteolysis of extracellular proteins.

Recent investigations suggest that granzyme B can be internalized in a M6PR-independent manner in vitro. However, granzyme B is secreted from cytotoxic granules as a complex with proteoglycan, and Veugelers et al have demonstrated that the M6PR is critical for granzyme B/proteoglycan-mediated apoptosis. We have treated SMCs with proteoglycan-bound granzyme B, and this induces SMC apoptosis with similar efficiency as purified granzyme B and is similarly not inhibited by M6P (data not shown). Although studies in certain types of cells from M6PR knockout mice have suggested that this receptor is not required for granzyme B internalization, M6P clearly attenuates SMC uptake of granzyme B because it attenuated SMC association with GrB-488. Recently, Gross et al have suggested that granzyme B can also be internalized into certain tumor cells by plasma membrane-bound Hsp70, and that this induces apoptosis in a perforin-independent manner. However, Hsp70 localizes to the cell surface only in certain tumor cells and minimal levels are present only within the cytosol of SMCs. Combined, these results strongly support the notion that granzyme B is acting extracellularly to induce SMC death. In addition, the long time point required for splenocyte-induced SMC death further supports the notion that the mechanism of SMC apoptosis involves the degradation of extracellular proteins and is not a mitochondrion-mediated process.

Although we are not aware of any reports implicating granzyme B proteolysis of extracellular substrates in the induction of apoptosis, certain reports demonstrate that granzyme B can degrade extracellular matrices synthesized by certain cells. Specifically, Sayers et al showed that the addition of purified rat granzyme B to certain tumor cell lines resulted in similar morphological changes, as we have described in this report, and that this led to a decrease in tumor cell proliferation. Cytotoxic T cells can also degrade the microenvironment of hematopoietic stromal cells. In addition to these existing findings, we have demonstrated that granzyme B induces the cleavage of multiple SMC extracellular proteins and have identified fibronectin as one of the granzyme B substrates. Fibronectin is important in cell survival of SMCs. Cleavage of fibronectin by mast cell chymase induces SMC apoptosis. Therefore, although the identity of other granzyme B extracellular substrates remains to be determined, cleavage of fibronectin likely contributes to granzyme B-induced SMC death. Interestingly, addition of granzyme B-induced SMC extracellular protein cleavage products does not induce SMC apoptosis. This is in contrast to the death-inducing properties of chymase-cleaved fibronectin. The reasons for this are unclear but may involve differences in the types of fibronectin fragments generated. Fibronectin fragments generated by granzyme B may not be capable of interfering with established SMC–extracellular protein interactions.

Induction of SMC death by granzyme B may have implications for vasomotor dysfunction and intimal remodeling in inflammatory vascular diseases. In arteritic diseases and allograft arteries, there is T cell-mediated medial SMC death that is accompanied by defects in SMC constriction. In atherosclerosis, both T cells and granzyme B localize to TUNEL-positive SMCs. The induction of SMC death in atherosclerosis contributes to plaque destabilization and rupture. Granzyme B may also play a role in arterial damage induced by neutrophils, because these leukocytes also express granzyme B. Also, mast cell chymase (which is secreted from granules in this cell type) has recently been implicated as a mediator of arterial damage in atherosclerosis. Because granzyme B is secreted from T cell granules, and because
chymase is secreted by mast cell granules, SMC apoptosis induced by the extracellular activity of granzyme B and mast cell chymase may represent a common mechanism through which granule-associated serine proteases contribute to arterial damage during inflammatory vascular diseases. Although a role for granzyme B in the induction of SMC anoikis and the resultant pathogenesis of vascular diseases has been suggested, to our knowledge, our current findings are the first to directly show that granzyme B can induce SMC death through the cleavage of extracellular proteins.

In summary, we have identified a perforin-independent mechanism of granzyme B-mediated cell death through the cleavage of extracellular proteins. This novel mechanism of granzyme B-induced apoptosis accounts for ~30% of SMC death induced by T cells, whereas 62% of T cell-induced SMC death is perforin-dependent. Granzyme B-induced cell death in the absence of perforin may be cell type–dependent, which has implications for the regulation of the granzyme-induced apoptotic pathways in vascular diseases and suggests that granzyme B may not be completely dependent on perforin for cytotoxicity. The latter may have implications for matrix remodeling that could effect SMC migration and/or atherosclerotic plaque stability.

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MATERIALS AND METHODS

Reagents

Human coronary artery SMC (Clonetics, San Diego, CA) were cultured in SmBM + 5% FCS and used up to the eighth passage. HeLa cells (ATCC) were cultured in DMEM + 10% FCS. Granzyme B was purified from the cytotoxic granules of YT Indy cells as described previously. Monoclonal caspase-3 antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), biotin and FAK antibodies from New England BioLabs (Beverly, MA), β-actin and fibronectin antibodies from Sigma, and collagen IV antibody from Weislab (Lund, Sweden). The inhibitors z-VAD.fmk and z-AAD.cmK were purchased from Enzyme Systems Products (Livermore, CA), and M6P purchased from Sigma.

Assessment of apoptosis

Chromatin condensation was assessed by staining SMC nuclei with the DNA dye Hoechst 33342. SMCs were treated with granzyme B for 24h and fixed in 10% neutral buffered formalin for 10 min. After washing gently in PBS, SMCs were permeabilized with 1% Triton X-100 for 15 min., incubated in a 1/1000 dilution of Hoechst 33342, and coverslipped in an aqueous mounting medium. Gentle washings were performed between each step. Chromatin condensation was visualized with fluorescence microscopy.

Phosphatidylserine (PS) outer membrane localization was determined by staining unfixed cells with Alexa 488-labeled Annexin V. An Annexin V-labeling kit (Molecular Probes, Eugene, OR) was used as per the manufacturer’s instructions, and Annexin V-
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positivity was visualized with fluorescence microscopy. All digital photomicrographs were obtained on a Spot camera. Quantitation of Annexin V-positive cells was performed in a blinded manner by counting the number of Annexin V-positive cells in three low power (80X) photomicrographs and then dividing by the total number of cells. Data is presented as an average ± SEM of three independent experiments.

*Silver staining*

Purified granzyme B (0.1 µg/mL and 0.2 µg/mL) was electrophoresed in a 4-12% bis-tris acrylamide gel at 200V for 35min in MES buffer (Invitrogen, Burlington, ON, Canada) as per manufacturer’s instruction. After rinsing the gel in water for 1 min., it was fixed in 40% methanol, 10% acetic acid and 50% deionized water for 30min. Silver staining was performed with the SilverQuest staining kit (Invitrogen) under standard conditions. In brief, the fixed gel was sensitized for 10 min., rinsed in 30% ethanol for 10 min., washed with deionized water for 10 min., stained for 30 min. and then finally washed with deionized water for 1 min. The gel was then immersed in developing solution for approximately 7 min. (until the appropriate band intensities were observed), at which point the stopping solution was added. The stained gel was washed with deionized water and imaged.

*Splenocyte killing assays*

Splenocyte killing assays were performed using Fisher rat aortic SMCs or concanavalin A (Con A)-treated rat splenocytes as targets and C57BL/6 (WT) or PKO splenocytes as effectors. Calcein release was used to quantitate SMC death. This method
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measures membrane permeability that occurs during necrotic cell death or late stage apoptosis. Calcein is a cell membrane impermeable fluorescent dye. Conjugation of calcein to acetoxymethyl (calcein, AM) facilitates membrane permeability of this dye. The AM is cleaved from calcein by intracellular methyl esterases, resulting in the intracellular localization of calcein subsequent to loading with calcein, AM. Compromization of the plasma membrane integrity during cell death results in the release of calcein into the supernatant and quantitation of this release by fluorometry was used to measure cell death.

Rat SMCs were grown to 70% confluence in 24-well TC dishes, washed twice with PBS, and loaded with 10 µg/mL calcein, AM in MCDB for 30 min. Calcein, AM was then removed and SMCs washed twice with PBS before incubation overnight in MCDB + 10% NCS. Activated splenocytes were added the following morning. The number of cells/well was calculated to be approximately 5 X 10^4 in the first experiment and this cell concentration/well was used in the subsequent experiments to calculate the effector:target (E:T) ratio. In experiments using rat splenocytes as targets, 5 X 10^6 rat splenocytes were treated with 5 µg/mL Con A for 24h prior to loading with calcein, AM as described above. This treatment was required to reduce spontaneous calcein release from the target cells.

To obtain effectors, WT or PKO mice were anesthetized with ketamine/xylene and the spleen was removed aseptically. Splenocytes were obtained by disruption of the spleen on a wire mesh with the blunt end of a 3 mL syringe in RPMI + 10% FCS. After centrifugation for 5 min. at 1300 rpm to isolate cells, red blood cells (RBC) were lysed
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with the addition of RBC lysis buffer (10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA (pH 8.0)) for 3 min. 10⁷ splenocytes were then reconstituted in 10 mL of medium and stimulated with 5 µg/mL Con A for 72h. Con A is a plant sugar that cross-links the CD3 molecules associated with the T cell receptor (TCR) and in this way polyclonally activates T cells. Activation of T cells through this mechanism has been shown to induce activation to a cytotoxic phenotype in a subset of T cells. Activated T blasts were morphologically identified and counted before addition of varying concentrations of activated T cells to SMCs in order to set up the various E:T ratios. After incubation of Con A-activated splenocytes with SMCs, the supernatant was removed, centrifuged for 5 min. at 1300 rpm to remove suspended cells, and calcein release quantitated with a Tecan GENios fluorescence microplate reader at Ex = 485 nm and Em = 535 nm. Spontaneous release was calculated on the supernatant of calcein, AM-loaded SMCs without the addition of splenocytes and was always less than 35%. Total calcein release was determined by lysis of SMCs with 0.1% Triton for 30 min. The following formula was used to calculate % specific calcein release:

\%
 Specific Calcein Release = (calcein release in sample – spontaneous release)/(total calcein release – spontaneous release)

Finally, in experiments using zAAD.cmk to inhibit granzyme B activity in T cells, 50 µM zAAD.cmk was added to Con A-activated splenocytes prior to incubation with rat SMCs. Fresh zAAD.cmk was added to the co-culture culture after 24h to ensure the maintained inhibition of granzyme B.
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*Cell lysis and Western blot*

SMCs were grown to 70% confluence on 60mm TC dishes, maintained in SmBM + 0.2% FCS for 48h, and treated with granzyme B (75 nM). After 24h, cells were washed 1X with cold PBS, and lysed in 400ul of SDS lysis/loading buffer. Western blot was performed as described previously \(^3,4\) using a 1:100 dilution of anti-caspase-3, 1:1000 dilution of anti-biotin, 1:200 dilution of anti-fibronectin, 1:500 dilution of anti-collagen, 1:1000 dilution of anti-vitronectin, 1:1000 of anti-FAK or anti-P-FAK, and 1:1000 dilution of anti-β-actin. The gel was stained with Gelcode Blue (Pierce Chemicals, Rockford, IL) to ensure equal loading.

*Granzyme B uptake assay*

Granzyme B was labeled with Alexa-488 (GrB-488)(Molecular Probes, Eugene, OR) as per the manufacturer’s instructions and as described previously \(^5\). SMCs grown on 96-well TC dishes were treated with GrB-488 for either 2h or 24h. Live SMCs were washed gently with PBS before visualization with fluorescence microscopy. Data are representative of 3 independent experiments.
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References


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JC Choy, VH Hung, AL Hunter, PK Cheung, B Motyka, IS Goping, T Sawchuk, RC Bleackley, TJ Podor, BM McManus, DJ Granville

Figure I. A. HeLa cells were treated with varying concentrations of granzyme B with (gray bars) or without (black bars) Ad5 for 24 h, and cell death quantitated with an MTS assay. B. Granzyme B preparations were silver stained to ensure that they were not contaminated with perforin. C. Granzyme B preparations were Western blotted for perforin.

Figure I - Choy et al.
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**Figure II.**
A. The morphology of SMCs and HeLa cells treated with granzyme B (75 nM) for 24h was assessed with phase contrast microscopy. Arrows denote SMCs that are rounding and detaching from the culture plate (160X). B. Phase contrast photomicrographs of the same field of view as that stained with Annexin V indicated that Annexin V-positive cells were displaying membrane blebbing (arrows; 320X). C. SMCs were treated with granzyme B and photomicrographs obtained at 12, 16, 20, 24, and 48h. Arrows denote SMCs displaying morphological characteristics of apoptosis, such as cell rounding and membrane blebbing (160X).
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**Figure III.** A. Activated mouse splenocytes were co-cultured with calcein-loaded rat SMCs and cell death assessed by specific calcein release. B. The kinetics of SMC death was then compared to that of splenocyte death. Activated mouse splenocytes (black bars) or activated syngeneic rat splenocytes (gray bars) were co-cultured with calcein-loaded rat splenocytes and calcein release quantitated at 6h.
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Figure IV. SMCs were treated for 24h with granzyme B, lysed in an SDS lysis buffer, and Western blot performed for caspase-3.
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![Figure V](image)

**Figure V**. SMCs were incubated for 2h or 24h with GrB-488 in the absence or presence of M6P (20mM), and the localization of this protease visualized with fluorescence microscopy.
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**A.**

Collagen IV

GelCode Blue

Vitronectin

GelCode Blue

**B.**

Figure VI. A. Western blots for collagen and vitronectin were performed on lysates and supernatants from untreated and granzyme B-treated SMCs. B. The supernatant from granzyme B-treated SMC extracellular proteins was then treated with z-AAD.cmk to block granzyme B activity, added to SMCs for 24 h, and cell viability quantitated with an MTS assay.