Regulation of leukocyte extravasation by Fas ligand expression on the vascular endothelium

血管内皮上のファスリガンドによる白血球血管外遊出の制御

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血管内皮上のファスリガンドによる白血球血管外浸潤の調節

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Background

Vascular endothelium and leukocyte extravasation

The monolayer of endothelial cells (ECs) that coat the luminal surface of the vessel wall have numerous physiological functions including prevention of coagulation, control of vascular permeability, maintenance of vascular tone, and regulation of leukocyte extravasation [1]. Leukocytes circulate within the network of blood vessels surveying tissues for pathogens, and rapidly accumulate at sites of infection and injury. To gain access to sites of inflammation, leukocytes pass the endothelial barrier and migrate out of blood vessels by a process that is known as extravasataion. Extravasation is a endothelium-dependent process that involves tethering, firm adhesion, and the migration of leukocytes to the subendothelial space [2]. Upon cytokine stimulation, the vascular endothelium induces the selectins (E-, P-, and L-selectin), the intercellular adhesion molecules (ICAM-1, -2, and -3) and the vascular cell adhesion molecule (VCAM-1) which interact with specific receptors on leukocytes [3]. Endothelial cells can also synthesize chemoattractants, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8, that are important in activation of adhesion receptors on leukocytes and in directing migration [4]. The tethered leukocytes adhere firmly through interactions with platelet-endothelial cell adhesion molecule (PECAM) expressed on the leukocyte and at the intercellular junctions of endothelial cells. These interactions enable leukocytes to pass between endothelial cells and penetrate the basement membrane with the aid of proteolytic enzymes. Leukocytes migrate through the subendothelial space to sites of infection under the influence of chemoattractant cytokines [2]. Once at an inflammatory site, neutrophils eliminate pathogens by phagocytosis, monocytes can differentiate into macrophages and also phagocytize pathogens, and T lymphocytes activate cellular and humoral immune responses.

In addition to the inflammatory response to pathogens, leukocyte extravasation is also believed to have important roles in processes of angiogenesis [5] and in the initiation of the inflammatory-fibroproliferative disorders of the vessel wall [1]. Therefore, the identification and characterization of new regulators of leukocyte extravasation may serve to elucidate aspects of a variety of pathological processes, and these findings may have broad implications in the development of novel therapies to treat these disorders.

Though much is known about the chemoattractant and adhesion molecules that regulate leukocyte recruitment in response to bacterial infection [6], relatively little is known about mechanisms that may actively control the transendothelial cell migration.

The Fas-Fas ligand system

Fas ligand induces apoptosis in cells that bear its receptor, referred to as Fas (also called CD95 and APO-1). The Fas receptor is a member of the TNF receptor family that is expressed on many cell types including leukocytes. In contrast to Fas, FasL expression is more restricted in its expression. The Fas-FasL system has been implicated in the regulation of physiological cell turnover, particularly in the immune system [7].

FasL can induce apoptosis in Fas-bearing cells [8], but Fas surface expression does not necessarily render cells susceptible to FasL-induced death signals [9, 10] and many cells expressing both Fas and FasL do not undergo suicide or fratricide under normal conditions [10, 11], but these cells can become dramatically sensitized to the Fas-mediated apoptosis in response to specific stimuli or injuries [12-15]. The increased sensitization to Fas/FasL-mediated suicide may play a role in regulation of physiological T cell number [10] and in pathological tissue destruction [16]. However, the mechanisms by which stimuli sensitize cells to Fas-mediated apoptosis remains to be elucidated.

Death-inducing signal downstream of Fas

Fas contains a cytoplasmic domain, referred to as the death domain, that is required to transmit an apoptotic signal [17]. The death domain of Fas receptor interacts with FADD (Fas-associated death domain), a linker protein [18, 19]. Caspases are cysteine proteases that play a central role in Fas-mediated apoptosis signaling pathway [20]. Upon ligand activation, Fas is oligomerized [9] and recruits Fas-associated death domain protein (FADD) and pro-FLICE (FADD-homologous ICE like protease) resulting in proteolytic activation of FLICE [21, 22]. Active FLICE is released into the cytosol and triggers a cascade of caspases [20]. FLIP (for FLICE-inhibitory protein) is a recently identified FADD-binding suppressor of apoptosis [23-25]. Multiple isoforms are predicted based upon the analysis of FLIP cDNAs [23-25], two of which designated $FLIP_L$ and $FLIP_S$ have been isolated from activated human peripheral blood leukocytes [23]. All isoforms of FLIP contain FADD binding domain but lack the active-center cysteine residue and

may function as dominant negatives for FLICE, thus blocking Fas-mediated apoptosis [23-25]. FLIP isoforms are expressed during the early stage of T-cell activation and disappear when T cells become susceptible to Fas ligand-mediated apoptosis [23, 26], suggesting that the levels of intracellular FLIP may determine sensitivity of the cells to Fas-mediated apoptosis. FLIP inhibits Fas-mediated apoptosis [23, 27] and an apoptosis-protective role has been found in the viral FLICE-inhibitory proteins [28, 29]. However, the role of FLIP is controversial in some cell types as its overexpression can induce apoptosis [23, 24, 30, 31].

Fas ligand expression and immune-privilege status

Although the expression of FasL was originally considered restricted to activated T cells and NK cells, FasL has been identified in other cell types including Sertoli cells and cells of the eye [32, 33]. It has been proposed that expression of functional FasL by some tissues contributes to their immune-privileged status by preventing the infiltration of inflammatory leukocytes [32, 33]. Recently, constitutive FasL expression has been detected on some tumor cells where it may function to induce apoptotic cell death in Fas-expressing immune cells when they attempt to enter the tumor [8, 34, 35].

Leukocyte extravasation and atherosclerosis

Atherosclerosis is believed to result from a chronic, localized immune response within the vessel [1]. Depending on the arteries involved, atherosclerotic lesions can cause myocardial or cerebral infarction, gangrene, or loss of function of the extremities. Elevated plasma concentrations of low-density lipoprotein (LDL) are associated with accelerated atherogenesis [1, 36], and recent evidence suggests that oxidative modification of LDL renders it more atherogenic [37]. Endothelial cells internalize OxLDL [38] and this has numerous detrimental effects on endothelial cell function including upregulation of adhesion molecules and production of MCP-1 [39]. Oxidized LDL has been identified in atherosclerotic lesions where it accumulates at high levels in sub-endothelial macrophages [40, 41]. In addition to the macrophage-derived foam cells, human atherosclerotic plaques also contain T cells and phenotypically modulated smooth muscle cells. The majority of T cells and macrophages in plaques are considered to be immunoactivated [42-45] and produce various cytokines and growth factors [46-49]. Vascular smooth muscle cells can respond to these factors by acquiring a "synthetic" phenotype and becoming proliferative.

Oxidized LDL and endothelial cell apoptosis

It is firmly established that elevated plasma concentrations of low density lipoprotein (LDL) are associated with accelerated atherogenesis, and clinical trials demonstrate that LDL lowering treatments reduce the risk of death in patients with coronary artery disease [36]. Recent evidence suggests that oxidative modification of LDL renders it more atherogenic [39] and oxidized LDL has been identified in atherosclerotic lesions [41, 50]. Oxidized LDL and its lipid constituents have numerous detrimental effects on endothelial cell function [51-53] including the induction of apoptosis [54-57]. However, the molecular mechanism by which oxidized LDL induces apoptosis of endothelial cells remains to be elucidated.

Study purpose

In an attempt to elucidate the mechanism by which vascular endothelium actively inhibits leukocyte extravasation in the absence of normal inflammatory stimuli, I found that vascular endothelial cells express functional FasL. I studied functional significance of the FasL expression on the vascular endothelial cells. Here, I illustrate how TNFa-regulated FasL expression by vascular endothelium modulates leukocyte extravasation. Furthermore, I show that adenovirus-mediated gene transfer of FasL to the denuded artery can effectively inhibit injury-induced vessel lesion formation and that the FasL gene transfer confers to smooth muscle a property normally confined to the endothelium - the ability to inhibit leukocyte infiltration of the vessel wall. Finally, I show that endothelial cells undergo Fas/FasL mediated suicide process in the presence of oxidized LDL. These results indicates that deregulated expression of FasL, Fas, or signaling pathway components of this system may be a feature of endothelial cell dysfunction in response to injurious agents leading to inflammatory-fibroproliferative disorders of the vessel wall.

Materials and Methods

Cells and reagents

Human aortic endothelial cells (HAECs) were obtained from Clonetics (San Diego, CA). Human microvascular endothelial cells (HMVECs) were provided by Dr. Joyce Bischoff (Children's Hospital, Boston, MA). Human umbilical vein endothelial cellls (HUVECs) were isolated as described [58]. All endothelial cells were cultured in EGM medium (Clonetics) containing 2% FBS, 10 ng/ml human EGF, 1.0 µg/ml hydrocortisone, and 12 µg/ml bovine brain extract and used for this study at less than 9 passages. The human T cell leukemia line, Jurkat clone E6-1, was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI medium 1640 with 10% fetal bovine serum. P815-huFas, mastocytoma cells P815 stably transfected with human Fas was kindly provided by Dr. Douglas Green. Human vascular smooth muscle cells (HUSMCs) were isolated from a saphenous vein obtained during coronary bypass surgery and cultured as described previously [59].

Recombinant human TNFα was purchased from R&D systems (Minneapolis, MN). LDL was isolated by sequential ultracentrifugation (d=1.019-1.063) from freshly drawn, citrated normolipidemic human plasma to which EDTA was added [60]. LDL was oxidized in the presence of 5 µM $CuSO_4$ for 24 hours at 25 °C and the degree of oxidation was assessed by the increase of mobility on 1% agarose gel (1.3 -1.5 versus native LDL) [60]. Different preparations of OxLDL displayed similar electrophoretic mobilities. Minimally modified LDL (MM-LDL) was prepared by dialyzing native LDL against 9 µM $FeSO_4$ in PBS for 72 hours at 4 °C as described [61]. The electrophoretic mobility increased 1.1 to 1.2 versus native LDL. Acetylation of LDL was performed with excess acetic anhydride as described [62]. L-α-lysophosphatidylcholine, lauroyl (LPC-C12:0), L-α-lysophosphatidylcholine, palmitoyl (LPC-C16:0), L-α-lysophosphatidylcholine, stearoyl (LPC-C18:0), L-α-phosphatidylcholine, β-lynoleoyl-γ-palmitoyl (PC-C18:2, 16:0), cholesterol, 7-ketocholesterol, and 25-hydoxycholesterol were purchased from Sigma (St. Louis, MO). Lypopolysaccaride (LPS) from E. coli 0.55 were purchased from Sigma (St. Luis,

MO). Endotoxin contamination in OxLDL was measured with the coagulation *Limulus* amebocyte lysate asssay using a commercially available kit (E-TOXATE, Sigma).

Adenoviral constructs.

A replication-defective adenovirus construct encoding murine FasL (Ad-FasL) was constructed by inserting a 943 bp cDNA containing murine FasL (a generous gift from S. Nagata) into the XbaI site of the plasmid pACCMV.pLpA that expresses transgenes from the cytomegalovirus promoter [63, 64]. Ad-βgal has the β-galactosidase gene under the control of the cytomegalovirus promoter [64]. Viral titer was measured by standard plaque assay using 293 cells. Viral titer was measured by standard plaque assay using 293 cells.

Immunoblot analysis of regulation of FasL expression

HUVECs, at approximately 90% confluence, were incubated with basal medium containing different concentrations of TNFα (0, 0.5, 5, 25 ng/ml) for 21 hours at 37°C. Culture medium was removed by washing twice with PBS, and the cells were lysed with a buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2mM PMSF in PBS. The protein content was measured with BCA protein assay reagent (Pierce, Rockford, IL). The cell lysates (15 μg for each lane) were analyzed by SDS PAGE using a 10% polyacrylamide gel and were transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in Tris-buffered saline (TBS), the membrane was incubated with an anti-human FasL mouse monoclonal antibody (1:1000, Transduction Laboratories, Lexington, KY), anti-VCAM-1 goat polyclonal antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-human α-tubulin mouse monoclonal antibody (1:2000, Calbiochem, San Diego, CA). Membranes were washed in TBS supplemented with 0.05% Tween20 (T-TBS) and incubated with horseradish peroxidase-conjugated sheep antibody to mouse Ig or goat Ig (1: 3000, Amersham, Arlington Heights, IL). After washing in T-TBS, antibody binding was detected using enhanced chemiluminescence (Amersham).

Flow cytometric analysis of FasL and Fas expression on cell surfaces

HAECs, at approximately 90% confluence, were incubated with either basal medium or 25 ng/ml TNFα at 37°C, 5% CO_2 for 7 hours. HAECs were detached from the culture plate with 0.5% EDTA and incubated with anti-FasL monoclonal antibody (A11, Alexis Corp., San Diego, CA) or with rat IgM. Cells were then washed and stained with FITC-conjugated anti-rat IgM antibody (Kirkegaard & Perry Laboratories, Gaithburg, MD). Immunofluorescence staining was analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). To determine Fas expression, HAECs were incubated with an FITC-conjugated anti-Fas monoclonal antibody (clone UB2, Immunotech, Westbrook, ME). To detect cell surface expression of FasL by VSMCs following Ad-FasL infection, primary cultured rat aortic VSMCs were infected with Ad-FasL at a multiplicity of infection (MOI) of 300 for 4 hours and incubated for an additional 12 hours. Cells were detached from the culture plate with 0.5% EDTA and stained with biotinylated anti-FasL monoclonal antibody (Alexis Corp., San Diego, CA) or mouse IgG, followed by the incubation with FITC-conjugated ExtrAvidin (Sigma, St. Louis, MO). To analyze endogenous expression of Fas on VSMCs, rat VSMCs were detached from the plates with 0.5% EDTA and incubated with an anti-Fas mouse monoclonal antibody (Transduction laboratories, Lexington, KY) or mouse IgG (Sigma), followed by the incubation with an FITC-conjugated goat antibody to mouse IgG (Biosource, Camarillo, CA). Immunofluorescence staining was analyzed by fluorescence activated cell sorter (FACS, Becton Dickinson, Mountain View, CA).

Flow cytometric analysis of apoptosis

To detect apoptosis of the Ad-FasL-infected VSMCs, flow cytometric profile of DNA content was analyzed. Rat VSMCs were infected with Ad-bgal or Ad-FasL at different MOIs (10 to 300) for 4 hours, after which the virus was removed. 48 hours after infection, cells were harvested by trypsinization, fixed with 70% ethanol and stained with propidium iodide and DNA content was analyzed by flow cytometry.

Cytotoxic assay

The ability of FasL on ECs to induce apoptosis in Fas-positive target cells was assessed by co-incubating the mastocytoma cells P815 stably transfected with human Fas (P815-huFas, kindly provided by Dr. Douglas Green) with HAECs using a technique described previously [65]. HAECs were plated in triplicate in 24-multiwell tissue culture plates and allowed to reach 90% confluency as a monolayer. HAECs were incubated with either basal medium or medium containing TNFα (10 ng/ml) at 37°C for 4

hours, after which HAECs were washed twice with PBS. P815-huFas cells were labeled with 10 μCi/ml ^{3}H-thymidine (NEN, Boston, MA) for 24 hours, washed twice in PBS and then incubated at 37°C for 8 hours to minimize spontaneous release. P815-huFas cells were applied to HAECs at a ratio of 1 : 4 (HAECs : P815-huFas) in the absence or presence of the anti-FasL antibody (10 μg/ml, clone 4H9, MBL, Nagoya, Japan), which is capable of neutralizing the cytotoxic activity of human FasL[66]. The 24-multiwell culture plates were centrifuged at 200*g* for 2 min. and incubated at 37°C for 18 hours. Cells were harvested by trypsinization. The amounts of the fragmented DNA and the retained DNA were measured by liquid scintillation counting as described [67]. The percentage of the specific DNA fragmentation was calculated as 100 × (spontaneous - experimental)/spontaneous. "Experimental" is counts per minute of the retained DNA in the presence of HAECs and "spontaneous" is counts per minute of the retained DNA in the absence of HAECs. All values are presented as mean ± S.E.M.

The cytotoxic activity of Ad-FasL-infected VSMCs was evaluated by measuring the DNA fragmentation of co-cultured cells as described previously[68]. Rat VSMCs were incubated with Ad-βgal, Ad-FasL or PBS (mock) at a MOI of 100 for 4 hours, washed twice in PBS, and incubated for an additional 16 hours. Rat VSMCs or Jurkat cells were labeled with 4 μCi/ml of ^{3}H-thymidine for 24 hours and incubated with medium for 2 hours to minimize spontaneous release. The ^{3}H-labeled rat VSMCs or Jurkat cells (target cells) were then applied to the infected rat VSMCs (effector cells) at a ratio of 1:2. After 7 hours incubation, cells were harvested and the radioactivity of the retained DNA was counted in a liquid scintillation counter. Percentage of specific DNA fragmentation was calculated as 100X (basal count-experimental count)/basal count. Basal count is the radioactivity of the retained DNA when only target cells were cultured.

Cytotoxicity was also tested by analyzing the ability of ECs to induce a DNA ladder in target cells. The P815-huFas cells were incubated with 100 μM 5-bromo-2'-deoxy-uridine (BrdU) for 9 hours. After washed in PBS, the P815-huFas cells were applied to HAECs and cultured for 18 hours. The total genomic DNA from both P815-huFas cells and HAECs was prepared from a well of a 6-well plate as

described [67] and loaded on a 1.5% agarose gel in 1x TBE buffer. The electrophoretically separated DNA was transferred onto a nylon membrane (Hybond-N, Amersham) and UV-crosslinked. The BrdU-labeled DNA from the P815-huFas cells was detected by using an anti-BrdU monoclonal antibody (clone BMC 9318, Boehringer Mannheim) as described for the immunoblot analysis.

Local administration of cytokines into rabbit ear central arteries

The effect of TNFα on the endothelial expression of FasL *in vivo* was determined using the rabbit ear central artery which is superficial and therefore easily accessible for isolation and injection [69]. New Zealand White rabbits (3500-4000 g) were sedated by intramuscular injection of xylazine (5 mg/kg) and then anesthetized by an intramuscular injection of ketamine (40 mg/kg) and acepromazine (1 mg/kg). The central artery of the rabbit ear was isolated by applying a tourniquet at the base of the ear, temporarily interrupting blood flow. The artery was then cannulated with a 25-gauge needle and one ml of TNFα diluted in PBS (50 ng/ml) or PBS was then infused into the isolated segment and incubated for 15 minutes. Rabbits were sacrificed by administration of pentobarbital at either 4 or 30 hours after the treatment. The arteries were harvested and snap-frozen in OCT compound (Miles Inc., Elkhart, IN). To study the effect of constitutive FasL expression on mononuclear infiltration, the isolated segment of a rabbit ear artery was incubated with 1×10^7 pfu of either Ad-FasL or Ad-βgal for 15 minutes. These replication-defective adenoviral constructs express the murine FasL or *E. coli* β-galactosidase genes from the cytomegalovirus promoter [63]. At 12 hours post-infection, the artery was treated with PBS or TNFα and the rabbits were sacrificed as described above.

Immunohistochemistry

Cryosections (5 mm thick) were mounted on microscope slides and fixed in 4% paraformaldehyde for 10 minutes. Sections were washed twice in PBS and blocked with 5% normal goat serum and 0.01% Triton-X in PBS for 1 hour at room temperature. Biotin-conjugated monoclonal antibody to FasL (1:2000, clone A11, Alexis Corp., San Diego, CA) in PBS with 1% goat serum and 0.01% Triton-X was added to the sections for one hour at room temperature. Sections were washed three times in PBS followed by the addition of alkaline phosphatase-conjugated

streptavidin for 5 minutes at 37°C. Sections were washed three times in PBS and antibody location was determined with the addition of Fast Red substrate (BioGenex Laboratories, San Ramon, CA). Color development was stopped by washing in distilled water. Distributions of ECs, T lymphocytes, and macrophages were revealed in adjacent sections using an anti-CD31 monoclonal antibody (1:100, clone JC/70A, DAKO, CA), an anti-CD3 monoclonal antibody (1:2000, clone 6B10.2, Santa Cruz Biotech, Santa Cruz, CA), and an anti-macrophages monoclonal antibody (1:1000, clone RAM11, DAKO, Carpinteria, CA), respectively, followed by avidin-biotin complex technique and Fast Red substrate. Expression of VCAM-1 was revealed by using a goat polyclonal antibody against VCAM-1 (1:1000, clone C-19, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were counter-stained with hematoxylin.

TUNEL staining

TdT-mediated dUTP nick end labeling (TUNEL) technique was employed to detect apoptotic cells in the rabbit ear artery. Cryosections (5 mm thick) were fixed in 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 in 0.1 % sodium citrate for 2 minutes at 4°C. Terminal deoxynucleotidyl transferase enzyme and dUTP conjugated to a fluorescein cocktail were added to the tissue sections according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, IN). Nuclei were counterstained with Hoechst 33258 (Sigma) and mounted for fluorescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Specimens were examined and photographed on a Nikon Diaphot microscope.

Balloon injury and gene delivery of rat carotid arteries

The left carotid arteries of adult male Sprague-Dawley rats (400-500g) were injured with a 2F embolectomy catheter (Baxter Edwards Healthcare Corp., Irvine, CA) as described [70]. Virus solution in 7.5% (wt/vol) Paloxamer 407 (BASF, Parsippany, NJ) was infused via a 24-gauge intravenous catheter, inserted just proximal to the carotid bifurcation into a temporarily isolated segment of the artery. The adenovirus solution was incubated for 20 min after which the viral solution was withdrawn and the cannula was removed. Rats were sacrificed 3 or 14 days after balloon injury. The infected artery was fixed by perfusion with 4% paraformaldehyde and embedded in paraffin. Cross sections (5-mm) from four separate left carotid arterial segments of each rat were stained with hematoxylin and eosin or with

Richardson's combination elastic tissue trichrome stain. The intimal, medial and luminal areas were measured by quantitative morphometric analysis using a computerized sketching program and expressed as mean ± SEM. The experimental protocol was approved by the Institutional Animal Care and Use Committee and complied with the "Guide for the Care and Use of Laboratory Animals". Rats were immunized against human adenovirus by intravenous injection of an adenoviral vector lacking E1 and containing no insert (1×10^9 pfu) via the tail vein. Two weeks after immunization, the left common carotid artery was balloon-injured, then infected with either Ad-FasL (1×10^8 pfu), Ad-βgal (1×10^8 pfu) or a mixture of Ad-FasL (1×10^8 pfu) and Ad-βgal (1×10^8 pfu). Rats were sacrificed at 3 days after infection. The infected artery was removed and snap-frozen in OCT embedding compound (Miles, Elkhart, IN). Cryosections (4 μm) were fixed in 2% paraformaldehyde for 10 min, rinsed in phosphate-buffered saline (PBS) and blocked with 5% goat serum and 0.01% Triton X-100 in PBS for 1 hour. A polyclonal antibody against CD3 (Sigma, St. Louis, MO) was added to the sections for 1 hour. After rinsing in PBS, biotinylated goat antibody to rabbit IgG (Biogenex, San Ramon, CA) was applied for 20 min, followed by alkaline phosphatase-conjugated streptavidin. Antibody complexes were detected by the FAST-RED system (Biogenex) followed by counter staining with Mayer's hematoxylin. To detect β-galactosidase expression, frozen section (4 μm) was fixed in 2% paraformaldehyde for 10 min. and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Sigma, St. Louis, MO) for 16 hours at room temperature followed by counter staining with hematoxylin. FasL expression was detected using a monoclonal antibody against FasL (Alexis Corp., San Diego, CA).

Detection of DNA fragmentation by agarose gel electrophoresis

HUVECs (1×10^6) were incubated in the presence or absence of native LDL (300 μg protein/ml), OxLDL (300 μg protein/ml), LPS (100 endotoxin units/ml), or a neutralizing anti-FasL antibody (23) (10 μg/ml, 4H9, MBL, Nagoya, Japan) for 36 hours. Attached cells and floating cells were combined and lysed in 0.33 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA, 0.2% TritonX-100) followed by

incubation with 0.1 mg/ml RNAaseA for 1 hour at 37 °C and 0.2 mg/ml proteinase K for 3 hours at 50 °C. Ethanol-precipitated DNA was resuspended in TE buffer, fractionated on 1.5% agarose gel in 1 × TBE buffer, and stained with ethidium bromide.

Detection of DNA fragmentation by TUNEL

70% confluent HUVECs were incubated in the presence or absence of OxLDL (300 μg protein/ml), a neutralizing anti-FasL antibody [66] (10 μg/ml, 4H9), or an agonistic anti-Fas antibody (0.5 μg/ml, CH11, MBL) for 16 hours at 37 °C, 5% CO_2. Attached cells harvested by trypsinization and floating cells were combined, fixed in 4% paraformaldehyde, permeabilized in 0.1 % Triton X-100, 0.1 % sodium citrate, and incubated with TdT-mediated dUTP nick end labeling (TUNEL) solution (Boehringer Mannheim, Indianapolis, IN) in the absence and in the presence of terminal deoxynucleotidyl transferase. After washed in PBS, fluorescence intensity was analyzed by FACS.

Cell viability assay

HAECs were cultured in a 96-well plate at 80% confluency and incubated in the presence or absence of OxLDL (300 μg protein/ml), LPC-C16:0 (55 μM), a neutralizing anti-FasL antibody (10 μg/ml, 4H9), or an agonistic anti-Fas antibody (0.5 μg/ml, CH11) for 18 hours. Cell viability was measured by means of MTT (dimethylthiazol-diphenyltetrazolium bromide) assay [66, 71] and % cell death was calculated as 100 × (1- viability of treated endothelial cells /viability of untreated endothelial cells).

HUVECs were also incubated with LPC-$C_{12:0}$ (100μM), LPC-$C_{18:0}$ (45 μM), PC-$C_{18:2, 16:0}$ (100 μM), cholesterol (200μM), 7ketocholesterol (200μM), and 25 hydoxycholesterol (200 μM), actylated LDL (300 μg protein/ml), or minimally modified-LDL (300 μg protein/ml) in the absence or presence of the neutralizing anti-FasL antibody (αFasL) for 24 hours. Cell viability was determined by means of MTT assay

Organ culture

Organ culture experiment was performed as described [72]. Carotid arteries were excised from male New Zealand white rabbit, cut into 1-2 mm long ring segments, and washed in PBS. The rings was cultured in M199 medium containing 0.5% FBS and OxLDL (100 μg protein/ml) in the absence or presence of the anti-FasL antibodies (4H9 10μg/ml and C-20 (Santa Cruz) 10 μg/ml) [66, 73] for 24 hours or 64 hours at 37 °C, 5% CO_2. The rings were washed in PBS and snap-frozen in OCT compound (Miles, Elkhart, IN). Cryosections (5 μm) from the rings harvested at 64 hours were stained for endothelial cells using an anti-CD31 monoclonal antibody (clone JC/70A, DAKO) as described [11]. Cryosections from the rings harvested at 24 hours was stained by dUTP nick end labeling (TUNEL) technique as described [11]. Nuclei were counterstained with propidium iodide (Boehringer Mannheim) and mounted for fluorescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Specimens were examined and photographed on a laser confocal microscope (ODYSSEY CLSM, Noran, Middleton, WI).

Aortic rings from male C57BL/6J mice and B6Smn-*gld*$^{fasL-/-}$ mice (Jackson Laboratories, Bar Harbor, ME) were incubated with OxLDL (200 μg protein/ml) in M199 medium containing 0.5% FBS for 72 hours and fix with 4% paraformaldehyde and embedded in paraffin. Sections (6 μm) were stained for CD31 (clone MEC 13.3, Pharmingen, San Diego, CA) with FAST RED chromagen (Biogenex, San Ramon, CA), followed by counterstaing with Mayer's Hematoxylin.

Anti-FLIP antibody

An antiserum against human FLIP was generated in New Zealand white rabbits against a peptide spanning amino acids 2-27 (SAEVIHQVEEALDTDEKEMLLFLCRD) synthesized using the multiple antigen technology [74]. The IgG fraction of the antiserum was isolated using E-Z-SEP kit (Pharmacia, Piscataway, NJ) and affinity-purified on the corresponding peptides coupled to Affi-Gel 15 gel (Bio-Rad, Hercules, CA).

Northern blotting and RNAase protection assay

90% confluent HUVECs were incubated with 60 μM LPC at 37 ^{0}C, 5% CO_2. Total RNA was isolated using RNeasy Mini kit (Qiagen, Santa Clarita, CA) at various time points. Northern blot

hybridization was performed as described [75]. Briefly total RNA was fractionated on 1% agarose-formaldehyde gel, blotted onto a nylon membrane, and UV-crosslinked. The short form of FLIP ($FLIP_S$) cDNA, 0.9-kb EcoRI fragment from PL296 plasmid, was labeled with α^{32}P-dCTP by using a random prime labeling kit (Amersham, Arlington Heights, IL) and hybridized with the membrane under high stringency conditions.

90% confluent HUVECs were incubated with 60 μM LPC at 37 ^{0}C, 5% CO_2. Total RNA was isolated using RNeasy Mini kit (Qiagen, Santa Clarita, CA) at various time points and used for ribonuclease protection assay and northern blotting. Ribonuclease protection assay was performed using multi-probe assay system (RiboQuant, Pharmingen, San Diego, CA). The RNase-protected bands were identified by using a standard curve of migration distance versus log nucleotide length with the undigested probes as markers.

Statistical analysis

All data are presented as mean ± SEM. Unpaired student's *t* test was used for statistical comparison of mean. A value of $p<0.05$ was considered to be significant.

Results

(1) TNFα regulation of Fas ligand expression on vascular endothelial cells

Regulation of endothelial cell FasL expression

To investigate the regulation of FasL expression on ECs, human umbilical vein endothelial cells (HUVECs) were subjected to Western immunoblotting assays using an anti-FasL monoclonal antibody. These analyses revealed a single band of 37 to 40 kDa, which had the same mobility as FasL from extracts of activated Jurkat cells [76] (Fig. 1A). No FasL was detected in human smooth muscle cell (HUSMC) lysate, but a FasL signal was detected in lysates prepared from HUSMCs infected with a replication-defective adenovirus expressing FasL from the cytomegalovirus promoter (Ad-FasL) [63, 64]. Treatment of HUVECs with TNFα downregulated the FasL expression in a dose-dependent manner (Fig. 1A). Immunoblot analyses also revealed that TNFα downregulated FasL expression on human aortic endothelial cells (HAECs) and human microvascular endothelial cells (HMVECs) (not shown). In agreement with previous findings [2, 3, 77], TNFα induced a dose-dependent upregulation of the cell adhesion molecule VCAM-1 (Fig. 1A).

Flow cytometric analyses were performed to examine FasL expression on the cell surface of vascular ECs. Consistent with data from the Western blot analyses, incubation with TNFα decreased the expression of FasL on HAECs (Fig. 1B) and HMVECs (not shown). EC expression of the FasL receptor, Fas, was also examined by flow cytometry (Fig. 1C). Fas expression was detectable on HAECs, but it was present at lower levels than on Jurkat cells (Fig. 1C). Despite Fas expression on ECs, these cells do not undergo apoptosis when exposed to Adeno-FasL or an anti-Fas antibody that can function as a receptor agonist (Fig. 2).

Cytotoxic activity of endothelial cell FasL

To determine whether FasL expression on ECs is functional, HAECs were co-cultured with P815-huFas target cells that were labeled with BrdU or ^{3}H-thymidine. After 18 hours co-culture, significant target cell DNA fragmentation was observed either by agarose gel electrophoresis and immunoblotting with BrdU-specific antibody (Fig. 3A) or by calculating the specific fragmentation of ^{3}H-thymidine-

labeled DNA (Fig. 3B). Prior treatment of the HAECs with TNFα, at a dose that downregulates FasL expression, significantly decreased the cytotoxic activity of HAECs (Fig. 3A and B). HAECs pre-infected with Adeno-FasL, which constitutively express FasL, displayed higher frequencies of target cell DNA fragmentation that was not inhibited by TNFα treatment (not shown). To further demonstrate that the cytotoxic activity of HAECs is mediated by Fas-FasL interaction, HAECs were incubated with a neutralizing anti-FasL antibody for 30 minutes prior to the co-culture assay. Treatment with antibody completely inhibited the cytotoxic activity of HAECs (Fig. 3B), similar to reports that this antibody can inhibit the cytotoxicity of both soluble FasL on W4 cells [66] and simian immunodeficiency virus-infected cells on Jurkat cells [78].

(2) Functional significance of Fas ligand expression on the vascular endothelium

FasL inhibits mononuclear cell infiltration

FasL expression was detected in the endothelium of the central artery of the rabbit ear as determined by co-localization with CD31, an established marker for vascular endothelial cells [79] (Fig. 4A). Analysis of FasL regulation was performed by applying a tourniquet at the base of the ear, to temporarily interrupt blood flow, and infusing TNFα or PBS. Following a 15 minute incubation, blood flow was restored and FasL expression was assessed by immunohistochemistry at 30 hours post-treatment. In the artery treated with TNFα, FasL expression by the endothelium was markedly downregulated relative to the PBS treated vessels (Fig. 4B). TNFα treatment also upregulated the expression of the adhesion molecule VCAM-1, and robust T lymphocyte and macrophage infiltration was observed under these conditions.

To determine the functional significance of FasL downregulation *in vivo*, rabbit ear central arteries were incubated for 15 minutes with Ad-FasL 12 hours prior to treatment with TNFα. At 30 hours post-cytokine treatment, FasL expression by the endothelium was readily detectable in the Adeno-FasL pre-treated vessels, but not in vessels pre-treated with the control construct Ad-βgal, a replication defective adenovirus expressing β-galactosidase (Fig. 5A). Though VCAM-1 expression was upregulated following treatment with TNFα, T cell and macrophage infiltration was markedly attenuated in vessels

that were transduced with Ad-FasL but not in the Ad-βgal-transduced vessels (Fig. 5A and Table 1). In control animals, Ad-FasL-infected vessels treated with PBS were negative for VCAM-1 expression and for T cell and macrophage infiltration (data not shown). TUNEL staining of the Ad-FasL-treated vessels at 4 hours post-cytokine treatment revealed mononuclear cells undergoing apoptosis while attached to the luminal surface of endothelium, while mononuclear cell apoptosis was not detected when arteries were pre-treated with Ad-βgal (Fig. 5B).

(3) Inhibition of neointima formation by Fas ligand gene transfer to the vessel wall

Adenovirus-mediated Fas ligand expression induces apoptosis in cultured VSMCs.

The efficacy of a replication defective adenovirus expressing Fas ligand (Ad-FasL) was first assessed *in vitro* using VSMCs derived from rat aorta. Cell surface expression of FasL by VSMCs was detected following infection with Ad-FasL (Fig. 6A). Cultures of rat VSMCs also express Fas (Fig. 6B) and thus are likely to undergo apoptotic cell death following infection. Analysis of the Ad-FasL-infected VSMC cultures revealed the presence of cells with hypodiploid DNA content indicative of apoptotic cell death (Fig. 6C), whereas infection with an adenovirus encoding β-galactosidase (Ad-βgal) did not change the DNA profile (Fig. 6D). Infection with Ad-FasL, but not Ad-βgal, also decreased cell number (not shown). The effect of Ad-FasL was dose-dependent with increasing amounts of the apoptotic hypodiploid DNA observed with increasing multiplicities of infection (Table 2). Similar levels of VSMC apoptosis were observed in low serum (0.5% FBS) and in high serum (15% FBS) media (not shown), indicating that the Ad-FasL is cytotoxic to proliferating as well as quiescent VSMCs.

Ad-FasL-transduced VSMCs induce apoptosis in uninfected VSMCs and Jurkat cells.

The ability of FasL-expressing VSMCs to induce apoptosis in uninfected Fas-positive target cells was assessed by incubating Ad-FasL infected VSMCs with non-transduced VSMCs or Jurkat cells that had been preincubated with ^{3}H-thymidine (Fig. 6E). After 7 hours co-incubation, appreciable levels of target cell DNA fragmentation were observed both in Jurkat and VSMC cultures. No DNA fragmentation was detected in target cells when incubated either with uninfected or Ad-βgal-infected VSMCs. Thus ectopic FasL expression by VSMCs can induce apoptotic cell death in neighboring non-transduced cells which express Fas.

Adenovirus-mediated Fas ligand expression inhibits neointima formation in balloon-injured rat carotid arteries. Ad-FasL was delivered to rat carotid arteries immediately following balloon injury to determine the effect of FasL expression on lesion formation. Injured rat carotid arteries develop a reproducible neointimal lesion that is dependent on VSMC migration and proliferation [80]. Morphometric analyses revealed that neointimal lesion formation was significantly reduced by treatment with Ad-FasL (Fig. 7A). At fourteen days post-injury robust neointimal lesions were detected in vessels treated either with saline or a relatively high dose of Ad-βgal (1×10^9 pfu). In contrast, lesion size was significantly reduced in arteries treated with Ad-FasL at doses ranging from 1×10^9 to 3×10^8 pfu (Fig. 7B). The effect of Ad-FasL on neointima formation was particularly potent when compared to other adenovirally-delivered cytotoxic or cytostatic genes, including herpes virus thymidine kinase [81,82], mutant or wild-type Rb [83,84], or other growth inhibitory genes [85,86], where effective doses typically range from 1 to 2×10^9 pfu.

At 14 days post-injury, the medial layers of the Ad-FasL-treated vessels appeared normal with regard to size and cellularity (Fig. 7). Balloon injury results in the immediate loss of VSMCs due to barotrauma-induced apoptosis, followed by rapid VSMC proliferation and repopulation of the medial layer [87,88]. Analyses of histological sections revealed normal VSMC density in the media at 3 days post-injury in the saline- and Ad-βgal-infected vessels. However, VSMC density was decreased by a factor of 3 at this time point in the vessels infected with Ad-FasL (Table 3). By 14 days post-injury, medial cell density had returned to normal levels in the Ad-FasL-treated vessels (Table 3), and FasL expression was no longer detectable by immunohistochemistry (data not shown), presumably because the FasL-expressing VSMCs had themselves undergone apoptosis by this time.

Since the systemic administration of anti-Fas antibody can cause severe liver damage and morbidity [64,89], we addressed the issue of systemic toxicity. The lowest effective dose of Ad-FasL examined here, 1×10^8 pfu, is more than 6000-fold below the lethal dose [64]. At the highest Ad-FasL dose, 3×10^8 pfu, no grossly detectable lesions were observed in tissue sections from liver, heart, lung, kidney, spleen, skeletal muscle, or the contralateral carotid artery at days 3 or 14 post-infection. Biochemical markers of liver function in serum at 3 days post-gene transfer did not significantly differ between a relatively high

dose of Ad-FasL ($1X10^8$ pfu) and non-treated groups. Serum levels of alkaline phosphatase were 165 ± 36 and 108 ± 21 IU/l in control and Ad-FasL-treated rats, respectively; glutamine pyruvate transferase levels were 59 ± 2 and 58 ± 8 IU/l in control and Ad-FasL-treated rats, respectively; and glutamine oxaloacetic transferase levels were 87 ± 8 and 137 ± 28 IU/l ($p>0.05$) in control and Ad-FasL-treated rats, respectively. Therefore, local delivery of Ad-FasL to the vessel wall can effectively inhibit injury-induced lesion formation without detectable toxicity to the major organs. In light of these observations, it may be possible to deliver a therapeutic dose of the FasL gene to the site of angioplasty and minimize systemic toxicity by expressing FasL from a conditional or smooth muscle-specific promoter.

(4) Inhibition of adenovirus-mediated T cell response by Fas ligand

Fas ligand gene transfer overrides the adenovirus-mediated T cell response.

FasL expression has been identified in immune-privileged tissues [90], and it has been proposed that it functions to kill Fas-bearing inflammatory cells as they enter the tissue [8, 32-34, 91]. Thus we tested whether ectopic FasL expression could modulate the cellular immune response to adenoviral antigen. T cell infiltration in the vessel wall was assessed in naive rats and in rats immunized with intravenous injection of an E1-deleted adenovirus that lacked a transgene. Little or no T cell infiltration was detected 3 days after transduction with Ad-βgal or Ad-FasL in naive rats (Fig. 8A). In contrast, immunized rats displayed marked T cell infiltration of the vessel wall following local delivery of Ad-βgal, but not Ad-FasL. However, T cell infiltration was essentially eliminated in immunized rats when Ad-βgal and Ad-FasL were co-administered to the vessel wall. These data suggest that the differential T cell response results from the proactive effects of FasL on the vessel wall rather than from differences in the immunogenic properties of the viral transgenes.

Transgene expression by viral constructs was also assessed in naive and immunized rats. The FasL transgene was expressed at similar levels at 3 days post-infection in Ad-FasL-transduced vessels of both immunized and naive animals. In naive animals, β-galactosidase expression was detected in Ad-βgal-transduced vessels, but expression levels were lower relative to FasL transgene due to the

inefficiency of the histochemical detection of β-galactosidase [92,93]. However, in contrast to FasL, no β-galactosidase expression was detected in the vessel walls of immunized rats following infection with Ad-βgal (Fig 8. B&C). β-galactosidase expression in immunized rats could be restored to levels similar to that in naive animals when injured vessels were simultaneously co-infected with Ad-βgal and Ad-FasL constructs. These results demonstrate that FasL can function in *trans* to create conditions that permit viral-mediated gene expression in immunized animals.

FasL expression can also produce a proinflammatory response through its ability to recruit neutrophils, limiting its potential usefulness for immunotherapy [64, 94-96]. However, no neutrophil infiltration was detected in the vessel wall following transduction with Ad-FasL at any dose examined. The basis for these differences in FasL function is not known, but it is possible that the neutrophil response in the vessel wall is inhibited by the expression of TGF-β, which can function to suppress neutrophil function [97-99]. This cytokine is detected in human vascular lesions, and is markedly upregulated in rat carotid arteries following balloon injury [100-102]. Alternatively, the transient nature of FasL expression in VSMCs or the extrusion of cellular debris into the lumen may minimize secondary inflammatory responses in the vessel wall.

(5) FasL/Fas-mediated endothelial cell apoptosis induced by oxidized LDL

OxLDL upregulates FasL expression on human endothelial cells

Vascular endothelial cells express both Fas and FasL [11]. To investigate whether OxLDL-induced endothelial cell death may involve changes in cell surface FasL or Fas expression, FACS analysis was performed on HUVECs treated with OxLDL or L-α-palmitoyl lysophosphatidylcholine (LPC-C16:0), a component of OxLDL. Incubation with OxLDL or LPC upregulated FasL expression on HUVECs (Fig. 9). In contrast, treatment with OxLDL or LPC did not detectably alter HUVEC cell surface Fas expression. Treatment of human aortic endothelial cells (HAECs) with OxLDL or LPC also led to an increase in cell surface FasL expression, while Fas expression remained unchanged (Data not shown).

Neutralizing anti-FasL antibody blocks OxLDL-induced apoptosis of cultured endothelial cells

Consistent with previous reports [56, 103, 104], HAECs treated with OxLDL displayed characteristics of apoptosis including cell shrinkage and nuclear condensation (Fig. 10A). The expression of the apoptotic phenotype was markedly attenuated when cultures were co-incubated with a neutralizing anti-FasL antibody. Similar observations were also made with HUVECs (data not shown). Interestingly, OxLDL-treated endothelial cells protected from death by anti-FasL antibody attained a distinctive elongated cell morphology (Fig. 10A), indicating that other actions of OxLDL are not blocked by the neutralizing anti-FasL antibody. Eight preparations of OxLDL were tested and all produced similar extents of apoptosis in HAECs and HUVECs.

Genomic DNA prepared from OxLDL-treated HUVECs showed DNA ladder, a well established marker of apoptosis (Fig. 10B). Consistent with the prevention of apoptotic phenotype, DNA fragmentation was markedly attenuated by co-incubation with the neutralizing anti-FasL antibody. Treatment with native LDL did not induce DNA fragmentation in HUVECs consistent with observations of others [103].

Endotoxin is a common contaminant of lipoprotein preparations [105]. Though a low level of endotoxin was detected in the OxLDL (0.3 endotoxin units/mg protein/ml), incubation with 100 endotoxin units/ml of LPS did not result in DNA fragmentation in HUVECs (Fig. 10B). Furthermore, LPC is free of detectable endotoxin, and this agent also induces Fas-mediated apoptosis in endothelial cells (see below). These data indicate that endothelial cell apoptosis induced by OxLDL is not due to endotoxin contamination.

HUVECs treated with OxLDL or LPC-C18:0, a major component of OxLDL, also showed decreased mitochondrial function in the MTT assay (Fig. 10C), another indicator of apoptotic cell death [66, 71]. Consistent with the data presented above, the neutralizing anti-FasL antibody inhibited the decrease in cell viability that was induced by incubation with OxLDL or LPC-C18:0. Minimally modified LDL (MM-LDL) is biologically quite different from native LDL and induces many detrimental effects on endothelial cells *in vitro* [106] and *in vivo* [61]. 300 μg protein/ml of MM-LDL induced endothelial cell and coincubation with the neutralizing anti-FasL antibody (4H9, 10 μg/ml) significantly reduced the cell death. These

results indicate that MM-LDL also induces endothelial cell apoptosis, at least in part, through Fas/FasL interaction. Incubation with acetylated LDL, cholesterol, oxidized cholesterols (7-ketocholesterol and 25-hydoxycholesterol), LPC-C12:0, L-α-phosphatidylcholine, β-lynoleoyl-γ-palmitoyl (PC-C18:2, 16:0) did not result in cell viability loss.

OxLDL induces endothelial cell apoptosis through Fas-FasL interaction in organ culture

To further study the physiological relevance of the OxLDL-induced endothelial cell apoptosis, we performed organ culture of arteries. TUNEL staining demonstrated endothelial cell apoptosis in the artery ring treated with OxLDL for 24 hours (Fig. 11A). However, the OxLDL-induced apoptosis was not observed in the artery co-incubated with the neutralizing anti-FasL antibodies. In the rabbit artery ring cultured for 64 hours, by an anti-CD31 immunostaining revealed intact endothelium layer, consistent with previous report [72] (Fig. 11B). However, in the ring treated with OxLDL, most of the endothelium layers were lost, a well establisher marker for endothelial cell [79]. Consistent with cell culture study, co-incubation with neutralizing anti-FasL antibodies prevented endothelial cell apoptosis and endothelium loss, indicating that the endothelial cell death in organ culture is also mediated by Fas-FasL interaciton.

To further confirm that OxLDL-induced endothelial cell apoptosis is mediated Fas/FasL interaction, we performed organ culture of aortic rings from *gld* or *lpr* mice that lack functional FasL or Fas, respectively (n=5 for each group). After 3 days incubation with OxLDL (150 μg/ml), endothelium from C57BL/6J wild type mice were destroyed (Fig. 12). However, aortic endothelia of *gld* or *lpr* mice were relatively resistant to OxLDL-induced cell death. 49.7 ± 6.6 % of the endothelium was disrupted in aortic rings from wild-type mice after treatment with 150 μg/protein OxLDL , but only 10.4 ± 1.1% or 9.9 ± 1.7% of the luminal surface lost endothelium in rings from *gld* or *lpr* mice, respectively ($p<0.05$, $p<0.05$). These data provide genetic evidence that the Fas/FasL interaction is essential for OxLDL induced endothelial cell apoptosis.

OxLDL sensitizes human endothelial cells to the Fas-mediated apoptosis

HAECs treated with OxLDL or LPC also showed decreased mitochondrial function in the MTT assay (Fig. 13 A), another indicator of apoptotic cell death [66, 71]. Consistent with the data presented above, the neutralizing anti-FasL antibody inhibited the decrease in cell viability that was induced by

incubation with OxLDL or LPC. Untreated HAECs were resistant to Fas-mediated apoptosis as indicated by the inability of the agonistic anti-Fas antibody to decrease mitochondrial function. However, decreased mitochondrial function could be induced in the presence of OxLDL or LPC by the agonsitic anti-Fas antibody when endogenous FasL was blocked by the neutralizing antibody.

HUVECs treated with OxLDL displayed DNA fragmentation by FACS analysis following TUNEL staining, an established marker of apoptotic cell death (Fig. 13B). Similar observations DNA fragmentation were also made with HUVECs treated with LPC and with HAECs treated with OxLDL or LPC (M. Sata and K. Walsh, unpublished data). Incubation with neutralizing anti-FasL antibody inhibited the OxLDL induced fragmentation of endothelial cell DNA (Fig. 13B), further indicating that the Fas/FasL interaction mediates cell death under these conditions. Consistent with previous reports [11, 107], untreated HUVECs were resistant to Fas-mediated apoptosis, since incubation with an agonistic anti-Fas antibody did not result in DNA fragmentation (Fig. 13B). Surprisingly, DNA fragmentation could be induced in the presence of OxLDL by the agonistic anti-Fas antibody when endogenous FasL was blocked by the neutralizing antibody. Collectively, these data suggest that treatment with OxLDL sensitizes endothelial cells to Fas-mediated apoptosis (Fig. 13C).

(6) Downregulation of endothelial FLIP expression by oxidized LDL

OxLDL downregulates FLIP level

To identify the molecule regulating endothelial cell sensitivity to Fas-mediated apoptosis, we initially determined the parameters influencing oxidized lipid-induced apoptosis. HUVECs were treated with different doses of OxLDL or LPC for 24 hours and mitochondrial function was assessed by the MTT assay, an indicator of cell death [66, 71]. Consistent with previous reports of cytotoxicity by these agents [56, 103], OxLDL at or above 300 µg protein/ml (Fig. 14A) and LPC at or above 60 µM (Fig. 14B) markedly decreased mitochondrial function. Lower doses of LPC were slightly stimulatory, consistent with reports that these agents can stimulate growth [108, 109].

To study regulation of FLIP under conditions of OxLDL- or LPC-induced endothelial cell apoptosis, a polyclonal antibody was generated against the 26 amino acid sequence of N-terminus of FLIP. This sequence is common among all of the reported isoforms [23-25]. Immunoblotting revealed that untreated HUVECs express a 55kDa immunoreactive protein which has an identical mobility to the

positive control, a extract from COS cells transfected with the $FLIP_L$ expression plasmid (Fig. 14C&D). Two other immunoreactive protein bands with electrophoretic mobilities similar to $FLIP_L$ were also detected. Multiple anti-FLIP, immunoreactive bands have also been detected in T cell extracts [23], and they may represent post-translationally modified FLIP polypeptides or alternatively spliced FLIP isoforms of slightly different molecular mass. Treatment with OxLDL or LPC markedly downregulated expression of the immunoreactive proteins migrating at or near the mobility of $FLIP_L$ in a dose-dependent manner and occurred at doses of OxLDL or LPC that induced endothelial cell death (Fig. 14A&B). Expression levels of the previously reported short FLIP isoform ($FLIP_S$) were very low or undetectable in HUVECs. A band larger than $FLIP_L$ was detected in HUVECs, suggesting another FLIP isoform. The Bcl-2 family of proteins functions as positive and negative regulators of apoptosis [110] including Fas mediated cell death [111]. In contrast to FLIP, OxLDL treatment had no effect on the expression of the pro-apoptotic protein Bax or the anti-apoptotic proteins Bcl-x or Bcl-2 (Fig. 14E).

OxLDL downregulates FLIP transcripts

The decrease in mitochondrial function by LPC was also time dependent with significant reduction occurring within 1 hr of exposure to 60 μM LPC (Fig. 15A). Similar time dependent decrease in mitochondrial function also occurred upon treatment with OxLDL, but the observed time course varied between the different preparations of this agent (not shown). .Northern blotting assay was performed to determine whether FLIP expression is regulated at the level of mRNA by oxidized lipids. Multiple FLIP transcripts were detected in HUVECs (Fig. 15B). Multiple transcripts have also been reported in peripheral blood lymphocyte, heart and skeletal muscle [23]. Treatment of HUVECs with LPC (60 μM) downregulated all FLIP transcripts. Consistent with the results of flowcytometry and Western blotting, FasL transcript increased by LPC (Fig. 15B). Time course of FLIP downregulation correlated with the time course of decreased HUVEC viability by LPC exposure. Furthermore, LPC did not affect the expression of genes encoding the death-signaling molecules, FLICE, FADD, FAP, FAF, DR3, TRAIL, TNFR-p55, or RIP (Fig. 15C). These data suggest that oxidized lipids specifically modulate FLIP levels, and that they do not have widespread effects on the expression of other apoptosis-regulatory molecules.

Exogeneous FLIP expression rescues LPC-induced endothelial cell apoptosis

To examine the functional significance of FLIP downregulation in sensitization of human endothelial cells to Fas-mediated apoptosis, we tested whether ectopic expression of FLIP could protect against LPC-induced apoptosis (Table 4). Using a co-transfection cell death assay [112-114], HUVECs were co-transfected with FLIP expression plasmid and β-galactosidase (β-gal) expression plasmid to mark the transfected cells. As expected, treatment with LPC-C16:0 reduced the number of viable β-galactosidase-positive cells. The FLIP expression plasmids significantly increased the number of viable β-galactosidase-positive cells compared to the pcDNA control plasmid or mock co-transfection, indicating that ectopic expression of $FLIP_L$ prevented endothelial cells from LPC-induced apoptosis. Consistent with observations that Bcl-X_L but not Bcl-2 protects Fas mediated apoptosis [111], ectopic expression of Bcl-X_L also protected LPC-induced endothelial cell apoptosis, while Bcl-2 did not.

Discussion

(1) Regulation of leukocyte extravasation by Fas ligand expression on the vascular endothelium

The recruitment of leukocytes at sites of inflammation is a multistep process that involves tethering, rolling, firm adhesion, and the migration of these cells to the subendothelial space [2]. Though much is known about the chemoattractant and adhesion molecules that regulate leukocyte recruitment in response to bacterial infection [6], relatively little is known about mechanisms that may actively control the transendothelial cell migration. Here we demonstrated functional FasL expression on the vascular endothelium and its downregulation upon exposure to the inflammatory cytokine TNFα. Adenovirus-mediated constitutive FasL expression by the endothelium markedly reduces the leukocyte extravasation that is induced by local treatment with TNFα. Under these conditions, VCAM-1 is upregulated resulting in the adherence of mononuclear cells, but these cells undergo apoptosis rather than diapedesis. These data suggest that the downregualtion of FasL by the endothelium is essential for TNFα-induced leukocyte extravasation.

In marked contrast to ECs, Fas-bearing VSMCs are highly sensitive to FasL-induced apoptosis, and the adenovirus construct expressing FasL is a potent inhibitor of neointimal hyperplasia when applied to arteries that have been denuded of endothelium by balloon-injury [63]. However, Adeno-FasL does not induce detectable apoptosis in cultured ECs nor in the endothelium of rabbit ear central arteries. Presumably ECs are resistant to Fas-mediated cell death either because they normally express relatively low levels of Fas, or they are unable to transmit an apoptosis-inducing signal following the engagement of Fas by FasL as has been suggested by others [107]. Thus, it is conceivable that the deregulated expression of FasL, Fas, or signaling pathway components of this system may be a feature of EC dysfunction in response to injurious agents leading to inflammatory-fibroproliferative disorders of the vessel wall.

Atherosclerosis is believed to result from a chronic, detrimental immune response at localized regions within the vessel [1]. Our data suggest that FasL may serve an atheroprotective function on the endothelium through its ability to induce apoptosis in mononuclear cells attempting to invade the vessel wall in the absence of normal inflammatory stimuli. Human atherosclerotic plaques contain significant

amounts of T lymphocytes and macrophages [1, 42, 45, 49, 115, 116]. TNFα is expressed by macrophages and smooth muscle cells at the sites of vascular injury, and this cytokine is thought to have an important role in the progression of atherosclerotic lesions [46, 117]. This process is particularly evident in transplant arteriosclerosis, a robust form of atherosclerosis that is responsible for the majority of deaths in heart transplant recipients surviving for one or more years [118]. Our data suggest that secretion of TNFα by activated cells within an atheroma [43, 46, 116] may downregulate FasL expression in adjacent normal endothelium, promoting more leukocyte extravasation and lesion growth. Consistent with this notion is the observation that blockade of TNFα reduces coronary artery neointimal formation in a rabbit model of cardiac transplant arteriosclerosis, while having no effect on the degree of myocardial rejection [119]. Therefore, these findings not only suggest an additional function of the vascular endothelium, but may provide insights about the pathogenesis of the atherosclerosis.

(2) Potential therapeutic utility of Fas ligand gene transfer to treat proliferative vascular disorders

Coronary atherosclerosis lesions are commonly treated by percutaneous transluminal coronary angioplasty (PTCA), a procedure where inflated balloon dilates the narrowed artery lumen. However, a significant fraction of these procedure fail due to post-angioplasty restensis that occurs within six months following the procedure [120, 121]. Restenosis results partly from inflammatory cell infiltration [122] and exuberant vascular smooth muscle cell proliferation [123]. These pathological process are particularly pronounced in restenosis that occurs within stents [124, 125], prosthetic devices inserted in vessels to prevent constrictive remodeling. Thus, much effort has been devoted toward both understanding the molecular pathways regulating vessel wall responses to acute injury and developing strategies to block post-angioplasty restenosis.

In this study, we showed that the Ad-FasL induces apoptosis in Fas-bearing vascular smooth muscle. Ad-FasL-transduced vascular smooth muscle cells also kill neighboring non-infected smooth muscle cells and T cells in co-culture assays. When this virus is introduced locally in balloon injured rat carotid arteries denuded of endothelium, a well characterized model that produces a vascular smooth muscle cell-derived lesion, Ad-FasL functions as a potent inhibitor of neointima formation [63]. In this

model, Ad-FasL is effective at doses as low as 1×10^6 infectious units per vessel, well below the dose required to produce systemic toxicity. We attribute the potency of Adeno-FasL to the ability of Fas ligand to induce apoptosis in a paracrine manner at the site of delivery, thereby limiting smooth muscle cell proliferation and lesion formation.

Though adenovirus is a promising vector for gene therapy through its ability to achieve high gene transfer efficiency, a major limitation is the T cell-mediated immune response to adenoviral antigens that induce the destruction of the infected cells [126]. For this reason, repeated administration of the recombinant virus is associated with robust immune responses and a low gene transfer efficiency both in animal models and in patients receiving repeated adenovirus-mediated gene therapy [127-129]. This issue is also a concern with adenovirus-mediated therapies for vascular disorders that they may employ just a single delivery of an adenoviral construct since a large portion of the patient population has developed immunity to several adenovirus serotypes. Furthermore, inflammatory responses are reported to occur when high doses of adenovirus are administered to laboratory animals that are normally sero-negative to adenovirus, and this can promote the formation of occlusive lesions in normal vessels [130, 131]. Our findings indicate that the localized expression of Fas ligand can allow adenovirus-harboring cells to evade immune destruction and may prevent the T cell response to repeated delivery of genes with viral vectors. In effect, this experiment suggests that adenovirus-mediated gene transfer of Fas ligand to the denuded vessel confers to smooth muscle a property normally confined to the endothelium - the ability to inhibit leukocyte infiltration of the vessel wall.

Therefore, ectopic Fas ligand expression in balloon-injured vessels serves two functions, to inhibit inflammation and reduce smooth muscle cell mass, suggesting that it may be particularly well suited for the treatment of restenosis.

(3) Oxidized LDL activates Fas-mediated endothelial cell suicide by downregulating intracellular caspase inhibitor FLIP

We showed that vascular endothelial cells express both Fas and FasL, and that these cells are normally resistant to Fas-mediated apoptosis [11]. Here, we demonstrate that oxidized lipids induce apoptosis in endothelial cells by activating the Fas death pathway. Oxidized lipid-induced endothelial cell apoptosis, identified by changes in cell shape, nuclear condensation, DNA fragmentation, and reduced

mitochondrial function, was inhibited by incubation with a neutralizing antibody to FasL. Neutralizing anti-FasL antibodies also inhibited oxidized lipid-induced endothelial cell death in cultured arterial rings, and aortic endothelia prepared from FasL- or Fas-deficient mice were resistant to OxLDL-induced death. Collectively, these data indicate that the Fas/FasL pathway is a key feature of apoptosis induced by acute exposure to oxidized lipid.

Endothelial cell apoptosis induced by moderate levels of copper-oxidized LDL, ferrous-oxidized (minimally modified) LDL, LPC-C16:0 or LPC-C18:0 was blocked by treatment with the neutralizing anti-FasL antibody. At higher levels of oxidized lipids (OxLDL > 400μg protein/ml, LPC-C16:0 > 80μM, or LPC-C18:0 > 60μM), cell death was unaffected by inhibition of the Fas pathway (data not shown), indicating that other apoptotic mechanisms are also triggered by these agents. In this study, cytotoxic effects of these agents were examined in 2% (cell culture) or 0.5% (organ culture) fetal bovine serum. Endothelial cells were more resistant to OxLDL in the presence of higher concentration of serum (>10%) or after pretreatment with high serum medium (data not shown). The presence of high density lipoprotein [132] or survival factors [133] in serum may modulate endothelial cell sensitivity to OxLDL-induced cytotoxicity.

Copper-oxidized LDL has been widely used to study the biological activity of OxLDL [56, 57, 60, 104], but a recent study suggests that copper-oxidized LDL may not represent the relevant form of *in vivo* modified LDL [134], indicating that one must exert caution in extrapolating *in vitro* findings with copper-oxidized LDL to the *in vivo* state. However, circulating OxLDL can be detected in blood of patients with hyperlipidemia using antibody raised against copper-oxidized LDL [135], and monoclonal autoantibodies from apolipoprotein E-deficient mice recognize epitopes in copper-oxidized LDL [136]. OxLDL and its components have also been detected in atherosclerotic plaque of humans [41], apolipoprotein E3-leiden transgenic mice [137], and Watanabe heritable hyperlipidemic rabbits [41, 50]. Taken together, these findings demonstrate that OxLDL is present *in vivo* and suggest that our results provide insights about the pathophysiology of OxLDL toxicity in endothelial cells.

Oxidized lipids influence the Fas/FasL pathway in endothelial cells in at least two ways. First, they increase cell surface expression of FasL with no detectable effect on the level of cell surface Fas expression. Second, they sensitize endothelial cells to death signals from the Fas receptor. This conclusion is indicated by the finding that an agonistic anti-Fas antibody can kill endothelial cells in the presence, but not absence, of oxidized lipids when endogenous FasL is inactivated by a neutralizing antibody. Endothelial cells are normally refractive to apoptosis in response to Fas ligand overexpression [11]. Therefore, these data show that oxidized lipids do not kill endothelial cells by upregulating FasL alone, but instead by increasing the responsiveness of endothelial cells to Fas ligation.

Recent evidence suggests that cells expressing both FasL and Fas can become dramatically sensitized to the Fas-mediated apoptosis in response to specific stimuli and injuries. There is increasing evidence that alterations in cancer cell sensitivity to Fas-mediated apoptosis is a key feature controlling tumor progression [8, 34, 35], and perturbations in the Fas/FasL cell suicide pathway appear to be important in determining the viability of transformed cells [12, 14, 15]. Ultraviolet- or γ-radiation-induced apoptosis is also reported to be mediated by an activation of the Fas/FasL pathway [13, 138], and it is well established that T lymphocyte number is controlled by a delayed sensitization to Fas/FasL-mediated suicide following activation [10]. The data presented here suggest that OxLDL enhances the sensitivity of endothelial cells to Fas-mediated cell suicide leading to a disruption of the endothelium. Since the status of endothelial cells is critical for vessel wall homeostasis [1], alterations in endothelial cell sensitivity to Fas-mediated apoptosis may play a role in vascular disease.

Here, I found that oxidized lipids downregulate FLIP protein. FLIP downregulation correlates with a loss of endothelial cell viability, and oxidized lipid-induced cell death was reversed by forced FLIP expression. Previously, FLIP regulation has only been described during T cell activation where it may control Fas-mediated suicide [23, 26]. The findings described herein suggest that FLIP levels may play a role in determining endothelial cell sensitivity to oxidized lipid-induced apoptosis, and they indicate that FLIP may be a target of agents that cause pathological cell death.

(4) Role of endothelial FasL expression in vessel wall

At this time it is difficult to assess the overall impact of the endothelial Fas/FasL system in atherogenesis. On one hand, endothelial FasL can function to inhibit leukocyte extravasation by inducing apoptosis in mononuclear cells invading the vessel wall in the absence of normal inflammatory stimuli [11]. In this context, FasL may have a protective role since abnormal leukocyte adhesion to the endothelium and extravasation is an early event in atherogenesis [1]. On the other hand, oxidized lipids can increase the sensitivity of endothelial cells to death signals from the Fas receptor. As injuries to endothelium trigger inflammatory processes, sensitization to Fas-mediated apoptosis may contribute to atherosclerosis that results from exaggerated hyperlipidemia. Therefore, further studies on Fas, Fas ligand, and the Fas-signaling pathway in endothelial cells may provide a mechanistic rationale for inflammatory cell accumulation that is characteristic of fibroproliferative disorders of the vessel wall.

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Table 1. Effect of constitutive FasL expression on mononuclear cell infiltration

	Saline	TNFα	Adeno-FasL +TNFα	Adeno-βgal +TNFα
T cell	0.2± 0.2	31.8± 12	1.5± 0.6	65.0± 23.4
Macrophage	0	4.0± 0.9	0.5± 0.5	7.0± 1.9

The number of mononuclear cells infiltrating into the media (Figs 4B and 5A) was counted in each cross section of the artery (n=4 for each group).

Table 2. Dose-dependent production of apoptotic sub-G1 DNA population in VSMCs by Ad-FasL infection.

Treatment	MOI	Sub-G1 DNA
Ad-FasL	10	3.3%
	30	9.2%
	100	35.2%
	300	49.2%
Ad-βgal	10	0.2%
	30	0.2%
	100	1.1%
	300	0.3%
Saline	—	0.6%

Table 3. Cellular density of media after balloon-injury (/mm^2).

cells/mm^2	saline	Ad-βgal (1×10^8pfu)	Ad-FasL (1×10^8pfu)
3 days after injury	3905 ± 419	3686 ± 335	1365 ± 224*
14 days after injury	3764 ± 494	3699 ± 279	3845 ± 263

Cellular density was calculated by determining the number of nuclei and the area of the media for each section (n=4 for each group). The results are expressed as mean ± SEM.

* $p<0.05$ vs. normal artery (3408 ± 124 /mm^2)

Table 4 Ectopic expression of FLIP protects endothelial cells from LPC-induced apoptosis.

Plasmid	Number of β-galactosidase-positive cells/well
mock	41.5 ± 6.0
pcDNA	39.3 ± 4.9
$FLIP_L$	111.5 ± 20.4
Bcl-X_L	119.0 ± 22.3
Bcl-2	40.5 ± 8.2

HUVECs cultured in 24-well plates in EGM medium were co-transfected with the test plasmid (0.15μg) and β-galactosidase expression plasmid (1.5μg) using the Superfect procedure (Qiagen). After 48 hours, HUVECs were incubated with 60μM LPC-C16:0 for 24 hours, and stained with X-gal.

Figure Legends

Figure 1. Regulated expression of FasL on human endothelial cells

A. Immunoblot analysis of FasL and VCAM-1 regulation in HUVECs. HUVECs were incubated for 21 hours with either basal medium or TNFα at 0.5 ng/ml, 5 ng/ml, or 25 ng/ml. Cell lysates (15 µg) were loaded onto 10% SDS-polyacrylamide gel and analyzed by Western blotting using anti-FasL (upper panel), anti-VCAM-1 (middle) or anti-α-tubulin antibody (lower). Lysate of PMA- and ionomycin-activated Jurkat cells and HUSMCs infected with Ad-FasL (multiplicity of infection of 100) served as positive controls for FasL. Lysate of uninfected HUSMCs were probed to demonstrate antibody specificity.

B. Flow cytometric analysis of FasL expression on HAECs. HAECs were incubated in either basal medium (left panel), or 25 ng/ml TNFα (right panel) for 6 hours. Endothelial cells were detached from the culture plate with 0.5% EDTA and incubated with anti-FasL monoclonal antibody (A11, Alexis Corp., San Diego, CA) (filled curve) or with rat IgM (open curve) in PBS with 10% FBS, followed by incubation with FITC-conjugated anti-rat IgM.

C. Flow cytometric analysis of Fas expression on HAECs and Jurkat cells. Cells were incubated with an FITC-conjugated anti-Fas monoclonal antibody (filled curve) or with an FITC-conjugated mouse IgG (open curve).

Figure 2. Endothelial cells are resistant to Fas-mediated cell death

HUVECs or Jurkat cells were grown in basal medium, harvested, fixed with 70% ethanol and stained with propidium iodide as described [63]. DNA content was analyzed by flow cytometry (**A&D**). HUVECs and Jurkat cells were incubated with an agonistic anti-Fas monoclonal antibody (0.5 µg/ml, clone CH11, MBL, Japan) for 16 hours prior to harvesting and analysis (**B&E**). HUVECs and Jurkat cells were infected with Adeno-FasL at a multiplicity of infection of 300 for 48 hours prior to harvesting and analysis (**C&F**).

Figure 3. Cytotoxic activity of endothelial cells to P815-huFas cells

A. P815-huFas cells were labeled with BrdU and applied to HAEC cultures that were pre-treated with either basal medium or TNFα. Genomic DNA from both P815-huFas cells and HAECs were loaded on

an 1.5% agarose gel (left panel). DNA was transferred onto a nylon membrane and the DNA fragmentation of P815-huFas cells was detected by a immunoblotting using anti-BrdU antibody (right panel). 1, 100 bp molecular size standard; 2, P815-huFas cells co-cultured with HAECs; 3, P815-huFas cells co-cultured with HAECs which have been pre-treated with TNFα (10 ng/ml) ; 4, P815-huFas cells alone.

B. P815-huFas cells were labeled with ^{3}H-thymidine and applied to HAECs that were pre-treated with either basal medium or TNFα (10 g/ml). Cells were harvested after 18 hours co-culture and the percentage of specific DNA fragmentation was calculated as described in Materials and Methods. A neutralizing anti-human FasL antibody (4H9) was applied to HAECs 30 minutes before co-culturing. All values are presented as mean ± S.E.M.

Figure 4. Regulated expression of FasL on vascular endothelial cell *in vivo*

A. A cryosection of the central artery of an untreated rabbit ear was stained for FasL and an adjacent section was stained for CD31, an antigen expressed by endothelial cells. A biotin-conjugated rat IgM was used as an isotype control from the anti-FasL antibody stain.

B. Central rabbit ear arteries were temporarily isolated by application of a tourniquet and incubated with either PBS or TNFα (50 ng) for 15 minutes. Rabbits were then sacrificed 30 hours after treatment and the arteries were harvested and snap-frozen in embedding compound. Cryosections were stained for FasL, VCAM-1, CD3 (T cells) or RAM 11 (macrophages, Mϕ). Bar, 10 μm.

Figure 5. Constitutive expression of FasL prevents TNFα-induced T cell and monocyte infiltration by inducing apoptosis.

A. Rabbit central ear arteries were isolated and infected with either Adeno-FasL (1×10^{7} pfu) or Adeno-βgal (1×10^{7} pfu) for 15 minutes prior to the restoration of blood flow. After 12 hours, the arteries were again isolated and incubated with TNFα (50 ng) for 15 minutes. Rabbits were sacrificed 30 hours after the TNFα treatment, and arteries were harvested. Cryosections were analyzed by

immunohistochemical methods for FasL, VCAM-1, T cells and macrophages as described in Methods. Bar, 10 μm.

B. Rabbit central ear arteries were infected with Adeno-FasL ($1X10^7$ pfu) or Adeno-βgal (1×10^7 pfu) for 15 minutes, and, after 12 hours, incubated with TNFα (50 ng) for 15 minutes. Arteries were harvested 4 hours after TNFα treatment and stained with Hoechst 33258 (blue) to detect total chromatin, and TUNEL (green) to detect fragmented chromatin. The internal elastic lamina containing autofluorescent elastin is visible with a filter specific for fluorescein. Bar, 25 μm.

Figure 6. Effect of Ad-FaL on VSMC viability in vitro. (**A**) FasLexpression in VSMCs at 12 hours post-Ad-FasL infection. Cultured rat aortic VSMCs were infected with Ad-FasL at a MOI of 100 for 4 hours. After 12 hours incubation, cells were harvested and stained with biotinylated anti-FasL monoclonal antibody (filled curve) or with mouse IgG (open curve) followed by FITC-conjugated ExtrAvidin. Immunofluorescence staining was analyzed by flow cytometry. (**B**) Endogenous expression of Fas on VSMCs. Rat VSMCs were harvested and incubated with an anti-Fas mouse monoclonal antibody (filled curve) or mouse IgG (open curve) followed by the incubation with an FITC-conjugated goat anti-mouse IgG. (**C**) Flow cytometric profile of DNA content after Ad-FasL infection. Rat VSMCs were infected with Ad-FasL at a MOI of 100 for 4 hours. (**D**) DNA profile of rat VSMCs after Ad-βgal infection at a MOI of 100 for 4 hours. 48 hours after infection, cells were harvested, stained with propidium iodide and DNA content was analyzed by flow cytometry [139]. For C and D the positions of the G0/G1, G2/M, and apoptotic sub-G1 (P_0) DNA populations are indicated. (**E**) Cytotoxicity of the Ad-FasL-transduced VSMCs toward uninfected T cells and VSMCs. Rat VSMCs were briefly incubated with saline, Ad-βgal, or Ad-FasL (effector cells), washed twice in PBS and then co-cultured with ^{3}H-thymidine-labeled-rat VSMCs or Jurkat cells (target cells). DNA fragmentation of the target cells were measured as described in Materials and Methods.

Figure 7. Effect of Ad-FasL on neointimal formation in rat carotid arteries two weeks after balloon injury. (**A**) Representative cross-sections from arteries treated with either saline or Ad-FasL (1×10^8 pfu) at the time of injury. Cross sections were stained with Richardson's combination elastic

tissue trichrome stain (upper) or with hemotoxylin and eosin (lower). Arrows indicate the internal elastic lamina. **(B)** Intimal (open bar) and medial (stippled bar) areas of carotid artery cross sections at 2 weeks post-injury (n=5 arteries for each group). The intimal/medial area ratio is reported in parentheses.

Figure 8. Effect of Ad-FasL on T cell infiltration and transgene expression in immunologically primed-rats. **(A)** In situ detection of T cell infiltration in carotid arteries of naive and preimmunized rats three days after balloon injury and viral infection. Viral solutions of Ad-βgal ($1 X10^8$ pfu), Ad-FasL ($1 X10^8$ pfu), or mixture of Ad-βgal and Ad-FasL ($1 X10^8$ pfu each) were introduced into the balloon-injured carotid arteries of naive or immunized rats (n=4 arteries for each group). Rats were immunized with an adenoviral vector lacking a transgene ($1X10^9$ pfu) two weeks prior to injury/infection. T cells were detected using an anti-CD3 polyclonal antibody (arrow). **(B)** Transgene expression in carotid arteries of naive and immunized rats three days after injury and viral infection. β-galactosidase and FasL expression in arteries infected with Ad-βgal (1×10^8 pfu), Ad-FasL (1×10^8 pfu), or mixture of Ad-βgal (1×10^8 pfu) and Ad-FasL (1×10^8 pfu). β-galactosidase was stained with X-gal (blue), while FasL expression was revealed using an anti-FasL monoclonal antibody (FAST-RED detection system). The Ad-βgal encodes a *E. coli* β-galactosidase containing a nuclear localization signal under the control of cytomegalovirus promoter. Arrows indicate β-galactosidase-expressing cells. **(C)** Mean transduction efficiencies of Ad-βgal (left panel) or Ad-FasL (right panel). Transduction efficiencies were calculated by determining the percentage of cells staining positive for the presence of transgenes (n=4 for each group). The results are expressed as mean ± SEM.

Figure 9. OxLDL upregulates the FasL expression on human endothelial cells.

90% confluent HUVECs were incubated with 150 μg protein/ml of OxLDL or 45 μM of L-α-palmitoyl lysophosphatidylcholine (LPC) for 13 hours. To detect Fas expression, HUVECs were detached from the culture plate with 0.5% EDTA and incubated with an FITC-conjugated anti-Fas antibody (filled curve) or an FITC-conjugated mouse IgG (open curve). To detect FasL expression, cells were incubated with an anti-FasL antibody (filled curve) or with a rabbit IgG (open curve) followed by

incubation with an FITC-conjugated anti-rabbit Ig antibody. Immunofluorescence staining was analyzed by FACS.

Figure 10. OxLDL-induced endothelial cell apoptosis is blocked by a neutralizing anti-FasL antibody.

A. Apoptotic phenotype induced by OxLDL 90% confluent HAECs were incubated with OxLDL (300 μg protein/ml) in the absence (middle) and presence (right) of a neutralizing anti-FasL antibody (αFasL). After 22 hours, cells were fixed in 4% paraformaldehyde, stained with Hoechst 33258 (Sigma), and observed under the microscope equipped with a phase-contrast and epifluorescence optics.

B. DNA fragmentation induced by OxLDL HUVECs (1×10^6) were incubated with native LDL (300 μg/ml), OxLDL (300 μg/ml), LPS (100 endotoxin units/ml), or a neutralizing anti-FasL antibody in the indicated combinations for 36 hours. The genomic DNA was isolated as described in Methods, fractionated on 1.5% agarose gel in 1 × TBE buffer, and stained with ethidium bromide.

C. Decrease in cell viability induced by OxLDL and other lipid components

HUVECs were incubated with OxLDL, minimally modified LDL (MM-LDL) (300 μg protein/ml), L-α-lysophosphatidylcholine, stearoyl (LPC-C18:0) (45 μM), actylated LDL (AcLDL) (300 μg protien/ml), L-α-lysophosphatidylcholine, lauroyl (LPC-C12:0) (100μM), β-lynoleoyl-γ-palmitoyl (PC-C18:2,16:0), cholesterol (200μM), 7keto cholesterol (200μM), or 25 hydoxycholesterol (25OH-cholesterol) (200 μM) in the absence or presence of the neutralizing anti-FasL antibody (αFasL) (10 μg/ml) for 24 hours. Cell viability was determined by means of MTT assay.

Figure 11 OxLDL induces endothelial cell apoptosis through Fas-FasL interaction in an organ culture of rabbit carotid artery.

Rings from rabbit carotid arteries were treated with OxLDL in the absence or presence of the neutralizing anti-FasL antibodies (αFasL, 4H9, 10μg/ml and C-20, 10 μg/ml) and harvested at 24 hours and 64 hours. Arteries harvested at 24 hours were stained with TUNEL (green) to detect fragmented

chromatin and propidium iodide (red) to detect total chromatin and examined by laser confocal microscopy (**A**). Apoptotic nuclei are shown as yellow with a filter for rhodamine and fluorescein. The internal elastic lamina containing autofluorescent elastin is visible with a filter specific for fluorescein. Bar, 25 μm. Arteries harvested at 64 hours were stained for CD31 using FastRed chromagen (**B**). Endothelium are indicated by arrows. Bar, 50 μm.

Figure 12 Endothelial cells from *gld* or *lpr* mice is resistant to OxLDL-induced apoptosis

Aortic rings from C57BL/6J wild type, *gld, lpr* mice are incubated with OxLDL 150 μg/ml for 72 hours, stained for CD31 and observed in lower (upper panels) or higher magnification (lower panels).

Figure 13 OxLDL sensitizes endothelial cells to Fas-mediated apoptosis.

(A&B) OxLDL and LPC decrease HAEC viability through sensitization to Fas-mediated apoptosis. HAECs were cultured in a 96-well plate at 80% confluency and incubated in the presence or absence of OxLDL (300 μg protein/ml) (panel A), LPC-C16:0 (55 μM) (panel B), a neutralizing anti-FasL antibody (αFasL), or an agonistic anti-Fas antibody (αFas) for 18 hours in combinations as indicated. Cell viability was determined by means of MTT assay [66,71] and % cell death was calculated as 100 × (1-viability of treated endothelial cells /viability of untreated endothelial cells).. Data are presented as mean ± S.E.M. **(C)** OxLDL induces DNA fragmentation in HUVECs through sensitization to Fas-mediated apoptosis. 70% confluent HUVECs were incubated in the presence or absence of OxLDL (300 μg protein/ml), a neutralizing anti-FasL antibody (αFasL), or an agonistic anti-Fas antibody (αFas) for 16 hours in combinations as indicated. Hamster IgG was used as an isotype-matched control for 4H9. Floating and attached cells were incubated with TdT-mediated dUTP nick end labeling (TUNEL) solution (Boehringer Mannheim) in the absence (open curves) and in the presence (filled curves) of terminal deoxynucleotidyl transferase. Fluorescence intensity of the TUNEL-positive DNA was analyzed by FACS. **(D)** OxLDL sensitizes endothelial cells to Fas-mediated apoptosis. Normal vascular endothelial cells are resistant to Fas-mediated apoptosis because they express high levels of cellular FLIP that blocks

Fas-signalling pathway stimulated by FasL or an agonistic anti-Fas antibody (Untreated, αFas). OxLDL downregulates FLIP levels causing endothelial cell apoptosis through Fas-mediated suicide pathway (OxLDL, OxLDL + IgG). In the presence of OxLDL, a neutralizing anti-FasL antibody blocks Fas-FasL interaction and prevents apoptosis (OxLDL + αFasL). In the presence of OxLDL, the agonistic anti-Fas antibody could induce apoptosis when endogenous FasL was blocked by the neutralizing anti-FasL antibody (OxLDL + αFasL + αFas).

Figure 14 Oxidized LDL-induced endothelial apoptosis is associated with downregulation of FLIP

(A&B) Oxidized lipids reduce viability of human endothelial cells in a dose-dependent manner. HUVECs were cultured in 96-well plate at 80% confluency and incubated with different concentrations of OxLDL (A) or LPC (B) for 16 hours. Cell viability was determined by means of MTT assay. Data are presented as mean ± S.E.M. **(C, D &E)** OxLDL or LPC downregulates FLIP, but not Bcl-2 family proteins, in human endothelial cells in a dose-dependent manner. HUVECs were treated with OxLDL (C & E) or LPC (D) at the indicated concentrations for 21 hours. Cell lysates (15μg) were loaded on 10% SDS-polyacrylamide gel and analyzed by Western blotting using the indicated antibodies. Extracts from COS cells transfected with $FLIP_L$ and $FLIP_S$ expression plasmids function as positive controls. Immunoblotting with α-tubulin antibody was performed to demonstrate equal loading of samples.

Figure 15 FLIP is downregulated at transcriptional level.

(A) LPC-C16:0 reduces viability of human endothelial cells in a time-dependent manner. HUVECs were treated with 60 μM of LPC-C16:0 and cell viablity was determined at indicated time points by means of MTT assay. **(B)**. LPC-C16:0 downregulates FLIP transcripts. .HUVECs were treated with LPC (60 μM) and total RNA was isolated at indicated time points. Northern blotting assay was performed using human $FLIP_S$ cDNA as a probe. **(C)** LPC does not affect levels of death-signaling molecules. HUVECs were treated with LPC (60 μM) and total RNA was isolated at indicated time points. Multi-probe ribonuclease protection assay was performed according to manufacture's instruction. The ribonuclease-

protected bands were identified by using a standard curve of migration distance versus log nucleotide length with the undigested probes as markers. Assays for L32 and glyceraldehyde 3-phophate dehydrogenase (GAPDH) serve to demonstrate equal loading of samples.

DR3, death receptor3; FAP, Fas-associated phosphatase; FAF, Fas-associated protein factor; TRAIL, TNF-related apoptosis inducing ligand; TNFR, Tumor necrosis factor receptor; TRADD, TNFR1 associated death domain protein; RIP, receptor interacting protein.

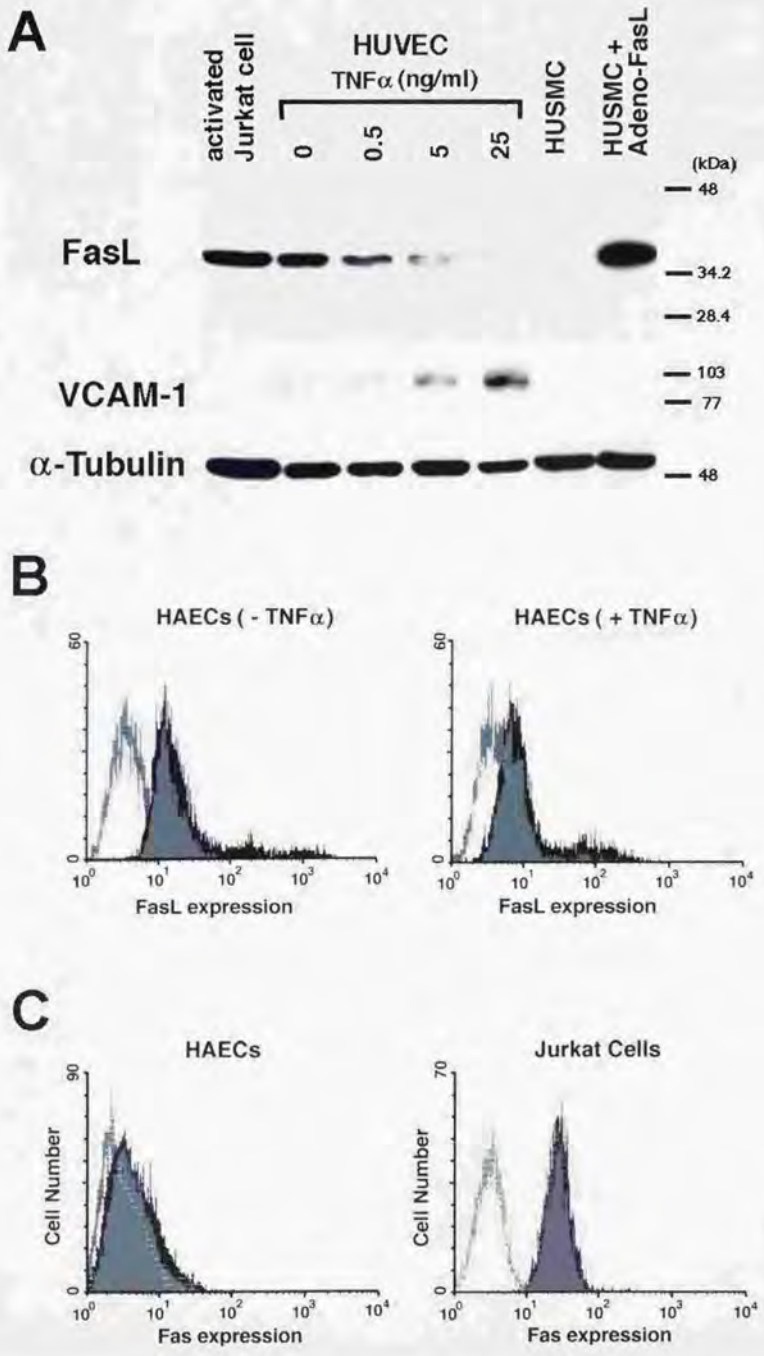
A
activated Jurkat cell
HUVEC
TNFα (ng/ml)
0
0.5
5
25
HUSMC
HUSMC + Adeno-FasL
(kDa)
48
FasL
34.2
28.4
103
VCAM-1
77
α-Tubulin
48
B
HAECs (- TNFα)
HAECs (+ TNFα)
60
0
10^0
10^1
10^2
10^3
10^4
FasL expression
C
HAECs
Jurkat Cells
90
70
Cell Number
Fas expression

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Figure 1

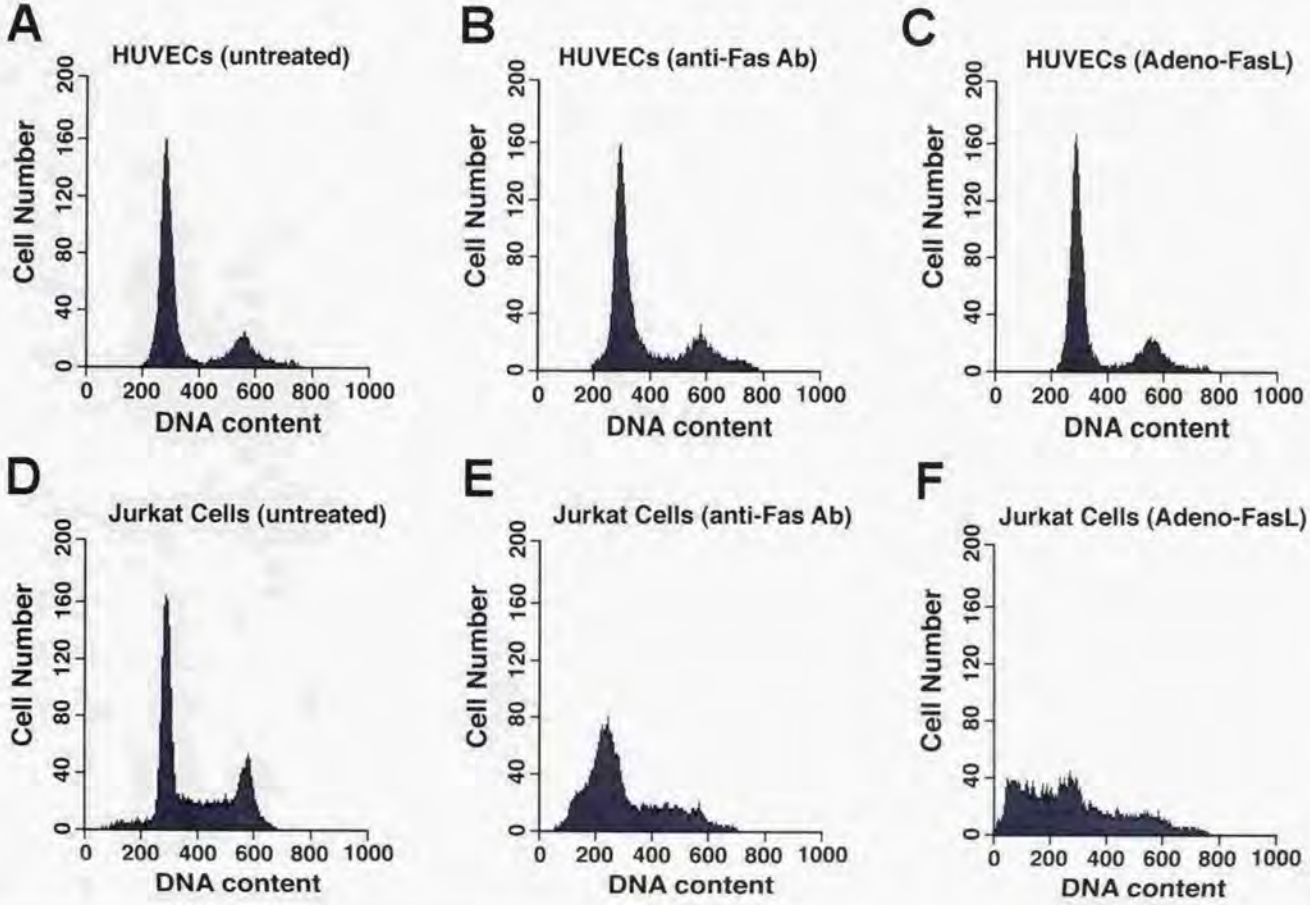
A
HUVECs (untreated)
B
HUVECs (anti-Fas Ab)
C
HUVECs (Adeno-FasL)
D
Jurkat Cells (untreated)
E
Jurkat Cells (anti-Fas Ab)
F
Jurkat Cells (Adeno-FasL)
Cell Number
0
40
80
120
160
200
0
200
400
600
800
1000
DNA content

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Figure 2

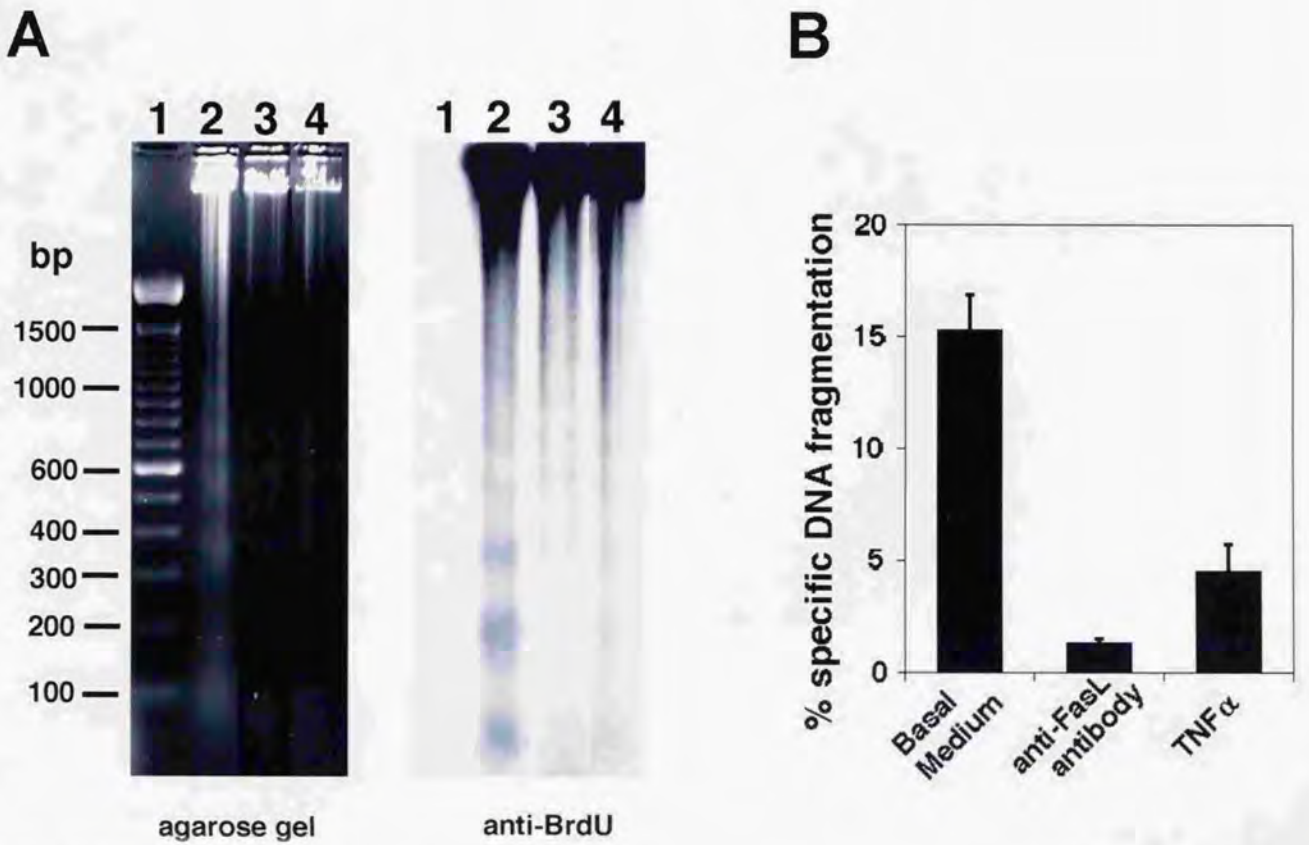
A
1 2 3 4
bp
1500
1000
600
400
300
200
100
agarose gel
1 2 3 4
anti-BrdU
B
% specific DNA fragmentation
20
15
10
5
0
Basal Medium
anti-FasL antibody
TNFα

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Figure 3

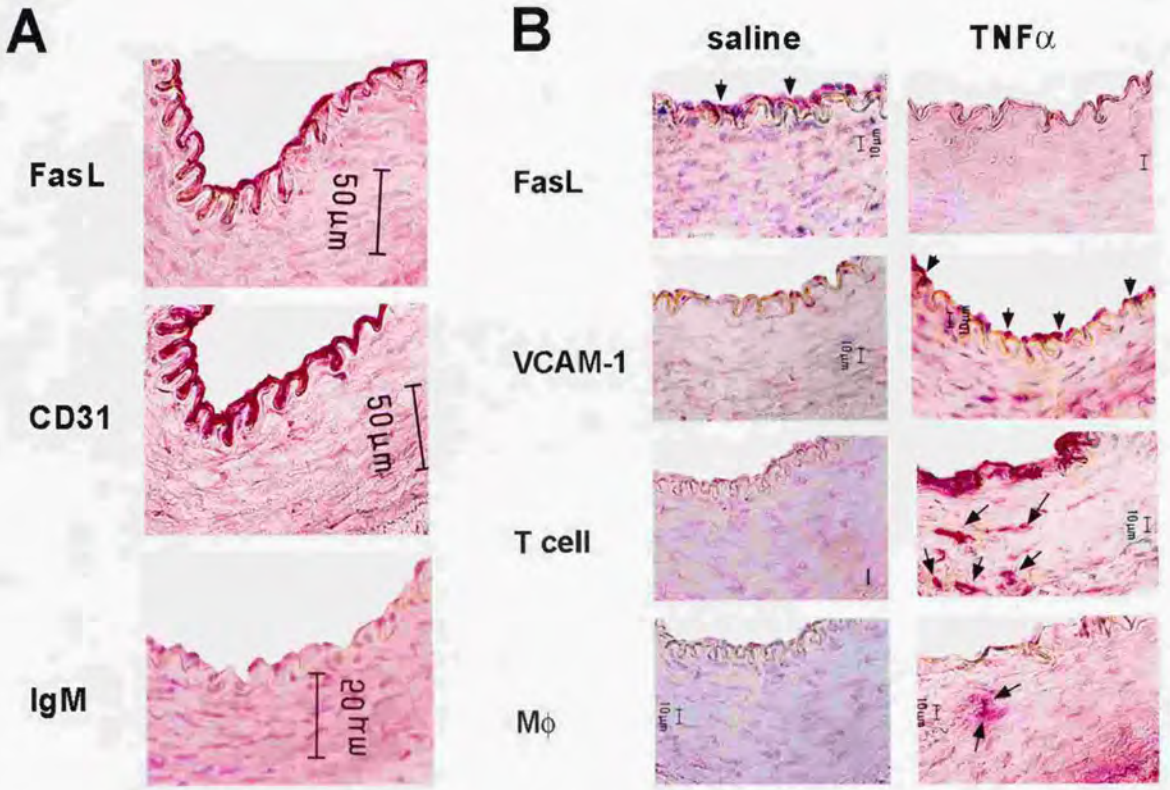
A
FasL
50μm
CD31
50μm
IgM
20μm
B
saline
TNFα
FasL
VCAM-1
T cell
Mϕ
10μm

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Figure 4

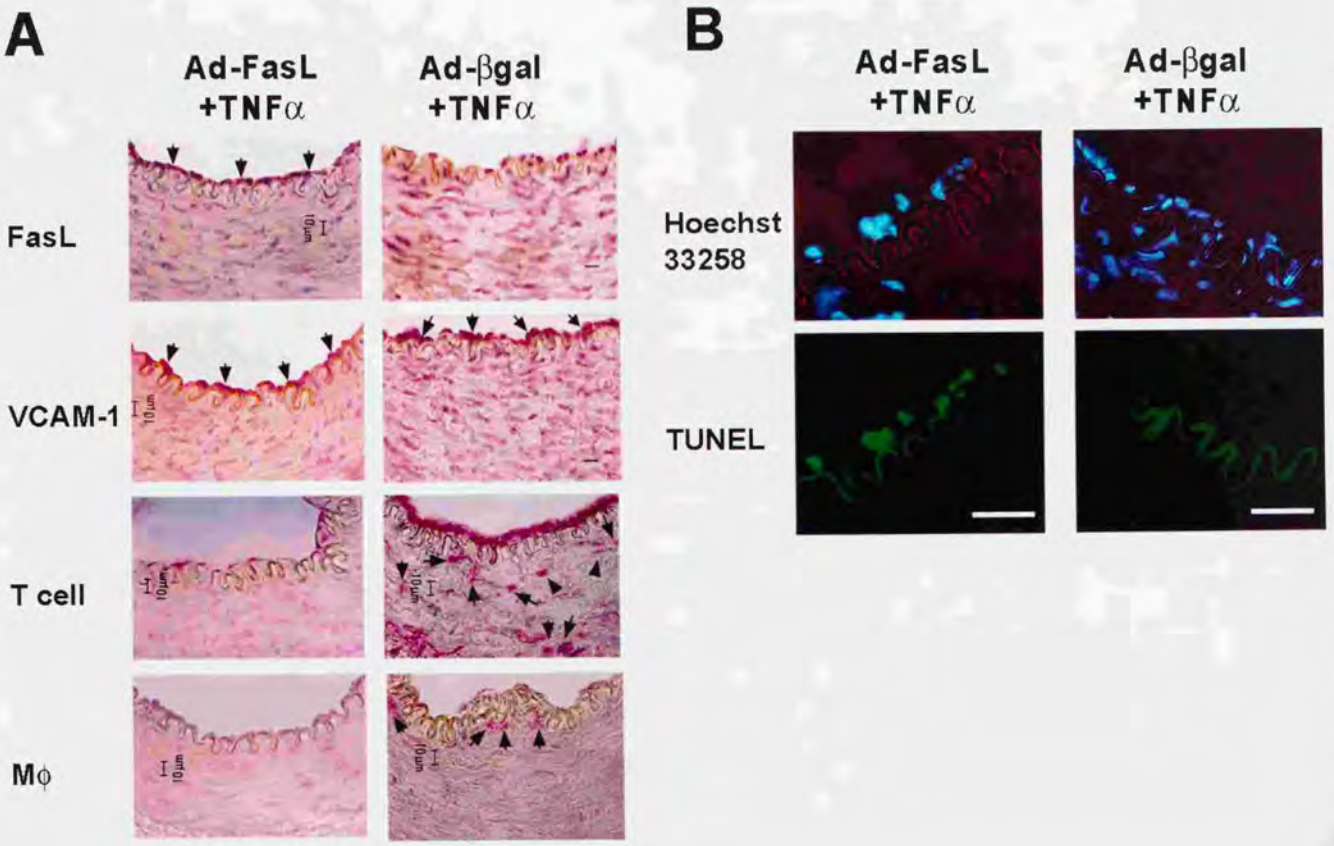
A
Ad-FasL +TNFα
Ad-βgal +TNFα
FasL
VCAM-1
T cell
Mϕ
B
Ad-FasL +TNFα
Ad-βgal +TNFα
Hoechst 33258
TUNEL

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Figure 5

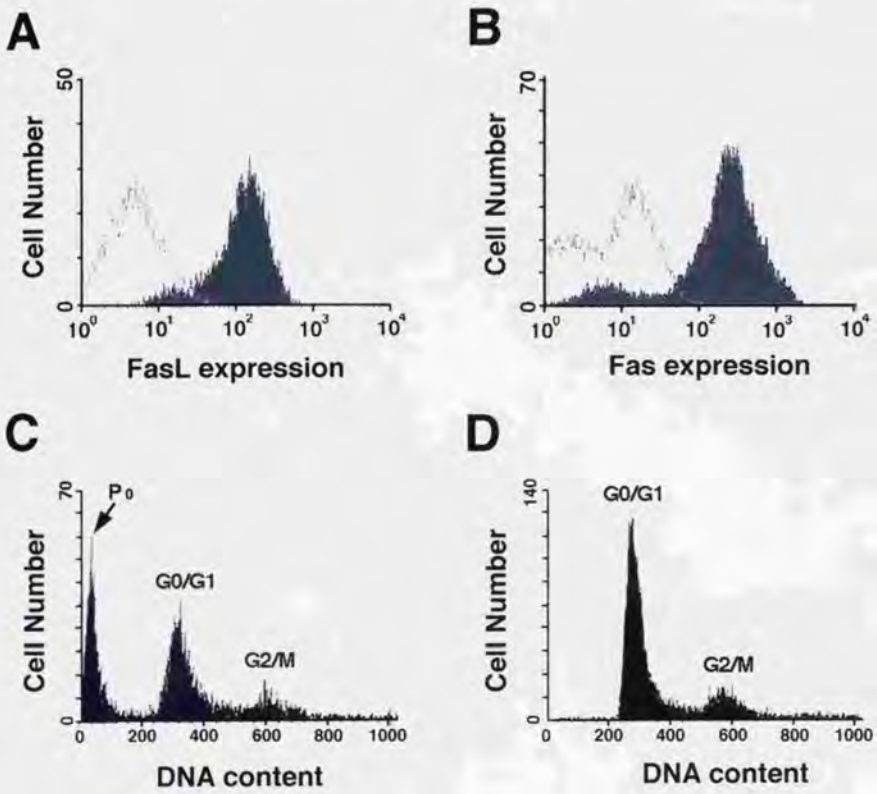
A
Cell Number
50
0
10^0 10^1 10^2 10^3 10^4
FasL expression
B
Cell Number
70
0
10^0 10^1 10^2 10^3 10^4
Fas expression
C
Cell Number
70
0
P0
G0/G1
G2/M
0 200 400 600 800 1000
DNA content
D
Cell Number
140
0
G0/G1
G2/M
0 200 400 600 800 1000
DNA content
E
DNA fragmentation (%)
30
25
20
15
10
5
0
-5
uninfected VSMCs
uninfected Jurkat cells
PBS
Ad-βgal
Ad-FasL

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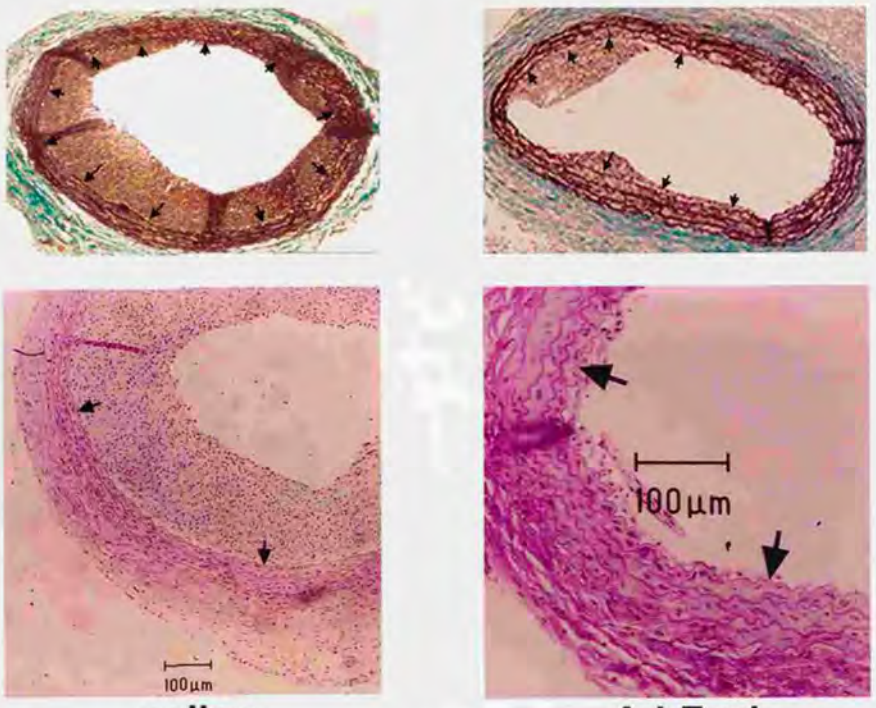
Figure 6

A


100μm
100μm
saline
Ad-FasL

B

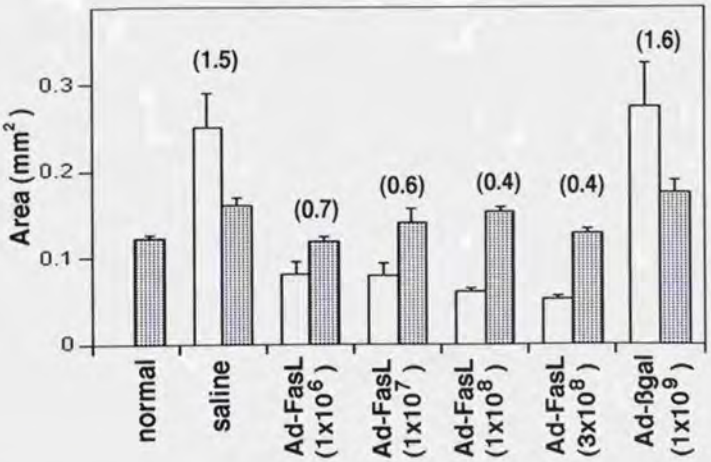

Area (mm²)
0
0.1
0.2
0.3
(1.5)
(0.7)
(0.6)
(0.4)
(0.4)
(1.6)
normal
saline
Ad-FasL (1x10⁶)
Ad-FasL (1x10⁷)
Ad-FasL (1x10⁸)
Ad-FasL (3x10⁸)
Ad-βgal (1x10⁹)

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Figure 7

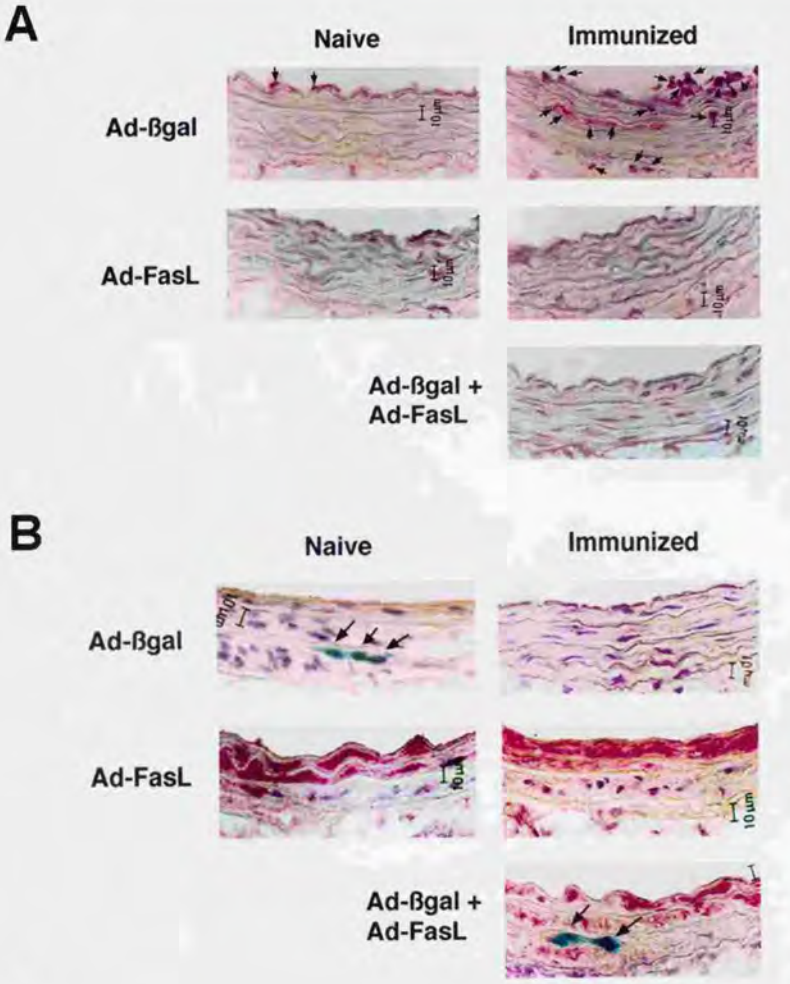
A
Naive
Immunized
Ad-ßgal
Ad-FasL
Ad-ßgal +
Ad-FasL
B
Naive
Immunized
Ad-ßgal
Ad-FasL
Ad-ßgal +
Ad-FasL
C
ßgal expression (%)
3.5
3
2.5
2
1.5
1
0.5
0
Naive (Ad-ßgal)
Immunized (Ad-ßgal)
Immunized (Ad-ßgal + Ad-FasL)
FasL expression (%)
25
20
15
10
5
0
Naive (Ad-FasL)
Immunized (Ad-FasL)
Immunized (Ad-ßgal + Ad-FasL)

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Figure 8

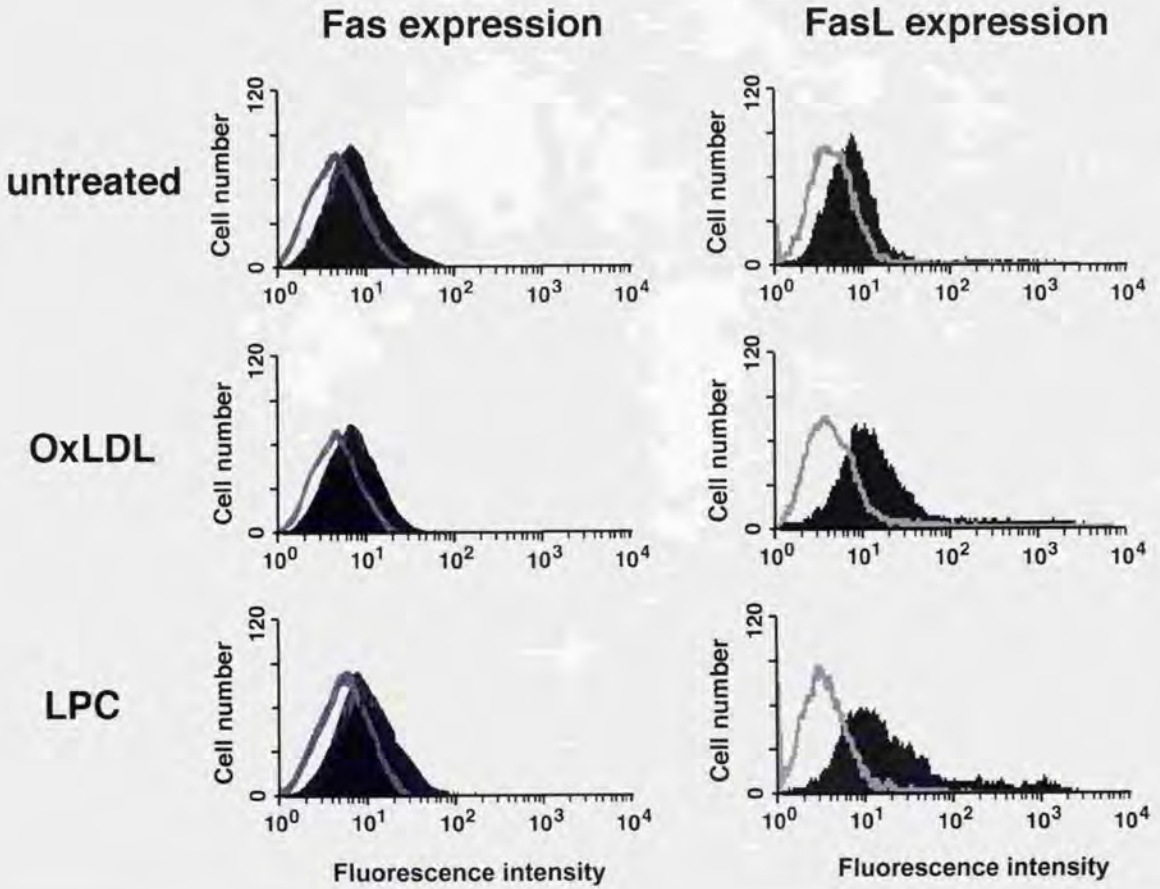
Fas expression
FasL expression
untreated
OxLDL
LPC
Cell number
120
0
10^0
10^1
10^2
10^3
10^4
Fluorescence intensity

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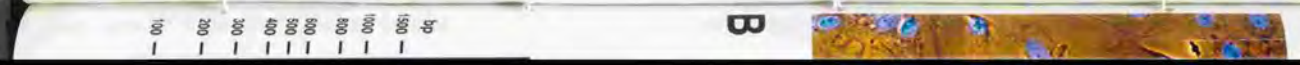
Figure 9



A
untreated
OxLDL
OxLDL + αFasL
B
marker
untreated
OxLDL
OxLDL + αFasL
native LDL
LPS
bp
1500
1000
900
800
700
600
500
400
300
200
100
C
Cell Death (%)
70
60
50
40
30
20
10
0
-10
-20
untreated
OxLDL
OxLDL + αFasL
MM-LDL
MM-LDL + αFasL
LPC-C18:0
LPC-C18:0 + αFasL
AcLDL
LPC-C12:0
PC-C18:2/C16:0
cholesterol
7keto-cholesterol
25OH-cholesterol

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Figure 10

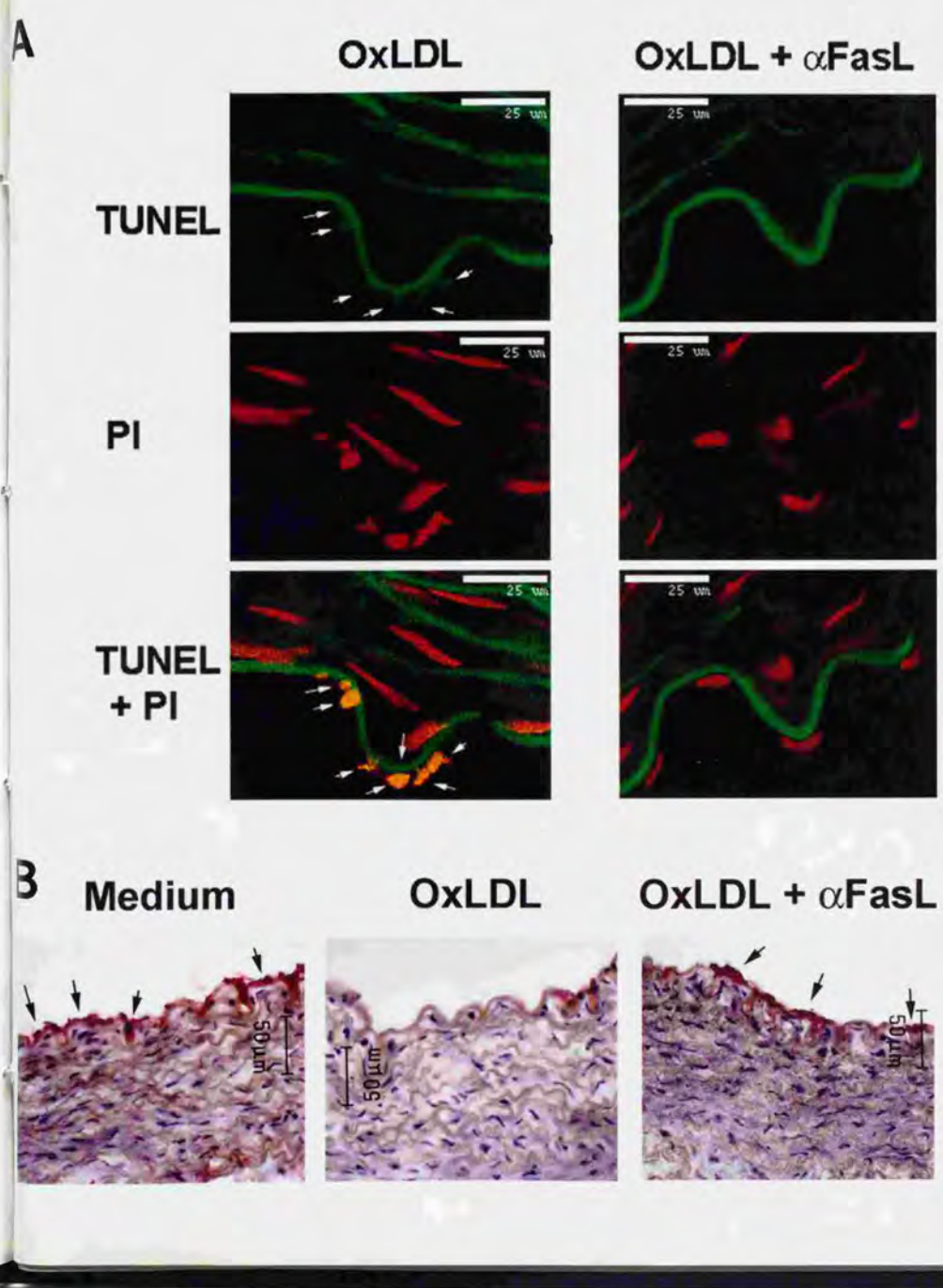
A
OxLDL
OxLDL + αFasL
TUNEL
PI
TUNEL
+ PI
25 um
B
Medium
OxLDL
OxLDL + αFasL
50µm

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Figure 11

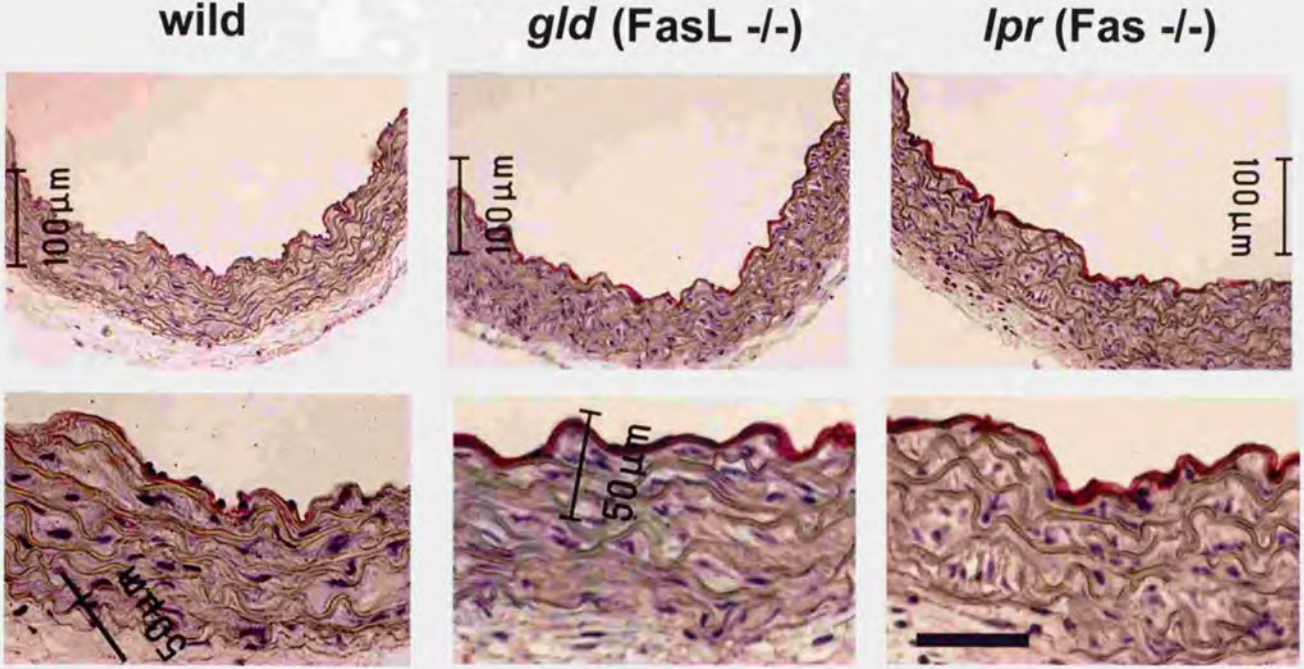
wild
gld (FasL -/-)
lpr (Fas -/-)
100µm
100µm
100µm
50µm
50µm

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Figure 12

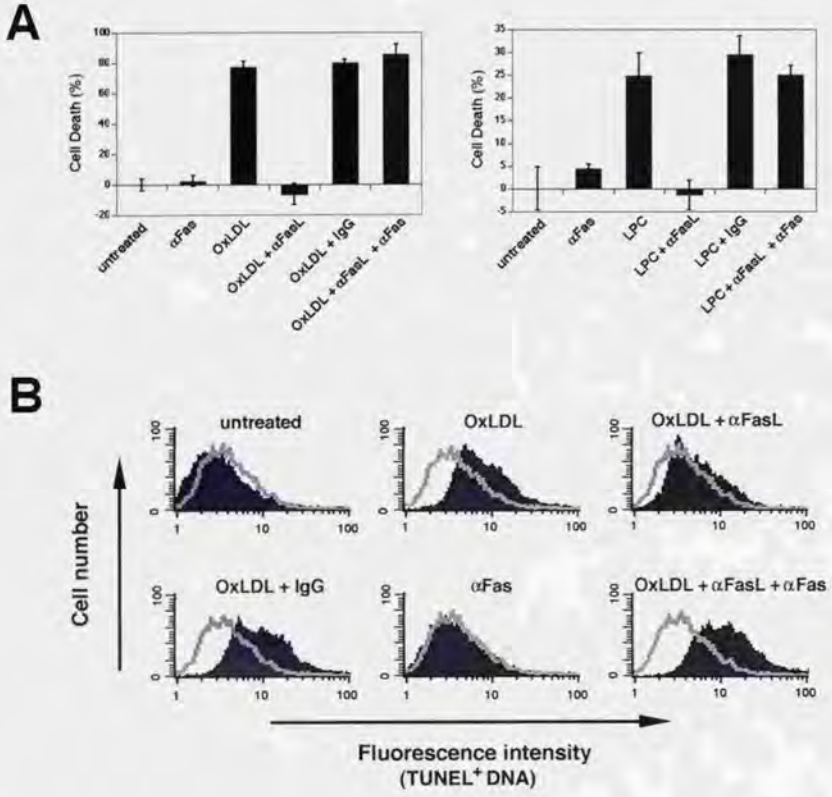
A
Cell Death (%)
untreated
αFas
OxLDL
OxLDL + αFasL
OxLDL + IgG
OxLDL + αFasL + αFas
Cell Death (%)
untreated
αFas
LPC
LPC + αFasL
LPC + IgG
LPC + αFasL + αFas
B
untreated
OxLDL
OxLDL + αFasL
OxLDL + IgG
αFas
OxLDL + αFasL + αFas
Cell number
Fluorescence intensity
(TUNEL+ DNA)
C
Untreated
OxLDL
OxLDL + α Fas L
Fas L
Fas
Survival
Death
Survival
OxLDL + IgG
α Fas
OxLDL + α Fas L
+ α Fas
Death
Survival
Death

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Figure 13

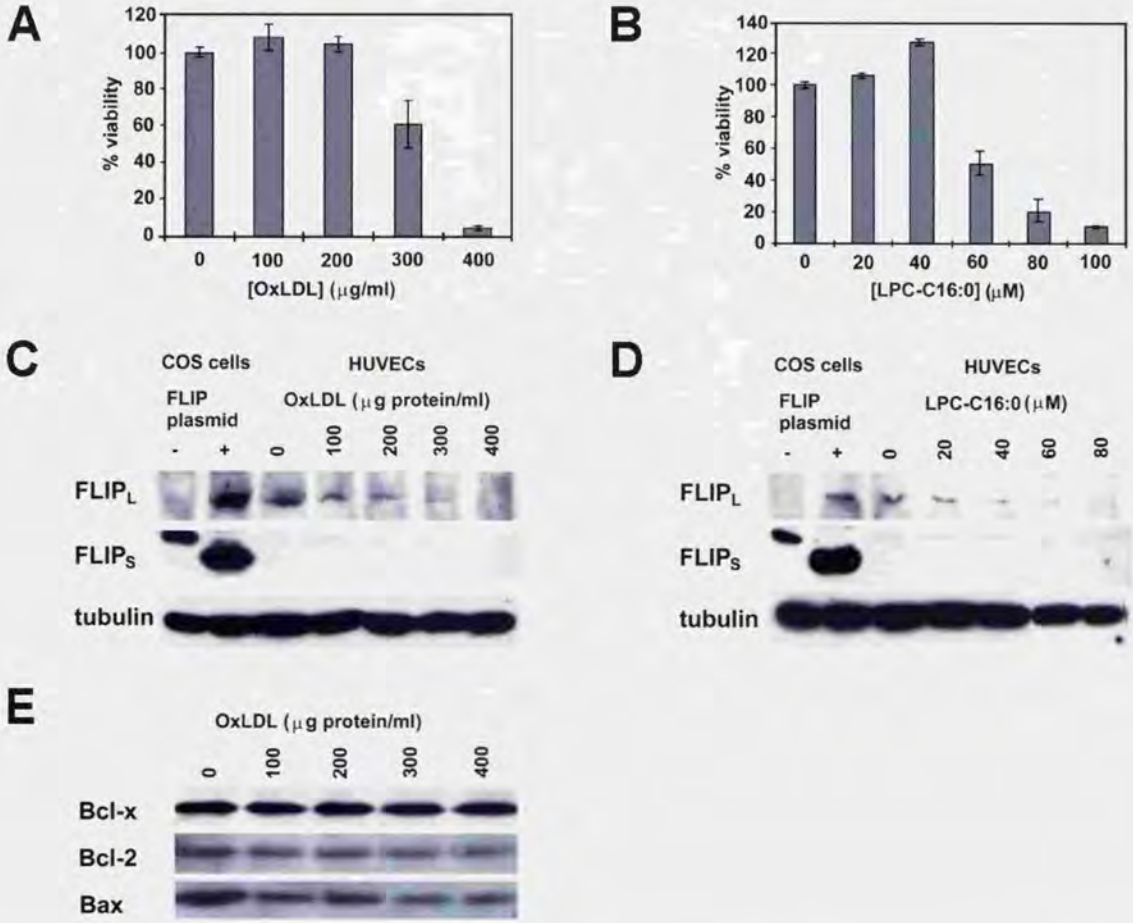
A
% viability
120
100
80
60
40
20
0
0
100
200
300
400
[OxLDL] (μg/ml)
B
% viability
140
120
100
80
60
40
20
0
0
20
40
60
80
100
[LPC-C16:0] (μM)
C
COS cells
HUVECs
FLIP plasmid
OxLDL (μg protein/ml)
-
+
0
100
200
300
400
FLIPL
FLIPS
tubulin
D
COS cells
HUVECs
FLIP plasmid
LPC-C16:0 (μM)
-
+
0
20
40
60
80
FLIPL
FLIPS
tubulin
E
OxLDL (μg protein/ml)
0
100
200
300
400
Bcl-x
Bcl-2
Bax

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Figure 14

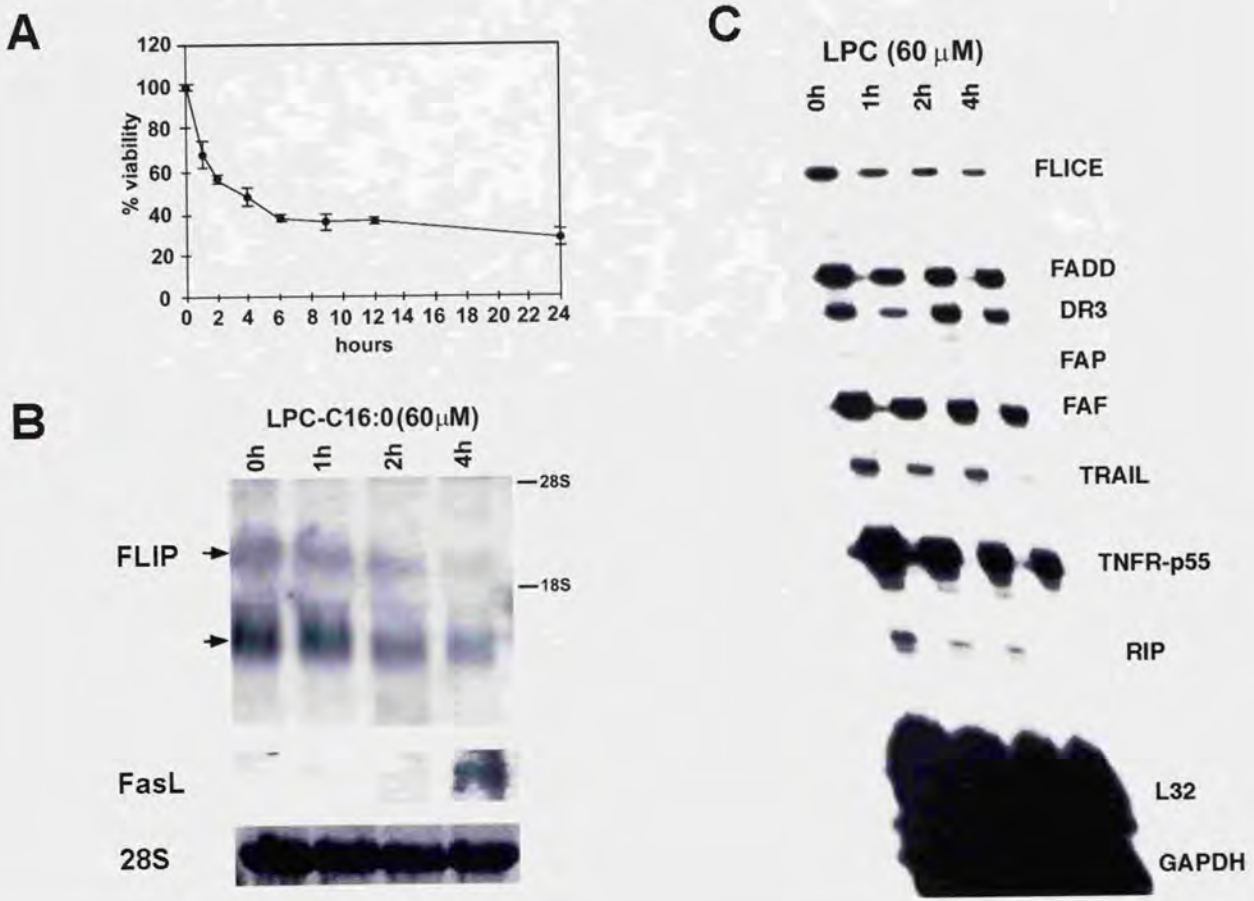
A
120
100
80
60
40
20
0
% viability
0 2 4 6 8 10 12 14 16 18 20 22 24
hours
B
LPC-C16:0(60μM)
0h
1h
2h
4h
28S
18S
FLIP
FasL
28S
C
LPC (60 μM)
0h
1h
2h
4h
FLICE
FADD
DR3
FAP
FAF
TRAIL
TNFR-p55
RIP
L32
GAPDH

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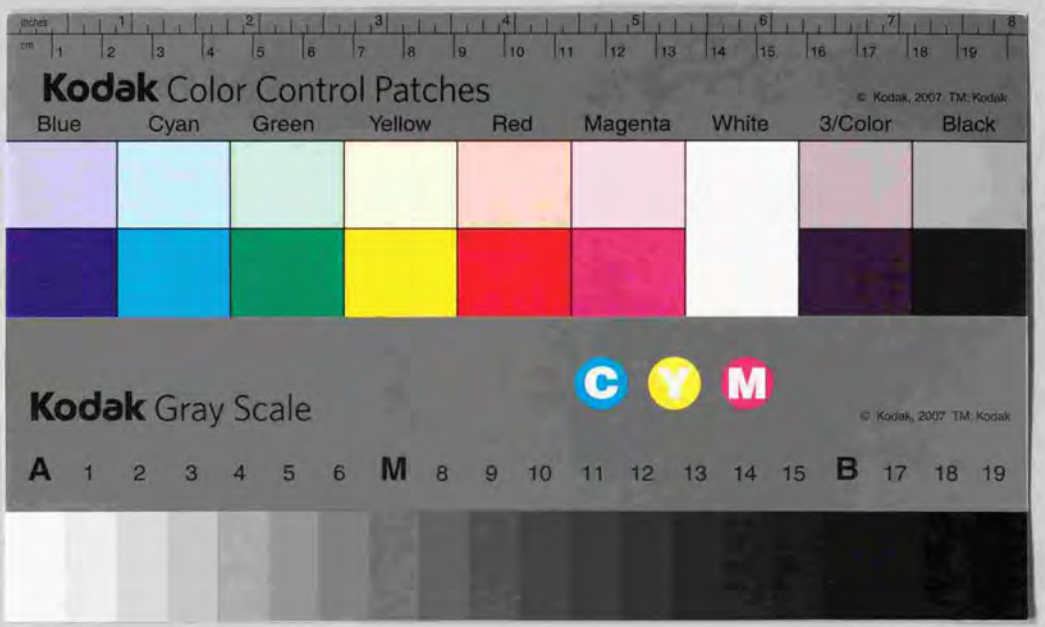
Figure 15


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
C
Y
M
Kodak Gray Scale
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19