Expression of Fas Ligand in Arteries of Hypercholesterolemic Rabbits Accelerates Atherosclerotic Lesion Formation

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Abstract—Fas ligand (FasL) is expressed by cells of the arterial wall and is present in human atherosclerotic lesions. However, the role of FasL in modifying the initiation and progression of atherosclerosis is unclear. To investigate the role of arterial FasL expression in the development of atherosclerosis, we first established a model of primary lesion formation in rabbit carotid arteries. In this model, infusion of adenoviral vectors into surgically isolated, nondenuded arteries of hypercholesterolemic rabbits leads to the formation of humanlike early atherosclerotic lesions. Expression of FasL in arterial endothelium in this model decreased T-cell infiltration and expression of vascular cell adhesion molecule-1 but did not affect expression of intercellular adhesion molecule-1. Intimal lesions grew more rapidly in FasL-transduced arteries than in arteries transduced with a control adenovirus that did not express a transgene. Total intimal macrophage accumulation was increased in FasL-transduced arteries; however, the proportion of lesion area occupied by macrophages was not elevated. The accelerated lesion growth was primarily due to the accumulation of intimal smooth muscle cells with a synthetic proliferative phenotype. There was no significant apoptosis in FasL-transduced or control arteries and no granulocytic infiltrates. Thus, the net result of elevated FasL expression is to accelerate atherosclerotic lesion growth by increasing lesion cellularity. Vascular expression of FasL may contribute to the progression of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2000;20:298-308.)

Key Words: adenovirus ■ carotid arteries ■ gene transfer ■ atherosclerosis ■ inflammation

The abnormal accumulation of cells in the arterial intima is a central aspect of the pathogenesis of atherosclerosis.1,2 Agents that eliminate intimal cells or prevent their accumulation may preserve or widen the arterial lumen, thereby maintaining or increasing blood flow and relieving ischemia. Elimination of intimal cells might be accomplished by interventions that promote cell death via apoptosis.3 Identification of the molecular mediators of apoptosis, along with the discovery that certain of these mediators are expressed in the arterial wall,4-8 has prompted speculation concerning the biological roles of proapoptotic molecules in maintaining the arterial phenotype and the possibility that enhanced expression of these molecules might limit or reverse intimal growth.9-11

Fas ligand (FasL) and its receptor, Fas (APO-1/CD95), are effective mediators of apoptosis.12 Both FasL and Fas are expressed in arterial tissue, including human atherosclerotic plaque,13-15 but their biological roles are unclear. It has been suggested that FasL expression in arterial endothelium limits lesion growth by promoting intimal smooth muscle cell (SMC) apoptosis and decreasing T-cell infiltration.11,16 However, a recent report of abundant FasL expression in clinically significant human atherosclerotic plaques15 suggests that FasL may not be atheroprotective and might instead play a proinflammatory or proliferative role. Indeed, although classically proapoptotic molecules, such as FasL, Fas, and Fas-associated death domain/Mort-1, are best known for their ability to promote cell death and downregulate the immune response, they can also mediate cell proliferation, activation, and inflammation.17-24

In the present study, we tested the hypothesis that increased expression of FasL by arterial endothelium would limit intimal growth. We considered that the most appropriate means for this test would be to overexpress FasL in the endothelium of a developing atherosclerotic lesion. Such a lesion should comprise SMCs, T cells, macrophages, and endothelial cells (ECs), the primary cell types in the atherosclerotic intima. Moreover, because systemic elevation of FasL expression would likely cause immune deficiency and hepatic dysfunction,12,25,26 thereby complicating the interpretation of experimental results, increases in FasL expression should be confined to a small segment of the arterial wall.

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Vascular Biology
Because no such animal model (ie, focal transgene expression in an atherosclerotic lesion with morphologically intact endothelium) has yet been described, we first developed a model of primary atherosclerotic lesion formation in uninjured carotid arteries of cholesterol-fed rabbits. We then used an adenoviral vector to express FasL in these lesions and measured lesion growth and cellular composition. Increased FasL expression decreased intimal T-cell infiltration and expression of vascular cell adhesion molecule-1 (VCAM-1), a marker of vascular cell activation. However, FasL expression did not affect the expression of intercellular adhesion molecule-1 (ICAM-1) and increased SMC proliferation and intimal mass. Our data suggest that the expression of FasL in the arterial wall may promote rather than retard atherosclerotic lesion growth.

Methods

Adenoviral Vectors

Three adenoviral vectors were used: AdFasL, AdNull, and AdRS-VnLacZ. AdFasL is an E1- and E3-deleted vector that contains a murine FasL cDNA driven by the cytomegalovirus (CMV) immediate-early enhancer/promoter. AdNull is identical to AdFasL but lacks the CMV-FasL expression cassette. AdRSVnLacZ is an E1- and E3-deleted vector that expresses the Escherichia coli β-galactosidase gene. The vectors were propagated, purified, titered, and stored as described. The absence of replication-competent virus was confirmed in all virus preparations by a polymerase chain reaction–based assay that is capable of detecting competent virus was confirmed in all virus preparations by a polymerase chain reaction–based assay that is capable of detecting.

For animal experiments, viral stocks were diluted in DMEM containing 1 mg/mL rabbit serum albumin (Sigma Chemical Co) to achieve a final concentration of 7.5 × 10¹¹ particles per milliliter. For AdNull, this corresponded to a concentration of 5.8 × 10¹⁰ plaque-forming units (pfu)/mL, with <0.1 mL infused in a 3- to 4-cm length of isolated carotid artery. To achieve equivalent viral doses in the AdNull and AdFasL groups, dilutions were based on particle titers (ie, particles per milliliter) rather than plaque titers. Use of particle titers was necessitated in part by difficulties in obtaining reliable plaque titers for the FasL virus, a problem that has also been reported by others. Use of particle titers was not a disadvantage, because particle titers are more objectively and reproducibly measured than plaque titers. In addition, the particle titer is a direct physical measurement of the total dose of virions in a preparation and therefore should be a more appropriate measure of the potential biological effects of adenoviral capsid proteins. Notably, the dose of adenovirus infused in these experiments (<2 × 10¹⁰ pfu/cm of artery length, based on pfu values of AdNull [pfu/cm values of AdFasL are even less]) is essentially identical to the dose of AdFasL, reported by Luo et al to inhibit intimal hyperplasia in the rat. If the increased diameter of rabbit versus rat arteries is taken into account, then the vector dose per cell in our experiments is likely lower than that reported by Luo et al. As additional controls, some arteries were infused with a “vehicle” solution, as described.

Cell Lines

Rabbit aortic ECs were a generous gift of Dr Mohamad Navab (University of California, Los Angeles). ECs were cultured on gelatin-coated flasks in EC growth medium (low-glucose DMEM [Life Technologies] with 15% FCS [HyClone]). Rabbit vascular SMCs were isolated from the iliac artery of a New Zealand White rabbit by explant culture and were grown in SMC growth medium (medium 199 [Life Technologies] with 20% FCS).

Animals

Experiments were performed with specific-pathogen–free adult male New Zealand White rabbits (2.5 to 3.5 kg, Charles River, Montreal, Quebec, Canada). Unless otherwise indicated, rabbits were fed High Fiber Laboratory Rabbit Diet No. 5326 (Dean’s Feed). Experimental protocols were approved by the Committee on Animal Research of the University of California San Francisco.

In some rabbits, hypercholesterolemia was induced by feeding 100 g/d of rabbit chow containing 0.25% cholesterol and 3% soybean oil (Ziegler Bros). Plasma cholesterol and triglyceride levels were determined with a clinical chemistry analyzer system (Abbott Spectrum, Abbott Laboratories). After 2 weeks, rabbits with plasma cholesterol levels ≤400 mg/dL were continued on the same diet. Rabbits with plasma cholesterol levels of 400 to 500 mg/dL were changed to a diet containing 0.125% cholesterol and 1.5% soybean oil. Rabbits with cholesterol levels >500 mg/dL were fed normal chow for 4 days and were then placed on a diet containing 0.125% cholesterol and 1.5% soybean oil. For 4 weeks, ~75% of all rabbits fed according to this protocol had plasma cholesterol of 400 to 700 mg/dL. Rabbits with cholesterol levels outside this range were withdrawn from the study. The preoperative diets were withheld for the day of surgery and resumed after surgery.

In Vivo Gene Transfer to Carotid Artery Endothelium

General anesthesia, isolation of common carotid arteries, infusion of adenoviral vectors or vehicle control solution, and wound closure were performed essentially as described. This protocol of adenoviral vector infusion into the lumen of isolated nondenuded large arteries results in endothelium–specific gene transfer. The surgical protocol used in the present study is essentially the same as we have used previously to examine the effect of adenoviral infusion on normal rabbit femoral arteries, with 2 exceptions: (1) in the previous study, the infusate was not aspirated after the incubation period but was released to the systemic circulation and (2) the amount of adenovirus was ~10% of that used previously. These adjustments were made to minimize viral toxicities while maintaining the dose at a level adequate to achieve significant gene transfer to the arterial wall.

Harvesting of Carotid Arteries

Arteries were harvested 1, 2, 7, 14, or 28 days after gene transfer. Vessels were either perfusion-fixed in situ and embedded in paraffin or snap-frozen in OCT medium. Carotid arteries were perfusion-fixed with 10% neutral-buffered formalin infused via the abdominal aorta at a distending pressure of 90 mm Hg. The common carotid arteries were then removed, placed in formalin for 2 hours, and stored in 70% ethanol. Each vessel was then divided into 10 equal-sized rings of ~3 mm in length. All 10 rings from each vessel were embedded side by side sequentially, in a single paraffin block. Sections (5 µm thick) were cut from each block for histochemical and immunohistochemical analysis.

Vessel segments for frozen section analysis were obtained essentially as described above, except that vessels were not perfused. Excised arteries were divided into four 8-mm rings. The rings were rinsed in 0.9% saline, placed side by side in OCT, snap-frozen by immersion in isopentane and liquid nitrogen, cut into 6-µm-thick sections, and stored at −20°C until use.

Histochemical and Immunohistochemical Staining

Serial paraffin-embedded sections were stained with hematoxylin and eosin, Movat pentachrome, and Leder stains, as well as antibodies specific for macrophages (RAM-11, 1:40 dilution, DAKO), smooth muscle actin (HHF-35, 1:50 dilution, Enzo Diagnostics), ECs (anti-CD31, JC70A, 1:30 dilution, DAKO), or proliferating cell nuclear antigen (PCNA, 1:40 dilution, Santa Cruz Biotechnology). Serial frozen sections were stained with antibodies to T cells (anti-CD5, KEN5, 1:25 dilution; Spring Valley Laboratories) and with VCAM-1 and ICAM-1 (Rb1/9 and Rb2/3 at 1:200 and 1:50 dilutions, respectively, from Dr Myron Cybulsky, University of Toronto, Toronto, Canada), and bound antibody was detected essentially as described.

The specificity of primary antibody binding was confirmed both by omitting the primary antibody and by substituting isotype-matched antibodies.
TUNEL and Transmission Electron Microscopy

Apopotic cells were detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) technique, essentially as described.27 Sections of involuting rat mammary gland were stained in parallel, as positive controls. Tissue preparation, processing, and transmission electron microscopy were performed as described.36

Vessel Morphometry

Movat pentachrome–stained sections from perfusion-fixed arteries were analyzed with a computerized morphometric imaging system (Image One, Universal Imaging Corp) to determine intimal area, medial area, and the intimal-to-medial (I:M) area ratio. Intimal and medial areas and the I:M area ratio were calculated from data generated by planimetry of the luminal surface, internal elastic lamina, and external elastic lamina. The percentage of intimal area staining positive for RAM-11 was determined by using the image analysis program to quantify the area within the intima of RAM-11–stained sections that contained the brown peroxidase reaction product. The percentage area of RAM-11 staining was then calculated by dividing this area by the total intimal area of the same slide. Results obtained with this technique were reproducible (interobserver correlation $r^2=0.98$). Morphometric results for each artery represent the mean calculated from measurements made on 8 evenly spaced cross sections per vessel, according to the Cavalieri method (Bolender et al36). The most proximal and distal of the 10 rings per vessel were excluded from analysis to avoid potential artifacts caused by the vascular clamps and the infusion cannula.

Quantification of Inflammation, Neointima Formation, and Cell Proliferation in Arterial Specimens

The degree of vascular inflammation was determined, in part, by evaluation of frozen sections immunostained for T cells, VCAM-1, and ICAM-1. The intensity of antibody staining was graded by 2 independent observers, each blinded to the identity of the sections. Staining intensity was scored semiquantitatively31: 0 indicated no staining; 1, rare positive cells or staining barely visible at low power ($>100$); 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; and 4, intense diffuse staining. The magnitude of intimal lesion formation was scored semiquantitatively by the same observers: 0 indicated no lesion; 1, partial circumferencence and $<3$ cells thick; 2, partial circumference and $>3$ cells thick; and 3, circumferential lesion. Four sections per vessel were scored by each observer, and the median of these 8 scores was used to generate a score for the entire vessel. The staining intensity and the lesion size scores given by the 2 observers were highly correlated ($r^2=0.93$ and 0.87). To determine the percentage of PCNA-positive intimal cells in an artery, total intimal cells and total PCNA-positive cells were counted in 4 evenly spaced high-power ($>400$) fields in each of 3 evenly spaced sections (12 fields per artery). The total number of intimal cells counted per artery ranged from 475 to 3014 per artery, for a total of 15,471 cells counted in 11 arteries.

Neutralizing Anti-Adenoviral Antibody Assay, Complete Blood Counts, and Liver Function Tests

Neutralizing serum antibodies to adenovirus type 5 were assayed before vector infusion and 14 days later, as described.38 Because systemic delivery of AdFasL (or release of soluble FasL from the arterial wall) might have systemic consequences, including lethal hepatic apoptosis,26 complete blood counts and liver function tests were performed 7, 14, and 28 days after vector infusion (IDEXX Laboratories).

FasL Expression and Cytotoxicity in Vascular Cells

Expression of FasL in AdFasL–transduced 293 cells and carotid arteries was evaluated by Western blotting, as described.39 Briefly, transduced arteries were harvested 24 hours after gene transfer and rinsed, and the luminal endothelium was scraped off with a scalpel.

Separate lysates were made of the endothelium and of the denuded artery and were analyzed independently. Complete and equal transfer of electrophoresed proteins was confirmed by staining membranes with the SYPRO Rose Protein Blot stain (Molecular Probes). To evaluate FasL-mediated toxicity in vitro, SMCs were infected with AdFasL or AdNull at a concentration of $5\times10^6$ particles per milliliter (or mock-infected with PBS) for 1 hour and incubated in growth medium for an additional 12 hours. Cells were examined by phase-contrast microscopy and were then harvested. Total genomic DNA was extracted by using the Easy SNAP DNA kit (Invitrogen) and subjected to electrophoresis on a 1.5% agarose gel, followed by ethidium bromide staining.

Cell viability and apoptosis were also evaluated by transducing SMCs and ECs with AdNull or AdFasL at $1\times10^6$ to $9\times10^10$ particles per milliliter for 1 hour. Eighteen hours later, the cells were examined by phase-contrast microscopy and stained with 5 μmol/L quinolinium,4-[3-methyl-2(3H)-benzoxazolylidene] methyl]-[3-(trimethylammonio)propyl]-diiodide (YoPro-1, Molecular Probes) for 45 minutes at 37°C. This dye is taken up only by nonviable cells and stains nuclear DNA. Apoptotic cells are identified by the presence of nuclear condensation and chromatin fragmentation.

Statistics

Results are reported as mean±SEM or median and range for data not normally distributed. Normally distributed data were compared with the unpaired t test. Data that were not distributed normally were compared with the Mann-Whitney rank sum test. Comparisons between multiple groups with normally distributed data were made by using 1-way ANOVA with controls for multiple pairwise comparisons by the Student-Newman-Keuls method. Semiquantitative scores of $>2$ groups were compared with the nonparametric Kruskal-Wallis ANOVA; the Dunn method was used to control for multiple pairwise comparisons.39 The strength of correlation ($r^2$) between semiquantitative scores of staining intensity given by independent observers was assessed by the Spearman rank order correlation. Differences were considered to be significant at $P<0.05$.

Results

Development of a Model of Focal Transgene Expression in an Atherosclerotic Lesion

We hypothesized that a combination of cholesterol feeding and local adenoviral vector infusion would foster focal atherosclerotic lesion development. The effect of FasL overexpression on the developing lesion could later be determined by inclusion, in the adenoviral vector, of a FasL expression cassette.

Four Weeks of an Atherogenic Diet Causes Hypercholesterolemia but Does Not Cause Carotid Atherosclerosis

Rabbits fed a standard diet had cholesterol levels of 27±2.5 mg/dL (n=17) and 25 mg/dL (n=25) at 1, 2, and 3 months, respectively. Rabbits fed the atherogenic diet for 4 weeks had cholesterol levels of 563±25 mg/dL (n=39). As noted by others (Dr D. Heistad, personal communication, 1998), una- operated rabbits fed this (or other atherogenic) diet(s) for 4 weeks did not develop carotid artery intimal lesions (n=4 and data not shown).

Hypercholesterolemia and Adenoviral Vector Infusion Make Additive Contributions to the Development of Atherosclerosis

To determine whether adenoviral infusion in hypercholesterolemic rabbits would produce carotid atherosclerosis, rabbits were fed either standard or atherogenic diets for 4 weeks and were then treated with bilateral carotid infusion of either vehicle or AdNull. Infusion of vehicle or AdNull in the presence or absence of hypercholesterolemia produced 4 strikingly different arterial morphologies at 4 weeks after...
Figure 1. Hypercholesterolemia and adenoviral infusion have distinct additive effects on the arterial wall. Sections are from carotid arteries harvested from rabbits fed the indicated diets (standard or high cholesterol) for 4 weeks. Arteries were then infused with vehicle or AdNull (no transgene) and harvested 4 weeks later. A to D, Movat pentachrome stain. E to H, RAM-11 immunostaining (detects macrophages) with hematoxylin counterstaining. Arrows indicate internal elastic lamina. Original magnifications ×100.
infusion. First, arteries infused with vehicle in the absence of hypercholesterolemia appeared essentially normal (Figure 1A and 1E). Second, arteries of hypercholesterolemic rabbits infused with vehicle contained foamy intimal lesions (Figure 1B) composed of macrophages (Figure 1F) and SMCs (data not shown). Vessels in this group harvested 2 weeks after vehicle infusion contained macrophages adherent to and below morphologically intact endothelium and a smaller SMC-containing intima (data not shown). Third, arteries of normocholesterolemic rabbits infused with AdNull had non-foamy lesions that were highly cellular but did not contain macrophages (Figure 1C and 1G). Fourth, arteries from hypercholesterolemic rabbits infused with AdNull contained larger lesions that were rich in foam cells and macrophages (Figure 1D and 1H). The luminal endothelium was morphologically intact in all arteries.

Quantitative morphometric analyses supported the impressions derived from the histological studies. Arteries from normocholesterolemic rabbits infused with vehicle (n = 7) had an I:M area ratio of 0.07 ± 0.01 (Figure 2A). Because the intima in these arteries was composed almost exclusively of a single layer of endothelium, this figure approximates the I:M ratio for a normal nondiseased rabbit carotid artery, as determined by planimetry. The I:M ratio for arteries from hypercholesterolemic rabbits infused with vehicle (n = 6) was 0.08 ± 0.01 (P < 0.5 versus vehicle, normal diet). The failure of planimetry to separate these 2 groups is likely due to the insensitivity of this technique to detect small lesions, in view of the fact that the 2 groups were easily differentiated both by histological criteria (see above) and by semiquantitative scoring of lesion size (data not shown). The I:M ratio of arteries from normocholesterolemic rabbits infused with AdNull was 0.15 ± 0.02 (n = 8, P < 0.05 versus vehicle only). Arteries from hypercholesterolemic rabbits infused with AdNull had a mean I:M ratio of 0.28 ± 0.02 (n = 7, P < 0.05 versus both AdNull alone and vehicle alone).

Macrophages were present only in arteries from hypercholesterolemic rabbits (Figure 1F and 1H) and represented a significantly larger percentage of lesion area in AdNull-infused than in vehicle-infused arteries (Figure 2B; 29 ± 5 versus 2.4 ± 1.5%, P < 0.05). Thus, adenoviral infusion accelerates both lesion growth and macrophage accumulation. These differences in lesion size and cellularity were not due to variability in plasma cholesterol levels, which were 598 ± 63 mg/dL (n = 6) at the time of carotid infusion and 454 ± 41 mg/dL (n = 3) at 4 weeks in the vehicle-infused rabbits versus 561 ± 46 mg/dL (n = 7) and 582 ± 155 mg/dL (n = 4) in the AdNull-infused rabbits (P > 0.5 at both time points).

**T-Cell Infiltration and VCAM-1 Expression Are Caused by Adenoviral Infusion, Not Hypercholesterolemia**

Significant T-cell infiltrates and expression of VCAM-1 and ICAM-1 were found in all AdNull-infused arteries (Figures 3 and 4). In contrast, vehicle-infused arteries (from either normocholesterolemic or hypercholesterolemic rabbits) contained essentially no T cells and no VCAM-1 expression (Figure 4A and 4B). Low-level ICAM-1 expression was present even in the vehicle-infused arteries and may be a response to the surgical procedure alone.

In summary, surgical manipulation and vehicle infusion in carotid arteries of hypercholesterolemic rabbits causes early atherosclerotic lesions that are composed largely of intimal macrophages and SMCs below morphologically intact endothelium 4 weeks after infusion. Infusion of AdNull instead of vehicle causes T-cell infiltration and VCAM-1 expression and increases macrophage and SMC accumulation as well as ICAM-1 expression. Thus, the arterial response to adenoviral infusion in hypercholesterolemic rabbits mimics the histopathology of early human atherosclerosis, in which T cells, macrophages, SMCs, and elevated adhesion molecule expression are all prominent features.1,40 The addition of adenovirus renders the lesions far more representative of human atherosclerosis than the lesions produced by hypercholesterolemia and buffer infusion alone. Carotid arteries from hypercholesterolemic rabbits infused with adenovirus are therefore a useful background on which to test hypotheses regarding the roles of modifier genes (such as FasL) in the genesis and progression of atherosclerosis.

**Expression of FasL in a Developing Atherosclerotic Lesion**

**Murine FasL Causes Apoptosis of Rabbit Vascular SMCs In Vitro and Can Be Expressed in Rabbit Endothelium In Vivo**

Cultured rabbit vascular SMCs were exposed to either AdNull or AdFasL (5 × 10⁹ particles per milliliter). Cells exposed to AdNull remained intact (Figure IA, which appears online at http://atvb.ahajournals.org/cgi/content/full/20/2/298/DC1) and could not be distinguished from mock-transduced cells (not shown). In contrast, SMCs exposed to AdFasL appeared pyknotic (Figure IB, which appears online at http://atvb.ahajournals.org/cgi/content/full/20/2/298/DC1). Agarose electrophoresis revealed fragmentation characteristic of apoptosis only in DNA harvested from the AdFasL-transduced cells (Figure IC,
which appears online at http://atvb.ahajournals.org/cgi/content/full/20/2/298/DC1). Apoptotic death of AdFasL-transduced SMCs was further confirmed by detection of chromatin fragmentation with a fluorescent DNA-binding dye (data not shown). Thus, murine FasL can trigger apoptosis in rabbit vascular cells. We next tested whether AdFasL could direct expression of FasL protein in vivo. As expected, Western blot analysis detected a protein of $\approx 40$ kDa in extracts of endothelium scraped from AdFasL-transduced arteries. This protein was either absent or present at a far lower level in extracts of endothelium from AdRSVnLacZ-transduced arteries (n=3 per group; Figure II, which appears online at http://atvb.ahajournals.org/cgi/content/full/20/2/298/DC1, and data not shown).

**FasL Expression in Developing Atherosclerotic Lesions Decreases T-Cell Infiltration and Vascular Cell Activation but Increases Lesion Size and Does Not Alter the Proportion of Lesion Macrophages**

Arteries in new cohorts of cholesterol-fed rabbits were transduced with either AdNull or AdFasL and harvested 2 days to 4 weeks later. As expected, AdNull-transduced arteries harvested at 14 days (n=6) showed robust infiltration of T cells and expression of ICAM-1 and VCAM-1 (Figure 5A to 5C). However, at 14 days, FasL-transduced arteries (n=5) had significantly fewer T cells in the intima and media and essentially no expression of VCAM-1 (Figure 5A and 5B; $P<0.05$ for CD5 and VCAM-1 scores, respectively). In contrast, ICAM-1 was expressed at similar levels in FasL-transduced and AdNull-transduced arteries (Figure 5C). The decreased inflammation in AdFasL-transduced arteries was likely not due to FasL-mediated systemic immunosuppression because AdNull and AdFasL-infused rabbits developed similarly high serum titers of anti-adenoviral antibodies ($\approx 1.512$ for all animals).

Notably, despite the paucity of T cells and the marked decrease in VCAM-1 expression, intimal lesions were present in all AdFasL-transduced arteries. Indeed, semiquantitative analysis showed that vascular lesions in frozen sections of the AdFasL arteries were larger than lesions in the AdNull arteries (Figure 5D, $P<0.05$). The more quantitative technique of planimetry, performed on perfusion-fixed arteries...
harvested at 7, 14, and 28 days (n=4 to 8 arteries for each group at each time point), confirmed that FasL gene transfer accelerated neointimal formation. At 7 days, the I:M ratio was 3-fold greater in FasL-transduced arteries than in AdNull-transduced arteries (0.22±0.03 versus 0.07±0.02). This difference persisted at 14 days (0.22±0.02 versus 0.07±0.02) but was no longer present at 28 days (0.30±0.03 versus 0.28±0.02). The increased lesion size in AdFasL arteries at 7 days was due to increased intimal cellularity: sections of AdFasL arteries had 680±52 sections in each of 5 or 6 arteries per group). The increased intimal cellularity was not due to increased plasma cholesterol, which was 552±172 mg/dL (n=4) at the time of carotid infusion and 952±125 mg/dL (n=4) at 4 weeks (P=0.1 versus AdNull at both time points).

To investigate the cellular mechanisms of FasL-induced neointimal formation, we performed several histochemical and immunohistochemical stains. The increased lesion size at 7 and 14 days was not due primarily to accelerated macrophage accumulation because the percentage of RAM-11–stained areas in all lesions was relatively low (5% to 15%) and not significantly different in arteries transduced with either AdFasL or AdNull (Figure 6A and 6B and data not shown). We also considered that injury to the vascular media (potentially resulting from FasL-induced apoptosis of medial SMCs) might stimulate neointimal formation. However, at 7 and 14 days, all AdFasL and AdNull arteries had morphologically intact SMC-containing mediae without significant TUNEL positivity (Figure 6E and 6F and data not shown). Finally, we considered that the neointima in AdFasL-transduced arteries might consist largely of neutrophils, because neutrophilic infiltrates are associated with ectopic FasL expression.20,21 However, the neointimas contained only rare neutrophils.

**FasL Expression Causes Accumulation of Proliferative Synthetic Intimal SMCs**

At 7 weeks after gene transfer, no intimal cells stained positively for smooth muscle actin (Figure 6E and 6F). However, at 14 and 28 days after gene transfer, the majority of intimal cells in both the AdNull and AdFasL-transduced arteries stained positively for smooth muscle actin expression (not shown). Therefore, the intimas of AdNull- and AdFasL-transduced arteries appear to be composed primarily of SMCs that are initially smooth muscle actin negative (ie, they are of the synthetic proliferative SMC phenotype).3,41 Examination of ultrathin sections supported this conclusion, in view of the fact that the majority of intimal cells had abundant rough endoplasmic reticulum and surrounding collagen fibrils, features associated with the synthetic SMC phenotype (Figure 6G and 6H).

To test whether FasL expression increased the proliferative rate of these intimal cells, we stained sections from 2- and 7-day arteries with an antibody to PCNA. Intimal lesions at 2 days were rare and small in all arteries, with only occasional cells staining positively for PCNA (not shown). Intimal cells in both the 7-day AdNull- and AdFasL-transduced arteries showed abundant PCNA expression (Figure 6I and 6J). However, the percentage of intimal cells expressing PCNA was significantly higher in AdFasL-transduced arteries (37±7.5% versus 15±6.3%, P<0.05). Medial SMCs in these arteries demonstrated only rare PCNA staining.

In summary, elevated FasL expression by arterial endothelium does not cause cell death or apoptosis of ECs or SMCs in vivo. In addition, FasL expression is not proinflammatory: T-cell infiltrates and VCAM-1 expression were decreased in FasL-transduced arteries, relative macrophage abundance and ICAM-1 expression were unaffected, and neutrophils were essentially absent. Rather, FasL expression increases the rate of development of intimal lesions at least in part by promoting intimal SMC proliferation. Notably, at the time points examined, the mitogenic effect of FasL expression is restricted to intimal rather than medial SMCs (Figure 6I and 6J).

**Discussion**

The advent of techniques to transfer and express genes in the arterial wall has enabled experiments that test the ability of potential “modifier” genes (such as FasL) to alter the development of atherosclerotic lesions. Selection of the optimal animal model for such experiments has been a challenge. The animal model described here permits testing of the function of modifier genes and also has several features that recommend it as an informative model of human atherosclerosis. First, the lesions develop below morphologically intact endothelium and without balloon-induced medial SMC death.
Second, early lesions are characterized by macrophages that adhere to and migrate within the intima. Third, phenotypically modulated SMCs and excess intimal lipid deposits are prominent components of the lesions. Fourth, lesion growth is cholesterol dependent. Fifth, inflammation (ie, T-cell infiltration and adhesion molecule expression) is a prominent feature, as is true “in human lesions in every stage of development.”

Additional practical features, evident in the present study, support the utility of the model: lesions are reliably localized at the site of infusion, facilitating sampling protocols; intragroup variability is low, permitting use of only 5 to 8 arteries per group to detect statistically significant 2- to 3-fold differences; and an efficient gene transfer vector (adenovirus) is part of the model (modifier genes can be inserted as expression cassettes, and the consequences of local overexpression of their protein products will be revealed.

Figure 6. Histology and ultrastructure of intimal lesions 7 days after transduction with AdFasL (A, C, E, G, H, and I) or AdNull (B, D, F, and J). Sections are immunostained for RAM-11 (macrophages, A and B), CD31 (endothelium, C and D), smooth muscle actin (E and F), and PCNA (proliferating cells, I and J) and counterstained with hematoxylin. Arrows indicate internal elastic lamina. Transmission electron micrographs (G and H) reveal luminal endothelium (e), intimal lipid deposition (Li), a medial SMC with a “contractile” appearance (m), the internal elastic lamina (arrow), rough endoplasmic reticulum (arrowhead), and extracellular collagen fibrils (asterisk) characteristic of the “proliferative/synthetic” SMC phenotype. Original magnifications ×100 (A to F and I to J). Bar=2 μm (G and H).
sclerotic plaques but only low level expression in normal arteries. Geng et al. found abundant FasL expression in 34 (100%) of 34 clinically significant human carotid atherosclerotic lesions could have ranged from severe inflammation, given the well-established associations between progres-

Our studies do not define a precise molecular mechanism through which FasL increases intimal SMC accumulation and preserves ICAM-1 expression. Three mechanisms are particularly worthy of consideration (Figure 7). First, FasL might activate ECs in an autocrine manner, resulting in secretion of mitogenic or chemotactic molecules. Second, FasL expression by ECs might cause T-cell apoptosis, and these apoptotic T cells (which may be removed by flowing blood, precluding detection by our TUNEL stains) may release mesenchymal growth factors. Third, soluble FasL released from transduced ECs may have direct mitogenic or chemotactic effects on SMCs. All of these mechanisms are consistent with well-described activities of the Fas/FasL system, including induction of cell proliferation and activation of mitogen-activated and cyclo-oxygenase-2-dependent kinases, which is itself capable of inducing ICAM-1 expression in SMCs as well as coronary intimal thickening, and release of mitogens from apoptotic SMCs. Further studies of the consequences of FasL-mediated signaling in ECs, SMCs, and vessel wall T cells will be important in discriminating among these possibilities. These studies will be most optimally executed either in vivo or in vitro systems in which cultured ECs and SMCs exposed to FasL behave similarly to their in vivo counterparts: they persist or proliferate (Figure 6C, 6E, and 6I) rather than undergoing apoptosis (Figure IB, which appears online at http://atvb.ahajournals.org/cgi/content/full/20/2/298/DC1). Finally, it is possible that systemic release of soluble FasL from transduced arteries might contribute to the generation of the observed phenotype. Complete blood counts and liver function tests performed at 7, 14, and 28 days after transduction revealed no difference between the AdFasL and AdNull groups and no evidence of systemic toxicity (data not shown); however, these negative data do not prove that there are no systemic effects of FasL expression.

The additive effects of adenovirus and hypercholesterolemia on lesion formation are notable (Figures 2 and 3). We previously reported that infusion of adenovirus in the brachial arteries of hypercholesterolemic monkeys enhanced lesion cellularity and intimal macrophage accumulation. The additive effects of adenovirus-induced inflammation and hypercholesterolemia are demonstrated even more conclusively in the present study. This additive effect is perhaps not surprising, given the well-established associations between progression of human atherosclerosis and markers of inflammation found in both tissue and serologic specimens.1,57 Our data suggest a cautious approach to intraluminal infusion of adenovirus in hypercholesterolemic humans.

In summary, experiments performed in a new model of primary atherosclerotic lesion formation suggest a novel atherogenic role for FasL. Future studies will be directed at elucidating FasL signaling pathways in vascular cells. The animal model itself will undoubtedly prove valuable for uncovering roles of other molecular modifiers of the atherosclerotic process.
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