

Systemic Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Delivery Shows Antiatherosclerotic Activity in Apolipoprotein E–Null Diabetic Mice

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Background—Although in vitro studies have suggested that tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) might be involved in vascular biology, its potential role in the pathogenesis and/or treatment of atherosclerosis has not been investigated.

Methods and Results—Both recombinant human TRAIL and an adeno-associated virus vector expressing human TRAIL were used to deliver TRAIL in apolipoprotein E (apoE)–null mice in which diabetes mellitus was induced by destruction of islet cells with streptozotocin. Diabetes in apoE-null mice was associated with a significant increase in atherosclerotic plaque area and complexity in the aorta as assessed by a marked increase in interstitial collagen, cellular proliferation, and macrophage infiltration and a focal loss of endothelial coverage. Repeated intraperitoneal injections of recombinant human TRAIL and a single intravenous injection of adeno-associated virus–human TRAIL significantly attenuated the development of atherosclerotic plaques in apoE-null animals. TRAIL also markedly affected the cellular composition of plaque lesions by inducing apoptosis of infiltrating macrophages and increasing the vascular smooth muscle cell content. Moreover, TRAIL promoted the in vitro migration of cultured human aortic vascular smooth muscle cells but not of monocytes or macrophages. Conversely, TRAIL selectively induced apoptosis of human cultured macrophages but not of vascular smooth muscle cells.

Conclusions—Overall, data from the present study indicate that atherosclerosis in diabetic apoE-null mice is ameliorated by systemic TRAIL administration and that adeno-associated virus–mediated TRAIL gene delivery might represent an innovative method for the therapy of diabetic vascular diseases. (*Circulation*. 2006;114:1522-1530.)

Key Words: atherosclerosis ■ diabetes mellitus ■ gene therapy ■ immunohistochemistry ■ plaque

Cardiovascular disease is currently the leading cause of death and illness in developed countries and accounts for up to 70% of mortality in patients affected by diabetes mellitus.¹ Atherosclerosis, which constitutes the single most important contributor to this growing burden of cardiovascular disease, can be considered a form of chronic low-grade inflammation resulting from interactions between modified lipoproteins, monocyte-derived macrophages, and the normal cellular elements of the arterial wall. This inflammatory process can ultimately lead to the development of complex lesions, or plaques, that protrude into the arterial lumen. Plaque rupture and thrombosis result in the acute clinical complications of myocardial infarction and stroke.^{2,3}

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Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is expressed as a type II TNF family

transmembrane protein, but its extracellular domain can be proteolytically cleaved from the cell surface and acts as a soluble cytokine interacting with 4 transmembrane receptors (TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4) belonging to the TNF-receptor (R) family.^{4,5} The high-affinity TRAIL-R1 and TRAIL-R2 contain cytoplasmic “death domains” and mediate proapoptotic signals but also can promote cell type–dependent prosurvival and proliferation signals.⁴ In this respect, it has been shown that both vascular endothelial cells^{6,7} and vascular smooth muscle cells⁸ (VSMCs) express TRAIL receptors and that recombinant TRAIL is able to promote their in vitro survival/proliferation by activating the Akt and extracellular signal-regulated kinase/mitogen-activated protein kinase pathways.^{7,8} However, under certain circumstances such as inhibition of the Akt pathway^{7,9,10} or direct cell-to-cell contact with TRAIL-expressing leukocytes,¹¹ TRAIL can induce apoptotic cell

Received February 20, 2006; de novo received June 5, 2006; revision received July 28, 2006; accepted July 31, 2006.

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Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/CIRCULATIONAHA.106.643841

death of both vascular endothelial cells and VSMCs. Of note, 2 recent *in vivo* studies have shown that the serum levels of TRAIL are significantly decreased in patients affected by or predisposed to cardiovascular diseases,^{12,13} suggesting that circulating soluble TRAIL may be involved in the physiopathology of cardiovascular disease. With these conflicting *in vitro* and *in vivo* data taken into account, the aim of the present study was to evaluate the biological effect of TRAIL administration *in vivo* in an experimental model of atherosclerosis, the apolipoprotein E (apoE)-null mice, in which diabetes mellitus was induced by streptozotocin injection. In these animals, atherosclerotic lesions take place over time and resemble in appearance and distribution those observed in humans.¹⁴ Both recombinant soluble TRAIL and an adeno-associated virus (AAV) human TRAIL (hTRAIL) vector were used to deliver TRAIL in diabetic apoE-null mice to evaluate its effect on the development and severity of atherosclerotic lesions.

Methods

Recombinant TRAIL and AAV-hTRAIL Construct

Recombinant (r) histidine 6-tagged hTRAIL_(114–281) was produced in bacteria as previously described.^{7,8} The coding sequence of the hTRAIL cDNA was obtained by reverse-transcriptase polymerase chain reaction (PCR) amplification and cloned under the control of the cytomegalovirus (CMV) promoter into an rAAV vector based on the pTR-UF5 construct.¹⁵ All plasmid manipulations were performed in XL10-Gold *Escherichia coli* cells (Stratagene, La Jolla, Calif) to ensure the integrity of the inverted terminal repeats, which was analyzed by *Sma*I digest of the resulting clones. One of the clones was expanded, checked by sequence analysis, and functionally tested by indirect immunofluorescence and Western blot by using a monoclonal antibody (Ab) anti-hTRAIL (R&D Systems, Minneapolis, Minn) in transfected Chinese hamster ovary cells. Infectious AAV vector particles and viral stocks were generated as previously described.¹⁵ Viral preparations used for animal transduction had titers between 1×10^{13} and 1×10^{14} viral genome particles per 1 mL.

Animals and Experimental Protocols

Animal care and treatment conformed to institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, December 12, 1987). Thirty-six 6-week-old apoE-null mice (The Jackson Laboratory, Bar Harbor, Me) were rendered diabetic by 5 daily intraperitoneal injections of streptozotocin (Sigma Chemical Co, St Louis, Mo) at a dose of 55 mg/kg and were observed for 3 months. Control mice (n=8) received citrate buffer alone and were processed in parallel to the diabetic mice. Diabetic animals were further randomized to receive hrTRAIL, the vehicle (AAV-hTRAIL or AAV-LacZ), or no treatment. For injection of rTRAIL, each mouse received an intraperitoneal injection of rTRAIL (3 μ g per mouse in a total of 200 μ L HEPES-buffered saline) or of an equivalent volume of vehicle (HEPES-buffered saline) every 3 weeks. For AAV injection, each mouse received a single intravenous injection of a total of 200 μ L purified AAV vector preparation (either AAV-hTRAIL or control AAV-LacZ). After 3 months, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and euthanized for blood tests and histological examination.

In each group of animals, serum glucose and glycosylated hemoglobin were determined as previously described.¹⁴ Mouse TNF- α and hTRAIL levels were measured in serum samples using sandwich-type ELISA kits (both purchased from R&D Systems) according to the manufacturer's instructions. Measurements were performed in duplicate.

Real-Time PCR

To examine AAV distribution, 4 animals were euthanized after 3 weeks by AAV injection, and total DNA was extracted from frozen tissue samples with Trizol reagent (Invitrogen, Milan, Italy) according to manufacturer's instructions. Total DNA was then used as a template for real-time PCR amplification to measure the levels of AAV construct and 18S genomic DNA. All amplifications were performed on a 7000 ABI Prism Instrument (Applied Biosystems, Foster City, Calif) using a predeveloped assay for the housekeeping gene 18S (Applied Biosystems) with appropriate probes and primer pairs chosen in the context of the CMV viral promoter sequence. Results were expressed as number of viral genome particles per ng of total DNA.

Evaluation of Atherosclerotic Plaques

To evaluate the atherosclerotic lesions, 2 complementary approaches were used: *en face* whole and histological section analyses. The *en face* approach was used to obtain information about the distribution and extent of atherosclerosis in the aorta, whereas microscopic analysis was used to evaluate lesion complexity as previously described.¹⁴ In brief, the entire aorta was opened longitudinally and stained with Sudan IV-Herxheimer's solution (Sigma), and lesion area measurements were performed by calculating the proportion of aortic intimal surface area occupied by the red stain in the arch, descending thoracic aorta, and abdominal aorta with the use of a video-based image analysis program (MCID, Imaging Research, St Catharines, Ontario, Canada).

Next, all aortic segments were embedded in paraffin, and 4- μ m-thick cross-sectional serial sections were obtained. To quantify luminal cross-sectional area involved by atherosclerotic plaque, 10 sections obtained every 20 μ m from the aortic arch were stained with hematoxylin and eosin and analyzed by light microscopy. For quantification of collagen content, aortic cross sections were stained with either Masson's trichrome or Sirius red as previously described.^{14,16} Atherosclerotic lesions were manually traced on the computer, with care taken to exclude normal-appearing media and to include only the intimal/subintimal atherosclerotic lesions, and analyzed with the MCID video-based image analysis program.

Immunohistochemistry and In Situ DNA Nick End Labeling Assay

After neutralization of endogenous peroxidase, paraffin sections of aorta were incubated with the following primary Abs: α -smooth muscle actin (α -SMA; smooth muscle cell marker), proliferating cell nuclear antigen (PCNA; both from DAKO, Copenhagen, Denmark; diluted 1:50), and CD34 (endothelial marker; from Serotec Ltd, Oxford, UK; diluted 1:400). Biotinylated immunoglobulins, diluted 1:200, were then applied as a secondary Ab, followed by horseradish peroxidase-conjugated streptavidin (DAKO; diluted 1:625). The staining was visualized by reaction with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Macrophage detection was performed by using the primary Ab for F4/80 (Serotec; diluted 1:200), followed by secondary anti-rat immunoglobulins (Vector Laboratories, Burlingame, Calif; diluted 1:200) and the CSA mouse amplification kit (Dako A/S), following manufacturer's instructions. After counterstaining with hematoxylin, all the sections were examined by light microscopy and digitized with a high-resolution camera, and quantification of immunostaining within the plaques was assessed with the MCID image analysis system.

Identification of apoptotic cells in the tissues was performed with the In Situ Cell Death Detection Kit (Roche Diagnostics Corp, Indianapolis, Ind) according to the manufacturer's instructions, followed by analysis with fluorescent microscope (Axiophot 100, Zeiss, Germany). After counterstaining with DAPI, the percentage of *in situ* DNA nick end labeling (TUNEL)-positive cells was calculated by counting TUNEL-positive cells per total nuclei within the plaque. For double-immunofluorescent labeling, sections were double-stained with the F4/80 (1:200 dilution) or α -SMA (1:200 dilution) Abs, followed by an Alexa Fluor 546-conjugated second-

TABLE 1. Characteristics of Mice at the End of the Study

	Control (n=8)	Diabetic+Vehicle (n=8)	Diabetic+rTRAIL (n=6)	Diabetic+AAV-hTRAIL (n=6)	Diabetic+AAV-LacZ (n=6)
Body weight, g	31.3±1.8	25.8±2.6	27.2±3.2	26.5±1.9	25.3±2.3
Serum glucose, mg/dL	116±19	522±101*	450±46*	540±61*	473±118*
HbA _{1c} , %	3.9±0.9	14.1±1.2*	12.3±1.3*	13.1±1.3*	13.2±1.4*
Serum TNF- α , pg/mL	2.5±2.9	5.9±3.8*	ND	ND	ND

Values in diabetic mice were comparable to those in diabetic mice+vehicle and thus are not reported in the table. Data are expressed as mean±SD and statistically (ANOVA) compared. HbA_{1c} indicates glycosylated hemoglobin; ND, not determined.

* $P<0.05$ vs control group.

ary Ab (1:200 dilution; Invitrogen) to visualize the macrophages and smooth muscle cells, respectively.

Cell Cultures

Human aortic VSMCs, purchased from BioWhittaker (Walkersville, Md), were cultured in SmGM3 medium (BioWhittaker) according to the manufacturer's instructions.

Peripheral blood samples were collected in heparin-coated tubes from healthy human blood donors, after informed consent was obtained, in agreement with institutional guidelines. After isolation of peripheral blood mononuclear cells by gradient centrifugation with lymphocyte cell separation medium (Cedarlane Laboratories, Hornby, Ontario, Canada), CD14⁺ monocytes were immunomagnetically selected (MACS microbeads, Miltenyi Biotec, Auburn, Calif). Freshly purified CD14⁺ monocytes (purity >90%) were either used immediately or cultured in RPMI medium for 7 days with 50 ng/mL macrophage-colony stimulating factor (Peprotech, London, UK) to allow their differentiation into macrophages.

Cell Migration and Apoptosis Assays

Cell migration in response to TRAIL was analyzed with a modified Boyden chamber assay as described previously⁸ by using 24-well plates with 5- to 8- μ m-pore-size transwell inserts (Costar, Cambridge, Mass). Cells (0.5 to 1×10⁶/mL) resuspended in migration buffer (M199 medium, 10 mmol/L HEPES, pH 7.4, 0.5% bovine serum albumin) were placed in the upper compartment of the transwell chambers. Control cytokines (CCL8 for monocytes and macrophages and platelet-derived growth factor for VSMCs, both purchased from R&D System) or rTRAIL was added in the lower chambers. After 2 hours of incubation at 37°C in 5% CO₂ atmosphere, filters were removed, and cells on the upper face of the membrane were scratched with a cotton swab to remove the nonmigrated cells. Cells on the lower face were fixed, stained with Mayer's hematoxylin solution, and counted in 5 high-power fields (×20). Assays were performed in triplicates.

The effect of TRAIL on cell viability was analyzed by light microscopy after Trypan blue dye staining. Moreover, apoptosis induction was quantified by annexin V-FITC/propidium iodide staining (Immunotech, Marseille, France), followed by flow cytometric analysis performed as described elsewhere.^{7,8}

Statistical Analysis

Data were analyzed by ANOVA and with the Mann-Whitney rank sum test. Group means were compared by the Bonferroni method. Data are shown as mean±SD unless otherwise specified. Statistical significance was defined as $P<0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Induction of Diabetes Mellitus in ApoE-Null Mice and Lack of Major Toxicity by Repeated hrTRAIL Administration

Diabetes mellitus was induced in apoE-null mice by streptozotocin injection. After 3 months, diabetic and control ani-

mals were analyzed for various parameters, as reported in Table 1, and then euthanized for pathological examination. Diabetic animals gained less weight than control mice and showed elevated levels of blood glucose, glycosylated hemoglobin, and serum TNF- α . En face dissection of aorta segments revealed that diabetes was associated with a significant increase in percentage of plaque area in the entire aorta (mean±SD: 7.8±1.6 versus 1.3±0.9 in diabetic versus control apoE-null mice respectively; $P<0.01$, rank sum test) and in each segment (mean±SD in arch: 21.1±5.9 versus 3.9±2.6, $P<0.01$; mean±SD in thoracic aorta: 3.1±0.4 versus 0.6±0.5, $P<0.01$; mean±SD in abdominal aorta: 4.2±1.7 versus 0.8±0.6, $P<0.01$, rank sum test).

Although in nondiabetic control mice most plaques were fatty streaks, in the aorta of diabetic mice, there were predominantly advanced lesions characterized by an asymmetrically thickened intima composed of a thin fibrous cap and a lipid-rich necrotic core (Figure 1A), an increase in the collagen content within the plaque clearly detected by the Sirius red polarization method (Figure 1B), and a focal lack of endothelial integrity, especially on the shoulder of atherosclerotic lesions, as documented by immunohistochemistry (Figure 1B).

After the induction of diabetes, the animals were randomized to be injected intraperitoneally with hrTRAIL or the control vehicle. It is important to note that hrTRAIL was detectable in the sera of apoE-null mice up to 4 days after intraperitoneal injection (Figure 2) and that repeated intraperitoneal injections of hrTRAIL (every 3 weeks) in apoE-null mice were safe, as demonstrated by the fact that diabetic animals treated with TRAIL did not show significant alterations in body weight (Table 1) or gross abnormalities at necropsic examination with respect to vehicle-treated diabetic animals.

Persistence of TRAIL Expression in AAV-hTRAIL-Treated ApoE-Null Mice

As an alternative strategy of TRAIL administration, we have constructed an AAV vector-expressing hTRAIL (AAV-hTRAIL) to analyze possible advantages of low but sustained in vivo expression of TRAIL compared with injections with soluble rTRAIL. The coding region of the hTRAIL was cloned in an AAV vector under the control of the constitutive CMV promoter, and efficiency of expression of the transgene was initially demonstrated in cell cultures by immunofluorescence and Western blot (Figure 3A). In this set of experiments, diabetic apoE-null animals received a single

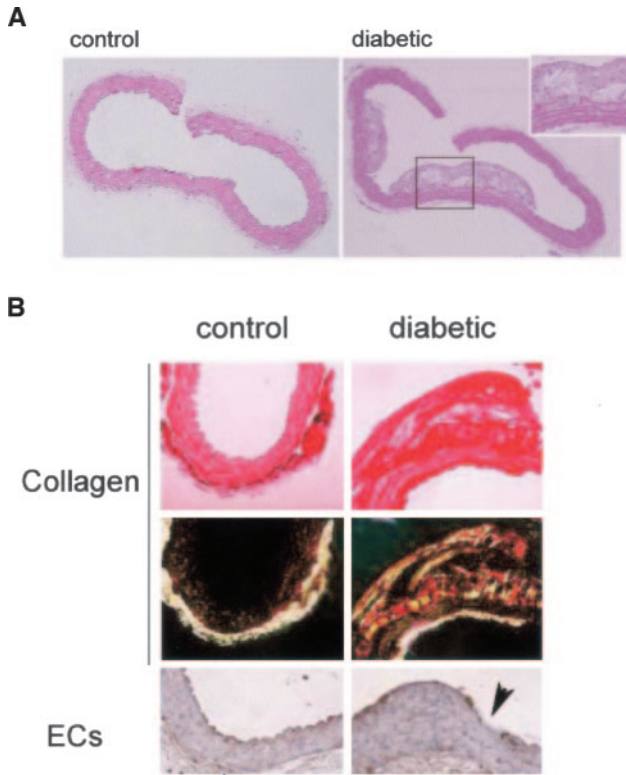


Figure 1. Increase in atherosclerotic lesions in the aorta of apoE-null mice after induction of diabetes. A, Representative hematoxylin and eosin-stained histological cross sections from aorta of control and diabetic apoE-null mice. Magnification $\times 10$. Inset represents higher magnification of a representative plaque (magnification $\times 30$). B, Representative analysis of the collagen content in the aortic intima detected by staining with Sirius red, followed by photography with a polarizing filter (top, middle). Interstitial collagen exhibits strong birefringence. Bottom, Representative immunostaining of endothelial cells (ECs). Arrowhead indicates the site of endothelial disruption. Magnification $\times 30$.

intravenous injection with high-titer (10^{12} viral genome particles) AAV-hTRAIL or AAV-LacZ through the tail vein. To assess the in vivo dissemination of AAV-hTRAIL, 4 diabetic animals were euthanized 3 weeks after injection, and viral (AAV) genomes were detected by real-time PCR in both lung and liver (Figure 3B). In vivo expression of hTRAIL by the AAV construct was documented by the detection of hTRAIL

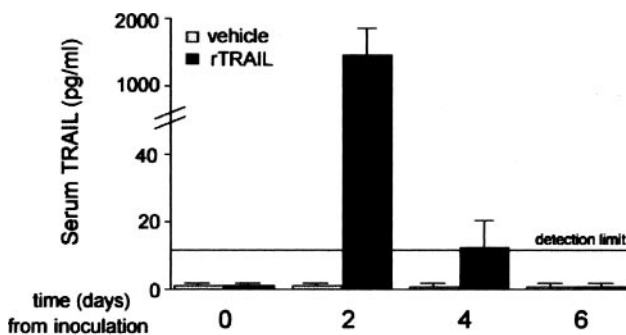


Figure 2. Serum detection of rTRAIL in diabetic apoE-null mice. Serum hTRAIL was analyzed by ELISA in diabetic apoE-null mice injected intraperitoneally with human rTRAIL or control vehicle. Data are expressed as mean \pm SD.

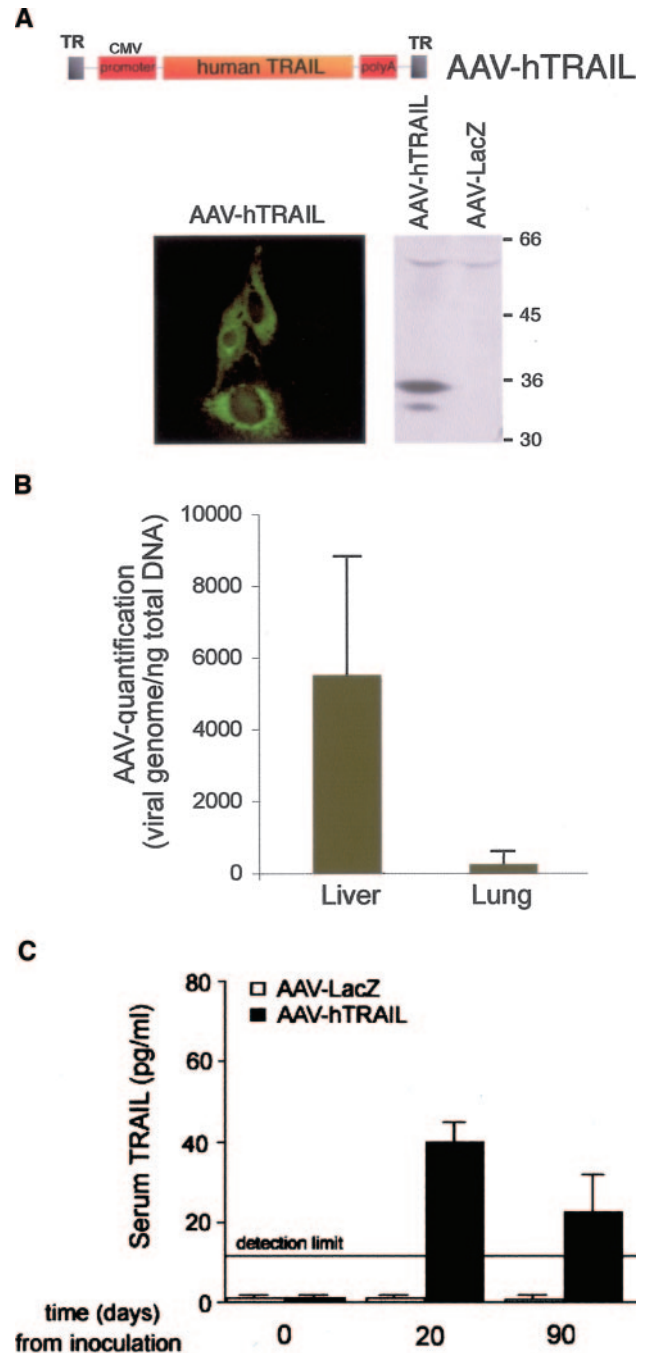


Figure 3. Delivery of hTRAIL cDNA to diabetic apoE-null mice using AAV vector. A, AAV vector used in this study. Expression of TRAIL was evaluated by immunofluorescence and Western blot in Chinese hamster ovary cells transduced with AAV-hTRAIL. B, Four mice injected with AAV-hTRAIL were euthanized 3 weeks after intravenous injection, and DNA was extracted from the indicated organs. Results are expressed as ratios between AAV and 18S RNA copy number. C, Serum hTRAIL was analyzed by ELISA in diabetic apoE-null mice injected intravenously with either AAV-hTRAIL or AAV-LacZ. Data are expressed as mean \pm SD. TR indicates terminal repeat sequences; CMV, cytomegalovirus immediate early promoter; and polyA, polyadenylation site.

in the sera of diabetic animals injected with AAV-hTRAIL but not with AAV-LacZ (Figure 3C). It is important to note that hTRAIL was detectable in the sera of AAV-hTRAIL-injected diabetic animals up to 3 months after AAV injection

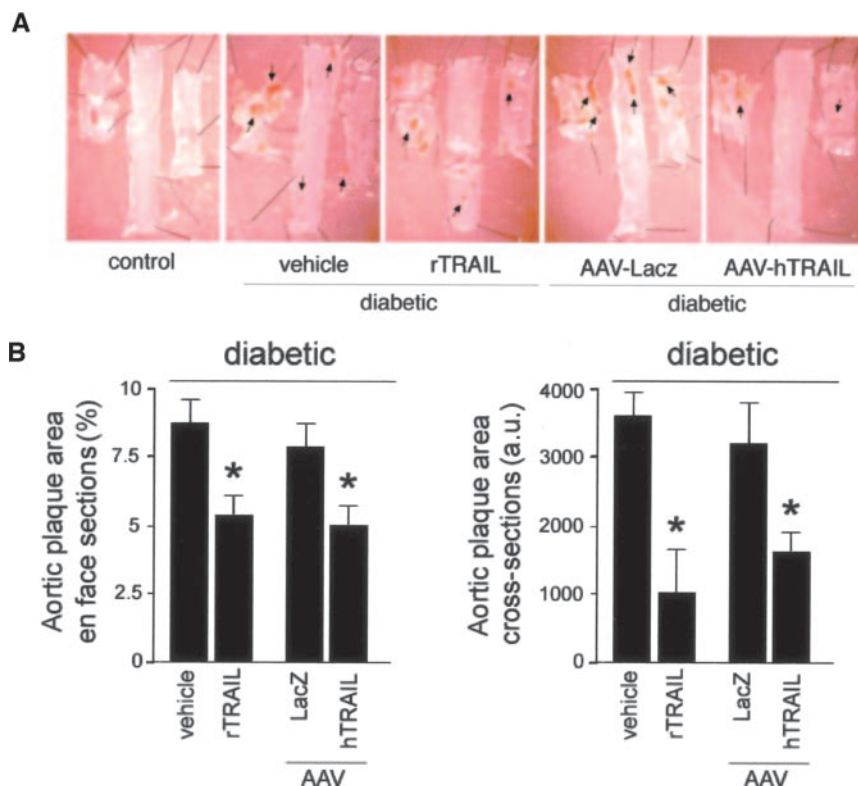


Figure 4. Effect of TRAIL on plaque formation in diabetic apoE-null mice. **A**, Representative examples of en face dissection of aortic arch and thoracic and abdominal aorta, stained with Sudan IV, showing atherosclerotic lesions (red, with arrows) in control and diabetic apoE-null mice treated with either vehicle or rTRAIL and with either control AAV vector (LacZ-AAV) or hTRAIL-AAV. **B**, Atherosclerotic involvement for each mouse was quantitatively determined in en face sections by measuring the proportion of aortic intimal surface area occupied by Sudan IV⁺ lesions in total aorta and by measuring cross-sectional atherosclerotic lesion surface area in hematoxylin and eosin-stained histological sections. Results are expressed as mean±SD. **P*<0.05 (rank sum test). a.u. indicates arbitrary units.

at the end of the experiments (Figure 3C). AAV-hTRAIL injection in apoE-null mice was safe and, as was also observed after soluble TRAIL administration, did not significantly alter body weight (Table 1) or cause gross abnormalities at necroscopic examination.

Attenuation of Atherosclerotic Plaque Development in Diabetic ApoE-Null Mice After In Vivo Administration of hrTRAIL or AAV-hTRAIL

Diabetic apoE-null mice injected with vehicle showed atherosclerotic plaques in all segments of the aorta without any significant difference with respect to untreated diabetic animals (data not shown). On the other hand, the apoE-null mice injected with rTRAIL exhibited a total plaque area, evaluated by both en face and cross-section analyses (Figure 4A and

4B), that was significantly (*P*<0.05) reduced compared with the mice injected with vehicle. Similarly, although the control AAV-LacZ construct did not significantly alter plaque area in diabetic apoE-null mice, AAV-hTRAIL significantly (*P*<0.05) decreased the total extension of the plaque area (Figure 4A and 4B).

We sought to investigate whether, besides decreasing the development of atherosclerotic lesions, TRAIL treatment also affected their cellular composition. For this purpose, serial plaque sections were analyzed by immunohistochemistry to quantitatively evaluate the total endothelial coverage, collagen content, PCNA-positive cells, VSMCs, and macrophages. As summarized in Table 2, atheroma lesions of diabetic apoE-null animals were characterized by significantly less (*P*<0.05) endothelial integrity and significantly more (*P*<0.05) collagen, PCNA-positive cells, and macro-

TABLE 2. Aortic Cellular Parameters Evaluated by Immunohistochemistry

	Control (n=8)	Diabetic+Vehicle (n=8)	Diabetic+rTRAIL (n=6)	Diabetic+AAV-TRAIL (n=6)	Diabetic+AAV-LacZ (n=6)
Endothelium	98.5±0.7	85.4±1.6*	94.2±1.3†	93.1±1.4‡	84.5±1.7*
Collagen	12.2±8.3	32.4±10.1*	20±9.4†	21.6±7.3‡	29.4±6.9*
PCNA	4.8±1.6	16.4±8.9*	3.1±2.6†	2.7±2.3‡	13.4±6.1*
VSMCs	7.2±4.6	13.3±6.6	21.5±8.8*	17.1±7.3	15.2±9.9
Macrophages	6.7±2.7	15.7±3.9*	5.5±3.2†	6.1±3.4‡	13.7±4.1*

Values in diabetic mice were comparable to those in diabetic mice+vehicle and thus are not reported in the table. Data are expressed as percentage stained area (mean±SD) in aortic arch sections and statistically (ANOVA) compared.

**P*<0.05 vs control group.

†*P*<0.05 vs diabetic+vehicle group.

‡*P*<0.05 vs diabetic+AAV-lacZ group.

phage infiltration compared with nondiabetic control animals. Both rTRAIL and AAV-TRAIL treatments were associated with a greater ($P<0.05$) integrity of endothelial layer on the plaque surface and a significant ($P<0.05$) reduction in PCNA-positive cells and, to a lesser extent, collagen content in the plaque of the diabetic animals (Table 2). With regard to the α -SMA-positive VSMCs, their number was increased in diabetic apoE-null animals compared with nondiabetic animals but not to a significant extent, at variance with collagen content, number of PCNA-positive cells, and macrophages (Table 2). More importantly, neither rTRAIL nor AAV-TRAIL reduced the amount of VSMCs found predominantly in the fibrous cap of the atherosclerotic lesions of apoE-null diabetic animals (Figure 5A). Rather, the plaque content of α -SMA-positive VSMCs showed a tendency to increase in diabetic animals treated with rTRAIL compared with both diabetic ($P=0.06$) and nondiabetic ($P<0.05$) animals (Table 2), possibly contributing to the stability of atherosclerotic plaques.^{17,18} On the other hand, both rTRAIL and AAV-hTRAIL significantly ($P<0.05$) decreased the number of plaque-infiltrating macrophages in diabetic apoE-null animals (Table 2 and Figure 5B). These findings appear particularly relevant because it has been proposed that plaque-infiltrating macrophages are involved in plaque destabilization through the production of metalloproteinases and possibly other tissue-injurious mediators.^{19–21}

Increase in Apoptosis in Plaque-Infiltrating Macrophages After In Vivo Administration of hrTRAIL

To evaluate whether the reduction in infiltrating macrophages observed in TRAIL-treated animals might involve the induction of apoptosis by TRAIL, TUNEL staining was applied to identify apoptotic cells in plaque cross sections of diabetic apoE-null mice treated or not treated with rTRAIL. As shown in Figure 6A, the number of TUNEL-positive apoptotic cells in atherosclerotic plaques was significantly ($P<0.05$) increased in diabetic TRAIL-treated animals. Double immunofluorescence staining performed by combining TUNEL with specific cell markers (F4/80 or α -SMA) confirmed that apoptosis occurred preferentially in infiltrating macrophages (Figure 6B). In fact, among the $15\pm6\%$ TUNEL-positive cells scored in plaque sections independently obtained from 4 TRAIL-treated animals, $11\pm4\%$ costained with the F4/80 macrophage marker, $1\pm0.8\%$ costained with the α -SMA VSMC marker, and $3\pm1.5\%$ were negative for both F4/80 and α -SMA. In this respect, it has been shown that a significant fraction of apoptotic cells within atherosclerotic plaques lose their differentiation marker.¹⁹ Similar findings were observed in animals treated with AAV-hTRAIL (data not shown).

Induction of Human VSMC Migration and Macrophage Apoptosis by rTRAIL

In the last group of experiments, the effect of TRAIL on the migration and apoptosis of human aortic VSMCs, as well as of freshly isolated monocytes and cultured macrophages, was investigated in vitro. Cell migration in response to TRAIL was analyzed with a modified Boyden chamber assay. As

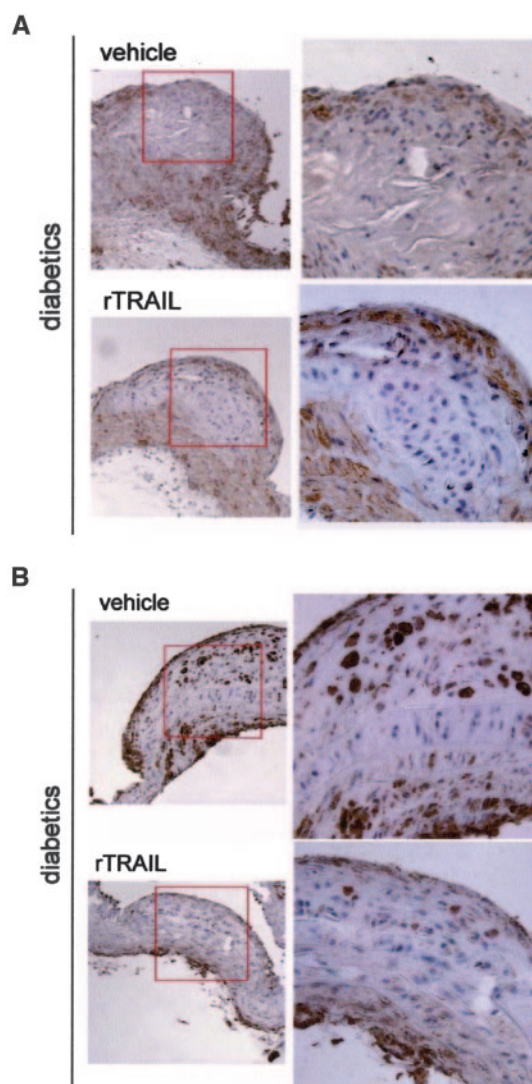


Figure 5. Histological characteristics of atherosclerotic plaques in TRAIL-treated diabetic apoE-null mice. Sections from aortic arch lesions were stained for VSMC (A) and macrophage (B) markers. Positive staining is shown as brown. Sections are counterstained with hematoxylin. Atherosclerotic plaques of diabetic TRAIL-treated apoE-null mice were characterized by α -SMA-positive cells located predominantly at the fibrous cap (A) and by fewer infiltrating macrophages (B) compared with vehicle treated apoE-null mice. Magnification $\times 20$. Insets represent higher magnification of the framed areas (magnification $\times 40$).

shown in Figure 7, TRAIL significantly ($P<0.05$) induced migration of VSMCs; on the other hand, TRAIL did not affect the migration of freshly isolated CD14⁺ monocytes or that of cultured macrophages. On the contrary, TRAIL did not induce apoptosis when added to either human aortic VSMCs or freshly isolated CD14⁺ monocytes, whereas it significantly ($P<0.05$) increased the percentage of apoptosis when added to cultured human macrophages (Figure 7).

Discussion

In the present study, we have demonstrated for the first time that systemic administration of rTRAIL has a protective role in vivo against atherosclerosis progression induced by diabe-

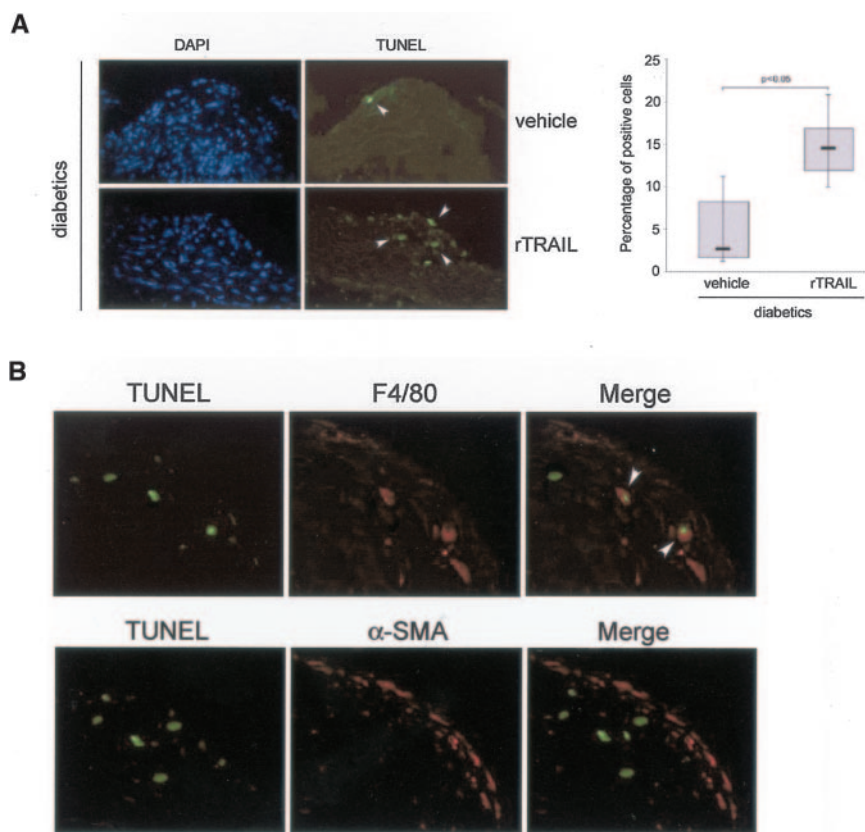


Figure 6. TUNEL staining in sections from aorta lesions. **A**, Representative sections showing TUNEL-positive apoptotic cells in aortic plaque (green, with arrowheads). After nuclear counterstaining with DAPI, the percentage of TUNEL-positive cells per total nuclei within the plaque was calculated in separate sections for each mouse injected with either vehicle or rTRAIL. Horizontal bars are median; upper and lower edges of box, 75th and 25th percentiles; and lines extending from box, 10th and 90th percentiles. $P < 0.05$ (rank-sum test). **B**, Aortic plaque sections from TRAIL-treated diabetic mice were double stained for TUNEL (green) plus the F4/80 or the α -SMA Abs, followed by an Alexa Fluor 546-conjugated secondary Ab (red) to visualize the macrophages and VSMCs, respectively. Arrowheads indicate apoptotic macrophages.

tes in the apoE-null mice model, in which atherosclerotic lesions resemble in appearance and distribution those observed in humans.¹⁴ Diabetes-accelerated plaque formation in the aorta of apoE-null mice was associated with a focal loss of endothelial coverage and a marked increase in collagen content and macrophage infiltration. Remarkably, TRAIL injection not only significantly attenuated the total extension of the plaques but also contributed to stabilize atherosclerotic plaques by preserving the endothelial cell coverage on the plaque lesions and by selectively decreasing the number of infiltrating macrophages in the atherosclerotic lesions.

Our data suggest that one of the key mechanisms by which TRAIL elicits plaque stabilization is the induction of apoptosis of infiltrating macrophages. In this respect, it has been shown that macrophages represent a key cellular component of innate immunity, which has been shown to promote atherosclerosis initiation and progression.^{19–22} In particular, during the initial or intermediate stages of atherosclerosis, accelerated apoptosis and rapid removal of apoptotic cells are beneficial, reducing the number of atherogenic and inflammation-prone macrophages within the plaque and therefore slowing lesion progression.^{2,3} On the other hand, macrophage lysis in advanced lesions has been involved in the generation of necrotic cores, which promote plaque instability.^{2,3} Moreover, in agreement with a previous study showing the induction of apoptosis in mouse macrophages by *in vitro* exposure to TRAIL,²³ we have shown for the first time that TRAIL induces apoptosis of human cultured macrophages but not of freshly isolated circulating monocytes.

TRAIL treatment did not decrease but rather showed a tendency to increase the number of VSMCs within the

atherosclerotic plaques in diabetic apoE-null mice. Because the loss of VSMCs in the fibrous cap represents a critical mechanism in transforming stable plaque into rupture-prone lesions,^{17,18} the enrichment of α -SMA-positive cells in the fibrous cap within the atherosclerotic plaque of TRAIL-treated diabetic apoE-null mice compared with vehicle-treated diabetic animals is relevant for plaque stability. Consistent with the *in vivo* data, we found that rTRAIL enhanced migration of human aortic VSMC without inducing apoptosis *in vitro*. Thus, it is possible that the increased number of VSMCs observed in the fibrous cap of atherosclerotic lesions in TRAIL-treated diabetic apoE-null mice is due to the ability of TRAIL to promote VSMC migration. It should be mentioned, however, that a recent study has indicated that TRAIL-expressing leukocytes, infiltrating human and mouse atherosclerotic plaques, might take part in the induction of VSMC apoptosis.¹¹ Although a clear-cut explanation for the discrepancy between our findings and those of Sato et al¹¹ is foreseeable, one possibility is that transmembrane TRAIL, expressed on the surface of infiltrating leukocytes, is more potent than soluble TRAIL in inducing VSMC apoptosis. An alternative, not mutually exclusive, explanation to reconcile these apparently contrasting results is that systemic rTRAIL might prevent the plaque infiltration by TRAIL-expressing leukocytes responsible for local VSMC apoptosis.

Although one must be cautious in extrapolating these experimental data to the clinical context, the potential clinical relevance of these results obtained in the apoE-null mice is corroborated by the findings that soluble TRAIL levels have

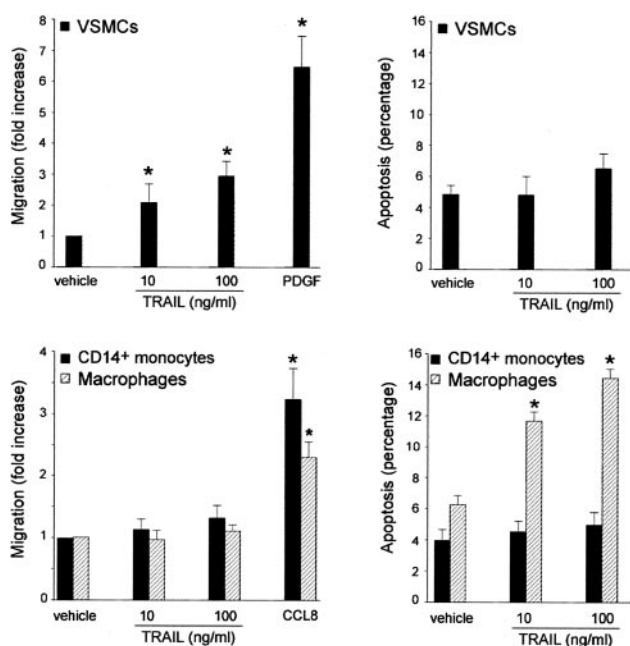


Figure 7. Migration and apoptotic response of primary human CD14⁺ monocytes, macrophages, and VSMCs to TRAIL. Cell migration in response to TRAIL or CCL-8 (100 ng/mL) or platelet-derived growth factor (10 ng/mL), used as positive controls, was evaluated by counting the number of migrated cells in 5 high-power fields and is expressed as the relative fold increase compared with spontaneous migration, determined in the absence of chemoattractant (vehicle). Apoptosis was quantitatively evaluated by flow cytometry after annexin V-FITC/propidium iodide staining. Data represent the mean \pm SD of 4 separate experiments. * $P < 0.05$ (rank sum test).

a tendency to decrease in the sera of patients affected by cardiovascular diseases.^{12,13} In fact, 2 recent studies carried out in patients with acute coronary syndrome revealed significantly lower soluble TRAIL serum levels compared with patients with stable angina or normal coronary arteries.^{12,13} Moreover, it has been shown that TRAIL serum levels negatively correlated with the level of C-reactive protein,¹³ a nonspecific “acute-phase” protein that represents a known marker of acute vascular events.²⁴ Because C-reactive protein serum levels are closely associated with plaque instability and oxidative stress,²⁵ these data further support a possible protective role of TRAIL in atherosclerosis and plaque instability.

Obviously, we are aware that treatment of vascular complications offers a number of problems in terms of efficacy, specificity, delivery, and duration that will be difficult to overcome with rTRAIL only. In this respect, the present demonstration that AAV can be used to systemically express and release TRAIL with pharmacological activity in the absence of immune or inflammatory reaction is particularly noteworthy, strongly suggesting that AAV represents a potential alternative to recombinant protein injection.

Another important aspect of the potential therapeutic efficacy of TRAIL for treating patients at risk of atherosclerosis is that the safety of rTRAIL in vivo has already been examined in various studies performed on both rodents and nonhuman primates.^{26–28} Recombinant TRAIL generally was

found to be well tolerated even when multiple doses were administered to animals, except for a mild anemia that probably was a result of its ability to impair erythroid differentiation.²⁹ It should be also reported that various phase 1 and 2 clinical trials using agonistic monoclonal Abs that engage the hTRAIL receptors TRAIL-R1 and TRAIL-R2 have provided encouraging results in cancer patients.³⁰ Therefore, our study indicates that TRAIL has to be considered a promising therapeutic agent not only for antitumor therapy but also for its antiatherosclerotic activity.

Source of Funding

This work was supported by grants (Drs Secchiero and Zauli) from Fondo di Incentivazione per la Ricerca di Base (Ministero dell'Istruzione, dell'Università e della Ricerca).

Disclosures

None.

References

- Booth GL, Kapral MK, Fung K, Tu JV. Recent trends in cardiovascular complications among men and women with and without diabetes. *Diabetes Care*. 2006;29:32–37.
- Glass CK, Witztum JL. Atherosclerosis: the road ahead. *Cell*. 2001;104:503–516.
- Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.
- Park SM, Schickel R, Peter ME. Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. *Curr Opin Cell Biol*. 2005;17:1–7.
- Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*. 1997;277:815–818.
- Zhang XD, Nguyen T, Thomas WD, Sanders JE, Hersey P. Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. *FEBS Lett*. 2000;483:193–199.
- Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D, Zauli G. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. *Circulation*. 2003;107:2250–2256.
- Secchiero P, Zerbini C, Rimondi E, Corallini F, Milani D, Grill V, Forti G, Capitani S, Zauli G. TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells. *Cell Mol Life Sci*. 2004;61:1965–1974.
- Pritzker LB, Scatena M, Giachelli CM. The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell*. 2004;15:2834–2841.
- Li JH, Kirkiles-Smith NC, McNiff JM, Pober JS. TRAIL induces apoptosis and inflammatory gene expression in human endothelial cells. *J Immunol*. 2003;171:1526–1533.
- Sato K, Niessner A, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. TRAIL-expressing T cells induce apoptosis of vascular smooth muscle cells in the atherosclerotic plaque. *J Exp Med*. 2006;203:239–250.
- Schoppet M, Sattler AM, Schaefer JR, Hofbauer LC. Osteoprotegerin (OPG) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) levels in atherosclerosis. *Atherosclerosis*. 2006;184:446–447.
- Michowitz Y, Goldstein E, Roth A, Afek A, Abashidze A, Ben Gal Y, Keren G, George J. The involvement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in atherosclerosis. *J Am Coll Cardiol*. 2005;45:1018–1024.
- Candido R, Allen TJ, Lassila M, Cao Z, Thallas V, Cooper ME, Jandeleit-Dahm KA. Irbesartan but not amlodipine suppresses diabetes-associated atherosclerosis. *Circulation*. 2004;109:1536–1542.
- Arsic N, Zentilin L, Zacchigna S, Santoro D, Stanta G, Salvi A, Sinagra G, Giacca M. Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. *Mol Ther*. 2003;7:450–459.
- Nakata Y, Maeda N. Vulnerable atherosclerotic plaque morphology in apolipoprotein E-deficient mice unable to make ascorbic acid. *Circulation*. 2002;105:1485–1490.
- Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J, Finn AV, Virmani R. The thin-cap fibroatheroma: a type of vulnerable plaque: the

- major precursor lesion to acute coronary syndromes. *Curr Opin Cardiol*. 2001;16:285–292.
18. Rossi ML, Marziliano N, Merlini PA, Bramucci E, Canosi U, Belli G, Parenti DZ, Mannucci PM, Ardissimo D. Different quantitative apoptotic traits in coronary atherosclerotic plaques from patients with stable angina pectoris and acute coronary syndromes. *Circulation*. 2004;110:1767–1773.
 19. Kockx MM. Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects. *Arterioscler Thromb Vasc Biol*. 1998;18:1519–1522.
 20. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res*. 2002;90:251–262.
 21. Lindstedt KA, Leskinen MJ, Kovanen PT. Proteolysis of the pericellular matrix: a novel element determining cell survival and death in the pathogenesis of plaque erosion and rupture. *Atheroscler Thromb Vasc Biol*. 2004; 24:1350–1358.
 22. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res*. 2002;91:281–291.
 23. Kaplan MJ, Ray D, Mo RR, Yung RL, Richardson BC. TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4⁺ T cell killing of antigen-presenting macrophages. *J Immunol*. 2000;164:2897–2904.
 24. Blake GJ, Ridker PM. C-reactive protein and other inflammatory risk markers in acute coronary syndromes. *J Am Coll Cardiol*. 2003;41:37S–42S.
 25. Kobayashi S, Inoue N, Ohashi Y, Terashima M, Matsui K, Mori T, Fujita H, Awano K, Kobayashi K, Azumi H, Ejiri J, Hirata K, Kawashima S, Hayashi Y, Yokozaki H, Itoh H, Yokoyama M. Interaction of oxidative stress and inflammatory response in coronary plaque instability: important role of C-reactive protein. *Arterioscler Thromb Vasc Biol*. 2003;23:1398–1404.
 26. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtry AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokhi Z, Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest*. 1999;104:155–162.
 27. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med*. 1999;5:157–163.
 28. Kelley SK, Harris LA, Xie D, Deforge L, Totpal K, Bussiere J, Fox JA. Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther*. 2001;299:31–38.
 29. Secchiero P, Melloni E, Heikinheimo M, Mannisto S, Di Pietro R, Iacone A, Zauli G. TRAIL regulates normal erythroid maturation through an ERK-dependent pathway. *Blood*. 2004;103:517–522.
 30. Cretney E, Shanker A, Yagita H, Smyth MJ, Sayers TJ. TNF-related apoptosis-inducing ligand as a therapeutic agent in autoimmunity and cancer. *Immunol Cell Biol*. 2006;84:87–98.

CLINICAL PERSPECTIVE

In the present study, we have demonstrated for the first time that systemic administration of recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein showed a protective role in vivo against atherosclerosis progression induced by diabetes mellitus in the apolipoprotein E-null mice model, in which atherosclerotic lesions resemble those observed in humans in appearance and distribution. TRAIL injections also contributed to stabilize the histological conformation of the atherosclerotic plaques by selectively decreasing the number of infiltrating macrophages. This observation is extremely important if we consider that vulnerable plaques in humans are associated with myocardial infarction and death. The potential clinical relevance of these results obtained in the apolipoprotein E-null mice is corroborated by the findings that soluble TRAIL levels tend to decrease in the sera of patients affected by acute coronary syndrome and show an inverse correlation with the levels of C-reactive protein. Because treating vascular complications with recombinant proteins creates a number of problems in terms of efficacy, specificity, delivery, and duration, it is particularly noteworthy that adeno-associated virus also can be used to systemically express and release TRAIL with pharmacological activity. From a therapeutic perspective, it is noteworthy that the safety of recombinant TRAIL and agonistic monoclonal antibodies that engage the human TRAIL receptors TRAIL-R1 and TRAIL-R2 has been already examined in various studies performed on nonhuman primates and in phase 1 and 2 clinical trials in cancer patients. Therefore, TRAIL must be considered a promising therapeutic agent not only for antitumor therapy but also for its antiatherosclerotic activity.

Systemic Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Delivery Shows Antiatherosclerotic Activity in Apolipoprotein E–Null Diabetic Mice

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Circulation. 2006;114:1522-1530; originally published online September 25, 2006;
doi: 10.1161/CIRCULATIONAHA.106.643841

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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