Interferon- γ Augments CD95(APO-1/Fas) and Pro-Caspase-8 Expression and Sensitizes Human Vascular Endothelial Cells to CD95-Mediated Apoptosis

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We have examined the effects of interferon (IFN)- γ on expression and function of CD95 (APO-1/Fas) and associated proteins in cultured human umbilical vein and dermal microvascular endothelial cells (HUVEC and HDMEC, respectively). Unstimulated cells express only low levels of CD95; IFN- γ produces a time- and concentration-dependent increase of CD95 in both cell types at the mRNA and cell surface protein levels. IFN- γ also produces an increase in expression of pro-caspase-8 (FLICE/MACH) but does not significantly change expression of either Fas-associated death domain (FADD) protein or cellular FLICE inhibitory protein (cFLIP), other proteins associated with the CD95 death-inducing signaling complex (DISC). Neither resting nor IFN- γ -treated EC express detectable CD95L mRNA or protein. Untreated HUVEC and HDMEC show minimal apoptosis when transduced to express CD95L. Treatment of CD95L-transduced cells with IFN- γ causes apoptosis within 24 to 36 hours that can be blocked by antagonistic anti-CD95 antibody or by the caspase-inhibitory peptide zVAD-FMK. The extent of apoptosis is increased by co-treatment with either the protein synthesis inhibitor cycloheximide or the phosphatidylinositol 3-kinase inhibitor LY294002. Untransduced HUVEC treated with IFN- γ also undergo CD95-initiated apoptosis when mixed with CD95L-transduced HUVEC or when incubated with pharmacologically activated cytolytic T lymphocytes. Overexpression of CD95 in HUVEC confers sensitivity to CD95L in the absence of IFN- γ -treatment. We conclude that IFN- γ induces sensitivity of endothelium to CD95L-mediated apoptosis, and that this response may result from increased expression of CD95 and/or pro-caspase-8. (*Am J Pathol* 2002, 161:1485-1495)

CD95 (APO-1/Fas), a member of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor family, is a cell-surface initiator of cell death.^{1,2} Clustering of CD95 molecules by binding of either CD95 ligand (CD95L), a type II integral membrane protein belonging to TNF family,³ or agonistic multivalent anti-CD95 Abs⁴ activates a cascade of cysteine aspartate-specific proteases (caspases) that culminates in apoptosis.⁵ Effector caspase activation is initiated by assembly of a death inducing signaling complex (DISC) which consists of clustered CD95, the Fas-associated death domain (FADD) adapter protein, the initiator caspase-8 (previously called FLICE or MACH)^{6,7} and cellular FLICE inhibitory protein (cFLIP),⁸ a regulatory protein that can either block or promote autoproteolytic conversion of pro-caspase-8 to caspase-8 within the DISC.⁹ Knock out mice that are deficient in cFLIP, which die as embryos are phenotypically indistinguishable from mice lacking FADD or pro-caspase-8; however, fibroblasts derived from cFLIP $-/-$ mice show increased caspase-8 activation.¹⁰ Once caspase-8 is activated, it may directly activate effector caspases leading to apoptosis, or *via* proteolytic activation of Bid, release cytochrome c from mitochondria, resulting in the autoproteolytic activation of pro-caspase-9, an alternative initiator of cell death.¹¹ The CD95/CD95L system has previously been shown to play a critical role both in the perforin-independent killing of infected, foreign or mutated targets by cytolytic T lymphocyte (CTL) and in physiological down-regulation of immune reactions.¹²⁻¹⁴ Consequently, defects in the CD95/CD95L pathway leads to both autoimmunity and to compromised host defense.^{12,14} Although CD95L expression is largely restricted to activated lymphocytes, expression of CD95L by tissue cells may create sites of immune privilege.¹⁵ Similarly, expression by tumor cells may induce apoptosis of invading, CD95-expressing activated T cells and inflammatory cells, thereby suppressing the anti-tumor immune response.^{12,16} These obser-

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ventions have led to the proposal that transduction of graft vascular endothelial cells (EC) to express CD95L might inhibit leukocyte extravasation and thereby protect allografts from cell-mediated rejection.¹⁷

EC in their resting state are poorly interactive with leukocytes. For this reason, immune and inflammatory processes depend on cytokine-mediated activation of vascular endothelium, a process involving synthesis and expression of new adhesion molecule and chemokines.¹⁸ In the absence of sensitizing agents [eg, a protein synthesis inhibitor such as cycloheximide (CHX) or a phosphatidylinositol 3-kinase (PI3-K) inhibitor] cytokines generally do not kill EC.¹⁹ However, cytokine-activated EC may become more susceptible to killing by neutrophils.²⁰ We wondered if cytokine-activated EC would become similarly sensitive to CD95L-mediated apoptosis, a death-inducing pathway for deletion of activated lymphocytes. Previous studies have reported that CD95-expressing human EC in their unstimulated state are resistant to injury by agonistic anti-CD95 antibody,²¹ by soluble CD95L,²² or by adenoviral-transduced cell-bound CD95L.²² Interferon- γ (IFN- γ), a cytokine associated with cell-mediated immunity has been reported to up-regulate CD95 expression on cultured human EC.^{21,22} Despite this change in CD95 expression, one group of investigators reported persistent resistance to both soluble CD95L and adenoviral-transduced CD95L.²² However, this conclusion may be confounded by technical considerations. For example, it is well established that cell-bound CD95L may deliver a potent apoptogenic signal to many cells that appear resistant to anti-CD95 antibody or soluble CD95L.^{13,23,24} This problem may not be adequately addressed by use of adenoviral gene delivery of cell bound CD95L because active adenovirus infection may coincidentally reduce the susceptibility of target cells to apoptosis mediated through death receptor pathways.²⁵ In the study reported here, we have re-examined this question using both large vessel and microvascular cultured human EC. We confirm that IFN- γ treatment increases expression of CD95 and also find up-regulation of pro-caspase-8, but not of FADD or cFLIP. Most significantly, we demonstrate that IFN- γ treatment does confer sensitivity to cell-bound CD95L expressed by retroviral transduced cells or on activated CTL, in both human large vessel and microvascular EC.

Materials and Methods

Reagents and Abs

Recombinant human IFN- γ was purchased from R&D Systems (Minneapolis, MN). Mouse IgG monoclonal antibodies (mAb) NOK-1 (anti-human CD95L) and DX2 (anti-human CD95) were purchased from BD PharMingen (San Diego, CA) and mouse IgM anti-human CD95 mAb (IPO4) was purchased from Kamiya (Seattle, WA). Mouse anti-human cFLIP mAb²⁶ was kindly provided by Dr Peter H. Krammer (German Cancer Research Center, Heidelberg, Germany). Anti-human class I MHC molecule mAb (W6/32) from a clone provided by Dr. Jack Strominger

(Harvard University, Cambridge, MA) and control non-binding mouse IgG Ab (K16/16) were prepared as ascites in our laboratory. Rabbit anti-pro-caspase-8 polyclonal antibody was purchased from BD PharMingen (San Diego, CA). Rabbit anti-FADD polyclonal antibody was purchased from Chemicon International (Temecula, CA). Propidium iodide (PI) and FITC-conjugated goat anti-mouse IgG (H+L) F(ab')₂ were purchased from Boehringer Mannheim (Indianapolis, USA). Horse radish peroxidase-conjugated donkey anti-mouse and anti-rabbit secondary Abs were purchased from Jackson ImmunoResearch (West Grove, PA). Phorbol myristate acetate (PMA), the calcium ionophore ionomycin, the pharmacological inhibitor of phosphatidylinositol-3 (PI-3) kinase LY294002 (LY) and the protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma (St. Louis, MO). The broad spectrum inhibitor of caspases zVAD-FMK was purchased from Calbiochem (San Diego, CA).

Cultured Cells

Human umbilical vein EC (HUVEC) and human dermal microvascular EC (HDMEC) were isolated and cultured in accordance with protocols approved by the Yale University Human Investigation Committee. HUVEC were isolated from 3 to 5 umbilical veins, pooled and serially cultured as previously described.²⁷ HUVEC cultures were used at passage levels 3 to 6. Such cultures uniformly express von Willebrand factor and CD31 and are free from detectable CD45⁺ contaminating leukocytes. HDMEC were isolated from normal adult breast skin from reduction mammoplasties and serially cultured as described previously.²⁸ HDMEC cultures were used at passage levels 3 to 7 and were uniformly positive for CD31, CD34, endoglin, and, following TNF-treatment, for E-selectin expression. A human CTL line was produced by repeated stimulation of peripheral blood mononuclear cells with allogeneic HUVEC derived from a single donor as described elsewhere.²⁹ By four to five weeks in culture, such a CTL line consists mostly of CD8⁺CD3⁺ T cells, and lysis of stimulator cells can be blocked by mAbs to class I MHC or to CD8. The JURKAT human leukemia cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 medium containing 10% fetal bovine serum.

Construction of and Transduction with Recombinant Retroviral Vectors

The plasmids pBX-hFL1 (human CD95L at *Xba*I site of pBluescript)³⁰ and pF58 (human CD95 at *Xho*I site of pBluescript)² were kindly provided by Dr. Shigekazu Nagata (Osaka Bioscience Institute, Japan). Using the following primers 5'-GGA TCC TAG ACT CAG GAC TGA GAA G-3'; 5'-CAA CAT TCT CGG TGC CTG TAA-3', CD95L cDNA was amplified and subcloned into a TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced. The *Eco*RI-*Not*I excisable DNA insert of CD95L was further subcloned into the LZRSpBMN-Z retroviral vector (kindly provided by Dr. G. Nolan, Stanford Univer-

sity, Palo Alto, CA). The retroviral vector DNA, containing human CD95L cDNA, was transfected into the Phoenix-Ampho packaging cell line (ATCC) by lipofection with Lipofectamine Plus Reagent (GibcoBRL, Grand Island, NY) and puromycin (Clontech, Palo Alto, CA)-resistant cells were derived which served as the source of retroviral stocks. HUVEC and HDMEC were transduced without drug selection as described previously.²⁹ The CD95 coding region was amplified with two primers: 5'-GGA AGC TCT TTC ACT TCG G-3'; 5'-GCG GCC GCT TTT CAA ACA CTA AT-3') and subcloned into pFB retroviral vector (Stratagene, La Jolla, CA) and transfected into packaging cell line PA317, kindly provided by Dr. G. Nolan, (Stanford University, Palo Alto, CA). G418 (Gibco-BRL)-resistant cells were derived and used as a source of retrovirus stock. Transduction of HUVEC was accomplished by three serial infections over 1 week. In brief, PA317 culture supernatant plus polybrene (5 μ g/ml) was incubated for 6 hours with 1×10^5 HUVEC at passage one, followed by replacement of the normal growth medium. The transduction was repeated the next day and the third day. The transduced HUVEC were carried in culture for additional 2 days and then 0.5 mg/ml G418 was used for the selection. In 2 weeks, the G418-resistant transduced HUVEC clones grew out and >95% expressed CD95. Multiple independent CD95-transduced HUVEC clones were pooled for use in all of the experiments described.

FACS Analysis of Cell Surface Protein Expression

Analytic flow cytometry was performed using a FACSort cytometer (BD Biosciences, Mountain View, CA) and analyzed using Cellquest software. Briefly, cells (0.25×10^6) were detached using trypsin, washed in cold buffer consisting of PBS, 1% BSA and 0.02% sodium azide, pelleted, suspended in 30 μ l of primary Ab, either 0.3 μ g of mouse anti-human CD95 mAb DX2, 1 μ g of mouse anti-human CD95L mAb NOK-1, or 1:200 diluted control non-binding mouse IgG Ab K16/16, and incubated on ice for 30 minutes with occasional shaking. After two washes, the cells were suspended in 30 μ l of FITC-goat anti-mouse F(ab')₂ Ab (1:100 dilution) and incubated in the dark for 30 minutes on ice. The cells were washed twice, resuspended in 0.5 ml PBS containing 0.02% sodium azide and subjected to flow cytometry immediately. Background fluorescence intensity was determined using a non-binding primary control mAb (K16/16) and corrected mean fluorescence intensities were calculated by subtracting the mean values of cells stained with the irrelevant primary mAb from the mean values of cells stained with specific primary mAb.

CD95 and CD95L Detection by RT-PCR

Transcripts encoding CD95 and CD95L were assayed by RT-PCR as described previously.³¹ Briefly, total RNA was isolated from the indicated cell types by Tri-Reagent (Molecular Research Center, Cincinnati, OH) according

to the manufacturer's recommended protocol. For reverse transcription, 10 μ g of RNA was converted into single-stranded DNA by a standard 50- μ l RT reaction using the ProSTAR First-Strand RT-PCR Kit (Stratagene, La Jolla, CA) according to the manufacturer's recommended protocol. 1/20 of the total cDNA product was amplified for 26 to 40 cycles in a 30- μ l reaction mixture consisting of 1 U of *Taq* polymerase (Roche, Indianapolis, IN) and 1 μ mol/L each of sense and antisense primers: CD95 (5'-ATG CTG GGC ATC TGG ACC CT-3' and 5'-CAA CAT CAG ATA AAT TTA TTG CCA C-3'); CD95L (5'-GGA TGT TTC AGC TCT TCC AC-3' and 5'-TCT TCC CCT CCA TCA TCA CC-3'); c-FLIP (5'-GAC CCT TGT GCT TCC CTA-3' and 5'-GTT AAT CAC ATG GAA CAA TTT CC-3'); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): (5'-AGA ACG GGA AGC TTG TCA TCA-3' and 5'-GAC CTT GCC CAC AGC CTT G-3'). PCR products were run on a 1.2% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination.

Quantitative RT-PCR Analysis by iCycler

Transcripts were quantified by real time RT-PCR. Total RNA was extracted from control or IFN- γ -treated HUVEC (using Tri-Reagent) and 2 μ g of RNA was converted into single-stranded DNA by a standard 50- μ l RT reaction (using ProSTAR First-Strand RT-PCR Kit) with the protocol described above. Real time quantitative RT-PCR was performed on an iCycler iQTM Multicolor Real Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. In brief, the cDNA generated from the reverse transcription reactions was amplified by PCR with the SYBR Green PCR Core Reagents (P/N 4304886) according to the recommended protocol. Each reaction contained 2.5 μ l of cDNA sample, 0.65 U AmpliTaq, 75 μ mol/L MgCl₂, 25 μ mol/L of all dNTPs, and 15 nmol/L primers (specific for CD95, pro-caspase-8 or β -actin) in a total volume of 25 μ l. The primers sequences used were as follows: CD95, sense 5'-TCC TCC AGG TGA AAG GAA AGC TAG G-3'; anti-sense 5'-AGA TTG TGT GAT GAA GGA CAT GGC-3' (product length 146 bp); pro-caspase-8, sense 5'-AGG AAA GTT GAC ATC CTG AAA A-3'; anti-sense 5'-GGA GAG TCC GAG ATT GTC ATT A-3' (product length pro-caspase-8 α , 127 bp; pro-caspase-8 β , 173bp). β -actin, sense 5'-TGC ACC ACA CCT TCT ACA ATG A-3'; anti-sense 5'-CAG CCT GGA TAG CAA CGT ACA T-3' (product length 158 bp). All primer pairs span at least one intron to exclude contamination of genomic DNA. Each PCR cycle consisted of a denaturation step at 95°C for 30 seconds, an annealing step at 58°C for 30 seconds and an elongation step at 72°C for 30 seconds. Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double-stranded (ds) DNA. The final RT-PCR products were electrophoresed on analytic gels to confirm amplification of only a single species of the correct size. Using the iCycler data analysis software (Bio-Rad), the PCR amplify cycle plot was constructed and the threshold cycle (Tc)

was determined for each well (the threshold cycle occurs when the level of fluorescence increases significantly above the background level of fluorescence measured in the early cycles of the amplification). In the IFN- γ -treated HUVEC, up-regulation of CD95 mRNA and of pro-caspase-8 mRNA were expressed as fold induction (FI), calculated as below:

$$FI = 2^{[(-Tc \text{ CD95 IFN-}\gamma) - Tc \text{ }\beta\text{-actin IFN-}\gamma) - (Tc \text{ CD95 control} - Tc \text{ }\beta\text{-actin control})]}$$

Immunoblotting

For immunoblotting, HUVEC (4×10^5 cells/sample) were washed twice with cold PBS, before the addition of 100 μ l of lysis buffer (10 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L EDTA) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN) and 1 mmol/L phenylmethylsulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN). Cells were maintained on ice and lysates were harvested by scraping. The protein concentration of each sample was determined using a Bio-Rad protein assay (based on the Bradford dye-binding procedure) as described by the manufacturer (Bio-Rad). For each lysate, 20 μ g of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to Immobilon PVDF membrane (Millipore, Bedford, MA). After transfer, the immunoblots were blocked with 5% nonfat dry milk in Tris-buffered saline before incubating overnight with primary antibody at 4°C. After washing, blots were further incubated with 1:5000 diluted secondary Ab (goat anti-mouse or anti-rabbit IgG HRP-conjugated) for 1 hour at room temperature. After additional washing, horse radish peroxidase activity was detected by chemiluminescence using SuperSignal West Dura (Pierce, Rockford, IL) according to the manufacturer's instructions.

Assessment of CD95L-Mediated Death

To quantify death induced in cultures of CD95L-transduced HUVEC or HDMEC, transduced cells were either untreated or treated with IFN- γ (100 ng/ml) for 24 to 36 hours in culture medium. Where indicated, inhibitory antibodies or zVAD-FMK were included in the medium during the entire treatment period. Death was assessed by propidium iodide (PI) exclusion. In brief, cells were harvested and suspended in fresh medium containing 25 μ g/ml PI for 5 minutes at 37°C and then subjected to analytic flow cytometry on a FACSort immediately after labeling. A light-scatter gate was set up to eliminate cell debris from the analysis. Cellular PI fluorescence signal was recorded on the FL2 channel and analyzed by using Cellquest software. In some experiments, CD95L-transduced HUVEC were used as effector cells for killing of untransduced HUVEC, CD95-transduced HUVEC or JURKAT cells. HUVEC.LZRS, lacking CD95L, were used as effector control. In these experiments, a one-to-one mixture of effectors and targets (either mock-HUVEC, IFN- γ -pre-treated HUVEC, CD95-transduced HUVEC, or JURKAT cells) were co-cultured in six well plates in the

absence or presence of inhibitory antibodies. Death was assessed by PI exclusion after the indicated time.

To quantify CD95L-mediated cytotoxicity of HUVEC induced by CTL, we used a calcein-acetoxymethyl ester dye release assay (Molecular Probes) as described previously.²⁹ In brief, CTL were preincubated with 10 ng/ml of PMA and 0.5 μ mol/L of ionomycin for 1 hour to accentuate the CD95L-killing mechanism in CTL,³² and then added at a 15:1 effector-to-target (E/T) ratio to calcein-loaded mock-treated or IFN- γ -treated HUVEC targets in 150 μ l/microtiter well in triplicates and incubated at 37°C for 5 hours in the absence or presence of inhibitory antibodies. Retained calcein was measured using a fluorescence multiwell plate reader (Cytofluor2; PerSeptive Biosystems; excitation wavelength 485 nm, emission wavelength 530 nm). Percent specific killing was calculated as:

$$[1 - (\text{sample retained} - \text{maximal retained}) / (\text{spontaneous retained} - \text{maximal retained})] \times 100\%$$

Characterization of Cell Death by Nuclear Morphology

To characterize the pattern of cell death, nuclear morphology was assessed by 4',6'-diamidinon-2-phenylindole (DAPI) staining and fluorescence microscopy. HUVEC-CD95L plated at 2.0×10^5 cells/ml in M199 medium with 20% FCS and 0.1% ECGS in gelatin-coated 6-well plates, were allowed to attach and then incubated for 25 hours with or without 100 ng/ml of IFN- γ . Cells were then harvested and spun onto glass slides by Cytospin (Cytospin 2, Shandon, Pittsburgh, PA) for 3 minutes at 800 rpm. Cells were fixed with 100% methanol for 3 minutes at room temperature, washed in PBS for 10 minutes, air dried, and embedded in mounting medium containing 0.05% DAPI. Cells were examined and photographed with a fluorescence microscope (Microphot FXA, Nikon, Japan).

Results

CD95 and CD95L Expression in Unstimulated Human EC

The goal of this study was to determine whether IFN- γ treatment could enhance the sensitivity of human EC to CD95L-mediated apoptosis. We first examined whether cultured HUVEC and HDMEC, prepared and characterized in our laboratory, basally express either CD95 or CD95L. The initial approach was a sensitive but non-quantitative RT-PCR to examine HUVEC and HDMEC for mRNA transcripts specifically encoding CD95 or CD95L. We could readily detect CD95 mRNA in both cell types but were unable to detect CD95L mRNA in either cell type (Figure 1A). As a positive control, CD95L mRNA was detected in a human CTL analyzed in parallel. We next measured surface protein expression by flow cytometry using mouse anti-human CD95 monoclonal antibody

Figure 1. Measurement of CD95 and CD95L expression by human EC. **A:** Assessment of CD95 and CD95L mRNA expression by RT-PCR. Total RNA from HUVEC, HDMEC, or a human CTL line (positive control) was isolated and mRNA encoding CD95, CD95L or GAPDH was detected by RT-PCR. DDW indicates a negative control lane in which double distilled water was substituted for RNA. **B:** FACS analysis of cell surface CD95 expression using anti-human CD95 mAb DX2 immunostaining. Specific staining is shown as filled histograms and staining with irrelevant, nonspecific primary antibody is shown as empty histograms. Numbers represent corrected mean fluorescence intensities. **C:** FACS analysis of cell surface CD95L expression using anti-human CD95L mAb NOK1 immunostaining. Data are depicted as in panel **B**. **D:** Cytotoxicity to JURKAT measured by PI exclusion. JURKAT cells were incubated with human EC cultures and/or anti-CD95 antibodies DX2 (IgG) and IPO4 (IgM) for 15 hours and cell viability was determined by FACS analysis. The results presented in each section of the figure are representative of three independent experiments.

DX2. As shown in Figure 1B, both HUVEC and HDMEC express CD95. The level of expression varied somewhat among different HUVEC cultures, but unstimulated EC consistently expressed CD95 at a level considerably below that expressed by the JURKAT cell line (Figure 1B). These observations are consistent with previous reports by others describing low CD95 expression by cultured human EC.^{21,22} Using the same flow cytometry approach, we detected no expression of CD95L on unstimulated HUVEC or HDMEC (Figure 1, A and C), although we could detect expression by a positive control, namely HUVEC transduced with a retroviral construct (LZRS.CD95L) encoding CD95L (HUVEC.CD95L; Figure 1C). The absence of CD95L on cultured human EC differs from previous reports from another laboratory.³³

We also used a CD95L-functional assay to assess endogenous CD95L protein expression by EC, namely killing of JURKAT cells. First we confirmed that the JURKAT cells were sensitive to anti-CD95 antibody-mediated cytotoxicity, demonstrated by loss of PI exclusion in response to anti-CD95 IgM antibody IPO-4 (Figure 1D). These JURKAT cells were also killed by co-culture with HUVEC.CD95L, which could be prevented by antagonistic anti-CD95 IgG antibody DX2 (Figure 1D). Only a small fraction of JURKAT cells were killed when incubated with either untransduced HUVEC or HDMEC for 16 hours and blocking antibody DX2 did not decrease the number of dead JURKAT cells in this assay (Figure 1D). We conclude from these studies that our cultured HUVEC and HDMEC express CD95 but not CD95L.

IFN- γ -Induced Up-Regulation of CD95 Expression in EC

We next examined whether CD95 can be up-regulated in human EC by exposure to IFN- γ . As shown in Figure 2, A

Figure 2. Cell surface CD95 expression by human EC after IFN- γ treatment. HUVEC (**A**) or HDMEC (**B**) were stimulated by IFN- γ at the indicated concentrations for 22 hours, and cell surface CD95 expression was determined by indirect immunofluorescence and FACS using anti-human CD95 mAb DX2. **C:** HUVEC were stimulated with IFN- γ 100 ng/ml at the indicated time periods and cell surface CD95 expression was determined by indirect immunofluorescence and FACS analysis. Specific staining is shown in the filled histograms and irrelevant primary mAb control is shown as empty histograms. The numbers in the panels represent corrected mean fluorescence intensities. The results presented are representative of three independent experiments.

Figure 3. Real-time RT-PCR assay of CD95 and pro-caspase-8 mRNA expression. HUVEC were incubated in the absence or presence of 100 ng/ml IFN- γ for 5 hours, 12 hours, and 24 hours. mRNA for CD95, pro-caspase-8 and β -actin were assessed using an iCycler iQTM Multicolor Real Time PCR Detection System as described in Materials and Methods. **A:** PCR amplify cycle plot. **a**, β -actin; **b**, CD95; **c**, pro-caspase-8; **d**, RNA controls without the RT procedure. **B:** Fold induction of CD95 and pro-caspase-8 mRNA, normalized relative to expression in the absence of IFN- γ . One of two independent experiments with similar results.

and B, we observed a dose-dependent increase of CD95 expression on HUVEC and HDMEC after treatment with IFN- γ for 22 hours, demonstrated by FACS. There was also some variation among the levels of expression achieved in different cultures; in general HDMEC appeared to be more responsive than HUVEC in this assay. No induction of CD95L could be detected in any of the samples tested (data not shown). We then examined the kinetics of CD95 expression on HUVEC after IFN- γ stimulation. CD95 expression on HUVEC increased steadily between 3 and 22 hours of treatment (Figure 2C). As shown in Figure 3, CD95 mRNA also progressively increased in IFN- γ -treated HUVEC, suggesting that the increase of surface CD95 protein expression is the result of increased biosynthesis following IFN- γ stimulation.

Effects of IFN- γ on Other DISC Components

The CD95 DISC contains FADD, caspase-8, and cFLIP in addition to CD95. Therefore, we examined the effects of IFN- γ on these proteins in HUVEC. As shown by immunoblotting, pro-caspase-8, but not FADD or cFLIP, is up-regulated in HUVEC after incubation with 100 ng/ml IFN- γ for 20 hours or 40 hours (Figure 4). Consistent with these protein data, pro-caspase-8 mRNA level in HUVEC is also elevated after incubation with 100 ng/ml IFN- γ for 5 hours, 12 hours and 24 hours (Figure 3, A and B), although the fold increase of pro-caspase-8 mRNA is smaller than that observed for CD95.

Figure 4. Immunoblot analyses of FADD, cFLIP, pro-caspase-8, and β -actin in HUVEC. HUVEC were stimulated with or without 100 ng/ml IFN- γ for 20 hours and 40 hours, and expression levels of FADD, cFLIP, pro-caspase-8, and β -actin were evaluated by immunoblotting as described in Materials and Methods. One of three independent experiments with similar results.

IFN- γ Sensitizes Human Vascular EC to CD95-Mediated Apoptosis

We next tested whether IFN- γ increased sensitivity to anti-CD95 antibodies (DX2 and IPO-4) using the same PI exclusion assay previously applied to JURKAT cells. No death was evident when HUVEC were treated by either of two anti-CD95 antibodies following treatment with 100 ng/ml IFN- γ for 40 hours (Table 1), consistent with two previous reports that human EC resist CD95 antibody-triggered cell death.^{21,22} However, since some CD95-expressing cells resist stimulation by CD95 antibody (and soluble CD95L), yet still undergo apoptosis when exposed to cell-bound CD95L^{13,23,24} we also tested if IFN- γ could sensitize human EC to CD95 death signals when triggered by cell-bound CD95L. We initially examined this possibility by treating CD95L-transduced EC with IFN- γ . As shown in Figure 5A, about 30% of transduced HUVEC.CD95L died after stimulation with 100 ng/ml IFN- γ for 30 hours, compared to about 5% cell death in replicate cultures not treated with IFN- γ . Moreover, IFN- γ only caused death of about 7% of mock-transduced HUVEC (HUVEC.LZRS) (Figure 5A). DAPI staining showed that IFN- γ -induced HUVEC.CD95L death is predominantly apoptotic, showing nuclear condensation and fragmentation (Figure 5B). This conclusion is consistent with the observation that CD95L-mediated death was completely blocked by the broad spectrum caspase inhibitor zVAD-FMK (Figure 5A). LZRS.CD95L transduced HDMEC showed an even greater sensitivity to IFN- γ treatment than HUVEC.CD95L (Figure 5C). Importantly, IFN- γ -induced cell death of HUVEC.CD95L and of

Table 1. Effects of Anti-CD95 Abs and IFN- γ on HUVEC Viability

Treatment	Dead HUVEC (%)
None	6.1
IFN- γ	9.9
IFN- γ + CD95 IgG Ab DX2	10.6
IFN- γ + CD95 IgM Ab IPO4	8.8

After incubation with IFN- γ and CD95 Abs for 40 hours, HUVEC viability was determined by PI exclusion and FACS analysis. The concentrations of IFN- γ and CD95 antibodies were 100 ng/ml and 3 μ g/ml, respectively. One of three independent experiments with similar results.

HDMEC.CD95L could be completely inhibited by addition of 3 μ g/ml antagonistic anti-CD95 mAb DX2 (Figure 5, A and C).

Although the experiments described above show clear induction of CD95-mediated apoptosis in IFN- γ -activated human EC, the killing level of HUVEC never approached

100%. Susceptibility to CD95 signaling can be augmented in some cell types by simultaneously reducing anti-apoptotic responses with agents such as the protein synthesis inhibitor CHX or the PI-3 kinase inhibitor LY.^{34–36} As shown in Figure 5D, co-treatment of HUVEC.CD95L with 0.5 μ g/ml CHX and 100 ng/ml IFN- γ greatly increased cytotoxicity in HUVEC.CD95L compared to treatment with IFN- γ or CHX alone, and the majority of the enhanced cytotoxicity was inhibited by anti-CD95 mAb DX2 (Figure 5D). Treatment with 25 μ mol/L LY showed similar results (Figure 5D). These experiments suggest that the CD95 apoptotic pathway in IFN- γ -treated human EC is actively inhibited by *de novo* protein synthesis or by PI-3 kinase signaling.

To address if IFN- γ -treated normal HUVEC (untransduced) could also be killed by CD95L, we performed a mixing experiment. In this case, untransduced HUVEC were pre-incubated with either medium alone, or with 100 ng/ml IFN- γ for 20 hours, washed and then co-cultured 1:1 with HUVEC.LZRS or HUVEC.CD95L for an additional 12 hours. Co-culture of IFN- γ treated HUVEC with HUVEC.CD95L resulted in a reproducible increase of dead cells compared to co-cultures with HUVEC.LZRS or to co-cultures of untreated HUVEC with HUVEC.CD95L (Figure 6A). Moreover, the HUVEC.CD95L-mediated cytotoxicity to IFN- γ -treated HUVEC was markedly inhibited by 3 μ g/ml antagonistic anti-CD95 antibody DX2 (Figure 6A).

Next we examined whether CD95L expressed by CTL could also kill IFN- γ -treated EC. Killing by CTL may be confounded by several issues, the two most important of which are that IFN- γ changes the surface of the target cells to improve recognition by CTL in ways which are unrelated to CD95 expression (eg, by enhanced class I MHC molecule, adhesion molecule and chemokine expression) and that CTL can lyse HUVEC by the perforin/granzyme B pathway whether or not the CD95L pathway is functional. We adopted an experimental strategy to circumvent these issues. Specifically, we preactivated our CTL by incubation with PMA and ionomycin for 1 hour before addition to target cells. This treatment causes discharge of prestored perforin and granzyme B before exposure to the target cell, reducing the contribution of this pathway. It also enhances the contribution of CD95L

Figure 5. IFN- γ -induced sensitivity of human EC to CD95-mediated apoptosis. **A:** HUVEC.CD95L were treated with 100 ng/ml IFN- γ for 30 hours, in the presence or absence of 3 μ g/ml anti-CD95 antagonistic mAb DX2, irrelevant IgG or the caspase inhibitor zVAD-FMK (50 μ mol/L) and the cell viability was determined by PI exclusion staining as described in Materials and Methods. Mock-transduced HUVEC.LZRS are shown as a negative control. The results presented here are representative of three independent experiments. **B:** HUVEC.CD95L were incubated with or without 100 ng/ml IFN- γ for 25 hours, and nuclear morphology was examined by DAPI-staining as described in Materials and Methods. Note the nuclear condensation and fragmentation in the IFN- γ -treated group, indicative of apoptosis. This experiment was performed twice with similar results. **C:** HDMEC.CD95L were treated with 100 ng/ml IFN- γ for 30 hours, in the presence or absence of 3 μ g/ml anti-CD95 blocking antibody DX2 or normal IgG, then the cell viability was determined by PI exclusion staining. One of three independent experiments with similar results. **D:** HUVEC.CD95L were stimulated by 100 ng/ml IFN- γ for 30 hours, in the presence or absence of 3 μ g/ml mAb DX2, 0.5 μ g/ml CHX or 30 μ mol/L LY, then the cell viability was determined by PI exclusion staining. Note that both CHX and LY enhance CD95L-mediated cytotoxicity to IFN- γ -treated cells. One of three independent experiments with similar results.

Figure 6. Effect of IFN- γ on the sensitivity of normal (untransduced) HUVEC to CD95L. **A:** Normal HUVEC were treated with 100 ng/ml IFN- γ for 20 hours, washed, and mixed with HUVEC.CD95L or HUVEC.LZRS (empty vector) at a 1:1 ratio, in the presence or absence of 3 μ g/ml anti-CD95 mAb DX2. After an additional 12 hour-incubation, the cell viability was determined by PI exclusion staining. One of three independent experiments with similar results. **B:** Normal HUVEC were treated with 100 ng/ml IFN- γ for 20 hours, washed and labeled with calcein-acetoxymethyl ester. Human CTL were preincubated with 10 ng/ml PMA and 0.5 μ mol/L ionomycin for 1 hour and then mixed with HUVEC at a 15:1 E/T ratio, in the presence or absence of 3 μ g/ml anti-CD95 mAb DX2 or isotype control Ab (mouse IgG). After a 5-hour incubation, the HUVEC viability was determined by measuring the retained calcein using a fluorescence multiwell plate reader as described in Materials and Methods. The unstimulated CTL clone showed less than 4% lysis in these experiments (data not shown). One of three independent experiments with similar results.

by causing translocation to and expression on the CTL surface of preformed CD95L molecules. Moreover, pre-activated CTL no longer require antigen recognition to be triggered, bypassing the effects of IFN- γ on target cell MHC molecule expression and probably reduce the role of inducible chemokines and adhesion molecules by causing LFA-1 on the CTL to assume a high affinity state. As shown in Figure 6B, PMA and ionomycin-activated CTL are more effective at lysis of IFN- γ -pretreated versus mock treated HUVEC and lysis is completely abrogated by DX2, the antagonistic anti-CD95 mAb. Cumulatively, these results demonstrate that human vascular EC do become susceptible to CD95-mediated apoptosis triggered by cell-bound CD95L when activated by IFN- γ .

Figure 7. Effect of CD95 overexpression on CD95L-induced cytotoxicity in HUVEC. CD95 expression on HUVEC, on HUVEC.pFB and on HUVEC.CD95 was determined by indirect immunofluorescence and FACS analysis using anti-human CD95 mAb DX2. The sensitivity of these three cultures to CD95-mediated apoptosis, was determined by PI exclusion staining after co-culture with HUVEC.CD95L effector at a 1:1 ratio. Similar results were obtained in three independent experiments.

Relationship of CD95 Expression Levels to CD95L Sensitivity

Thus far we have shown that IFN- γ treatment results in CD95 up-regulation and in enhanced susceptibility to killing by membrane-bound CD95L. However, we have not rigorously demonstrated that these changes are causally related. To explore this question, we generated HUVEC cultures which over-express CD95 by retroviral transduction. As shown in Figure 7, HUVEC transduced with retroviral construct pFB-CD95 (HUVEC.pFB-CD95) expressed much higher levels of CD95 than mock-transduced HUVEC (HUVEC.pFB). When these EC were co-cultured with HUVEC.CD95L, there was an increase of dead cells in the HUVEC.pFB-CD95 populations (18.7%) compared with HUVEC pFB- HUVEC (4.3%). Once again, this killing was mediated by CD95 since it could be inhibited by antagonistic anti-CD95 antibody DX2 (Figure 7).

Discussion

While the function of the CD95-CD95L system in the apoptosis of lymphocytes is well established, its role in non-lymphoid tissues is less clear.^{37,38} Our data demonstrate that human EC can be sensitized by IFN- γ and killed by membrane-bound CD95L, although not by an agonistic CD95 antibody. Microvascular cells seem to be even more responsive than large vessel cells based on our comparison of HUVEC and HDMEC. The mechanism behind the IFN- γ -mediated up-regulation of CD95 death pathway in human EC may be due to induction of CD95 and pro-caspase-8, although additional actions of IFN- γ cannot be excluded. Notably, we did not observe IFN- γ -mediated reduction in the level of cFLIP.

The level of apoptosis we observed in IFN- γ -treated HUVEC in the absence of sensitizing agents was modest, eg, about 30%. Both CHX and LY294002 further enhanced CD95-mediated HUVEC apoptosis after IFN- γ pretreatment (Figure 5D). These agents have been reported to sensitize cells to apoptosis through down-regulation of cFLIP expression.^{39,36,40} We have confirmed that CHX does reduce cFLIP expression in HUVEC, but we have found that LY-treated EC, like IFN- γ -activated EC, retain cFLIP expression (L.A. Madge et al, manuscript in preparation). There has been considerable recent controversy regarding the function of cFLIP, with some groups reporting that cFLIP proteins act as activa-

tors of apoptosis pathways and others finding that they inhibit apoptosis.¹² The current evidence seems to suggest that cFLIP may be either pro-apoptotic or anti-apoptotic depending on the cellular context.¹² Of note, cFLIP knockout mice have a phenotype resembling that of FADD or pro-caspase-8 knockout mice, but fibroblasts isolated from knockout mice embryos show enhanced caspase-8-dependent lysis.¹⁰ In any event, our findings of IFN- γ -induced sensitivity to CD95L show that it is unnecessary for cFLIP to be down-regulated in HUVEC to allow CD95-based apoptosis to occur.

The limited degree of susceptibility of IFN- γ -treated HUVEC to CD95L in the absence of sensitizing raises two questions. First, is this level of killing significant? We believe it is because a 30% loss of EC in a large vessel is likely to produce thrombosis *in vivo*. Moreover, the susceptibility of microvascular EC is much higher. Second are there *in vivo* equivalents to metabolic inhibitors or PI-3 kinase inhibitors? This is less clear, but the actions of these drugs do raise the possibility of enhanced EC sensitivity to CD95L in pathological settings.

IFN- γ has previously been reported to induce expression of CD95 in cultured human EC. We have confirmed this finding and also show that pro-caspase-8 is induced at the mRNA and protein levels in HUVEC stimulated by IFN- γ . An effect of IFN- γ on pro-caspase-8 expression has recently been reported in human breast tumor cell lines MCF-7 and MDA-MB231.⁴¹ Pro-caspase-8 is rapidly recruited to a DISC on ligation of CD95 at the cell surface, by either CD95L or agonistic CD95 antibodies,^{6,7} where it is autoproteolytically processed to generate the active caspase-8, and transduction of pro-caspase-8 has been reported to induce apoptosis in certain tumor cell lines.^{6,7} According to the induced-proximity model for caspase-8 activation, locally high concentration of the pro-caspase zymogen within the DISC leads to autoprocessing resulting in active caspase-8.⁴² It is therefore possible that the increased expression of CD95 and pro-caspase-8 in IFN- γ -treated HUVEC might facilitate recruitment and thus activation of caspase-8 on death receptor activation. Similarly high levels of death receptor expression may cause spontaneous activation independent of the presence of ligand. A high level of spontaneous apoptosis may explain why we had difficulty producing CD95-transduced HUVEC with LZRSpBMN-Z retroviral vector. The approach we eventually used, involving selection of clones, may have inadequately selected for cells with lower degrees of spontaneous activation.

The level of basal CD95 expression and the levels induced by IFN- γ show variability among HUVEC cultures as illustrated in Figures 1B and 2. We have not noted changes in these responses of HUVEC within individual cultures with passage level. This is not surprising since other cytokine-induced molecules show variability in their basal and inducible levels, apparently determined by genetics.⁴³ We also noted that a greater response of HDMEC than HUVEC to IFN- γ . This is consistent with a previous report that cardiac microvascular EC are more susceptible than coronary artery EC, measured as IFN- γ -induced expression of MHC class II molecules.⁴⁴ In our

studies, we have focused on a role of CD95 as a receptor for signals delivered by CD95L. It is formally possible that CD95L could be a receptor for signals provided by CD95, potentially mediating signals from EC to be delivered to T cells. However, no specific signaling pathways have been shown to be activated by clustering of CD95L within T cells, reducing the likelihood of significant reverse signaling *in vivo*.

Even though IFN- γ -treated HUVEC are susceptible to cell-bound CD95L (Figures 5–7), we found that they continued to resist agonistic anti-CD95 antibodies (Table 1). This difference probably reflects the higher efficacy of signaling by cell-bound CD95L. Antibody agonists and natural ligands may actually stimulate different CD95 signaling pathways.⁴⁵ More recent studies comparing physiological CD95L with an anti-CD95 antibody have suggested that membrane-bound CD95L or aggregated soluble CD95L are better able to cluster CD95 molecules.⁴⁶ It is possible that CD95-expressing cells which are resistant to anti-CD95 antibody, might express molecules that block CD95 aggregation, such as homologues of silencer death domains (SODD), a cytoplasmic inhibitor of spontaneous TNF-R1 aggregation.⁴⁷

Our results demonstrating that IFN- γ -treated HUVEC and HDMEC are susceptible to retrovirus-transduced CD95L differ from those reported previously by others, involving HUVEC that are adenovirus-transduced to overexpress CD95L.²² We do not know whether this difference resides in the state of the EC cultures or whether it can be attributed to differences in the retroviral versus the adenoviral approach. Replication defective retroviruses stably integrate into the target cell genome, and express only the transduced gene product and do so at the physiological levels. In contrast, replication-defective adenoviruses persist as metabolically active virions in the cytoplasm, express viral as well as transduced proteins. Adenovirus vectors typically express transduced proteins at superphysiological levels. Overexpression of CD95L may lead to shedding of this protein, and soluble CD95L may act as an antagonist of cell-bound CD95L. Active adenoviruses may trigger an anti-viral response that could alter cellular physiology. Also adenoviral gene products, such as E1B19K, may actively antagonize death receptor-mediated apoptosis.²⁵ A possible objection to results obtained by the retroviral approach is that retroviral integration at particular genome sites may disrupt cellular functions. Our approach, using a population-based infection rather than drug selection of particular clones,²⁹ avoids this issue. In any event, we not only demonstrated IFN- γ -induced sensitivity to transduced CD95L, but also to CD95L expressed by activated CTL, a natural source of this molecule.

Our findings also differ from a previously published report that normal cultured human EC basally express CD95L.^{22,33} Using three experimental approaches, RT-PCR, flow cytometry, and a functional assay (based on triggering CD95-mediated JURKAT apoptosis), we failed to detect any CD95L expression by HUVEC or HDMEC (Figure 1, A, C, and D). The absence of CD95L in cultured human EC is consistent with the general conclusion that CD95L expression is restricted to a few tissues such

as lymphoid organs, lung, small intestine and testis, whereas kidney, liver, skeletal muscle and heart do not express CD95L at all.^{3,48} A possible source of experimental error may be the choice of anti-CD95L antibodies used in the previous analyses. CD95L antibodies used in many studies have been now shown to lack specificity. For example, mouse mAb (mAb33) from Transduction Labs and rabbit anti-human CD95L antibodies C-20 and N-20 from Santa Cruz Biotechnology stain CD95L-transfected and untransfected cells to a similar extent.⁴⁹ The studies by Sata et al used mAb33 and C-20 to show CD95L expression by HUVEC.^{22,33} However, these investigators also used three other reagents against mouse CD95L that are thought to cross-react with human CD95L. It therefore seems most likely that vascular EC may express CD95L under conditions present in their cultures but lacking in our culture system. For example, endothelial growth factors at a high concentration in the medium may activate EC and induce CD95L as reported by Suhara et al.³⁶ However, the key questions, as yet unanswered, are whether human EC can express CD95L *in vivo* and if so, under what circumstances. Our data suggest that if human EC do express CD95L *in vivo*, then exposure to IFN- γ could produce injury through a CD95-CD95L based pathway of cell suicide or fratricide, without requiring CD95L expressed by neutrophils, macrophages and activated T cells.

Finally, the results reported here are relevant to transplantation. There is now substantial experimental evidence to indicate that IFN- γ is an important mediator of chronic graft vasculopathy in coronary arteries,^{50,51} although the mechanism of this effect is unclear. Human allograft coronary arterial EC express HLA-DR molecules, a response dependent on IFN- γ .⁵² In this context, it is interesting to note that both CD95 expression and apoptosis of EC have been observed in graft coronary arteries undergoing chronic rejection.⁵³ Thus it is reasonable to suppose that CD95 and pro-caspase-8 expression in allogeneic EC, like HLA-DR molecule expression, are enhanced by IFN- γ exposure. In this manner, IFN- γ may promote EC injury, triggering a fibro-proliferative repair process that contributes to graft vasculopathy. Our findings of IFN- γ -mediated sensitivity to CD95L also suggest that considerable caution is in order for pursuit of the strategy of introducing exogenous CD95L into arterial EC as a mean to protect human allografts from rejection reactions.¹⁷

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