1 Introduction

Endothelial cells and macrophages/MΦ are members of the reticuloendothelial system and share properties such as phagocytosis, antigen presentation, and involvement in coagulation and inflammatory mechanisms [1-3]. Monocytes are continuously produced in the bone marrow, but MΦ do not normally accumulate to large numbers in peripheral tissues. The molecules regulating monocyte production have been intensively studied, but the mechanisms eliminating the approximately 1 x 10^10 monocytes produced daily [4] have not been well characterized. Similarly, the endothelium undergoes remodeling during development, inflammation and wound healing, with proliferation followed by endothelial cell death [5]. Again, signals promoting endothelial cell proliferation have been intensively studied, but signals that mediate endothelial cell death have not been well characterized.

One possible clue to mechanisms involved in MΦ and endothelial cell elimination comes from studies on T cells. We reported that MΦ undergo programmed cell death, or apoptosis, after activating CD4+ T cell clones [4], although the mechanism triggering the apoptotic response was not identified. One molecule potentially involved in this response is Apo-1/Fas. Fas, a member of the TNF receptor gene family, is a 43-kDa membrane protein that can transmit an apoptosis-inducing signal [6]. Interestingly, activated T cells express a functional Fas ligand [7], raising the possibility that T cells induce MΦ apoptosis through Fas. However, Fas expression and function on MΦ has not been well characterized. Similarly, T cells lyse endothelial cells in an MHC-specific fashion [8]. Since MΦ and endothelial cells share the function of antigen presentation, it is possible that Fas-mediated apoptosis is involved in the elimination of both cell types.

In these studies we used confocal laser microscopy, flow-cytometric analysis and immunoblotting to compare Fas expression and function on MΦ and endothelial cells. We demonstrate that a subset of endothelial cells and MΦ express the Fas molecule. In addition, IFN-γ is shown to cause an increase in Fas expression on both cell types. However, ligation of endothelial cell Fas does not result in cell death, while MΦ readily undergo apoptosis following Fas ligation. These observations may have relevance to understanding mechanisms regulating homeostasis of both cell types.

2 Materials and methods

2.1 Endothelial cell culture and treatment

Cultures of human umbilical vein endothelial cells (HUVEC) were established according to the method of Jaffe [9] and maintained as previously described [10]. Cultured cells were identified as endothelial on the basis of typical morphology at confluence, and by positive immunofluorescence with antibody to human von Willebrand factor (CalBiochem, San Diego, CA). Proliferation of HUVEC was induced with basic fibroblast growth factor (10 ng/ml, Synergen, Boulder, CO) supplemented with bovine lung heparin 0.1 μg/ml (Sigma Chemical Co., St. Louis, MO). Growth factors were withdrawn 24 h preceding assessment of Fas expression and other assays. Where indicated, media was replaced with fresh medium containing recombinant human IFN-γ 10-1000 U/ml (Boehringer Mannheim, Indianapolis, IN) for 18-24 h. Fixation was performed with 1% formaldehyde in PBS for 18 h at 4°C.
2.2 Isolation of MΦ and treatment

PBMC were isolated from the venous blood of healthy volunteers and MΦ isolated by adherence as described [4, 11]. MΦ were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with FCS, penicillin and streptomycin as before [4, 11]. Where indicated, IFN-γ was added as described above.

2.3 Detection of Fas by flow cytometry

Monoclonal antibodies against Fas were obtained from Kamiya Biochemicals (Japan) or Upstate Biotechnology (Lake Placid, NY). Both antibodies are IgM and of murine origin, and recognize the same epitope on Apo-I/Fas. For flow-cytometric detection, cells were incubated with anti-Fas (5 μg/ml) in standard buffer (PBS supplemented with 1% horse serum and 0.1% sodium azide) at room temperature for 1 h, washed with standard buffer, then incubated with 1:250 dilution of FITC-conjugated goat anti-mouse Ig (GAM-FITC) (Coulter, Hialeah, FL) for 1 h at room temperature. Negative controls included isotype-matched myeloma proteins (MOPC 104e, from Sigma) and positive controls included a mAb to framework determinants on class I MHC molecules (AMAC, Westbrook, ME). FITC-labeled cells were detected using an Epics Elite flow cytometer (Coulter) equipped with a 15-mW argon laser.

2.4 Quantitation of Fas using confocal laser cytometry

For confocal imaging, freshly isolated MΦ or primary HUVEC cultures were seeded into gelatin-coated two-well Lab-Tek coverglass chambers (Nunc, Inc., Naperville, IL) and incubated as described above. Confocal imaging was performed on an ACAS 570 laser cytometer (Meridian Instruments Inc., Okemos, MI) using a 1600-μm pinhole and an argon laser set at 100 mW with scanning strength set at 5%, with a 10% neutral density filter. FITC was excited at 488 nm and the emission detected with a photo-multiplier tube using a 575-nm dichroic short pass and 530-nm band pass filter. A 360-μm × 360-μm area was scanned at 1 μm step size and 32 samples/point. At least five areas randomly selected in each sample were analyzed and each experimental condition was measured in triplicate. Average fluorescence per cell was obtained by dividing the total integrated value by the number of data points in that cell. All experiments were repeated at least twice.

2.5 Measurement of MΦ cytotoxicity

MΦ cytotoxicity was measured by 51Cr release as previously described [4, 11]. MΦ apoptosis was confirmed by isolating DNA fragments followed by fractionation through agarose gels as described [4]. Briefly, 5 × 105 MΦ were cultured for 18 h with 2.5 μg/ml anti-Fas or an isotype-matched myeloma protein (MOPC 104e). The cells were recovered, washed, then lysed with buffer containing 0.2% Triton X-100. Chromatin was sedimented by centrifugation, and fragmented DNA precipitated from the cytosolic fraction with ethanol. DNA was extracted and purified from the precipitate, then fractionated by electrophoresis through 1.2% agarose gels and stained with ethidium bromide.

2.6 Measurement of endothelial cytotoxicity from Fas

Killing of endothelial cells was measured using a fluorescent technique that assesses survival of viable cells. Calcein AM (Molecular Probes, Eugene, OR) is a non-fluorescent non-polar esterase substrate that passively crosses cell membranes, and is cleaved by cytoplasmic esterases releasing a polar fluorescent product that is retained by cells with intact plasma membranes. Only viable cells generate and retain fluorescent calcein, the basis of the viability test.

HUVEC were seeded at 20,000 cells per well into gelatin-coated (Sigma, 0.2% for 10 min) 96-well tissue culture plates (Corning, Corning, NY), and allowed to form confluent monolayers for 48 h before being used. Cells were exposed to Fas antibody or other stimuli in culture medium. After incubation the cells were washed by replacing the medium twice with PBS. Cells were then exposed to 1 μM Calcein AM in PBS for 30 min at 37°C, and fluorescent emission detected with a CytoFluor 2300 plate fluorometer (Millipore Inc., Bedford, MA), using 485 nm excitation, and 530 nm emission filters. Microwells containing known numbers of cells were used to generate a standard curve of fluorescence versus cell number. This technique is capable of accurately quantitating viable cells over a range from 200 to 100,000 cells per well (unpublished data).

2.7 DNA analysis of apoptosis by flow cytometry

HUVEC were first incubated with IFN-γ for 18 h as described above to augment Fas expression. Control and IFN-γ-treated cells were then incubated with anti-Fas (2.5 μg/ml) at 37°C for 1 h. Where indicated, the anti-Fas was cross-ligated with anti-IgM (5 μg/ml) (Sigma) for 4 and 18 h. Cultured MΦ were similarly treated with anti-Fas, but without anti-IgM. All cells were then washed with PBS and stained with 10 μM Hoechst 33342 at 37°C for 30 min. Hoechst 33342 was excited at 325 nm by a 30-mW He-Cd laser, and fluorescence detected with a 470-nm dichroic long pass filter together with a 460-nm band pass filter.

2.8 Immunoblotting

HUVEC for immunoblotting were grown to confluence in gelatin-coated 10-cm diameter tissue culture dishes (Corning). Selected dishes were treated with IFN-γ 100 U/ml for 24 h. Culture medium was removed by washing twice with PBS, and the cells lysed in situ with 1 ml of 0.5% sodium dodecyl sulfate containing 1 μg/ml leupeptin, 1 μg/ml apropin, 10 μg/ml soy bean trypsin inhibitor, and 1 μg/ml pepstatin. After 30 min at 4°C, the lysate was collected into a syringe, the DNA was sheared by passing repeatedly through a 26-gauge needle, the protein content measured with BCA protein assay reagent (Pierce, Rockford, IL), and kept frozen at -80°C.

Aliquots (100 μg) of lysates (representing approximately 106 cells) were electrophoresed through a 4–20% linear gradient SDS polyacrylamide gel, and electrotransferred to nitrocellulose membranes (0.45 μm, Schleicher and Schuell, Keene, NH). Residual protein binding sites were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS, 0.15 M sodium chloride, 0.02 M Tris, pH 7.6). After
washing, Fas antibody or a control IgM myeloma protein was added in fresh blocking solution at 10 μg/ml, and incubated at room temperature for 8 h. Membranes were washed in TBS supplemented with Tween 20 0.05% (TTBS), incubated 1 h at room temperature with horseradish peroxidase-conjugated rabbit antibody to mouse IgM (Cappel, Malvern, PA) diluted 1/3000, the blot again washed with TTBS, and antibody binding detected using enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL).

3 Results
3.1 Cultured endothelial cells and MΦ express the Fas antigen

Initial experiments determined if cultured HUVEC and MΦ expressed the Fas antigen. To exclude the possibility that cells released from the culture plates with proteolytic enzymes have altered Fas expression, adherent cells were first analyzed in situ using confocal laser microscopy, then results compared to conventional flow cytometric analysis. In Fig. 1a and b, Fas expression on HUVEC and MΦ is quantitated by laser microscopy. A subset of HUVEC express Fas which in serial experiments, expression was of a relatively low level. MΦ expressed relatively more Fas. Fig. 1c and d shows a similar experiment using flow cytometry to measure Fas expression. Compared to negative controls, approximately 18% of the HUVEC expressed Fas, while 36% of the MΦ expressed Fas.

It has been reported that IFN-γ can increase Fas expression in some cell types [12], so the effect of this cytokine on HUVEC and MΦ Fas expression was examined. Fig. 2 shows that 100 U/ml IFN-γ causes a small (≤ threefold) but reproducible increase in Fas (mean channel fluorescence 0.78 on untreated HUVEC, and 2.27 on HUVEC treated with 100 U/ml IFN-γ for 48 h) using flow cytometry. Similar

Figure 2. Relative expression of Fas on HUVEC after incubation in control medium for 48 h (A), or treated with IFN-γ, 100 U/ml for 4 h (B), 24 h (C) or 48 h (D). Flow-cytometric analysis shows reactivity of HUVEC with MOPC 104e (solid line, anti-Fas (bold-face line) and anti-HLA (dotted line).
results were seen using 10 and 1000 U/ml IFN-γ (not shown). Note that class I MHC also increases in expression following IFN-γ treatment, as described earlier [13]. IFN-γ induced a similar increase in MΦ Fas expression as well (mean fluorescence 1.45 on untreated MΦ and 2.74 on MΦ treated with 1000 U/ml IFN-γ for 24 h). In a confirmatory experiment, performed using confocal laser microscopy, HUVEC were treated with 10–1000 U/ml IFN-γ for 24 h. Again, a distinct (two- to threefold) increase in Fas expression relative to untreated cells was seen (not shown).

Figure 3. MΦ death induced with anti-Fas. MΦ were labeled with 

\[ ^{51} \text{Cr} \], then incubated 18 h with the indicated concentrations of anti-Fas or MOPC 104e. Radioactive 

\[ ^{51} \text{Cr} \] release was then measured as described in Sect. 2.5. Results represent the mean ± SEM of quadruplicate determinations.

3.2 Anti-Fas is cytotoxic to MΦ but not to endothelial cells

The ability of anti-Fas to induce apoptosis was then compared using MΦ and HUVEC. Fig. 3 compares MΦ cytotoxicity induced by MOPC 104e with anti-Fas as measured by chromium release. A maximum in cell death of approximately 30% was induced by 2.5 μg/ml anti-Fas, equivalent to the number of cells expressing Fas. No further increase was seen if the MΦ were pretreated for 18 h with IFN-γ (not shown). DNA fragmentation studies demonstrated the expected nucleosome polymer “ladder”, confirming that MΦ death was mediated by apoptosis (not shown). MΦ cultured alone demonstrated a low-level spontaneous apoptosis, as described by others [14]. The addition of anti-Fas increased apoptosis as measured by DNA fragmentation. In contrast to the effect on MΦ, no cytotoxic effect was seen on HUVEC treated with up to 5 μg/ml anti-Fas, a saturating concentration of antibody as shown by fluoro-immunoassay (results not shown). These experiments were performed with and without IFN-γ pretreatment to increase HUVEC Fas expression, with no inducible cytotoxicity. Additional experiments were performed to determine whether a cytotoxic effect of Fas ligation could be exposed by co-treatment with a range of other potentially cytotoxic agents. Exposing HUVEC to Fas antibody (5 μg/ml) did not have any additional effect on the cytotoxicity induced by treating HUVEC with cycloheximide or actinomycin D over a dose range from 0.0001 to 10 μg/ml, and for times ranging from 4 to 24 h.

The effect of anti-Fas was also compared on MΦ and HUVEC using flow cytometry to measure DNA fragmentation (Fig. 4). In agreement with the studies using ethidium bromide-stained gels, the MΦ cultured for 18 h demonstrated a small population (13%) spontaneously undergoing apoptosis (Fig. 4a). The addition of anti-Fas increased the proportion of cells with degraded DNA to

Figure 4. Flow-cytometric DNA analysis of the effects of anti-Fas on MΦ and HUVEC. (A) MΦ were incubated with MOPC 104e for 18 h, then incubated with PI and analyzed by flow cytometry. G1 refers to cells in phase G1 of the cell cycle. (B) HUVEC were incubated with 100 U/ml IFN-γ for 18 h and anti-μ for 4 h, then similarly treated. (C) IFN-γ-treated HUVEC were incubated with anti-μ for 18 h and similarly stained. (D) MΦ were incubated with anti-Fas for 18 h. Sub G1 identifies the apoptotic population with less than 2 N DNA. (E) IFN-γ-treated HUVEC were incubated with anti-Fas for 1 h, washed, then incubated with anti-IgM for 4 h. (F) IFN-γ-treated HUVEC were incubated with anti-Fas for 1 h, washed, then cultured with anti-IgM for 18 h. No apoptosis is seen.
In contrast, 5 μg/ml anti-Fas did not induce apoptosis in IFN-γ treated HUVEC at 4 (Fig. 4e) or 18 h (Fig. 4f). In these experiments, the anti-Fas was cross-linked with anti-IgM to address the possibility that the low epitope density of Fas on HUVEC prevented the cross-linking required to induce apoptosis [15]. Since HUVEC Fas did not transmit an apoptosis-inducing signal, we addressed the possibility that the anti-Fas antibody was detecting a non-Fas-related cross-reactive protein. Immunoblotting was used to confirm HUVEC Fas expression. Fig. 5 shows an immunoblot of untreated and IFN-γ treated HUVEC, reacted with anti-Fas. The expected Fas antibody-reactive band at 43-kDa was seen on the HUVEC blots. No reactivity was seen in this or any other region with the use of an equivalent concentration of control IgM antibody. An additional band was seen at 105-kDa, consistent with a previous report of multiple immunoreactive isoforms of the Fas antigen [16]. The larger band was of considerably less intensity than the 43-kDa band, but the significance of this is difficult to interpret due to the expected relatively incomplete transfer of high molecular weight proteins from the gel to the supporting membrane.

Figure 5. Immunoblots of HUVEC with anti-Fas. HUVEC were cultured in the presence or absence of IFN-γ for 24 h. Cell lysate protein was electrophoresed (100 μg) through a 4–20% linear gradient SDS-PAGE gel, electrotransferred to nitrocellulose, and probed with either an IgM myeloma protein or anti-Fas. Lanes a and c: untreated HUVEC. Lanes b and d: IFN-γ-treated HUVEC. Lanes a and b: anti-Fas. Lanes c and d: control IgM.

4 Discussion

Previous investigators have demonstrated Fas expression by a variety of tissues and cell lines, including lymphocytes [17], heart, liver, lung, ovary [12], some hematopoietic [6] and non-hematopoietic cell lines [18], and a wide variety of epithelial cells [19]. Our experiments demonstrate that a subpopulation of HUVEC and freshly isolated monocytes/MΦ also constitutively express the Fas antigen. An earlier report used immunohistochemistry to show that the in situ microvasculature within the CNS, skeletal muscle and placenta express a variable amount of Fas [19], while endothelial cells from other tissues do not. The same authors also demonstrated that sinus histiocytes and "starry sky" MΦ expressed the Fas antigen, but freshly isolated monocytes/MΦ and HUVEC were not examined.

Fas expression on HUVEC and MΦ was increased by treatment with IFN-γ. The increase was not large in magnitude (two- to threefold), but was reproducible in serial experiments and using two imaging techniques. This finding is also consistent with other reports documenting the effects of IFN-γ on Fas expression in other cells [12]. However, despite expression of Fas on HUVEC, and its up-regulation with IFN-γ, we were unable to induce endothelial apoptosis using anti-Fas. A similar finding has recently been reported in certain malignant cell lines [18] and in freshly isolated T and B cells [17]. It is clear that endothelial cells undergo apoptosis in some circumstances, such as stimulation with TNF [20], indicating that failure of Fas ligation to induce apoptosis is not due to an inherent inability of this cell type to undergo an apoptotic response. In the present studies, the concentration of anti-Fas used was greater than that needed to trigger apoptosis in human MΦ, suggesting that failure to induce apoptosis was unlikely to be due to non-saturating amounts of anti-Fas. The possibility that Fas cross-linking was required, and that the low epitope density of Fas on HUVEC prevented cross-linking, was considered. However, adding anti-IgM to induce cross-linking had no effect on apoptosis, arguing against this possibility. Additional studies demonstrated no additive effect with cycloheximide or actinomycin D. Others have reported that the Bcl-2 gene product can inhibit apoptosis induced by some signals [21], but more recent reports demonstrate that Bcl-2 at best only partially inhibits Fas-induced apoptosis [18, 22], making this an unlikely explanation for our findings. A20 inhibits apoptosis, but is expressed only transiently in HUVEC following TNF treatment [23, 24], Bcl-x also inhibits apoptosis [25], but is not expressed in HUVEC (V. Dixit, personal communication). At this time, the reason that Fas is expressed in HUVEC, but does not induce apoptosis, is unknown. Possibilities include a requirement for a second signal, that the endothelial Fas molecule expressed represents a variant and nonfunctional form of the Fas molecule, or that the cells express other molecules which could be inhibiting Fas-induced apoptosis. Since the integrity of the vascular endothelium is important to the normal functioning of blood vessels, it is perhaps not surprising that Fas function is tightly regulated, to prevent inadvertent cell death and subsequent vascular pathology.

In contrast to freshly isolated B and T cells and cultured endothelial cells, freshly isolated monocytes, as well as cultured monocytes/MΦ, underwent apoptosis following Fas ligation. The reason why MΦ should express a functional Fas molecule, while unstimulated T cells, B cells and cultured endothelial cells do not, is not completely clear. However, recent studies suggest a possible explanation. We have reported that MΦ die by apoptosis after activating CD4+ T cell clones [4]. We proposed that this mechanism is involved in both preventing continued activation of CD4+ cells after the source of the initial antigenic challenge has
been removed, and in preventing the accumulation of MΦ which would otherwise occur from the large number of monocytes produced daily by the bone marrow [26]. Since activated T cells have recently been shown to express the Fas ligand [7], it is possible that MΦ Fas is stimulated by T cells, triggering apoptotic death.

It is also possible that T cell-triggered MΦ apoptosis has a pathogenic role in systemic lupus erythematosus (SLE). In contrast to recent findings in the MRL/lpr mouse [27], apoptosis actually appears to be increased in patients with active SLE. Numerous reports document that SLE patients have nucleosome-sized DNA fragments circulating in peripheral blood, and that PBMC from SLE patients will spontaneously release nucleosome-sized DNA fragments [28–32]. Furthermore, we have reported that T cells from patients with active SLE will spontaneously lyse autologous MΦ [11]. It is possible that Fas-ligand interactions occurring between T cells and MΦ contribute to the release of DNA fragments seen in these patients.

In summary, ligation of the Fas molecule has different effects on MΦ and endothelial cells. The differences in effects suggest that different mechanisms are involved in the elimination of these cells in vivo.

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5 References