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Effect of Redox Modulation on Xenogeneic Target Cells: The Combination of Nitric Oxide and Thiol Deprivation Protects Porcine Endothelial Cells from Lysis by IL-2-Activated Human NK Cells

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Evidence suggests that NK cells contribute to the pathogenesis of delayed rejection of vascularized xenografts, and NK cells have been suggested to participate in hyperacute xenograft rejection. Endothelial cells have been shown to be the primary target of the recipient’s immune responses that mediate both hyperacute and delayed xenograft rejection. Under conditions of oxidative stress induced by thiol deprivation, but not under normal conditions, pretreatment of porcine aortic endothelial cells (PAECs) with the NO donor, S-nitroso-N-acetyl-penicillamine, dramatically inhibited killing of PAEC target cells by IL-2-activated human NK cells. This same combined treatment reduced both surface expression and mRNA levels of E-selectin. Moreover, anti-E-selectin mAb, but not Ab to VCAM-1, protected PAEC from lysis by human IL-2-activated NK cells in a dose-dependent manner. These findings suggest that expression of porcine E-selectin is important for the cytotoxicity of PAEC mediated by activated human NK cells and may be involved in the redox-mediated modulation of that cytotoxicity. It is known that NF-κB activation is required for transcription of E-selectin, and the current data show that the suppression of E-selectin expression by S-nitroso-N-acetyl-penicillamine pretreatment and thiol deprivation was associated with reduced NF-κB DNA-binding activity in PAEC. These data suggest that the regulation of porcine E-selectin may be important for modulating delayed xenograft rejection and that manipulation of cellular redox systems may provide a means to protect xenogeneic endothelial cells from NK cell-mediated cytotoxicity. The Journal of Immunology, 2001, 166: 4106–4114.

Vascularized xenografts that survive beyond hyperacute rejection or acute vascular rejection or cellular xenografts that may not be susceptible to hyperacute rejection or acute vascular rejection may elicit a delayed cell-mediated rejection response several days to weeks after transplantation (1–3). This delayed rejection of vascularized xenografts is characterized by progressive infiltration of mononuclear cells, including NK cells, development of focal infarcts, interstitial hemorrhages, widespread coagulation within the microvasculature, and cessation of graft function (4, 5). NK cells have been implicated as mediators of rejection in concordant and discordant xenograft rodent models in vivo and in vitro (6–9) and may participate in the human anti-porcine rejection response (10–12). NK cells appear important in the rejection of porcine hearts transplanted into baboons (13) and have been reported to lyse porcine target cells with or without activation, in the presence or absence of human serum containing natural Abs (1, 10, 11, 14, 15). These findings suggest that NK cells may contribute to the pathogenesis of xenograft rejection.

Endothelial cells (ECs)2 in vascularized xenografts are the first target of recipient’s xenogeneic immune response and are activated to express adhesion molecules and to secrete cytokines during acute and delayed xenograft rejection (1, 16, 17). Pigs have been suggested as a primary potential source of xenografts for use in humans, but porcine ECs are susceptible to lysis by human NK cells in vitro (14, 18, 19), and, conversely, human NK cells activate porcine ECs (20, 21). Therefore, it is important to gain a further understanding of the interaction between porcine ECs and human NK cells to begin to devise means by which delayed xenograft rejection may be inhibited or prevented.

Redox regulation has been implicated in various biologic processes including signal transduction, gene expression, cell proliferation, cell cycle progression, and apoptosis (22–25). The ubiquitous physiological thiol, glutathione, is a common biological reducing agent (26) and has been reported to play an important role in regulating immunity, including killing activity (27) and lymphocyte proliferation (28) and modulating the activation of ECs (29). Depletion of thiol reducing agents through the manipulation of medium components has been used as a means to model oxidative stress in cell culture (25, 28, 30, 31).

The nitrogen free radical, NO, has a role in many physiological functions, including smooth muscle relaxation, neurotransmission, and apoptosis (32–35) and has also been reported to have cytoprotective effects (36–39). Adherence of leukocytes to endothelium is

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2 Abbreviations used in this paper: EC, endothelial cell; SNAP, S-nitroso-N-acetyl-penicillamine; CYS(+), medium containing cystine and glutathione; GSH, reduced glutathione; CYS(−), medium deficient in cystine and glutathione; PAEC, porcine aortic endothelial cell; iNOS, inducible NOS; NOS, NO synthase; eNOS, endothelial NOS; GSSG, glutathione disulfide, oxidized glutathione.
inhibited by NO (40, 41), and De Caterina et al. (42) reported that NO decreased activation of EC induced by cytokines, whereas others have reported that endogenous NO protected EC from cytokine-mediated and oxidant-mediated cytotoxicity (38, 39). We have reported that endogenous NO protected human NK cells from activation-induced apoptosis (36).

We now report on the effects of the intracellular redox status on porcine EC activation. We demonstrated that the pretreatment of EC with an NO donor under conditions of oxidative stress caused by thiol deprotection prevents porcine ECs from IL-2-activated human NK cells, and we have examined the mechanism of this effect.

Materials and Methods

**Abts and other reagents**

Human rIL-2 was kindly provided by Amgen (Thousand Oaks, CA). Human rTNF-α was purchased from R&D Systems (Minneapolis, MN), 5-nitroso-N-acetyl-penicillamine (SNAP) was obtained from Calbiochem (San Diego, CA). Anti-human CD62E mAb was purchased from Chemicon International (Temecula, CA) and has been shown to cross-react with porcine CD62E (E-selectin) (43). Anti-porcine VCAM-1 mAb (5F3) was kindly provided by Dr. Jean-Francois Bouhours (Institut National de la Sante et de la Recherche Medicale, France).

**Porcine EC cultures**

DMEM with 4.5 g/L glucose was purchased from BioWhittaker (Walkersville, MD). Standard DMEM medium containing cysteine and glutathione (CYS (+) medium) contained 20% heat-inactivated FCS (HyClone, Logan, UT), 1 mM pyruvate, 10 mM HEPES, 200 μM cysteine, and 50 μM penicillin-streptomycin. DMEM without cysteine and reduced glutathione (GSH) (CYS (-) medium) was from Life Technologies (Gaithersburg, MD). FCS for CYS (-) medium was dialyzed against PBS to remove small molecular mass molecules including thiols.

Porcine aortic endothelial cells (PAECs), obtained from BioWhittaker and cultured in EB2, a medium formulated for EC (Clonetics, Walkersville, MD), were used from passages 3 to 10. Monolayers that were cultured in CYS (+) medium was dialyzed against PBS to remove small interexperiment differences, likely due to inter-
donor variability, were sometimes observed.

**Cytotoxicity assay**

The standard 51Cr release assay was performed as described (27) in CYS (+) medium, using four E:T ratios in quadrupling dilutions of effector cells beginning with a ratio of 20:1 viable cells determined by trypan blue exclusion. Effector cells were IL-2-activated NK cells and target cells were PAEC obtained after treatments as indicated. IL-2-activated NK cells cultured in CYS (+) medium were used in assays to measure blocking of cytotoxicity by mAbs. Data were expressed as lytic units in 10^3 cells, in which 1 lytic unit was the number of effector cells required to lyse 20% of the target cells, and were obtained by fitting the titration curves to scale families of curves, as described previously (45).

**Flow cytometric analyses of expression of E-selectin and VCAM-1**

The cell surface expression of the adhesion molecules E-selectin and VCAM-1 on PAEC was quantified by flow cytometric analysis. Briefly, PAEC monolayers were washed with HBSS without calcium and incubated with a trypsin/EDTA solution (0.25 mg/ml; Clonetics) until the cells detached from the culture dish. Cells were washed with FACS buffer (HBSS with 1% FCS and 0.1% Na3HCO3) and incubated with anti-human CD62E mAb (E-selectin) or anti-porcine VCAM-1 mAb (46) for 30 min on ice. mAbs were used at a final concentration of 10 μg/ml. After washing with FACS buffer, cells were fixed with 1% PFA-conjugated, 0.1% paraformaldehyde. Cells were then washed extensively and cultured at 1 × 10^5 cells per well in CYS (+) medium in 96-well plates for 24 h, and nitrite/nitrate concentrations determined in the supernatants.

**RT-PCR analysis of mRNA**

mRNAs encoding for Porcine E-selectin, VCAM-1, Fas, inducible NO synthase (iNOS), and endothelial NOS (eNOS) were identified by RT-PCR. Total RNA was isolated from cells using TRIzol (Life Technologies, Gaithersburg, MD), according to the manufacturer’s instructions. The RNA was then reverse-transcribed to cDNA and amplified by PCR. The sequences of the primer pairs in this experiment were as follows: porcins β-actin, used as an internal control (sense: 5′-ATG TTT GAG ACC TTC AAC ACG CCG G-3′, antisense: 5′-GCA GGA CTC CAT GCC CAG GAA GGA G-3′); porcine E-selectin (5′-GAC TGC GGC AAG TGG AAT GAT GAG-3′, 5′-CAT CAC CAT TCT GAG GAT GGC CCA C-3′); porcine VCAM-1 (5′-ATG CCG AAG AAT AAC ACG CCG G-3′); porcine Fas (5′-CTG TCA GCC ATG CCC TTC TGG CAA ACG-3′, 5′-GCC CAT CAG TAG TTT GGA TTA CTT CTG-3′); porcine iNOS (5′-GACC GAC TGG ATT TGG TG-3′, 5′-GTT GGT GAG TTC TTT CAG CAT-3′); and porcine eNOS (5′-CCG AGC CAA CGT GAG GAT CAT CTG-3′, 5′-GCC CAC CAC GTG ATC TAT CTC A-3′). Amplifications were performed using a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, CT), using 24 cycles (E-selectin and β-actin), 20 cycles (VCAM-1) (94°C for 30 s, 58°C for 1 min, 72°C for 30 min), 30 cycles (Fas) (94°C for 30 s, 58°C for 30 s, 72°C for 30 s), 28 cycles (iNOS), or 24 cycles (eNOS) (94°C for 1 min, 60°C for 2 min, 72°C for 30 min). PCR products were loaded by electrophoresis in 1.5% agarose gels, stained with ethidium bromide for visualization under UV light. Gels were scanned and densitometry was performed using NIH Image software.

**EMSA**

Nuclear extracts were prepared from cells cultured with the indicated treatments, as described by Ueda et al. (47). The NF-κB oligonucleotide (5′-AGT TGA GGC GCC TGG AGC C-3′) was used as a probe to measure NF-κB-binding activity. dsDNA probe was synthesized in the CBER Core Facility Services (Center of Biologies Evaluation and Research, Food and Drug Administration, Bethesda, MD) and end-labeled with [γ-32P]ATP (10 μCi/ml; Amersham Pharmacia Biotech) using T4 polynucleotide kinase. Binding reactions were performed in 10 μl buffer (12 mM HEPES (pH 8.0), 60 mM KCl, 4 mM MgCl2, 1 mM EDTA, 12% glycerol, 1 mM DTT, and 0.5 mM PMSF) containing 10 fmol of labeled probe, 1 μg poly(dI:dC) dsDNA (Amersham Pharmacia Biotech), and 5 μg of each nuclear extract for 30 min at 20°C. Electrophoresis was performed through 5% polyacrylamide gels in 0.5× TBE buffer. For competition assays, 50-fold excess amounts of appropriate unlabeled oligonucleotides were added to the binding reaction mixture. Gels were dried and bands were visualized by autoradiography. Autoradiograms were scanned and densitometry was performed using NIH Image software.

**Measurement of NO oxidation products, nitrite plus nitrate (nitrite/nitrate) by Griess reaction using nitrate reductase**

NO formed by cells oxidizes to NO 2 and later to NO 3 . The amount of NO formed in the supernatants.

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The intracellular total glutathione and intracellular glutathione disulfide (GSSG) levels were measured using a Glutathione Assay Kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions in cells that had been homogenized in 10% metaphosphoric acid. The total glutathione content was calculated on the basis of the standard curve obtained with known amounts of GSH. The same method was used to assay for GSSG before derivatization of GSH by adding 0.5 μL of 2-vinylpyridine/50 μL of supernatant. The cellular GSH level was calculated as the difference between total glutathione and GSSG.

### Results

**Pretreatment with the NO donor, SNAP decreased susceptibility of thiol-deprived PAEC to killing by IL-2-activated human NK cells**

Evidence has shown that TNF-α, a cytokine that is active across species, is produced by cells that infiltrate rejecting xenografts (48) and human TNF-α can activate porcine EC when transplanted into immunodeficient mice (49). Therefore, TNF-α would be expected to be an important factor in vivo in determining xenograft survival. In addition, rejection of transplanted organs is associated with oxidative stress (50) and compromised antioxidant status (51), and the ischemia-reperfusion injury in transplanted vascularized organs is also associated with conditions of oxidative stress (52). These data suggest that oxidative stress would be an important consideration in xenotransplantation. Therefore, we hypothesized that the combination of TNF-α treatment and oxidative stress could influence the susceptibility of PAEC to lysis by human NK cells, cells that are likely to play a role in rejection of xenografts.

We found that treatment of PAEC with human TNF-α significantly enhanced their susceptibility to cytotoxicity mediated by IL-2-activated human NK cells (Fig. 1). In the presence of complete CYS(+) medium, pretreatment of PAEC with the NO donor SNAP slightly reduced their vulnerability to killing by IL-2-activated human NK cells compared with PAEC treated only with TNF-α, although this effect was not significant (Fig. 1). In contrast, pretreatment with SNAP under conditions of thiol deprivation in CYS(−) medium, as a model of oxidative stress, dramatically inhibited this cytotoxicity compared with that against TNF-α-treated PAEC in either medium (Fig. 1). Culture in CYS(−) medium alone resulted in a slight but nonsignificant increase in NK cell cytotoxicity against nonstimulated PAEC, compared with CYS(+) medium. Killing of TNF-α-treated PAEC that had been cultured under thiol-depleted conditions and pretreated with SNAP was suppressed to the same level of nonsimulated PAEC cultured in CYS(+) medium. The data suggest that NO in the presence of TNF-α and oxidative stress may decrease the susceptibility of PAEC to lysis by IL-2-activated NK cells.

**Decreased surface expression of E-selectin by PAEC treated with SNAP under conditions of thiol deprivation**

Because TNF-α activates EC to produce and express adhesion molecules (46, 49, 53), we analyzed the expression of E-selectin and VCAM-1 on the surface of PAEC under conditions of oxidative stress with and without pretreatment with SNAP (Fig. 2). Of nonstimulated PAEC cultured in CYS(+) medium, 4 ± 2% expressed E-selectin and 74 ± 12% VCAM-1. Treatment with TNF-α significantly increased the proportion of cells expressing both E-selectin and VCAM-1 on PAEC to 72 ± 5% and 98 ± 2%, respectively (p < 0.0001 and p < 0.02, respectively). Culture in CYS(−) medium reduced the proportion of cells expressing E-selectin compared with CYS(+) medium, but had no effect on VCAM-1 expression. Pretreatment with SNAP of TNF-α-treated EC cultured in thiol-depleted medium, but not in thiol-sufficient medium, significantly decreased the proportion of E-selectin-expressing cells compared with that in TNF-α-stimulated PAEC cultured under either condition (Fig. 2A). This change in E-selectin expression correlated well with the changes observed in the vulnerability of PAEC to cytotoxicity mediated by IL-2-activated NK cells, i.e., reduced E-selectin expression was associated with reduced susceptibility to lysis. In contrast, the proportion of cells expressing VCAM-1 was basically unaltered under the different conditions with only a marginal decrease seen in cells that had undergone SNAP pretreatment in thiol-insufficient CYS(−) medium (Fig. 2B). These findings suggest that the decreased vulnerability of NO-treated TNF-α-activated PAEC to lysis by human IL-2-activated NK cells is associated with E-selectin expression.

**Blocking Ab to porcine E-selectin inhibited human NK cell cytotoxicity against PAEC**

To investigate whether expression of porcine E-selectin was causally involved in determining the vulnerability of PAEC to IL-2-activated human NK cell-mediated cytotoxicity, we performed blocking studies using anti-E-selectin mAb. We found that anti-E-selectin specifically inhibited IL-2-activated human NK cell cytotoxicity against PAEC in dose-dependent manner compared with control IgG1 (Fig. 3). However, even at the highest concentration (10 μg/ml), anti-human E-selectin mAb did not inhibit the cytotoxicity by >50%. This level of cytotoxicity was approximately equivalent to that of non-TNF-α-stimulated PAEC cultured in thiol-sufficient medium and exhibited an inhibitory effect of similar magnitude to that of NO (see Fig. 1). It was not possible to judge inhibition by higher concentrations of specific Ab because greater concentrations of the control IgG1 became inhibitory (data not shown). In contrast to results with anti-E-selectin, anti-VCAM-1 did not inhibit the killing of PAEC by IL-2-activated EC cultured in CYS(−) medium, which is consistent with the decreased expression of VCAM-1 on PAEC under conditions of thiol deprivation. The data suggest that NO in the presence of TNF-α and oxidative stress may decrease the susceptibility of PAEC to lysis by IL-2-activated NK cells.

**Measurement of intracellular glutathione concentration**

The intracellular total glutathione and intracellular glutathione disulfide (GSSG) levels were measured using a Glutathione Assay Kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions in cells that had been homogenized in 10% metaphosphoric acid. The total glutathione content was calculated on the basis of the standard curve obtained with known amounts of GSH. The same method was used to assay for GSSG before derivatization of GSH by adding 0.5 μL of 2-vinylpyridine/50 μL of supernatant. The cellular GSH level was calculated as the difference between total glutathione and GSSG.
human NK cells. These findings suggest that IL-2-activated human NK cells recognize porcine E-selectin but not VCAM-1 and that this recognition is instrumental in the killing of TNF-α-activated PAEC by IL-2-activated human NK cells. The suppression of cytotoxicity by E-selectin mAb suggests a correlation between the SNAP-mediated reduction of E-selectin-expressing cells and the SNAP-mediated reduction in NK cell cytotoxicity against PAEC. These findings support the notion that the NO-induced reduction in E-selectin-positive cells may be responsible for the inhibition of IL-2-activated human NK cell cytotoxicity by SNAP pretreatment under thiol-deprived conditions.

**Attenuated expression of E-selectin mRNA by SNAP pretreatment of PAEC under conditions of thiol deprivation**

To determine whether the effect of SNAP pretreatment under conditions of thiol deprivation in CYS(−) medium might be related to altered gene expression, we examined the mRNA levels of both

**FIGURE 2.** Expression of E-selectin and VCAM-1 on PAEC. Surface expression of E-selectin (A) and VCAM-1 (B) were measured by flow cytometry on unstimulated PAEC and on PAEC that had been stimulated with TNF-α in CYS(+) or CYS(−) medium with and without pretreatment with the NO donor, SNAP. Data are means ± SE of results from five to six independent experiments. *, p < 0.02.

**FIGURE 3.** Inhibition of the IL-2-activated human NK cell-mediated cytolysis of PAEC with anti-human E-selectin mAb (A), but not with anti-porcine VCAM-1 mAb (B). PAEC treated with TNF-α and cultured in thiol-sufficient CYS(+) medium were used as target cells in blocking experiments with anti- E-selectin, anti-VCAM-1 or control Ab. Nonstimulated PAEC and TNF-α-stimulated PAEC, also both in CYS(+) medium, were controls, with the latter used to normalize results. PAEC that had been SNAP-pretreated and TNF-α-stimulated in thiol-insufficient CYS(−) medium were used as target cells as a positive control for inhibition of lysis. PAECs were detached by trypsinization (0.25 mg/ml), washed with HBSS, and experimental groups were treated with various concentrations of anti-human E-selectin mAb, anti-porcine VCAM-1 mAb, or control mAb (10 μg/ml) for 30 min on ice. After washing cells with PBS, they were used as target cells in a cytotoxicity assay. Data were calculated as lytic units and are expressed as the ratio of lytic activity against TNF-α-stimulated PAEC cultured in CYS(+) medium. Lytic activity by IL-2-activated NK cells obtained from the three to five different subjects ranged from 94.1 to 4640.9 LU in 10⁷ cells against control TNF-α-stimulated PAEC target cells in CYS(+) medium. Data shown are means ± SE of results of three to five independent experiments. *, p < 0.05.
E-selectin and VCAM-1 using RT-PCR (Fig. 4). Under the RT-PCR conditions used, E-selectin mRNA was below the limit of detection in nonstimulated PAEC but was markedly increased by TNF-α stimulation. However, whereas SNAP-pretreatment of PAEC in CYS(−) medium greatly attenuated the expression of E-selectin mRNA, SNAP-pretreatment in CYS(+) resulted in basically unchanged E-selectin mRNA expression, compared with that of TNF-α-stimulated PAEC in either medium. In contrast, VCAM-1 mRNA expression was detected under all conditions of TNF-α stimulation, thiol deprivation, and SNAP pretreatment, although a possible marginal increase after TNF-α stimulation is consistent with that seen by flow cytometry. None of the conditions used produced any discernable change in the level of β-actin transcripts. Because engagement of Fas is one mechanism by which activated NK cells lyse target cells (54), we also examined whether NO in the presence of thiol deprivation might alter Fas expression. However, no change in expression of Fas mRNA in PAEC was detected under these conditions (Fig. 4). These findings suggest that NO in the presence of thiol deprivation exerts its inhibitory effect on E-selectin mRNA levels.

Decreased expression of iNOS by SNAP pretreatment of PAEC under conditions of thiol deprivation

To investigate the possibility that endogenous NO produced by PAEC might determine, in part, the vulnerability of PAEC to cytotoxicity mediated by IL-2-activated human NK cells, we examined the expression of mRNA encoding iNOS and eNOS using RT-PCR and measured NO production by PAEC using the Griess reaction. As shown in Fig. 5A, culture in CYS(−) medium suppressed the expression of iNOS mRNA under all conditions of stimulation with TNF-α and SNAP. If any change was stimulated by SNAP pretreatment (i.e., addition of NO), it was a marginal decrease in iNOS expression in TNF-α-stimulated cells to nonstimulated levels in CYS(−) medium. As expected, eNOS mRNA levels did not change regardless of culture or treatment conditions. As shown in Fig. 5B, no change in supernatant NO in PAEC cultures was detected under any combination of thiol deprivation, TNF-α activation, and SNAP pretreatment. These findings suggest that endogenous NO produced by PAEC iNOS does not modulate human NK cell cytotoxicity.

FIGURE 4. Expression of porcine E-selectin mRNA was inhibited in PAEC by pretreatment with SNAP before TNF-α stimulation in CYS(−) medium. After culture in CYS(+) or CYS(−) medium for 24 h, PAEC were untreated or treated with SNAP (1 mM for 1 h), treated for 4 h with TNF-α (10 ng/ml), and harvested for RT-PCR. The data are representative of three independent experiments. A, The level of E-selectin (CD62E) transcripts, but not VCAM-1, Fas, or β-actin transcripts, is reduced by SNAP pretreatment of TNF-α-stimulated PAEC in thiol-insufficient CYS(−) medium. The level of VCAM-1 transcripts is similarly increased by TNF-α treatment regardless of SNAP pretreatment and thiol availability. B, The levels of E-selectin mRNA are shown and are expressed relative to the density of the β-actin bands for the same sample. Band densities were determined by scanning the photograph on an Epson Perfection 120U scanner and using the NIH Image program.

SNAP pretreatment of PAEC cultured under thiol insufficiency inhibited NF-κB DNA binding

Because the expression of E-selectin and VCAM-1 mRNA caused by TNF-α is associated with NF-κB activation in human EC (55, 56) and because E-selectin transcription requires NF-κB activation (57), we examined whether NF-κB DNA-binding activity is altered in TNF-α-activated PAEC by NO under conditions of thiol insufficiency. The results showed that TNF-α treatment increased the level of NF-κB DNA-binding activity in extracts of PAEC cultured in both CYS(+) and CYS(−) medium, compared with nonstimulated PAEC (Fig. 6). Although SNAP pretreatment with CYS(+) medium did not decrease NF-κB DNA-binding activity, SNAP pretreatment in CYS(−) medium almost completely suppressed NF-κB DNA binding in PAEC (Fig. 6). These data support the conclusion that pretreatment of PAEC with NO in thiol-depleted medium inhibits NF-κB DNA-binding activity in PAEC, which could cause decreased expression of E-selectin.

Changes of intracellular glutathione levels in PAEC

To analyze the relationship between the intracellular redox status and the activation of PAEC, we examined intracellular total glutathione levels and GSSG (oxidized glutathione) levels. Levels of GSH were calculated as the difference between the total and oxidized forms. Thiol deprivation almost completely abolished all intracellular glutathione in all treated cells (Table I). Total glutathione levels and GSSG levels were unaffected by TNF-α or SNAP treatments in PAEC cultured in thiol-sufficient medium. GSSG concentration was <10% of the total glutathione concentration under all conditions. The reduced level of glutathione in PAEC cultured in thiol-depleted medium was associated with a decreased ability for NF-κB in such cells to be activated in response to TNF-α and with the inhibition of E-selectin expression. However, no such correlation was seen in response to pretreatment with the NO donor, SNAP.

Discussion

The worldwide shortage of available human organs for allotransplantation coupled with advances in the development of effective immunosuppression have fostered a renewed interest in xenotransplantation as a potential alternative approach for the treatment of
seriously ill human recipients. The most commonly suggested animal source is the pig, because of its size and its historical proximity to humans and also because of the capability to produce pigs in a controlled environment (58). However, the response of the human immune system to porcine tissues or organs presents a significant and formidable barrier to the widespread use of xenotransplantation (59). Mounting evidence suggests a role for NK cells in the delayed, and possibly even the acute rejection of xenografts (6–8, 17, 60), and specifically supports the notion that they are important in the human anti-pig rejection response (1, 10–15, 61, 62). EC in vascularized xenografts are important targets of the host’s xenogeneic immune response and become activated to express adhesion molecules and secrete cytokines during acute and delayed xenograft rejection (1, 16, 17). In addition to the activation of porcine EC by cytokines such as TNF-α or IL-1β, they also become activated by direct contact with human NK cells (18). Control of the interaction between human NK cells and porcine EC would, therefore, be expected to be extremely important for increasing the likelihood of survival of a porcine xenograft in a human recipient.

In the present study, we confirmed that human TNF-α increased the expression of E-selectin and, to a lesser extent, VCAM-1, expression by PAEC, suggesting that PAECs are activated by human TNF-α to express certain adhesion molecules, consistent with previous reports (46, 49). Importantly, TNF-α-activated PAECs were more susceptible to lysis by IL-2-activated human NK cells compared with nonstimulated PAEC, implicating EC activation, and possibly expression of adhesion molecules, E-selectin, in particular, in their increased vulnerability to lysis mediated by IL-2-activated human NK cells. We found that pretreatment of TNF-α-treated PAEC with SNAP, an NO donor, marginally decreased the susceptibility of PAEC to lysis by IL-2-activated human NK cells.
by \( \sim 25\% \). However, this inhibition of lysis was much greater, reflecting a \( \sim 60\% \) reduction in cytotoxicity, when, in addition to SNAP pretreatment, PAECs were subjected to oxidative stress by culture under conditions of thiol deprivation. Pretreatment of PAEC with SNAP in thiol-depleted medium also significantly inhibited expression of E-selectin, but not VCAM-1, compared with that of TNF-\( \alpha \)-stimulated PAEC in either thiol-sufficient or -depleted medium, correlating with the effect of these treatments on the susceptibility of PAEC to lysis by IL-2-activated human NK cells. Interestingly, oxidative stress may be an important consequence of transplantation. For example, rejection of transplanted organs is associated with oxidative stress (50) and compromised anti-oxidant status (51), and ischemia-reperfusion injury in transplanted vascularized organs also is associated with conditions of oxidative stress (52).

We further showed that lysis of TNF-\( \alpha \)-activated PAEC by IL-2-activated NK cells was blocked by anti-human E-selectin mAb, of the actin cytoskeleton. Similarly, Fiorentini et al. (73) reported one and membrane blebbing accompanied by altered organization of the actin cytoskeleton. Interestingly, oxidative stress may be an important consequence of transplantation. For example, rejection of transplanted organs is associated with oxidative stress (50) and compromised anti-oxidant status (51), and ischemia-reperfusion injury in transplanted vascularized organs also is associated with conditions of oxidative stress (52).

We further showed that lysis of TNF-\( \alpha \)-activated PAEC by IL-2-activated NK cells was blocked by anti-human E-selectin mAb, and this inhibition reduced killing to the level seen using TNF-\( \alpha \)-activated, SNAP-pretreated PAEC cultured in CYS(−) medium. In contrast, anti-porcine VCAM-1 mAb did not block the lysis of TNF-\( \alpha \)-activated PAEC by IL-2-activated NK cells. These results suggested that cytotoxicity of PAEC mediated by IL-2-activated NK cells involves the recognition of porcine E-selectin on PAEC by the counterreceptor on IL-2-activated human NK cells. Consistent with this is the report by Pinola et al. (63), who showed that human NK cells adhered to human ECs through E-selectin. However, the participation of other surface molecules in this process is suggested because anti-E-selectin did not completely inhibit the killing of PAEC by IL-2-activated human NK cells. That human VLA-1, the human ligand for VCAM-1, can recognize porcine VCAM-1 is suggested by the finding that VCAM-1 is involved in the migration of human lymphocytes across porcine EC layers (64). However, our data suggest that this interaction is not important in the lysis of PAEC by IL-2-activated human NK cells. Other recognition structures on human NK cells for porcine EC may include CD2 (62) or leukocyte integrins (65), which may be involved in the recognition of PAEC by human NK cells. It also has been suggested that human NK cells somehow recognize the \( \alpha \)-galactosyl epitope expressed on pig cells (66) recognized by natural Abs and responsible for hyperacute rejection of xenografts in appropriate model systems (67–69). Although the capacity of human lymphocytes to recognize porcine MHC has been reported, this has been only for recognition by CD4+ T cells that resulted in proliferation of those human T cells (70). Compatibility between human and porcine adhesion molecules has been observed for many combinations of receptors and counterreceptors (71).

Interestingly, TNF-\( \alpha \)-treated porcine ECs cultured in CYS(−) medium appear to be as sensitive to lysis by IL-2-activated human NK cells as similar cells cultured in CYS(+) medium (Fig. 1), despite the fact that the former express lower levels of E-selectin (Fig. 2). This result may be due, at least in part, to the higher baseline cytotoxicity against PAEC target cells cultured in CYS(−) medium as evidenced by the increased cytotoxicity mediated against CYS(−)-cultured nonstimulated PAEC compared with CYS(+)-cultured nonstimulated PAEC (Fig. 1). This observation is consistent with reports that increased cellular damage has been observed under conditions of oxidative stress associated with reduced levels of thiols. Such damages, although not being apoptotic themselves, might serve to make target cells more susceptible to the induction of apoptosis. For example, Van Gorp et al. (72) reported that human ECs treated with tert-butyl hydroperoxide or hydrogen peroxide exhibited transient oxidation of glutathione and membrane blebbing accompanied by altered organization of the actin cytoskeleton. Similarly, Fiorentini et al. (73) reported that anaerobic bacteria that induce oxidative stress and a significant depletion of intracellular glutathione caused epithelial cells to round up and undergo breakdown of cytoskeletal actin and that treatment with N-acetylcysteine reversed the effect while correcting the intracellular glutathione level. These reports suggest the possibility that the increased vulnerability of PAEC cultured in CYS(−) medium to lysis by human IL-2-activated NK cells may be due to altered cytoskeletal organization.

We also investigated the mechanism by which the inhibition of E-selectin expression might proceed in PAEC pretreated with SNAP before TNF-\( \alpha \)-stimulation under conditions of oxidative stress in thiol-deficient medium. The steady-state level of E-selectin mRNA was most markedly reduced by SNAP pretreatment in CYS(−) medium, but not in CYS(+), suggesting an effect at the mRNA level or upstream thereof. Because NF-\( \kappa \)B activity is required for transcription of E-selectin (57), we investigated whether NF-\( \kappa \)B activation is modulated by SNAP treatment under conditions of oxidative stress, i.e., those conditions that affected E-selectin mRNA levels. Our data showed that the decreased mRNA level was associated with the suppression of NF-\( \kappa \)B DNA-binding activity in PAEC that had been cultured in CYS(−) medium and pretreated with SNAP before TNF-\( \alpha \) activation, suggesting that this change in NF-\( \kappa \)B expression is responsible for the decreased E-selectin expression under these conditions.

The TNF-\( \alpha \)-induced expression of E-selectin and VCAM-1 is reported to be associated with NF-\( \kappa \)B activation in human ECs (55, 74). Others have found that treatment with NO donors, such as SNAP, suppressed the NF-\( \kappa \)B DNA-binding activation in human EC (75) and vascular smooth muscle cells (76). In the current study, SNAP pretreatment alone was not sufficient to inhibit NF-\( \kappa \)B DNA-binding activity in TNF-\( \alpha \)-activated porcine ECs. Marked reduction of NF-\( \kappa \)B activity of stimulated PAEC was only observed with SNAP pretreatment together with oxidative stress. The difference between our findings and those of others may be attributable to differences in SNAP treatment and/or differences between species.

In the present study, we investigated the mechanism by which SNAP pretreatment under conditions of thiol depletion inhibited NF-\( \kappa \)B DNA-binding activation of PAEC by measuring intracellular glutathione levels in treated PAEC. As we have seen previously with other cell types (27), culture in CYS(−) medium significantly reduced the intracellular glutathione levels in PAEC. These decreased glutathione levels were associated with a diminished activation of NF-\( \kappa \)B DNA-binding activity in PAEC upon TNF-\( \alpha \) activation. These findings are consistent with reports that diethyl maleate, a glutathione-depleting agent, attenuated NF-\( \kappa \)B DNA nuclear translocation and E-selectin expression in LPS-stimulated HUVECs (77) and that change of intracellular glutathione levels affects NF-\( \kappa \)B DNA-binding activity in human ECs (78). However, our data suggest that changes in intracellular glutathione do not explain the suppression of NF-\( \kappa \)B DNA-binding activity of PAEC in response to pretreatment with the NO donor, SNAP. Induction of heat shock protein, HSP70 (79), decreases the activation of NF-\( \kappa \)B DNA-binding activity in epithelial cells (79), but we have not been able to detect induction of HSP70 in response to NO treatment of PAEC (data not shown).

Our results suggest that combined treatment of NO donors with oxidative stress may serve as a means to protect porcine ECs from lysis by human IL-2-activated NK cells through the suppression of activation NF-\( \kappa \)B DNA-binding activity and consequent inhibition of E-selectin expression. A combined treatment with thiol-depleting agents and NO donors may provide a means to protect EC in porcine vascularized xenografts from delayed xenograft rejection. However, the complicated effects, both anticipated and
unanticipated, that such treatment might have in vivo would first require well-designed animal studies.

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