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Protection of porcine endothelial cells against apoptosis with interleukin-4

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Abstract

Background—Apoptosis is crucial for tissue development and homeostasis, and insufficient apoptosis is pivotal in cancer pathogenesis. Apoptosis may also be important in tissue injury and in this case, it is of interest to induce protection against apoptosis. In organ transplantation, apoptosis has been implicated in acute vascular rejection (AVR); in xenotransplantation, the inducers of apoptosis of relevance in AVR, such as tumor necrosis factor- α (TNF- α), also cause endothelial cell (EC) activation. We have previously shown that interleukin (IL)-4 and IL-13 induced protection in porcine ECs against activation and apoptosis triggered by TNF- α . Now we define signaling processes activated by IL-4 in porcine ECs and mechanisms required for IL-4-induced protection against apoptosis.

Methods—Porcine aortic ECs were used as primary cultures or as virus-induced immortalized cells derived from galactosyl transferase-deficient (Gal^{-/-}) or wild-type pigs. ECs were stimulated with porcine IL-4, either extrinsically or transduced with recombinant adenovirus (adeno) IL-4, and analyzed using immunoblotting. Apoptosis was induced with TNF- α plus cycloheximide and assessed using neutral red uptake or flow cytometry. The role of various signaling proteins in IL-4-induced protection was established using pharmacologic inhibitors and siRNA downregulation of protein expression.

Results—IL-4 induced similar degrees of phosphorylation of STAT6 in all 3 types of ECs, and STAT6 was phosphorylated through Jak3. IL-4 induced phosphorylation of Bad through Jak3. Stimulation of ECs with IL-4 caused protection of ECs against apoptosis with an absolute requirement of Jak3/STAT6 activation and major participation of mammalian target of rapamycin complex 2 (mTORC2), Akt, and extracellular signal-regulated kinase 1/2. IL-4 caused no increase in EC levels of protective proteins hemoxygenase-1, inhibitor of apoptosis protein, heat shock protein 70, Bcl-2, and Bcl-xL. ECs transduced with adenoIL-4 exhibited strong and durable

protection from apoptosis. Gal^{-/-} ECs were as susceptible to induction of protection with IL-4 as wild-type ECs.

Conclusions—IL-4 induces activation of Jak3/STAT6 and phosphorylation of Bad in porcine ECs, ultimately resulting in effective protection of the ECs from apoptosis. Delineation of downstream signals activated by IL-4 that are required for induction of protection suggests possible sites of intervention to design effective therapeutic agents. This is of interest because substances such as IL-4 have pleiotropic effects and cannot be used directly due to potential deleterious effects. Inducing resistance to apoptosis in porcine vascular endothelium may be important to facilitate xenograft survival and accommodation.

Keywords

accommodation; apoptosis; cytoprotection; endothelial cells; IL-4; inflammation

Introduction

Apoptosis is a regulated process of cell death that can be triggered or modulated by many agents, including certain cytokines. While regulation of apoptosis is an important component of normal homeostasis, impaired or excessive apoptosis may play a major role in the pathogenesis of many diseases. For example, the inability of cells to undergo apoptosis is considered to be of central importance in cancer progression [1–3]. In contrast, apoptosis can be excessive and contribute to vascular injury, as in atherosclerosis, ischemia-reperfusion injury, and organ allograft and xenograft rejection [3–9]. In many of these processes, the target of injury is the vascular endothelium; therefore, in situations of excessive apoptosis, it may be desirable to induce a protective state against its development.

With regard to xenotransplantation, apoptosis of endothelial cells (ECs) is one consequence of various mechanisms that cause graft rejection [7,10]. Organs from genetically modified donor pigs are significantly less susceptible to rejection by a primate recipient but injury still occurs, leading to the process known as acute vascular rejection (AVR) [11–14]. The initial target in AVR is the vascular endothelium, ultimately resulting in microvascular inflammation and thrombosis followed by ischemia, apoptosis, or necrosis, and subsequent graft rejection [7,10,15]. As such, it is then of interest to make the vascular ECs resistant to apoptosis to improve organ survival, either directly or through accommodation, broadly defined as the survival of an organ graft in the presence of effectors of graft rejection [15–18]. A vascularized organ graft that expresses extensive cytoprotection at the time of grafting may be poised to respond with accommodation to additional protective stimuli from the recipient. In contrast, an unmodified graft is usually unable to respond with accommodation because its endothelium rapidly becomes activated toward inflammatory and procoagulant pathways [7,12]. Intrinsic EC resistance against injury and apoptosis in pig-to-human models has been achieved using various substances, including certain cytokines [19,20] and the adaptor protein Lnk [21].

In our previous studies, we have shown that the cytokines IL-4 and IL-13 are strong inducers of protection of porcine ECs against the consequences of immunologic agents that mediate graft rejection, including antibody (ab), complement, and tumor necrosis factor- α

(TNF- α) [19,20,22,23]. These agents may cause EC activation, production of IL-1 α , increased expression of E-selectin and tissue factor, intravascular coagulation, cell retraction and gap formation, and apoptotic or necrotic cell death. We have reported that many of these processes, including apoptosis triggered with TNF- α and necrotic cell death caused by the membrane attack complex (MAC) of complement, were prevented by pretreatment of the ECs with IL-4 or IL-13. We have reported that the IL-4-induced protection against killing by the MAC is intrinsic and requires activation of phospholipid synthesis, in association with preservation of mitochondrial structure and function [19,20,22,23]. We have also reported that pig iliac arteries transduced with human IL-4 recombinant adenovirus (adenoIL-4) were protected from the severe injury seen in control arteries when perfused with human blood [20].

With the knowledge that IL-4 and IL-13 have multiple protective effects on ECs, it became important to understand the signaling pathways that are involved in induction of protection. This knowledge would then allow the development of approaches to achieve protection by intervening downstream in the activation process. This strategy would avoid having to use a cytokine such as IL-4 or IL-13 that are known to have multiple pleiotropic effects, including certain effects that may be deleterious to a transplant or in various vascular diseases [24,25]. Therefore, in the present study, we investigate the signaling pathways that IL-4 activates in porcine ECs and then define the principal signaling proteins that are required for IL-4-induced protection against apoptosis mediated by TNF- α . Given that the protection induced using exogenous IL-4 lasts only ~48 h, we also studied the efficacy of transducing ECs with adenoIL-4. Finally, as organs from galactosyl transferase-deficient (Gal^{-/-}) pigs have been shown to have extended survival when transplanted in non-human primates, we investigated the initial activation mechanism and efficacy of IL-4 protection in Gal^{-/-} ECs.

Materials and methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), M199, L-glutamine, heat-inactivated fetal bovine serum (FBS), antibiotics, and other cell culture reagents were purchased from Invitrogen Life Sciences (Carlsbad, CA, USA). Pig rIL-4 and TNF- α were purchased from R&D Systems (Minneapolis, MN, USA). Jak3 inhibitor PF-956980, mTOR inhibitor PP242, cycloheximide (CHX), neutral red, and general chemicals were from Sigma (St. Louis, MO, USA). Jak inhibitor 1, MEK1/2 inhibitor, Erk inhibitor, SB203580, and rapamycin were from EMD Chemicals (Gibbstown, NJ, USA). Akt inhibitor triciribine was from Cayman Chemical (Ann Arbor, MI, USA). Abs cross-reacting with the following pig proteins were used: STAT6, phospho-STAT6_{tyr641} (p-STAT6), phospho-Akt_{ser473} (p-Akt), and phospho-Bad_{ser112} (p-Bad) (Cell Signaling, Beverly, MA, USA); Bad (BD Bioscience, San Diego, CA, USA); HSP70 (Stressgen; Enzo Life Sciences, Plymouth Meeting, PA, USA); HO-1 (Millipore, Bedford, MA, USA); Bcl-xL (Invitrogen); Bcl-2 (C-2), Akt (H-136), and horseradish peroxidase-conjugated donkey secondary antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Cross-reactivity of the antibodies with pig antigens was either stated by the manufacturer or established using a positive control with the corresponding antigen of the immunizing species run in parallel with the pig antigen of interest using

immunoblotting. Anti-pig glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the BCA protein assay kit were from Pierce (Rockford, IL, USA).

Endothelial cells

Pig ECs were explanted from pig aortae, cultured, and identified as previously described [19,26]. Experiments were performed with cells in passages 3 to 7, 2 to 3 days post-confluence, in 48-well gelatin-coated tissue culture plates for apoptosis assessed by vital dye, in 24-well plates for apoptosis assessed by flow cytometry, or in 6-well plates for immunoblotting (Costar, Cambridge, MA, USA). All incubations were carried out at 37 °C in 5% CO₂. Porcine aortic immortalized wild-type EC line PEDSV.15 and Gal^{-/-} EC line PEG-K28 were obtained from David H. Sachs (Massachusetts General Hospital, Boston, MA, USA) [27]. The cells were maintained in M199 containing 10% heat-inactivated FBS, L-glutamine, penicillin/streptomycin, and 50 µg/ml endothelial mitogen (Biomedical Technologies Inc, Stoughton, MA, USA). Experiments were performed with immortalized cells in passages 4 to 12, 1 day post-confluence in medium without endothelial mitogen.

Treatment of endothelial cells with cytokines

ECs were made quiescent by incubation with DMEM containing 1% FBS (1% FBS/DMEM) overnight and then incubated with IL-4 at 1 to 20 ng/ml in the same medium for 40 to 48 h or other time periods as indicated. Inhibitors were added as a 50× solution directly to the wells and incubated, usually for 1 h, and the incubation continued after addition of a 50× solution of IL-4. After the incubation period, the supernatant was removed and the cells were washed twice and tested for sensitivity to apoptosis or lysed for isolation of protein or mRNA [19]. IL-4-treated cells retained a normal capacity to take up the vital dye neutral red, indicating that their viability had not been impaired [19].

Induction and measurement of apoptosis

To induce apoptosis, pretreated ECs were washed twice with 1% FBS/DMEM, incubated for 2 h with 20 ng/ml porcine TNF-α plus 3 µg/ml CHX [28] in 1% FBS/DMEM, and then washed twice with Hank's balanced salt solution (HBSS). Apoptosis was measured by flow cytometry to analyze the population distribution of EC DNA content after propidium iodide staining or, in most experiments, by a vital dye uptake assay [19]. The vital dye neutral red in 10% FBS/DMEM was added to each well for 2 h. The cells were then washed once with HBSS and lysed with 1% acetic acid/50% ethanol. The cell lysates were transferred to a microwell plate and read at 540 nm using a V_{max} microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Percent-specific apoptosis was calculated using a control treated with medium instead of TNF-α, as follows: % apoptosis = $\left[\frac{\text{test OD}}{\text{control OD}} - 1 \right] \times 100$. Results are normalized to the average medium control of multiple experiments performed in triplicate. The amount of cell death in the inhibitor controls compared with the respective control without inhibitor was <25%. We have validated the use of neutral red to measure the degree of apoptosis caused by TNF-α/CHX at the concentration and incubation time that we used in our current studies. With neutral red, similar percentage killing was obtained as when apoptosis was measured by flow cytometry or Hoechst staining for microscopy; the ECs also showed activation of caspase-3 [19] and release of mitochondrial cytochrome c, both characteristic of apoptotic cell death.

Preparation of siRNAs and transfection of endothelial cells

21-nucleotide siRNAs to target the mRNA of porcine genes for STAT6 (GenBank HM135386) and β -actin (GenBank DQ845171) were designed and synthesized by Applied Biosystems (Foster City, CA, USA). One day before transfection, ECs were plated at 12 500 cells/cm² in 10% FBS/DMEM, using 0.5 ml/well of a 48-well plate or 5 ml/well of a 6-well plate. When the cells were 30 to 50% confluent, the medium was removed and replaced with antibiotic-free 10% FBS/DMEM (0.2 ml/well of a 48-well plate or 2.5 ml/well of a 6-well plate) and the siRNAs were diluted in OptiMEM (Invitrogen) and transfected into the cells with Lipofectamine RNAiMax reagent (Invitrogen) following the protocol provided by the manufacturer. After 4 h, the transfection mixtures were removed and replaced with 10% FBS/DMEM containing antibiotics and incubated for 3 days to confluence before incubation with 10 ng/ml IL-4 for 48 h; cell viability assessed using neutral red uptake was >90%. Knockdown for the desired protein was assessed by immunoblotting.

Protein and mRNA assessment

For protein immunoblot analysis, the pretreated ECs were lysed in RIPA buffer containing 50 mM tris-buffered saline, pH 6.8, 1% Triton-X100, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Complete; Roche, Indianapolis, IN, USA). Cell extracts were prepared using a standard procedure, and 10 to 20 μ g of protein was electrophoresed under reducing conditions in 4 to 12% NuPage gels (Invitrogen) and transferred onto nitrocellulose membranes (Millipore). After blocking, the membranes were incubated with a primary ab, and following incubation with secondary ab, bands were visualized by chemoluminescence using Lumi-glo (Cell Signaling). Blots were stripped with ReBlot PLUS (Millipore) and retested as required. The amount of protein signal was determined from densitometry using Molecular Analyst (BioRad, Hercules, CA, USA). For mRNA assessment, RNA isolation and Northern blot analysis were performed as described before, using 10 μ g RNA [29,30]. The amounts of RNA in loaded samples were normalized by hybridization with a β -actin cDNA insert. The amount of probed mRNA and β -actin signal was determined from densitometry using NIH Image. EC cytokine and chemokine expression was examined using RiboQuant multiprobe porcine RNase protection assay kits (pCK; BD Biosciences), using housekeeping genes L32 and GAPDH as internal controls. The RNase protection assay was carried out according to the manufacturer's protocol using 20 μ g total RNA. Normal porcine spleen and liver and yeast RNA were used as controls.

Adenoviral transduction

Confluent EC monolayers were infected with adenoIL-4, which is known to be replication-defective [31,32], at a multiplicity of infection (MOI) of 25 to 100 pfu/cell in 1% FBS/DMEM during 3 h at 37 °C, or adeno control lacking the insert, as previously described [20]. The ECs were then washed and incubated in growth medium. After 2 to 7 days of incubation at 37 °C, the cell-free supernatants were recovered and stored at -80 °C for measurement of IL-4 by ELISA, according to the manufacturer's instructions (Endogen; Pierce). The ECs were then washed and tested for sensitivity to apoptosis.

Statistical analysis

The degrees of inhibition of apoptosis in IL-4 treated samples and in the medium control were calculated and used to determine the significance of the change in EC sensitivity to apoptosis after treatment with inhibitors or siRNA. P-values were obtained using a two-sample t-test assuming equal variance. Results of quantitative experiments are expressed as means $\pm$ SEM of multiple independent experiments, as indicated in the figure legends.

Results

IL-4 activates Jak3/STAT6 and Bad in porcine endothelial cells

We have previously shown that IL-4 induces protection from apoptosis of porcine ECs elicited by TNF- α [19]. As it is known that the initial cellular response to binding of IL-4 to its receptor is the activation of the Jak/STAT6 pathway, which may be involved in control of apoptosis [33–35], we first investigated whether IL-4 would trigger Jak/STAT6 activation in porcine ECs. We incubated ECs with IL-4 for different periods of time and found that IL-4 induced a time-dependent phosphorylation of STAT6, with maximum phosphorylation at 0.5 to 1.0 h (Fig. 1A). We then investigated the role of Jak in phosphorylation of STAT6 using “Jak inhibitor 1”, which is known to inhibit the activity of Jak1, Jak2, and Jak3 [36], and the Jak3 specific inhibitor PF-956980 [35]. As shown in Fig. 1B, both inhibitors reduced the IL-4-induced phosphorylation of STAT6 to a similar extent, suggesting that Jak3 is the main kinase involved in STAT6 activation in porcine ECs. Given that the proapoptotic protein Bad plays a central role in apoptosis, we then asked whether incubation of ECs with IL-4 for different periods of time would result in phosphorylation of Bad. We found that IL-4 caused a time-dependent phosphorylation of Bad, with maximum phosphorylation at 2 h (Fig. 1C). Moreover, Bad phosphorylation was impaired when the incubation with IL-4 was performed in the presence of Jak inhibitors (Fig. 1D). These results demonstrate that IL-4 activates porcine ECs through the Jak3/STAT6 pathway and that Jak3 is critical for Bad phosphorylation and its consequent inactivation.

We then asked whether stimulation of porcine ECs with IL-4 would regulate the expression of protective genes at both the mRNA and protein levels. Using mRNA analysis, we found that, after incubation with IL-4 for 1, 4, and 20 h, there was no significant upregulation of A20 and inhibitor of apoptosis protein (IAP), and a moderate increase in Bcl-xL at 20 h only (results not shown). Immunoblots of extracts from ECs stimulated with IL-4 or medium for 0, 1, 6, 24, and 48 h showed similar levels of protein expression for Bad, heat shock protein 70 (HSP70), and heme oxygenase-1 (HO-1), and progressively reduced Bcl-2 and Bcl-xL expression at 24 to 48 h of incubation with IL-4 (Fig. 1E). In addition to these findings on expression of proteins that are regarded as protective, using an RNA-protection assay, we also found that mRNA for cytokines IL-1 α , IL-2, IL-6, IL-8, IL-10, IL-12, IL-18, TNF- α , and IFN γ did not change in IL-4-treated ECs; there was only moderate upregulation of MCP-1 (results not shown).

Induction of protection from apoptosis with IL-4 in porcine endothelial cells requires Jak3/STAT6

Given our findings that IL-4 activates Jak3/STAT6, we first investigated the role of Jak using the Jak pan inhibitor “JAK inhibitor 1” as well as the Jak3-specific inhibitor and found that both interfered with IL-4-induced protection against apoptosis (Fig. 2A). The role of STAT6 in protection was investigated using siRNA to downregulate STAT6 expression, which was confirmed by immunoblotting (Fig. 2B). ECs expressing reduced expression of STAT6 exhibited no protection from apoptosis when treated with IL-4. As control, we used siRNA for β -actin (Fig. 2B), which did not interfere with protection from apoptosis. These findings demonstrate that, in porcine ECs, IL-4-induced protection requires Jak3/STAT6 activation.

Role of mTOR, Akt, and MEK/Erk in protection

As mammalian target of rapamycin (mTOR) has been implicated in the control of apoptosis [37], we used the mTOR inhibitor rapamycin and found that ECs exposed to rapamycin for a short time period (1 h) before the addition of IL-4 did not exhibit significant changes in degree of protection from apoptosis (results not shown). However, ECs exposed to rapamycin for a longer time period (18 h) before the addition of IL-4 demonstrated a dose-dependent partial impairment of protection (Fig. 3A). The mTOR system consists of two different protein complexes, mTORC1 and mTORC2, and rapamycin is able to inhibit mTORC1 after a short time of incubation (1 h) while inhibition of mTORC2 requires a much longer exposure to rapamycin (18 h) [37,38]. Therefore, our results suggest a role for mTORC2 in protection from apoptosis. To confirm the participation of mTOR in protection, we also used PP242, which is highly specific for mTOR, inhibiting both mTORC1 and mTORC2 [39], and obtained results similar to those using rapamycin with a prolonged incubation time (Fig. 3B). This inhibitor also increased the sensitivity to apoptosis of the ECs that were not treated with IL-4. Akt phosphorylation was impaired when the incubation with IL-4 was performed in the presence of Jak inhibitors. Finally, we demonstrated that inhibition of mTOR with PP242 markedly reduced IL-4-induced phosphorylation of Akt, suggesting a role of mTOR in promoting protection through activation of Akt (Fig. 3C).

We have previously reported that induction of protection with IL-4 in part required activation of phosphatidylinositol-3 kinase (PI3K) as inhibition of PI3K with LY294002 partially interfered with protection [19]. Therefore, we now asked whether Akt activation was required for protection using the specific Akt inhibitor triciribine and found significant inhibition of protection at 10 μ M (Fig. 3D), demonstrating an important role of Akt in protection from apoptosis. We then asked whether mitogen-activated protein kinases (MAPKs) participated in IL-4-induced protection and found that inhibitors of MEK1/2 (results not shown) and extracellular signal-regulated kinase 1/2 (Erk) suppressed IL-4-induced protection from apoptosis (Fig. 3E). In contrast, inhibition of p38 MAPK with SB203580 failed to interfere with protection (Fig. 3F); however, inhibition of p38 MAPK reduced in a dose-dependent manner the level of apoptosis caused by TNF- α in the absence of IL-4, suggesting that in pig ECs, the p38 MAPK pathway is a positive regulator of apoptosis.

ECs transduced with adenoIL-4 exhibit strong and durable protection from apoptosis

In previous studies, we have shown that incubation of porcine ECs with exogenous IL-4 was usually incomplete and protection lasted ~48 h. Therefore, we now used adenoIL-4 constructs to investigate whether transfer of the IL-4 gene into the ECs would induce efficient and durable protection from apoptosis. We found that ECs transduced with adenoIL-4 (Fig. 4A), but not ECs transduced with adeno control, exhibited protection from apoptosis elicited with TNF- α . This protection increased with time after transduction, reaching ~80% protection from apoptosis in 7 days. The degree of protection from apoptosis was proportional to the MOI used to transduce the ECs (results not shown). ECs transduced with adenoIL-4 released IL-4 into the super-natant, in parallel with induction of protection (Fig. 4B), as we have shown previously in experiments using adeno-IL-4 to induce protection against killing of porcine ECs by human complement [20].

Gal^{-/-} ECs are as susceptible to IL-4-induced activation of STAT6 and protection as wild-type ECs

Gal^{-/-} pigs that do not express galactose-alpha1, 3-galactose (Gal) antigens are currently being used in experimental xenotransplantation studies as donors for primate recipients. Therefore, it is of great interest to define the biologic responses of Gal^{-/-} ECs to mediators of injury in comparison with ECs derived from wild-type pigs. To this end, we first compared the effect of IL-4 on Gal^{-/-} and wild-type ECs in regard to IL-4-induced activation of STAT6. As shown in Figs 1A and 5A, we found no difference in the time required for maximum phosphorylation, 0.5 to 1 h for all three cell lines. We then compared IL-4-induced protection from apoptosis and found that all three cell lines were protected to the same extent (Fig. 5B).

Discussion

In our current study, we first investigated the activation of signaling mechanisms that take place in porcine ECs stimulated with the cytokine IL-4, and then, we delineated signaling pathways required for induction of protection by IL-4 against apoptosis elicited by TNF- α . We found that IL-4 activates ECs through the classical IL-4 response Jak3/STAT6 pathway, as IL-4 induced time-dependent phosphorylation of STAT6 and pharmacologic inhibition of Jak3 prevented STAT6 phosphorylation. Most importantly, we also found that IL-4 caused time-dependent phosphorylation of Bad, which required Jak3 activation. It is known that Bad, once phosphorylated, loses the ability to bind Bcl-2 and Bcl-xL, releasing these proteins to express their anti-apoptotic effect [40,41]. This suggests that our previously reported protective effect of IL-4 from apoptosis [19] may be due to the release of Bcl-2 and Bcl-xL from their sequestration by Bad, as a downstream consequence of Jak3 activation. We also found that stimulation of ECs with IL-4 causes no significant upregulation in the expression of gene products of A20, IAP, Bcl-xL, Bcl-2, HSP-70, and HO-1. Bcl-xL and Bcl-2 protein expression was decreased at 24 to 48 h in comparison to controls, a time period when protection was observed. Levels of Bcl-xL mRNA, however, were not decreased. These findings suggest that the reduced Bcl-xL and Bcl-2 protein levels might reflect metabolic changes in these proteins due to their utilization in the face of inactive Bad. However, in another study, preservation of Bcl-2 levels have been reported to be associated

with the anti-apoptotic effect of IL-4 on B cell chronic lymphocytic leukemia cells, when apoptosis occurred spontaneously during incubation in vitro [42]. Others have reported that human umbilical cord ECs stimulated with IL-13 exhibited protection from apoptosis in association with upregulation of A20 mRNA [43]. Using other models, it was often found that cytoprotection was mediated through upregulated expression of HO-1 [44,45] or HSP70 [46,47]. Overexpression of the adaptor protein Lnk has been shown to protect porcine ECs from activation and apoptosis induced by TNF- α by acting on PI3K, Akt, and Erk [21,48].

In our previous study documenting that incubation of ECs with IL-4 or IL-13 induced protection of ECs from apoptosis elicited by TNF- α , we had found that this protection was in part dependent on PI3K [19]. We now suggest that a more complete understanding of the signaling processes required for induction of protection by IL-4 is important for several reasons. First, apoptosis mediated by substances released from infiltrating cells such as activated monocytes and macrophages is a major component in the pathophysiology of AVR of allografts and xenografts and other vascular and inflammatory diseases [5,7,10], conditions that may benefit by reducing the cellular response to agents that cause apoptosis. Second, we and others have shown that IL-4 and IL-13, in addition to protection from apoptosis, can induce protection from various mediators of inflammatory and degenerative injury, including injury caused by the MAC of complement [19,20,31,32,49], making it important to define whether single or multiple mechanisms mediate protection from each process. Third, new approaches are needed to obtain protection by intervening downstream in the activation process that results in protection as it is known that stimulation of ECs with IL-4, in addition to inducing protection from apoptosis and complement killing, also activates the ECs to express molecules that may be harmful to a transplant or in vascular diseases. For example, it is known that, in ECs, IL-4 induces the expression of certain adhesion molecules and cytokines [24,25,50] and may be promitotic [51], although more recent studies suggested that IL-4 inhibits mitosis [52]. We previously found that IL-4 does not induce proliferation in monolayers of porcine ECs [23]. Finally, it is known that in certain pathologies, primarily cancer, IL-4 may play a role contributing to impairment of apoptosis, and therefore, the presence or the effect of IL-4 may need to be suppressed. In this context, Jak inhibitors are being tested for possible treatments in certain cancers and autoimmune diseases [53,54].

In defining mechanisms required for induction of protection from apoptosis we now found that activation of the Jak3/Stat-6 pathway is an absolute requirement for IL-4-induced protection, as shown before in IL-4-induced protection from apoptosis of B lymphocytes [33,55]. Pharmacologic inhibition of Jak3 and downregulation of STAT6 expression completely prevented the induction of protection from apoptosis by IL-4. As inhibition of Jak3 abrogated phosphorylation of Bad, our results suggest that multiple downstream signals may result in release of the anti-apoptotic proteins Bcl-2 and Bcl-xL from their complex with unphosphorylated Bad. We also found that several signaling proteins contribute to induction of protection with IL-4. We have used the mTOR inhibitor rapamycin for different incubation times as in this manner, it is possible to distinguish between the participation of mTORC1 and mTORC2 [37]. Our findings suggest that mTORC2 but not mTORC1 regulates the response of ECs to IL-4 stimulation and consequent protection. In addition to our results with rapamycin, we found that the stronger and highly specific mTOR inhibitor

PP242 also inhibited protection. In agreement with previous reports that mTORC2 regulates Akt activity [37], we found that mTOR is critical for activation of Akt by IL-4 and that inhibition of Akt partially interferes with induction of protection. We also found that the MEK-Erk pathway is required for induction of protection with IL-4. On the other hand, inhibition of p38 MAPK appears not to interfere with protection, suggesting that p38 MAPK is possibly not involved in IL-4-induced protection from apoptosis. However, a firm conclusion that p38 MAPK is not involved in protection can not be made because using a high dose of the inhibitor, there was a marked reduction in the susceptibility of the cells to TNF- α -induced apoptosis. Interestingly, however, inhibition of p38 MAPK reduced the level of apoptosis caused in ECs by TNF- α in the absence of IL-4, suggesting that, in pig ECs stimulated with TNF- α , the p38 MAPK pathway is predominantly a positive regulator of apoptosis, likely through a stronger effect on the pro-apoptotic p38 α isoform than on the anti-apoptotic β isoform [56]. It was reported previously that IL-4-induced activation of human ECs does not involve NF- κ B [57]. We performed experiments using specific inhibitors of NF- κ B, and our results indicate that the protective effect of IL-4 is also NF- κ B-independent (unpublished).

We have previously shown that porcine ECs incubated with IL-4 during 24 to 48 h were partially protected from apoptosis, a protection that lasted about 2 days [19]. In our current study, we found that IL-4 gene transfer resulted in more efficient and durable protection (at least 7 days), suggesting that transfer of the IL-4 gene into a vascularized organ could result in protection from apoptosis that may be caused by TNF- α and possibly other apoptogenic substances. We now also confirmed our previous finding that the gene transfer resulted in production of the cytokine [20]. We have previously shown that transduction of pig iliac arteries in vivo with adenoIL-4 produced IL-4 and, 4 to 8 days after gene transfer, induced protection of the vascular endothelium from the severe complement-mediated injury that occurred in unmodified pig arteries when perfused with human blood [20]. Thus, IL-4, and likely IL-13, could offer simultaneous protection from complement-mediated EC injury and apoptosis mediated by substances released from infiltrating cells such as activated monocytes and macrophages, as may occur in vascular diseases and hyperacute rejection and AVR of allografts and xenografts.

Since the advent of Gal^{-/-} pigs, many studies have shown that organs from these animals, especially when bred to pigs that overexpress complement regulators, exhibit prolonged survival in non-human primate recipients, but eventually are rejected by a vascular rejection mechanism. In the context of our current study, therefore, it became important to define IL-4-induced protection in ECs derived from Gal^{-/-} pigs. We found that, in Gal^{-/-} ECs, IL-4 induced a time-dependent STAT6 phosphorylation and a degree of protection against TNF- α apoptosis entirely similar to those responses in wild-type ECs. These findings suggest that our conclusions regarding IL-4 activation and protection from apoptosis using wild-type ECs may be applicable to Gal^{-/-} ECs.

In conclusion, in this study, using porcine ECs, we found that the signaling responses elicited by stimulation with IL-4 comprise activation of Jak3/STAT6 and inactivation of Bad. We showed that the IL-4-induced resistance against apoptosis triggered by TNF- α requires activation of Jak3/STAT6 and inactivation of Bad, and that MEK/Erk, PI3K, Akt,

and mTORC2 are required for induction of maximal protection. Together with our previous findings, it is now apparent that the IL-4-mediated mechanisms that result in protection against various forms of injury are different depending on the type of injury. For example, while in protection from apoptosis caused by TNF- α , signaling through MEK/Erk and mTORC2 is important, these pathways appeared not to be involved in protection from necrotic killing by complement [19] (and unpublished observations), which relies to a major extent on activation of lipid biogenesis [23]. We have also shown that transduction of ECs with adeno-IL-4 is a useful model for studying mechanisms of protection because of the sustained production of IL-4 and the consequent strong and durable protection from apoptosis. Finally, we found that ECs that do not express the Gal antigen are activated by IL-4 through similar mechanisms as wild-type ECs and likewise exhibit similar degrees of IL-4-induced protection from apoptosis. Further analysis of the mechanisms mediating induction of protection of the endothelium will be necessary before designing approaches with potential for applications in prevention or treatment of allograft and xenograft rejection and in various vascular diseases.

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Abbreviations

ab	antibody
adeno	recombinant adenovirus
AVR	acute vascular rejection
CHX	cycloheximide
DMEM	Dulbecco's Modified Eagle's Medium
EC	endothelial cell
Erk	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
Gal	galactose- α 1,3-galactose
Gal^{-/-}	galactosyl transferase-deficient
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
HO-1	heme oxygenase-1
HSP70	heat shock protein 70

IL	interleukin
Jak	Janus kinase
MAC	membrane attack complex of complement C5b-9
MAPK	mitogen-activated protein kinase
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
IAP	inhibitor of apoptosis protein
Pfu	plaque-forming units
PI3K	phosphatidyl inositol-3 kinase
STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline
TNF-α	tumor necrosis factor- α

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Fig. 1.

IL-4 activates Jak3/STAT6 and Bad in porcine endothelial cells (ECs), but does not upregulate the expression of protective proteins. ECs were incubated with 10 ng/ml IL-4 for the times indicated, and cell extracts were prepared for immunoblotting. (A) IL-4 induces time-dependent phosphorylation of STAT6. (B) Jak inhibitor 1 and PF-956980, a Jak3 inhibitor, reduce STAT6 phosphorylation by IL-4. ECs were incubated with the indicated amounts of inhibitor for 1 h before adding IL-4 for 1 h. (C) Time-dependent induction of p-Bad in ECs incubated with IL-4. (D) Jak inhibitor 1 and PF-956980 reduce Bad phosphorylation by IL-4. ECs were incubated with the inhibitor for 1 h before adding IL-4 for 2 h. (E) Effect of IL-4 on expression of various proteins known to have protective properties. Bars represent ratios of band density of indicated protein to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control (means of two experiments for Bad, Bcl-2, and HO-1, values of single experiments for Bcl-xL and HSP70). Results in A--D are representative of 2 to 3 independent experiments. All incubations in these and subsequent experiments have been performed at 37 °C.

Fig. 2.

Induction of protection from apoptosis with IL-4 in porcine endothelial cells (ECs) requires Jak3/STAT6. (A) Abrogation of IL-4-induced protection from apoptosis using Jak inhibitor 1 or Jak3 inhibitor. ECs were incubated for 1 h with various doses of inhibitor before adding 10 ng/ml IL-4 for 48 h. After washing, in this and subsequent experiments, the ECs were tested for sensitivity to apoptosis elicited by incubation for 2 h at 37 °C with tumor necrosis factor- α /cycloheximide. (B) Abrogation of IL-4-induced protection from apoptosis using siRNA for STAT6. ECs were transfected with 10 nm siRNA targeting STAT6 or β -actin and then incubated with 10 ng/ml IL-4 for 48 h. The ECs were then tested for sensitivity to apoptosis or used to prepare cell extracts for immunoblotting. Bar graphs are means $\pm$ SEM of results from three independent experiments performed in triplicate; * $P < 0.001$ comparing degree of IL-4-induced inhibition in experimental samples vs. IL-4-induced inhibition in control samples. The immunoblot is representative of four independent experiments.

Fig. 3.

Participation of mTOR, Akt, and ERK, but not p38 mitogen-activated protein kinase (MAPK), in induction of protection from apoptosis. (A) Effect of rapamycin on IL-4-induced protection. Endothelial cells (ECs) were incubated with rapamycin for 18 h, and then, medium or IL-4 (10 ng/ml) was added for 48 h, and sensitivity to apoptosis was assessed. (B) Effect of PP242 on IL-4-induced protection. ECs were incubated with PP242 for 1 h, and then, medium or IL-4 (10 ng/ml) was added for 48 h, and sensitivity to apoptosis was assessed. (C) Role of Jak3 and mTOR in IL-4-induced Akt phosphorylation. ECs were incubated with Jak inhibitor 1, Jak3 inhibitor PF-956980, or PP242 for 1 h, and then, medium or IL-4 (10 ng/ml) was added for 2 h, and immunoblotting of cell extracts was performed. (D) Effect of triciribine on IL-4-induced protection. ECs were incubated with triciribine for 1 h, and then, IL-4 (10 ng/ml) or medium was added for 48 h. After washing, the apoptosis assay was performed. (E) Induction of protection requires Erk. ECs were incubated for 1 h with Erk inhibitor and then, medium or IL-4 (10 ng/ml) was added for 48 h. The ECs were tested for sensitivity to apoptosis. (F) Induction of protection does not require p38 MAPK. ECs were incubated with p38 inhibitor SB203580 for 1 h and then, medium or IL-4 (10 ng/ml) was added for 48 h, and the cells were tested for sensitivity to apoptosis. Bar graphs are means $\pm$ SEM of results from four independent experiments performed in triplicate; * $P < 0.01$ comparing degree of IL-4-induced inhibition in experimental samples vs. IL-4-induced inhibition in control samples. The immunoblots are representative of two independent experiments.

Fig. 4.

Endothelial cells (ECs) transduced with adenoIL-4 secrete IL-4 and exhibit durable protection from apoptosis. ECs were transduced with 100 multiplicity of infection adenoIL-4, or adenovirus control, or exposed to medium alone. After the days of incubation indicated, the cells were (A) tested for sensitivity to apoptosis induced with tumor necrosis factor- α and assessed by flow cytometry, and (B) IL-4 was measured by ELISA in the supernatants. The results are representative of two independent experiments performed in duplicate.

Fig. 5.

IL-4 phosphorylates STAT6 and induces protection from apoptosis in Gal^{-/-} endothelial cells (ECs) to a similar extent as in wild-type ECs. (A) Immortalized aortic porcine wild-type PEDSV.15 ECs and immortalized aortic porcine Gal^{-/-} ECs were incubated with 10 ng/ml IL-4 for the times indicated and then analyzed by immunoblotting. (B) Wild-type primary ECs, PEDSV.15 ECs, and Gal^{-/-} ECs were incubated with IL-4 for 48 h, and then tested for sensitivity to apoptosis. Apoptosis of immortalized cells was induced with 10 ng/ml tumor necrosis factor- α plus 1 μ g/ml cycloheximide. Results are means $\pm$ SEM of four independent experiments performed in triplicate, $P < 0.001$ comparing degree of apoptosis in IL-4-treated samples vs. medium control samples for each cell type.