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TNF- α is Critical for Ischemia-induced Leukostasis, But Not Retinal Neovascularization nor VEGF-induced Leakage

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Abstract

Vascular endothelial growth factor (VEGF) and tumor necrosis factor- α (TNF- α) show significant overlap with regard to their effects in the eye. It has been postulated that VEGF-induced leukostasis, breakdown of the blood-retinal barrier, and ischemia-induced retinal neovascularization may be mediated, at least in part, through TNF- α . In this study, we used mice deficient in TNF- α to test our hypothesis. Compared to wild type mice, TNF- α -deficient mice showed an 80% reduction in leukocyte accumulation in retinal vessels after intravitreal injection of VEGF, and 100% reductions after intravitreal injections of interleukin-1 β (IL-1 β) or platelet-activating factor (PAF). The increase in retinal vascular permeability induced by injection of PAF was significantly reduced in mice lacking TNF- α , but VEGF- and IL-1 β -induced leakage was unaffected. Compared to wild type mice with oxygen-induced ischemic retinopathy, TNF- α -deficient mice with ischemic retinopathy showed significantly reduced leukostasis and mild reduction in vascular leakage, but no significant difference in retinal neovascularization. These data suggest that TNF- α mediates VEGF-, IL-1 β -, and PAF-induced leukostasis and vascular leakage mediated by PAF, but not leakage caused by VEGF or IL-1 β . Ischemia-induced retinal neovascularization, which has previously been shown to require VEGF, does not require TNF- α and is unaffected by attenuation of leukostasis.

Keywords

tumor necrosis factor- α ; leukostasis; blood-retinal barrier; retinal neovascularization; vascular endothelial growth factor; interleukin-1 β ; platelet activating factor

Introduction

Retinal ischemia occurs in diabetic retinopathy, retinopathy of prematurity, retinal vein occlusions, and almost all retinal diseases complicated by retinal neovascularization (NV), and the causal link between retinal ischemia and retinal NV is well-established. It is also clear that vascular endothelial growth factor (VEGF) is increased in ischemic retina and is necessary for

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retinal NV to occur (Adamis et al., 1994; Aiello et al., 1994; Miller et al., 1994; Pierce et al., 1995; Ozaki et al., 2000). What is not clear is whether other proteins with proangiogenic activity in other settings contribute to retinal NV and whether the angiogenic and vasopermeability activities of VEGF are dependent on the interaction of VEGF with another factor.

Fibroblast growth factor-2 (FGF-2) is a strong stimulator of endothelial cell proliferation *in vitro* and stimulates NV in some other tissues, but does not contribute to ocular NV (Tobe et al., 1998b; Yamada et al., 2001). The situation regarding tumor necrosis factor- α (TNF- α) is even more complex. It was identified and isolated because of antiangiogenic activity; when injected into tumors, it causes tumor vessels to regress resulting in tumor necrosis (Carswell et al., 1975; Old, 1985). Toxicity precludes systemic administration of TNF- α for the treatment of tumors, but it is possible to reduce toxicity to acceptable levels and obtain remarkable benefit from the antiangiogenic effects of TNF- α by isolated limb perfusion, ligand-targeted therapy, or intratumor gene transfer targeted by radiation (Lienard et al., 1992; Borsi et al., 2003; Mauceri et al., 1996). So, it is quite clear that TNF- α has antiangiogenic effects, but it may also have proangiogenic effects in some situations. Despite its inhibition of endothelial cell proliferation *in vitro*, sustained release of TNF- α in cornea or injection of 10^5 units of recombinant TNF- α into the vitreous cavity of rabbits causes cellular infiltration and NV in the cornea (Frater-Schroder et al., 1987; Rosenbaum et al., 1988), possibly by induced expression of other proangiogenic proteins such as interleukin-8, VEGF, and FGF-2 (Yoshida et al., 1997). In cultured vascular endothelial cells, TNF- α induces expression of VEGF receptor 2 and neuropilin-1 (Giraud et al., 1998). In mice, subcutaneous implantation of a pellet containing a low dose (0.01–1 ng) of murine recombinant TNF- α stimulated angiogenesis, while implantation of a pellet containing a high dose (1–5 μ g) inhibited angiogenesis demonstrating opposite effects depending upon the concentration (Fajardo et al., 1992). This paradox may be explained in part by the ability of TNF- α to activate 2 intracellular signaling pathways in endothelial cells, one leading to apoptosis (Tartaglia et al., 1993; Grell et al., 1994) and one that promotes survival and proliferation through activation of nuclear factor-kappa B (NF- κ B) (Song et al., 1997; Hsu et al., 1996). In addition, TNF- α recruits inflammatory cells, which stimulate NV in some situations and inhibit it in others (Ishida et al., 2003; Lang and Bishop, 1993). Finally, the ability of TNF- α to induce expression of proangiogenic molecules can result in different effects depending upon the makeup of the local cell population and its response to TNF- α . Therefore the effect of TNF- α in various tissues and disease processes is difficult to predict and must be determined by experimentation.

Increased levels of TNF- α have been demonstrated in proliferative retinopathies (Limb et al., 1996) and in animal models of retinal NV (Armstrong et al., 1998; Camussi et al., 1991; Majka et al., 2002). These increased levels of TNF- α may be collaborating with VEGF to stimulate retinal NV. TNF- α may also contribute to the process in other ways. For instance, leukocytes have been shown to play a role in the pathogenesis of ischemic retinopathies and TNF- α is a chemoattractant for leukocytes (Majka et al., 2002). TNF- α also causes breakdown of the blood-retinal barrier (BRB) (Derevjanik et al., 2002), which may be related to its stimulation of leukostasis, and therefore TNF- α may contribute to the excessive permeability seen in ischemic retinopathies. In this study, we sought to explore how the activities of TNF- α in the eye relate to those of VEGF and determine if and how TNF- α contributes to ischemic retinopathies.

Materials and Methods

Mice

Mice were treated in accordance with the recommendations of the Association for Research in Vision and Ophthalmology and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. TNF α knockout mice (Strain *Tnf^{tm1Gkl}*), which were

generated by Pasparakis et al. (Paparakis et al., 1996), were purchased from The Jackson Laboratory (Bar Harbor, ME). These TNF α -deficient mice demonstrated no TNF α bioactivity in sera and no TNF α mRNA expression in macrophages following LPS stimulation, but these mice were viable and fertile and showed no apparent phenotypic abnormalities (Paparakis et al., 1996). For controls, we used both the B6129SF2/J strain (Jackson Laboratory stock #101045), which is an F2 hybrid resulting from a cross between C57BL/6J and 129S1/SvImJ, thus having a comparable background to the TNF α knockout line, and the C57BL/6 mice, which were crossed with the chimeric animals described above.

Intraocular injections

Intraocular injections were performed with a Harvard pump microinjection apparatus and pulled glass micropipets. Each micropipet is calibrated to deliver 1 μ l of vehicle upon depression of a foot switch. Mice were anesthetized with 25 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 4 mg/kg xylazine (Vedco, St. Joseph, MO) and pupils were dilated with 1% tropicamide. Under a dissecting microscope, the sharpened tip of a micropipet was passed through the sclera just behind the limbus into the vitreous cavity, and the foot switch was depressed. To establish the time course and dosage required to elicit the maximum retinal vascular permeability for PAF, 1 μ l 10^{-3} , 10^{-4} , or 10^{-5} M solutions were administered intravitreally into one eye with the other eye remaining untreated, which we previously found to show no difference from intravitreal injection of an equal volume of PBS (Derevjanik et al., 2002). The BRB was assessed at 3, 6, 24, and 48 hours. Statistical significance was calculated using a paired t-test. The optimal times and dosages for VEGF and IL-1 β were previously established (Derevjanik et al., 2002). The optimal doses of VEGF (10^{-6} M), IL-1 β (10u), and PAF (10^{-3} M) for inducing BRB breakdown were administered to assess leukostasis and BRB breakdown 6 hours after intravitreal injection, which was the optimal time determined for each of these agents. TNF α was also injected at the optimal dose (10^{-5} M) and at 10^{-6} M and evaluated for comparison of its proinflammatory activity at the optimal time (24 hours) (Derevjanik et al., 2002).

Quantitative measurement of leukostasis

Leukocytes were labeled with fluorescein isothiocyanate (FITC) as previously described (Joussen et al., 2001), except that 2.5 ml of a 40 μ g/ml solution of (FITC)-conjugated Concanavalin A (Vector Labs, Burlingame, CA) was used for perfusion. Retinal flat mounts were prepared as previously described (Tobe et al., 1998a), examined with the Axioskop microscope, and images were digitized. The total numbers of leukocytes adhering to the retinal vessels were counted at 200X by the same investigator (SAV), with the investigator being masked as to the nature of the specimen.

Oxygen-induced ischemic retinopathy (OIR)

Litters of 7-day old mice were exposed to an atmosphere of 75% oxygen in an airtight incubator for 5 days, after which they were placed in the relative hypoxia of room air for 5 days, inducing OIR (Smith et al., 1994).

Quantitative assessment of the blood-retinal barrier (BRB)

Quantitative measurement of breakdown of the BRB was measured as previously described (Derevjanik et al., 2002). Mice were sedated and given an intraperitoneal injection of 1 μ Ci/gram body weight of [3 H]mannitol at 6 or 24 hours, depending on the optimal time determined for each agent. One hour after injection, the mice were sedated and perfused with physiological saline. Retinas from both the treated and untreated (control) eyes were rapidly removed, cleaned of other tissue, and dissected free from lens, vitreous, and any RPE that has extruded, and then are placed within pre-weighed scintillation vials. The thoracic cavity was opened and

the left superior lobe of the lung was removed and placed in another pre-weighed scintillation vial. A left dorsal incision was made and the retroperitoneal space was entered without entering the peritoneal cavity. The renal vessels were clamped with a forceps and the left kidney was removed, cleaned of all fat, and placed into a pre-weighed scintillation vial. All liquid was removed from the vials and remaining droplets were allowed to evaporate over 20 minutes. The vials were weighed and after addition of 1 ml of NCSII solubilizing solution, incubated overnight in a 50°C water bath. The solubilized tissue was brought to room temperature and decolorized with 20% benzoyl peroxide in toluene in a 50°C water bath. After re-equilibrating to room temperature, 5 ml of Cytoscint ES and 30µl of glacial acetic acid was added and the vials were stored for several hours in darkness at 4°C to eliminate chemoluminescence. Radioactivity was counted with a Wallac 1409 Liquid Scintillation Counter. The CPM/mg tissue measured for lung, kidney, treated retina, and untreated retina was used to calculate retina/lung, retina/kidney, and lung/kidney leakage ratios. The ratios obtained for retinas treated with a particular agent at a specific concentration and time points were compared to those for untreated or saline-treated retinas.

Quantitative assessment of retinal NV

Sections of retinas from treated and untreated eyes were histochemically stained with *Griffonia simplicifolia* isolectin-B4 (GSA; Vector Laboratories, Burlingame, CA), to visualize vascular endothelial cells (Ozaki et al., 2000). To perform quantitative analysis, serial sections were cut 60 µm apart, stained for GSA, and examined with an Axioskop microscope (Zeiss, Thornwood, NY). The images were digitized using a 3 CCD color video camera (IK-TU40A, Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to measure the area of GSA-stained cells on the surface of the retina. A total of 13 sections from each eye were measured and the mean constituted a single experimental value for each eye.

Results

Leukostasis and breakdown of the BRB is induced by TNF-α, VEGF, IL-1β, and PAF

Along with TNF-α, VEGF, IL-1β, and PAF have been implicated in leukostasis in the retina. It has been hypothesized that leukostasis resulting in damage to vascular endothelium is the mechanism by which VEGF causes BRB breakdown (Joussen et al., 2001). We sought to correlate these activities for each of the agents. We previously showed that intravitreal injection of VEGF, IL-1β, and TNF-α caused breakdown of the BRB, with maximum effects achieved by injection of 1 µl of 10⁻⁶ M for VEGF, 10⁻⁵ M for TNF-α, or 10 units for IL-1β (Derevanik et al., 2002). Similar studies were performed for PAF and it was found to be a less potent inducer of BRB breakdown than the other agents (Figure 1). Maximum leakage occurred 6 hours after intravitreal injection of 1 µl of 10⁻³ M PAF.

For each agent, the dose that caused maximal breakdown of the BRB was injected into the vitreous cavity, and after the post-injection time for maximum BRB breakdown for each agent (6 hours for VEGF, IL-1β, and PAF; 24 hours for TNF-α) the number of leukocytes adherent to retinal vessels were counted. In wild type mice, each of the agents caused significant leukostasis compared to controls (Figure 2). TNF-α, which induced the greatest retinal vascular leakage with a peak at 24 hours post-injection, also demonstrated the greatest pro-inflammatory response (1019±352 leukocytes/retina compared to 11±3 leukocytes/retina for PBS-treated controls) at a dosage of 10⁻⁵ M, which is the dosage that promoted the maximum retinal vascular permeability. At this concentration, the leukocytes were difficult to accurately count, since many had aggregated to form clusters. To more accurately count the adherent leukocytes, the dosage of TNFα was reduced to 10⁻⁶ M, which yielded 281±81 leukocytes/retina.

TNF- α mediates leukostasis induced by IL-1 β and PAF and most of it induced by VEGF

To determine if any of the agents might be working indirectly through induced expression of TNF- α , injections of the various agents were performed in *Tnfa* knockout mice. The results were dramatic, because VEGF-induced leukostasis was significantly reduced by about 80% and leukostasis induced by IL-1 β or PAF was no different from that in PBS-treated mice, indicating that it was totally blocked (Figure 2E).

TNF- α mediates BRB breakdown induced by PAF, but not that caused by VEGF or IL-1 β

Intravitreal injection of 1 μ l of 10^{-6} M VEGF or 10 units of IL-1 β caused very similar increases in vascular leakage relative to injection of 1 μ l of PBS in mice deficient in TNF- α and wild type mice (Figure 3). In contrast, the vascular leakage caused by PAF was significantly attenuated in TNF- α -deficient mice.

Oxygen-induced ischemic retinopathy (OIR)

In wild type mice with OIR, there was a significant, roughly two-fold increase in the number of adherent leukocytes in retinal vessels (Figure 4A). Mice deficient in TNF- α with OIR had significantly less adherent leukocytes in retinal vessels than wild type mice with OIR, implicating TNF- α in the leukostasis that occurs in ischemic retinopathies. There was also a modest, but significant (roughly 25%) reduction in breakdown of the BRB in *Tnfa* knockout mice with OIR compared to wild type mice with OIR (Figure 4B), but there was no significant difference in BRB integrity between *Tnfa* knockout and wild type mice maintained in room air ($P=0.14$ for retina/lung leakage ratio and $P=0.21$ for retina/kidney leakage ratio). There was also no significant difference ($P=0.39$) in the amount of retinal NV that occurred in *Tnfa* knockout mice with OIR compared to wild type mice with OIR (Figure 4C).

Discussion

In this study, we investigated the activities of TNF- α in the retina and explored its role in ischemic retinopathies. The most prominent activity of TNF- α in the eye is induction of leukostasis. In fact, in the absence of TNF- α , IL-1 β and PAF fail to cause leukostasis and the ability of VEGF to induce leukostasis is reduced by 80%, indicating that these other factors cause leukostasis indirectly through TNF- α . Injection of TNF- α into the eye also causes extensive breakdown of the BRB. While IL-1 β and VEGF show a markedly reduced ability to induce leukostasis in mice deficient in TNF- α , they show no change in their ability to cause breakdown of the BRB, indicating that neither TNF- α nor leukostasis are involved in their compromise of the BRB. This is consistent with other studies that have demonstrated that VEGF and IL-1 β cause breakdown of the BRB by opening tight junctions between vascular endothelial cells and increasing vesicular transport (Luna et al., 1997). Since TNF- α is able to increase levels of VEGF (Nabors et al., 2003; Hangai et al., 2006), it is reasonable to postulate that VEGF contributes to mediating the effects of TNF- α on the BRB. There is also evidence that VEGF induces TNF- α from the recent report showing that anti-VEGF treatment inhibited the expression of TNF- α (Tsuchihashi et al., 2006). Leukostasis could be facilitated through the induction of TNF- α and in *Tnfa* knockout mice, leukostasis mediated by VEGF and other proinflammatory factors is suppressed. Since VEGF still promotes BRB breakdown in the absence of retinal leukostasis, this activity is not dependent on leukostasis or TNF- α . Thus it appears that these 3 factors collaborate to cause leukostasis and BRB breakdown, with TNF- α playing a primary role with regard to leukostasis and VEGF playing a primary role with regard to BRB breakdown and NV. Additional work is needed to clearly define the role of IL-1 β .

The actions of TNF- α in the retina differ from its effects in a sarcoma model in which the ability of VEGF to promote excessive vascular permeability is dependent upon TNF- α (Clauss et al.,

2001). This provides another example of how proteins can play significantly different roles in different vascular beds (Campochiaro, 2006).

One week after the onset of diabetes, mice have mild breakdown of the BRB that is attributable to leukostasis (Joussen et al., 2001), which is mediated by VEGF (Ishida et al., 2003). The relevance of this early hyperglycemia-induced change to diabetic retinopathy which takes years to develop is uncertain. Our data demonstrate that despite the ability of leukostasis to cause mild BRB breakdown, it provides only a modest contribution to the vascular leakage that occurs in ischemic retinopathy. This is not surprising since there are high levels of VEGF in ischemic retina and VEGF acts directly on vascular endothelial cells to cause leakage (Luna et al., 1997). Similarly, in view of the direct action of VEGF on endothelial cells to stimulate NV, it is not surprising that the significant reduction in leukostasis in TNF- α -deficient mice with ischemic retinopathy has no significant impact on retinal NV. This does not preclude contributions by inflammatory cells that have extravasated into the retina, since it has been demonstrated that they contribute by producing VEGF (Grunewald et al., 2006). However, it appears that TNF- α is not required for that contribution of inflammatory cells, despite its prominent role in the stimulation of leukostasis.

Previous studies have disagreed with respect to the role of TNF- α in ischemic retinopathies. Gardiner et al. (Gardiner et al., 2005) found that compared to wild type mice, *Tnfa* knockout mice with ischemic retinopathy developed less retinal NV, whereas Ilg et al. (Ilg et al., 2005) found that *Tnfa receptor* knockout mice with ischemic retinopathy showed no difference in the amount of retinal NV at P17 and showed slower regression of the NV so that at P21 there was more NV than in wild type mice. Our data are consistent with the findings of the latter study, and together they suggest that TNF- α is not a major contributor to ischemia-induced retinal NV. Recent clinical trials have suggested that antagonists of VEGF provide substantial benefit to patients with ocular NV. It is important to find other therapeutic agents that provide added benefit when used in combination with VEGF antagonists. It does not appear likely that antagonists of TNF- α are likely to fill this role, but the central role of TNF- α in leukostasis supports their continued evaluation in patients with uveitis (Ohno et al., 2004; Lindstedt et al., 2005; Kahn et al., 2006).

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Figure 1. Platelet activating factor (PAF) induces breakdown of the blood- retinal barrier (BRB) Breakdown of the BRB was measured as described in Methods following intraocular injection of PAF (1 μ l) into one eye while the contralateral eye remained untreated, which was found to be no different than a PBS injection of the same volume. Maximum BRB breakdown resulted 6 h after a 1 μ l injection of 10^{-3}M PAF. Significant BRB breakdown also occurred at 3 and 24 h after receiving the same dosage and at 48 h after receiving 10^{-4}M PAF (* $P \leq 0.04$; ** $P \leq 0.01$). At other time intervals and dosages significant BRB breakdown did not occur.

Figure 2. TNF- α is critical for leukostasis induced by VEGF, IL-1 β , and PAF

Retinal leukostasis was assessed as described in Methods after intraocular injection of 1 μ l of: (A) PBS, (B) 10^{-6} M VEGF, (C) 10U IL-1 β , or (D) 10^{-5} M TNF α . Leukocytes were labeled with FITC-conjugated Con-A. Few or no leukocytes adhered to the retinal vessels following injection of PBS, a moderate number of leukocytes adhered to the retinal vessels after VEGF or IL-1 β treatment, and pronounced leukostasis occurred following injection of TNF α . (E) Compared to wild type mice, the total number of adherent leukocytes in the retinal vasculature 6 hours after injection of 1 μ l of 10^{-6} M VEGF, 10u IL-1 β , or 10^{-3} M PAF was significantly reduced. VEGF-induced leukostasis was reduced by about 80% and that due to IL-1 β or PAF was completely blocked.

Figure 3. TNF- α is not critical for BRB breakdown induced by VEGF or IL-1 β

The retina/lung leakage ratio (RLLR) was calculated as described in Methods following intraocular injection of VEGF (10^{-6} M), IL-1 β (10U), or PAF (10^{-3} M) in *Tnfa* knockout mice or wild type mice expressed as a percentage relative to age-matched controls receiving an intraocular injection of PBS. The dotted line represents the values for PBS-injected controls. Absence of TNF α prevents the increase in BRB breakdown induced by PAF ($P=0.0006$), but not that induced by VEGF or IL-1 β .

Figure 4. In ischemic retinopathy, absence of TNF α reduces leukostasis and minimally reduces BRB breakdown, but does not reduce retinal neovascularization

(A) Retinal leukostasis was significantly greater in wild type mice with ischemic retinopathy than in *Tnf α* knockout mice with ischemic retinopathy at P17 ($P=0.04$). There was no significant difference when the mice were maintained in room air ($P=0.11$) or when comparing *Tnf α* knockout mice with and without ischemic retinopathy. (B) Breakdown of the BRB occurred in both *Tnf α* knockout and wild type mice with ischemic retinopathy, but was significantly suppressed in *Tnf α* knockout mice regardless of whether the leakage ratio was expressed relative to the lung (R/L) or the kidney (R/K). There was no significant difference between *Tnf α* knockout and wild type mice maintained in room air (RA). (C) There was no significant

difference ($P=0.39$) in the amount of ischemia-induced retinal neovascularization in *Tnfa* knockout mice compared to wild type mice.