Pigment Epithelium–Derived Factor: A Potent Inhibitor of Angiogenesis

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In the absence of disease, the vasculature of the mammalian eye is quiescent, in part because of the action of angiogenic inhibitors that prevent vessels from invading the cornea and vitreous. Here, an inhibitor responsible for the avascularity of these ocular compartments is identified as pigment epithelium–derived factor (PEDF), a protein previously shown to have neurotrophic activity. The amount of inhibitory PEDF produced by retinal cells was positively correlated with oxygen concentrations, suggesting that its loss plays a permissive role in ischemia-driven retinal neovascularization. These results suggest that PEDF may be of therapeutic use, especially in retinopathies where pathological neovascularization compromises vision and leads to blindness.

Angiogenesis, the growth of new blood vessels from preexisting ones, is under tight regulation in most healthy tissues, in part because of the influence of naturally occurring inhibitors that prevent new vessel growth (*1*). The disruption of such control mechanisms plays an essential role in the development of a variety of diseases, from arthritis to cancer (*2*). In the healthy mammalian eye, vessels are normally excluded from the cornea and the vitreous, compartments that have been shown to have antiangiogenic activity (*3*, *4*). Failure to exclude vessels from the cornea is associated with loss of visual acuity, opacification, and abnormal healing (*4*). In the retina, excessive neovascularization underlies ischemic retinopathies such as proliferative diabetic retinopathy and age-related macular degeneration (*5*), currently the leading causes of blindness in the western world.

In studies aimed at identifying antiangiogenic factors in the eye that might be regulated by the retinoblastoma tumor suppressor gene (*Rb*), we fractionated media conditioned by a retinoblastoma cell line that had been infected with a retrovirus expressing the wild-type *Rb* gene, WERI-Rb-27R (*6*). A protein purification scheme (*7*) resulted in a 1000- to 1250-fold enrichment of antiangiogenic activity and a single 50-kD band on a silver-stained protein gel. Protein microsequence analysis showed that this protein was identical to the previously described pigment epithelium–derived factor (PEDF) (*7*). PEDF (*8*) was first purified from the conditioned media of human retinal pigment epithelial cells as a factor that induced neuronal differentiation of cultured Y79 retinoblastoma cells (*9*). PEDF is neurotrophic for cerebellar granule cells, inhibits microglial growth (*10*), and is also referred to as early population doubling level cDNA (EPC-1), reflecting its up-regulation during cell cycle phase G_0 in young but not in senescing cultured fibroblasts (*11*). The protein shares sequence and structural homology with the serine protease inhibitor (Serpin) family but does not inhibit proteases (*12*). The antiangiogenic activity purified from WERI-Rb-27R conditioned media was likely due to PEDF and not to a minor contaminant, as it was retained when the protein was recovered as a single band from an SDS-polyacrylamide gel (*13*) and it was neutralized by antibodies raised against either recombinant PEDF or a PEDF peptide (Fig. 1A).

Biochemically purified as well as recombinant (*14*) forms of PEDF potently inhibited neovascularization in the rat cornea (Fig. 2, A and B). In vitro, PEDF inhibited endothelial cell migration in a dose-dependent manner with a median effective dose (ED_{50}) of 0.4 nM (Fig. 1B), placing it among the most potent natural inhibitors of angiogenesis in this assay (*15*), slightly more active than pure angiostatin (Fig. 1B), thrombospondin-1 (ED_{50} of 0.5 nM), and endostatin (ED_{50} of 3 nM). At doses of 1.0 nM or greater, PEDF also inhibited basic fibroblast growth factor (bFGF)–induced proliferation of capillary endothelial cells by 40% (*13*).

PEDF inhibited endothelial cell migration toward every angiogenic inducer we tested, including platelet-derived growth factor, vascular endothelial growth factor (VEGF), interleukin-8, acidic fibroblast growth factor, and lysophosphatidic acid (*15*). It showed some specificity for endothelial cells, inhibiting the migration of microvascular cells cultured from the bovine adrenal gland or human dermis and those from the umbilical vein. In contrast, it did not inhibit the migration of human foreskin or lung fibroblasts, aortic smooth muscle cells, oral keratinocytes, or neutrophils toward stimulatory cytokines, even when PEDF was present at concentrations 10 times that needed to inhibit endothelial cells (*15*).

Neutralizing PEDF antibodies reduced the

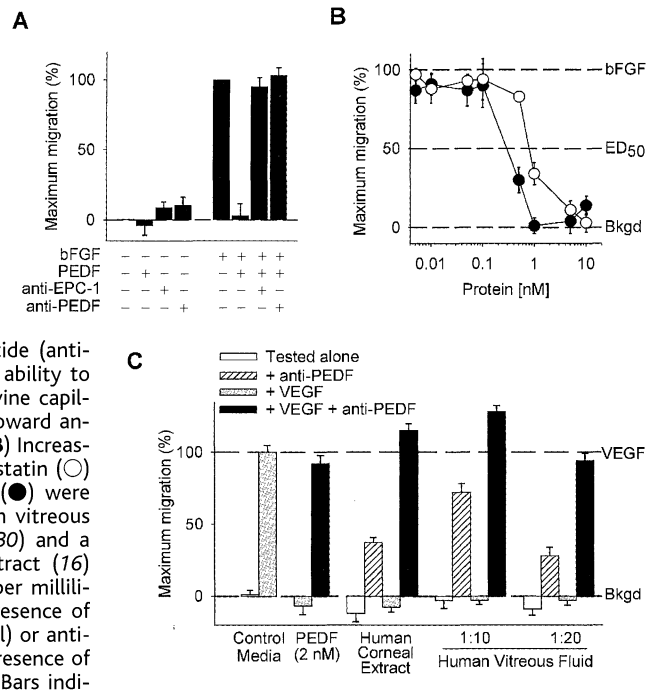

Fig. 1. Inhibitory activity of purified PEDF on migration of cultured endothelial cells and requirement of PEDF for antiangiogenic activity of human vitreous fluid and corneal extracts. (**A**) PEDF (0.1 μg/ml) purified from WERI-Rb-27R (*6*) medium was tested alone or in combination with antibody against recombinant PEDF (anti–EPC-1; 20 μg/ml) or against a PEDF peptide (anti-PEDF; 1 μg/ml) (*29*) for its ability to inhibit the migration of bovine capillary endothelial cells (*26*) toward angiogenic bFGF (10 ng/ml). (**B**) Increasing concentrations of angiostatin (○) or recombinant PEDF (*14*) (●) were tested as in (A). (**C**) Human vitreous fluid diluted as indicated (*30*) and a human corneal stromal extract (*16*) (used at 10 μg of protein per milliliter) were assayed in the presence of the inducer VEGF (0.1 ng/ml) or anti-PEDF (1 μg/ml) (or in the presence of both VEGF and anti-PEDF). Bars indicate standard error of mean (SEM) of five separate experiments (A). For (B) and (C), data from one representative experiment are shown with standard errors.

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inhibition of endothelial cell chemotaxis by stromal extracts (*4*, *16*) prepared from human (Fig. 1C), mouse, and bovine corneas (*13*). Similarly, removal of PEDF with antibody linked to protein A beads completely eliminated the antiangiogenic activity in bovine (*13*) and human stromal extracts (Fig. 2, A and B). Furthermore, addition of neutralizing antibodies to PEDF, in the absence of exogenous angiogenic inducers, stimulated the invasion of new vessels into the rat cornea (Fig. 2, A and B). This appeared to be due to local blockade of PEDF, which unmasked endogenous angiogenic stimulatory activity in the cornea (Figs. 1C and 2, A and B). Antibody to PEDF alone did not stimulate endothelial cell migration in vitro (Fig. 1A), and no neovascularization was observed in rat corneas when the antibody was preincubated with the PEDF peptide 327 to 343 against which it was raised (Fig. 2, A and B). The PEDF peptide alone was neutral in angiogenic assays (Fig. 2A).

Like the cornea, the vitreous humor is antiangiogenic (*3*) and generally devoid of vessels, and it also contains high concentrations of PEDF (*8*, *17*). We found that removal of PEDF from vitreous fluid abrogated its antiangiogenic activity and revealed an underlying angiogenic stimulatory activity (Figs. 1C and 2, A and B). The level of PEDF in the vitreous was sufficient to inhibit endothelial cell migration even in the presence of 4 ng of VEGF per milliliter of vitreous (*13*), a concentration similar to that found in vitreous fluid obtained from patients with proliferative diabetic retinopathy (*18*). Transforming growth factor β (TGFβ) has been postulated to be an inhibitor of ocular neovascularization (*19*). However, in our experiments, neutralization of TGFβ isoforms 1, 2, and 3 did not alter the antiangiogenic activity of vitreous fluid or corneal extracts in vitro (*15*) or induce corneal neovascularization in vivo (Fig. 2A).

In neonates, changes in ambient oxygen concentration can regulate the vascular density of the retina. This effect is usually attributed to changes in the level of the angiogenic inducer VEGF, which is up-regulated when oxygen is limiting and down-regulated when it is in excess (*20*). To determine if PEDF is also regulated by oxygen, we exposed newborn mice to 75% oxygen (hyperoxia) from postnatal day 7 to day 12, a condition that leads to the development of undervascularized retinas (*21*) and a decline in VEGF mRNA (*22*). The retinas of eight of nine mice exposed to hyperoxia stained strongly for PEDF at day 12 (Fig. 3B), whereas none of 10 untreated animals remaining at normoxia (21% oxygen) showed PEDF staining (Fig. 3A). In untreated animals, levels of PEDF during retinal development followed a pattern that might be expected for an angiogenic inhibitor. PEDF immunostaining was absent or weak in three of three animals before day 18 (Fig. 3, A and C), when retinal vasculature is developing (*23*), but strong in four of four mice (Fig. 3D) at day 21 and in six of six

A

Sample	bFGF (0.15 nM)	anti-PEDF (20 μg/ml)	anti-TGFβ (50 μg/ml)	Positive corneas/ total implanted
1. PBS	–	–	–	0/2
2. PBS	+	–	–	8/8
3. PBS	–	+	–	5/5
4. PBS	–	–	+	0/2
5. PEDF peptide	–	–	–	0/2
6. PEDF peptide	–	+	–	1/4[a]
7. rPEDF	–	–	–	0/2
8. rPEDF	+	–	–	0/3
9. pPEDF	–	–	–	0/3
10. pPEDF	+	–	–	0/3
Before PEDF removal				
11. Vitreous	–	–	–	0/4
12. Vitreous	+	–	–	0/4
13. Vitreous	–	–	+	0/3
14. Vitreous	+	–	+	0/3
15. Cornea extract	–	–	–	0/3
16. Cornea extract	+	–	–	1/4[a]
After PEDF removal				
17. Vitreous	–	–	–	6/6
18. Cornea extract	–	–	–	4/4
19. Cornea extract	+	–	–	3/3

[a]One cornea gave a mild response with a few sprouting vessels that did not reach the pellet.

B

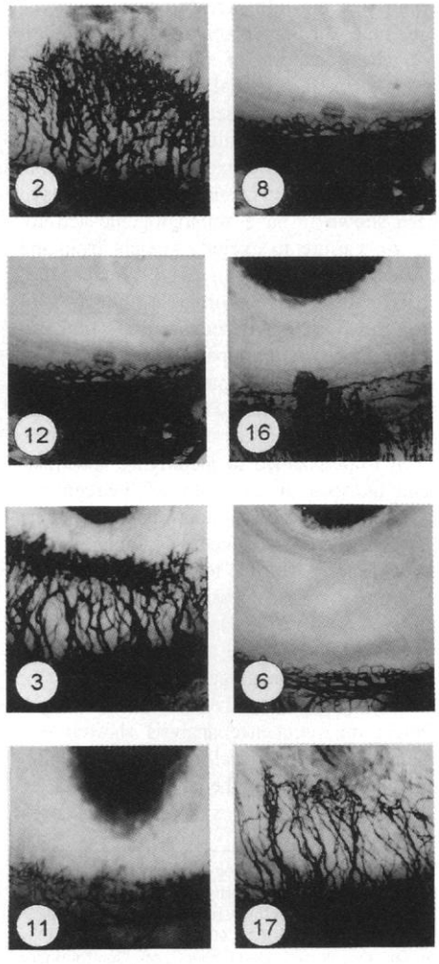

Fig. 2. Inhibition of neovascularization by purified PEDF and by PEDF present naturally in normal human vitreous and cornea. (**A**) Recombinant (rPEDF) or purified (pPEDF) PEDF (8 nM), PEDF peptide 327 to 343 (200 μg/ml), undiluted vitreous fluid, or corneal extract (used at 200 μg of protein per milliliter) was incorporated with vehicle (PBS) and the indicated additions into Hydron pellets that were implanted into the avascular rat cornea. Vigorous ingrowth of vessels from the limbus toward the pellet by 7 days was scored as a positive response (*28*). Where noted, anti-PEDF (*29*) linked to protein A beads was used to remove PEDF from vitreous fluid and corneal extract. (**B**) Representative photos of corneal responses from (A) shown at ×13 magnification.

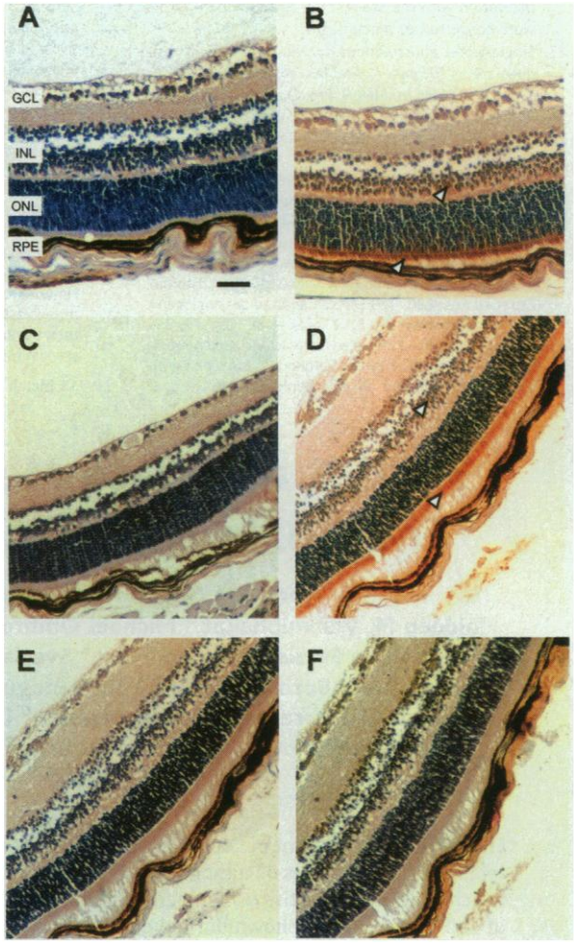

Fig. 3. Induction of PEDF protein expression by hyperoxia in the neonatal mouse. Retinas were harvested at postnatal day 12 (P12) (**A** and **B**), P18 (**C**), or P21 (**D**) from C57BL/6 mice that had been maintained at ambient oxygen (A, C, and D) or exposed to 75% oxygen from P7 to P12 (B) and stained for PEDF. Note accumulation of PEDF (arrowheads), as indicated by reddish-brown color. Control sections directly adjacent to (D) were stained without primary antibody (**E**) or after preincubation of primary antibody with PEDF peptide 327 to 343 (**F**). Retina layers indicated in (A) include retinal pigment epithelium (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Mouse eyes were fixed in formalin within 1 to 5 min of harvest. For immunostaining, paraffin-embedded sections were incubated with anti-PEDF (*29*) and visualized with ABC methods (Vectastain Elite; Vector Labs, Burlingame, California). Scale bar, 25 μm.

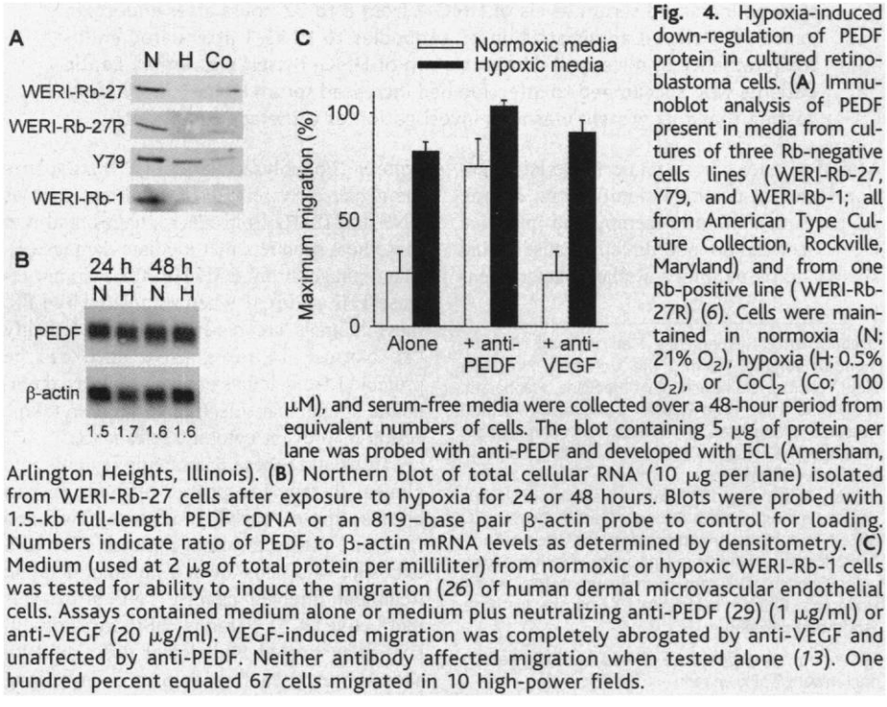

Fig. 4. Hypoxia-induced down-regulation of PEDF protein in cultured retinoblastoma cells. (**A**) Immunoblot analysis of PEDF present in media from cultures of three Rb-negative cells lines (WERI-Rb-27, Y79, and WERI-Rb-1; all from American Type Culture Collection, Rockville, Maryland) and from one Rb-positive line (WERI-Rb-27R) (*6*). Cells were maintained in normoxia (N; 21% O_2), hypoxia (H; 0.5% O_2), or $CoCl_2$ (Co; 100 μM), and serum-free media were collected over a 48-hour period from equivalent numbers of cells. The blot containing 5 μg of protein per lane was probed with anti-PEDF and developed with ECL (Amersham, Arlington Heights, Illinois). (**B**) Northern blot of total cellular RNA (10 μg per lane) isolated from WERI-Rb-27 cells after exposure to hypoxia for 24 or 48 hours. Blots were probed with 1.5-kb full-length PEDF cDNA or an 819–base pair β-actin probe to control for loading. Numbers indicate ratio of PEDF to β-actin mRNA levels as determined by densitometry. (**C**) Medium (used at 2 μg of total protein per milliliter) from normoxic or hypoxic WERI-Rb-1 cells was tested for ability to induce the migration (*26*) of human dermal microvascular endothelial cells. Assays contained medium alone or medium plus neutralizing anti-PEDF (*29*) (1 μg/ml) or anti-VEGF (20 μg/ml). VEGF-induced migration was completely abrogated by anti-VEGF and unaffected by anti-PEDF. Neither antibody affected migration when tested alone (*13*). One hundred percent equaled 67 cells migrated in 10 high-power fields.

adults, when neovascularization of the retina is essentially complete (*23*). Highest PEDF levels were seen in the photoreceptor cell layer, the most avascular layer of the retina.

To further investigate the effect of oxygen regulation on PEDF, we maintained retinoblastoma tumor cells in low oxygen (0.5%) or in chemical agents that simulate hypoxia (*24*). As expected, hypoxia induced a 9.5 ± 4.8-fold rise in the level of VEGF in conditioned media as measured by enzyme-linked immunosorbent assay and reduced the level of PEDF by 11.8 ± 4.7-fold (Fig. 4A). The responses of Rb-negative retinoblastoma cells and of revertants reexpressing Rb were similar (Fig. 4A). No difference in PEDF mRNA levels was detected among hypoxia-treated and untreated cells (Fig. 4B), suggesting that hypoxic regulation of PEDF occurred at the translational or posttranslational level.

Medium conditioned by hypoxic tumor cells was more angiogenic than that conditioned by normoxic tumor cells (Fig. 4C). Hypoxia reduced the concentration of medium needed to induce 50% of maximal endothelial cell chemotaxis from 4.0 to 0.3 μg of total protein per milliliter. Neutralization of VEGF, which made only a minor contribution to the angiogenic activity of these cells, did not reduce the angiogenic activity of the hypoxic conditioned media, but neutralization of PEDF made normoxic tumor media as angiogenic as that derived from hypoxic cells (Fig. 4C). Consistent with these in vitro studies, tumor cells present in 12 out of 12 human retinoblastoma pathologic specimens failed to stain for PEDF, presumably in part because of limited oxygen in the tumor environment (*25*), whereas adjacent normal retina was positive.

In summary, PEDF is likely to contribute to the regulation of blood vessel growth in the eye by creating a permissive environment for angiogenesis when oxygen is limiting (as it is in tumors and in retinopathies) and an inhibitory environment when oxygen concentrations are normal or high. Given its high potency and the broad range of angiogenic inducers against which it can act, PEDF may prove to be a useful therapeutic for pathologic ocular neovascularization as well as for retinoblastomas, where its dual activities of inducing cell differentiation (*9*) and inhibiting angiogenesis may be particularly effective.

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7. PEDF was purified from WERI-Rb-27R (*6*) serum-free conditioned media by sequential steps consisting of dialysis (molecular mass cutoff, 30 kD) against distilled water, 60 to 95% ammonium sulfate precipitation, step elution from lentil lectin Sepharose 4B (Pharmacia) with 0.5 M α-methyl-D-mannopyranoside, and elution from a HiTrap heparin Sepharose column (Pharmacia) with increasing NaCl gradient. Purification was monitored by an endothelial cell migration assay (*26*), and the yield was 17.5%. Edman degradation of proteolytically derived internal peptides of the protein yielded two unambiguous sequences (TSLEDFYLDEERTVRVPMMXD and IAQLPLTGXM) (*27*). A BLAST protein homology search revealed that PEDF contains identical sequences.
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14. Human PEDF cDNA was engineered by polymerase chain reaction to encode a COOH-terminal hexahistidine tag, cloned into pCEP4 (Invitrogen), and transfected into human embryonic kidney cells. Recombinant PEDF was purified from the conditioned media with the Xpress Protein Purification System (Invitrogen).
15. See supplemental figures, available at www.sciencemag.org/feature/data/1040070
16. For preparation of stromal extract, corneas were freed of associated epithelium and as much of the endothelium as possible, washed extensively in ice-cold phosphate-buffered saline (PBS, pH 7.4), and minced into small fragments that were incubated for 24 hours in PBS containing 0.5 mM phenylmethanesulfonyl fluoride. The extract was filter sterilized, stored at −80°C, and tested in migration assays at a final concentration of 10 μg of protein per milliliter.
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26. Migration assays were performed in quadruplicate for each sample with bovine adrenal capillary endothelial cells or human dermal microvascular endothelial cells (Clonetics, San Diego, CA) as described (*28*). To combine multiple experiments, we first subtracted background migration (Bkgd) toward vehicle (0.1% bovine serum albumin) and then normalized data by setting maximum migration toward inducer alone to 100%. All experiments were repeated two to five times. Statistics were performed on raw data before normalization with the Student's *t* test. Standard errors were converted to percentages.
27. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; X, any amino acid; and Y, Tyr.
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29. PEDF antipeptide antibody (anti-PEDF) was raised in rabbits against a peptide containing PEDF amino acids 327 to 343, conjugated to Keyhole-limpet hemocyanin, and affinity-purified on a peptide column. Polyclonal antisera against bacterial recombinant PEDF/EPC-1 (anti–EPC-1) [B. R. DiPaolo, R. J. Pignolo, V. J. Cristofalo, *Exp. Cell Res.* **220**, 178 (1995)] and the antiangiogenic protein angiostatin [M. S. O'Reilly *et al.*, *Cell* **79**, 315 (1994)] were gifts. Purchased reagents included neutralizing anti-VEGF (Genzyme, Cambridge, MA), pan antibodies to TGFβ, and all angiogenic inducers (R & D Systems, Minneapolis, MN) except lysophosphatidic acid (Sigma). All proteins and antibodies were extensively dialyzed against PBS before use in biological assays.
30. Human vitreous fluid was withdrawn from three cadaveric eyes (refrigerated within 1.4 to 4.5 hours of death) obtained from individuals without ocular disease. Fluid was frozen until used. Fresh vitreous fluid was obtained from bovine and mouse eyes.
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HMG-1 as a Late Mediator of Endotoxin Lethality in Mice

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Endotoxin, a constituent of Gram-negative bacteria, stimulates macrophages to release large quantities of tumor necrosis factor (TNF) and interleukin-1 (IL-1), which can precipitate tissue injury and lethal shock (endotoxemia). Antagonists of TNF and IL-1 have shown limited efficacy in clinical trials, possibly because these cytokines are early mediators in pathogenesis. Here a potential late mediator of lethality is identified and characterized in a mouse model. High mobility group–1 (HMG-1) protein was found to be released by cultured macrophages more than 8 hours after stimulation with endotoxin, TNF, or IL-1. Mice showed increased serum levels of HMG-1 from 8 to 32 hours after endotoxin exposure. Delayed administration of antibodies to HMG-1 attenuated endotoxin lethality in mice, and administration of HMG-1 itself was lethal. Septic patients who succumbed to infection had increased serum HMG-1 levels, suggesting that this protein warrants investigation as a therapeutic target.

Mortality rates for systemic bacterial infection have not declined significantly, despite advances in antibiotic therapy and intensive care. Bacteria do not directly cause lethal shock and tissue injury. Rather, bacterial endotoxin (lipopolysaccharide, LPS) stimulates the acute, early release of cytokines such as TNF and IL-1β from macrophages, and it is these host products that mediate damage (*1*). Macrophages from C3H/HeJ mice do not release TNF and IL-1 when stimulated by LPS; these animals are resistant to LPS lethality (*2*). Normal, LPS-responsive mice can be protected from lethal endotoxemia by therapeutic agents that selectively inhibit cytokine action or prevent cytokine release (*3*).

Translating these pathogenic insights into clinical therapy has proved difficult, in part because these "early" mediators (TNF and IL-1) are released within minutes after LPS exposure (*4*). Thus, even a minimal delay in treatment directed against TNF or IL-1 is ineffective (*3, 5*). Paradoxically, LPS-responsive mice treated with lethal doses of LPS succumb at latencies of up to 5 days, long

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