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VEGF deprivation-induced apoptosis is a component of programmed capillary regression

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SUMMARY

The pupillary membrane (PM) is a transient ocular capillary network, which can serve as a model system in which to study the mechanism of capillary regression. Previous work has shown that there is a tight correlation between the cessation of blood flow in a capillary segment and the appearance of apoptotic capillary cells throughout the segment. This pattern of cell death is referred to as synchronous apoptosis (Lang, R. A., Lustig, M., Francois, F., Sellinger, M. and Plesken, H. (1994) *Development* 120, 3395-3404; Meeson, A., Palmer, M., Calfon, M. and Lang, R. A. (1996) *Development* 122, 3929-3938). In the present study, we have investigated whether the cause of synchronous apoptosis might be a segmental deficiency of either oxygen or a survival factor. Labeling with the compound EF5 in a normal PM indicated no segmental hypoxia; this argued that oxygen deprivation was unlikely to be the cause of synchronous apoptosis. When rat plasma was used as a source of survival factors in an in vitro PM explant assay, inhibition of vascular endothelial growth factor (VEGF) all but eliminated the activity of plasma in suppressing apoptosis. This argued that VEGF was an important plasma survival factor. Furthermore, inhibition of VEGF in vivo using fusion proteins of the human Flk-1/KDR receptor resulted in a significantly increased number of capillaries showing synchronous apoptosis. This provides evidence that VEGF is necessary for endothelial cell survival in this system and in addition, that VEGF deprivation mediated by flow cessation is a component of synchronous apoptosis.

Key words: Apoptosis, Vascular regression, Tissue remodelling, Tissue regression, VEGF, Capillary, Flk-1, Flt-1

INTRODUCTION

Despite the potential value of an understanding of its mechanism, the means by which capillaries regress is not currently understood. A number of different systems have been used to assess capillary regression at the cellular level. These have included the regression that follows growth-factor-induced angiogenesis in the cornea (Ausprunk et al., 1978) and developmental systems where capillary regression is a component of a normal developmental program. Studies in the chick have characterized the capillary regression associated with formation of the precartilaginous condensations that will eventually give rise to bone in the limb (Feinberg et al., 1986; Latker et al., 1986; Hallmann et al., 1987). Since regression of the interdigit is associated with vascular regression, this too has served as a subject of experimental analysis designed to characterize the process at the cellular level (Hurle et al., 1985). The temporary ocular capillary networks collectively referred to as the hyaloid vessel system have also been used to assess the morphological events associated with capillary regression (Szirmai and Balazs, 1958; Jack, 1972; Balazs et al., 1980; Latker and Kuwabara, 1981).

The PM is a transient ocular capillary network (Mann, 1928; Matsuo and Smelser, 1971) that has proven a versatile subject of experimental analysis. This structure resides in the anterior chamber on the anterior surface of the lens and iris diaphragm and, in rodents, regresses in the second week after birth, presumably as an adaptation to allow efficient light transmission to the retina. Since this structure can be visualized vitally (Meeson et al., 1996), can be examined in whole-mount preparation (Lang et al., 1994) and is accessible via injection (Diez-Roux and Lang, 1997), it offers a number of advantages. Specifically, previous analysis has suggested that regression of the PM involves programmed cell death (PCD) with two distinct causes. The first apoptotic events occurring in capillary regression (Lang et al., 1994) are macrophage-dependent (Diez-Roux and Lang, 1997; Lang and Bishop, 1993), while a second phase of apoptosis, accounting for most of the cell death, appears to be dependent upon flow stasis (Meeson et al., 1996).

Much progress has been made in identifying molecular mediators of vasculogenesis and angiogenesis. These include the tyrosine kinase receptors Flk-1 (de Vries et al., 1992) and Flt-1 (Shibuya et al., 1990) and their ligand vascular endothelial growth factor (VEGF) (Klagsbrun and Rifkin, 1987; Plate et al., 1994; Neufeld et al., 1994; Ferrara and Davis-Smyth, 1997). In *flt-1* (Fong et al., 1995) and *flk-1* (Shalaby et al., 1995) gene targeted mice, embryonic death occurs in homozygotes at embryonic day 8.5-9.5. The phenotype of *flk-1* null mice shows that signaling through this receptor is essential for the very early steps in the formation of endothelial cells in blood islands and embryonic vessels (Shalaby et al., 1995). In contrast, in *flt-1* null mice, endothelial cells do differentiate but form vessels of abnormal morphology (Fong et al., 1995). This argues that the cellular responses to Flt-1 and Flk-1 signaling may be different. Mice that are missing even one functional copy of the *VEGF* gene die early in gestation with little development of the vascular system (Carmeliet et al., 1996; Ferrara et al., 1996). The death of *VEGF* homozygous null embryos at a similar stage of development to that of the Flt-1- and Flk-1-targeted animals has suggested that VEGF is the predominant ligand for Flt-1 and Flk-1 during development of the vascular system (Carmeliet et al., 1996; Ferrara et al., 1996). A number of culture studies show that one function of VEGF is to stimulate endothelial cell survival (Katoh et al., 1995; Watanabe and Dvorak, 1997). Some experimental manipulations performed in vivo have also led to the same conclusion. In rodent models of retinopathy of prematurity, injection of VEGF into the vitreous can suppress the PCD associated with regression of retinal capillaries stimulated by hyperoxia (Alon et al., 1995). The suppression of PCD by excess VEGF argues that one of its normal in vivo functions is to stimulate endothelial cell survival.

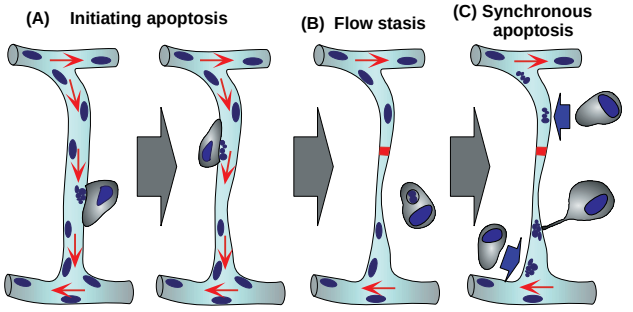

Fig. 1. A model to describe the cellular events occurring during PM regression. Current evidence suggests that programmed capillary regression occurs through a two-step mechanism. The model suggests that the first apoptoses of capillary cells are dependent upon the macrophage (Diez-Roux and Lang, 1997; Lang and Bishop, 1993). This event is termed initiating or isolated apoptosis (A). We have also shown that isolated apoptosis ultimately results in lumen restriction and a block to plasma flow within a capillary segment (Meeson et al., 1996). Red arrows indicate flow and the red bar, a block to flow (B). We have suggested that capillary cells die subsequently with a synchronous pattern because they are denied survival factors present in plasma (C). The large blue arrows indicate the likely chemotactic response of macrophages to apoptotic cells. This figure has been published previously (Lang et al., 1994; Lang, 1997).

The pattern of PCD (Wyllie et al., 1980) observed during PM regression has suggested a cellular mechanism to explain the regression process (Lang et al., 1994; Fig. 1). We have shown in two different studies (Diez-Roux and Lang, 1997; Lang and Bishop, 1993) that the first cell death in the PM is dependent on macrophages. These events are discerned histologically as isolated apoptotic capillary cells in otherwise normal segments and have been referred to as initiating apoptosis (Fig. 1A). We have previously demonstrated that initiating apoptosis can result in flow stasis when the capillary lumen becomes narrow (Fig. 1B; Meeson et al., 1996). In turn, the model proposes that lack of flow causes the synchronous pattern of apoptosis that has previously been observed, perhaps due to plasma survival factor deprivation (Lang et al., 1994; Fig. 1C).

In the current study, we have determined whether withdrawal of VEGF stimulation might be one element of programmed capillary regression; this suggestion arises directly from the model described. Our results show that all the components necessary for VEGF signaling are present in the cells of the PM during the phase of regression and that PM cells are responsive to VEGF signaling. Since inhibition of circulating VEGF results in a significantly increased number of capillaries showing synchronous apoptosis, the data provide evidence that, when flow stasis occurs, cell death will result in part from VEGF deprivation.

MATERIALS AND METHODS

Animal breeding

Sprague-Dawley rats were obtained from Taconic Farms (German Town, NY). Ages of rats were recorded as days postconception for experimental purposes but converted to days after birth in this publication. The day of birth was assumed to be the 22nd day of gestation. Thus, postconception day 31.5 (PC31.5) is equivalent to 9.5 days after birth (A9.5). *flt-1* and *flk-1* mutant mice (Fong et al., 1995; Shalaby et al., 1995) were generously provided by Dr Janet Rossant. All animals were maintained according to institutional guidelines.

Trans-corneal injection

Trans-corneal injection was performed as previously described (Diez-Roux and Lang, 1997). Recombinant VEGF 165 was either prepared as previously described (Cohen et al., 1992) or was obtained commercially (Genzyme). A volume of 0.5 μl of VEGF in phosphate-buffered saline (PBS) supplemented with 0.1% (1 μg/μl) bovine serum albumin was injected transcorneally at A9.5 and A11.5. PM regression was assessed at A13.5. The estimated volume of the aqueous is 5 μl. Each batch of VEGF was tested for activity by determining whether it would support the growth of human umbilical vein endothelial cells in culture.

In vivo inhibition of VEGF

Soluble VEGF receptors were generated either as an alkaline phosphatase (Tessler et al., 1994) or immunoglobulin fusion protein. To inhibit circulating VEGF, a mass of 85 μg was injected intraperitoneally using solutions that were in the range of 0.5-1 mg/ml. Soluble receptors were injected either at A7.5 (KDR-AP) or A8.5 (KDR-Ig) and the impact on PM regression assessed 48 hours later. Testing for levels of bacterial lipopolysaccharide was routinely performed on these preparations and shown to be at negligible levels.

Histological procedures

The PM/iris diaphragm complex was dissected from the rat eye as previously described (Lang et al., 1994). Apoptosis was recognized as the characteristic formation of chromatin fragments in apoptotic bodies (Wyllie et al., 1980) after nuclei were stained with Hoechst 33528 (Polysciences) or as cells that labeled positive with a modified version (Lang et al., 1994) of the terminal deoxyuridine nick end labeling (TUNEL) technique (Gavrieli et al., 1992). Tissues expressing β-galactosidase were stained according to standard procedures (Song et al., 1996).

Detection of hypoxia

Hypoxic conditions were detected using a previously established method (Lord et al., 1993) that relies on the oxygen-dependent formation of macromolecular adducts of a pentafluorinated derivative of etanidazole [EF5; 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide]. Since EF5 adducts can be detected with an antibody (ELK2-4), standard immunofluorescence methods can be used to detect tissue in which hypoxia existed. Both EF5 and the detection antibody ELK2-4 were generously provided by Dr Cameron Koch.

Immunoblotting

Immunoblotting for detection of VEGF in neonatal rat plasma was performed according to standard procedures (Harlow and Lane, 1988) using chemiluminescence visualization reagents (Amersham). The primary antibody, used at a dilution of 1:5000, was a rabbit polyclonal anti-mouse VEGF antiserum kindly provided by Dr Gera Neufeld. Protein A-HRP (Kirkegaard and Perry Laboratories Inc.) was used at a 1:5000 dilution.

Ex vivo assay for capillary cell survival activities

Survival stimuli for capillary cells of the PM were assessed using an explant assay. PMs were dissected from the eyes of rats at A9.5 and placed in culture medium comprising Dulbecco's modified Eagle's medium (Gibco BRL, NY) supplemented with neonatal rat plasma from A8.5 (at either 5% or 50%), heparin at 20 ng/ml, recombinant VEGF 165 (at 25 ng/ml, where applicable), VEGF antiserum (at a dilution of 1:200, where applicable) or combinations thereof. Neonatal rat plasma from any age between A5 and A14 was found to be effective in the assay. PM explants were cultured for a period of 6 hours prior to fixation and quantitation of apoptosis.

Plasma was harvested from neonatal rats by first injecting 50 units heparin subcutaneously and 1 hour later performing cardiac puncture with a heparin-coated needle and syringe under anaesthesia. Cells were centrifuged from whole blood (2500 revs/minute, 4°C, 20 minutes), the plasma fraction harvested and filter sterilized before use. This procedure prevented blood clotting and platelet aggregation.

RESULTS

Cessation of capillary flow results in synchronous apoptosis

Previous studies on the mechanism of programmed capillary regression had indicated a strong correlation between flow stasis and the induction of PCD in an entire capillary segment (referred to as synchronous apoptosis) (Meeson et al., 1996; Lang et al., 1994; Fig. 1). To ask directly whether cessation of flow caused synchronous apoptosis, we incubated intact enucleated rat eyes from A7.5 at 37°C. Enucleation ensured that plasma flow was eliminated but retained the normal aqueous environment for the PM. The percentage of capillaries showing a synchronous pattern of apoptosis was quantitated over a 16 hour time course and showed that there was a dramatic increase in the appearance of such capillaries between 8 and 12 hours (Fig. 2) with the proportion of capillaries showing a synchronous pattern of apoptosis reaching 100%. This experiment provided a simple demonstration that preventing flow resulted in the synchronous pattern of apoptosis observed during normal PM regression (Lang et al., 1994) and was consistent with earlier correlative evidence (Meeson et al., 1996).

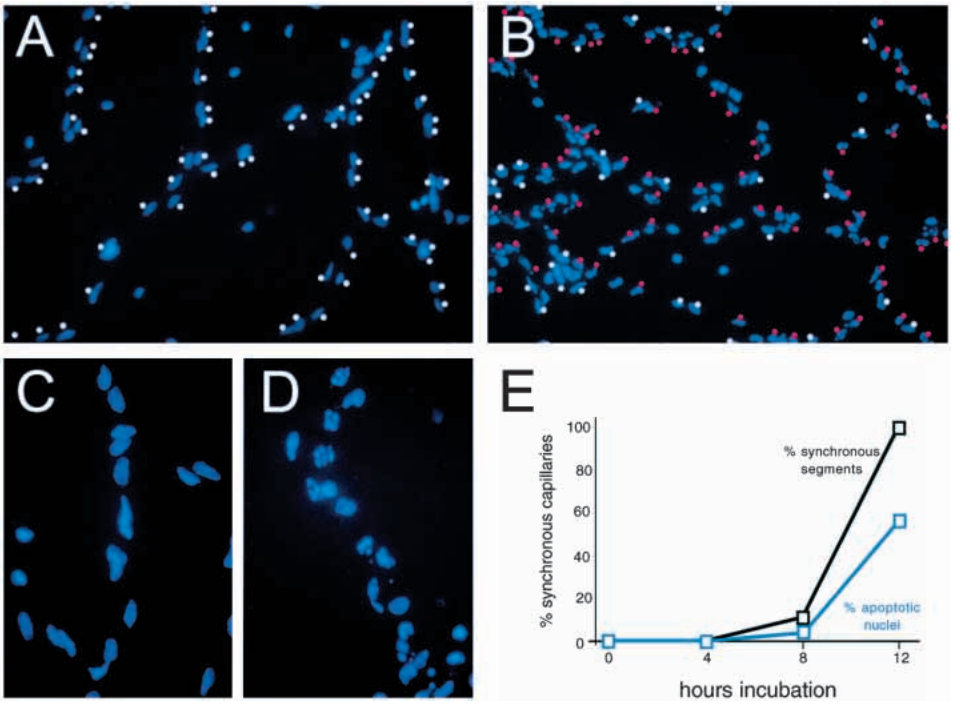

Fig. 2. Imposed cessation of blood flow in the PM causes a synchronous pattern of apoptosis. (A) Wild-type PM at A9.5. (B) PM from A9.5 rat after the enucleated eye was cultured for 12 hours. In both A and B, each cell with apoptotic morphology is marked by an adjacent red dot. Each non-macrophage with normal morphology is marked by a white dot. (C) Higher magnification of chromatin-labeled normal PM. (D) Higher magnification of chromatin-labeled PM after 12 hours of incubation in the absence of blood flow. Apoptotic morphology in almost all capillary cells is observed. (E) The % of total cells showing apoptotic morphology (blue line) and the proportion of segments showing synchronous apoptosis (black line) over the 12 hour time course of eye explant culture.

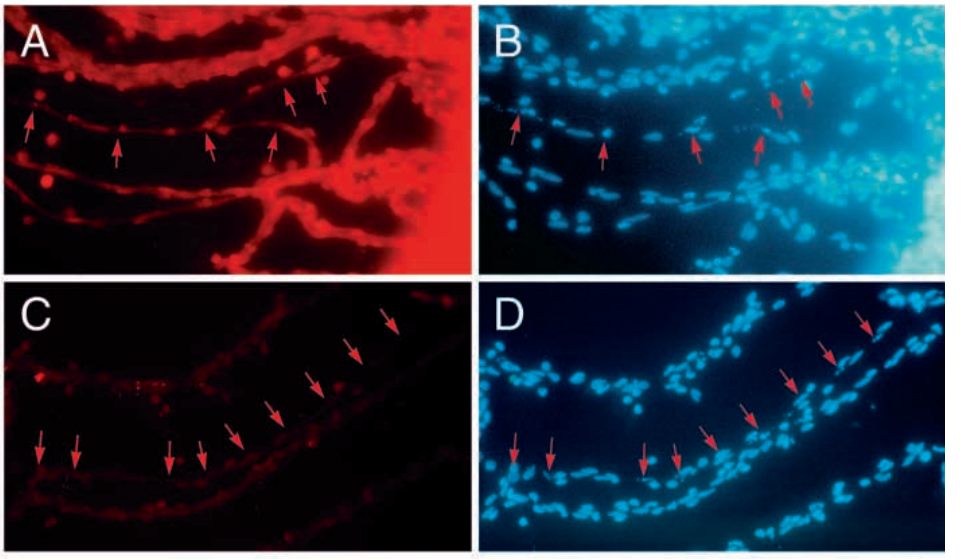

Fig. 3. PM capillary segments do not appear hypoxic. (A) PM from A7.5 labeled for hypoxic cells after hypoxia was purposefully generated. The strong red labeling is indicative of antibody binding to the modified form of EF5 (Lord et al., 1993). (B) The PM from A illuminated for detection of the chromatin dye Hoechst 33528. (C) A normal PM labeled for hypoxic cells. (D) The PM shown in C, illuminated for detection of the chromatin dye Hoechst 33528. Capillary segments undergoing synchronous apoptosis are indicated by the red arrows in all panels. Both membranes were labeled in the same experiment and the exposure times for the micrographs in A and C were identical.

Regressing capillaries of the PM do not appear hypoxic

Possible mechanisms to explain synchronous apoptosis included deprivation of either oxygen or a survival factor as a consequence of flow stasis. To test whether capillary cells of the regressing PM were hypoxic, we took advantage of a technique for specifically labeling hypoxic cells (Lord et al., 1993). The method relies on the conversion of the compound EF5 into an antibody-detectable product in hypoxic but not normal cells and has been accepted as a sensitive method for detecting conditions of hypoxia in vivo (Graeber et al., 1996; Koch et al., 1997). When control PMs were made hypoxic, both normal capillaries and those showing a synchronous pattern of apoptosis labeled positive with the anti-EF5 antibody (Fig. 3A,B). In contrast, neither intact nor regressing capillaries in normal PMs showed significant levels of labeling (Fig. 3C,D). This argued that hypoxia was not generated on a segment-by-segment basis after flow stasis during normal capillary regression. This further suggested, despite the limitations of the assay, that hypoxia was unlikely to be the cause of synchronous apoptosis.

Plasma and aqueous contain a survival factor for PM cells

If synchronous apoptosis results from survival factor deprivation due to cessation of blood flow (Fig. 1), several testable predictions are identifiable. First, an endothelial cell survival activity should be found in the circulation in the plasma fraction and, second, cell death that is a consequence of flow cessation might be prevented through provision of one or more survival factors or hastened through their inhibition.

To test the first of these predictions, we established a simple PM explant assay. This involved dissection of the PM from the anterior chamber of the eye and its incubation in culture medium. Since vascular connections are severed in this dissection, cessation of blood flow to all capillaries of the structure results. When cultured in standard medium, apoptosis of most cells within the capillaries occurred in 6 hours as determined by the TUNEL assay (Fig. 4A). To determine if an endothelial cell survival activity was present in plasma, PMs were cultured in the presence of 50% plasma from an A8.5 rat for a period of 6 hours and this resulted in the survival of most cells within the structure (Fig. 4B). Quantitation of TUNEL-positive cells after 6 hours of culture in the presence of either 5% or 50% rat plasma (Fig. 4C) confirmed that 50% plasma was sufficient to markedly reduce the number of apoptotic cells. 5% plasma was not sufficient to generate a significant change under these assay conditions (Fig. 4C). In all cases, plasma was harvested in the presence of heparin in order to prevent the aggregation of platelets and the release of platelet contents. Testing of fetal bovine aqueous showed that this too was a source of one or more survival factors (Fig. 4C). These data argued that both plasma and aqueous contained a survival factor active for cells of the PM.

VEGF is an endothelial cell survival stimulus in plasma

As a first step in identifying candidates for the survival factor(s) present in rat plasma, we performed the PM explant assay in the presence of basic FGF (not shown), PDGF (not shown) or VEGF (Fig. 4C) and showed that all had survival-stimulating activity. Furthermore, polyclonal anti-VEGF antiserum, but not control rabbit serum, could greatly suppress the survival stimulating activity of 50% plasma (Fig. 4C). Since anti-VEGF antiserum returned PCD to close to control levels, it was likely that VEGF was an important survival factor. To confirm the presence of VEGF in plasma, we performed immunoblotting on samples harvested from rats at postnatal days 8.5 and 11.5 (during the regression phase) as well as those from adults. The isoforms VEGF120, VEGF164 and VEGF188 were readily detected in plasma from rats of all ages (Fig. 4D) albeit with some non-reproducible variations in band intensities. These data are consistent with recent evidence showing that VEGF can be detected at low levels in human plasma from which platelet contents have been excluded (Maloney et al., 1998).

It has been established by histological analysis that bovine embryos have a PM that regresses and that the associated cell death is apoptosis (A. M. and R. A. L., unpublished). Due to the larger size of the anterior chamber in the eyes of these animals, it was possible to obtain sufficient aqueous to test for the presence of VEGF via immunoblotting. Immunoreactive species were present in all samples examined and appeared equivalent in size to VEGF isoforms 120 and 164 (Fig. 4D). VEGF in the

rabbit aqueous has recently been quantitated and shown to decrease from approximately 750 pg/ml to approximately 190 pg/ml over the period of time that the PM regresses (Yanagawa et al., 1998). Combined, these studies indicate significant levels of different VEGF isoforms are distributed both intraluminally and extraluminally with respect to capillaries of the PM.

To determine whether receptors for the VEGF signaling pathway were expressed in the endothelial cells of the PM, we used the *flt-1* and *flk-1* mutant mice that express bacterial *lacZ* in place of the receptor gene (Fong et al., 1995; Shalaby et al., 1995). X-gal staining of dissected PMs from heterozygous *flt-1* and *flk-1* mice revealed that both receptor genes were expressed (Fig. 5B,C). The presence of VEGF in the circulation and aqueous, combined with the expression of VEGF receptors argued that, for cells of the PM, VEGF signaling could be a factor in regulating their survival.

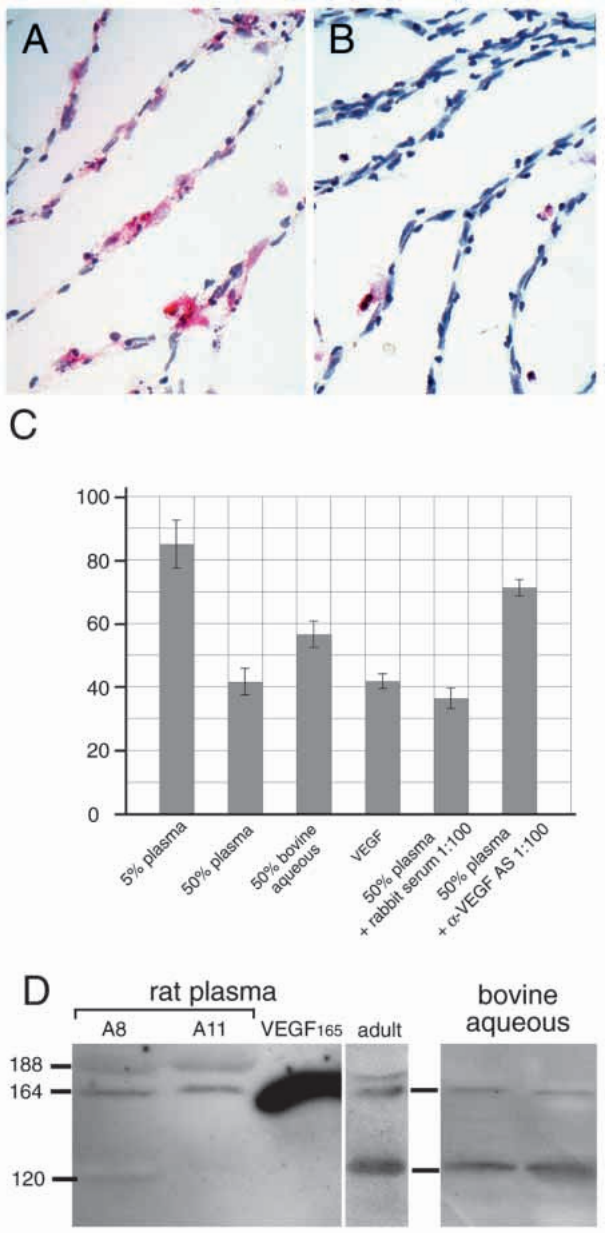

Fig. 4. VEGF is in the plasma and the aqueous. (A) TUNEL labeling of an explanted PM after 6 hours culture in DMEM + 5% neonatal rat plasma. (B) As in A, but with 50% neonatal rat plasma. (C) Quantification of the % apoptotic cells in PMs under the conditions indicated. The error bars represent standard errors of the mean. (D) Immunoblot detection of VEGF isoforms in rat plasma (left panels) and bovine aqueous (right panel). 188, 164 and 120 refer to the number of residues present in the different VEGF isoforms in the rat. The heavy band in the track labeled VEGF165 is recombinant human VEGF included as a positive control.

Cells of the PM are sensitive to VEGF signaling

As one means of determining whether cells of the PM were sensitive to VEGF signals, we asked if the $Flk^{+/lacz}$ and $Flt^{+/lacz}$ (heterozygous knockout) animals had measurable defects in this structure. Since PMs from wild-type (Fig. 5A) and $Flk^{+/lacz}$ and $Flt^{+/lacz}$ mice (Fig. 5B,C) were readily prepared in whole mount, we quantified the number of capillary junctions from postnatal days 4.5 to 9.5 (Fig. 5D). This showed that, in wild-type mice, the complexity of the membrane did not change from A4.5 to A6.5 but that, subsequently, capillary regression was indicated by a reduction in the number of capillary junctions between A6.5 and A7.5. Interestingly, both $Flk^{+/lacz}$ and $Flt^{+/lacz}$ membranes were less complex prior to the beginning of regression and showed, when compared with wild-type membranes, 33% ($Flt^{+/lacz}$) and 67% ($Flk^{+/lacz}$) reductions in the number of capillary junctions at A4.5 (the micrographs of membrane preparations shown in Fig. 5A-C do not represent membrane complexity adequately as capillary density varies). Like wild-type membranes, both $Flk^{+/lacz}$ and $Flt^{+/lacz}$ membranes showed a reduction in complexity from A6.5 to A7.5 indicating that the basic regression machinery was functional. The hypocellularity of $Flk^{+/lacz}$ and $Flt^{+/lacz}$ PMs is presumably indicative of a defect in formation of this structure and shows that PM cells are responsive to VEGF signaling in vivo.

Excess VEGF has a mild effect in inhibiting PM regression in vivo

Since the VEGF receptor heterozygous mutant animals have poorly developed PMs, it would be invalid to use these animals to draw conclusions about the role of VEGF in regulating synchronous apoptosis. For this reason, we have used a more direct approach to asking whether VEGF is involved. Specifically, trans-corneal injection (Diez-Roux and Lang, 1997) allowed us to determine whether excess VEGF was sufficient to suppress synchronous apoptosis in vivo. Given evidence that biological responses to growth factors may follow a bell-shaped concentration-dependence curve, we injected VEGF in three different amounts.

Recombinant human VEGF of the 165 amino acid isoform was injected into the anterior chamber of rats at A9.5 and A11.5. VEGF was injected at 1, 10 and 20 ng (in 0.5 μl) and the degree of PM regression assessed at A13.5. The level of VEGF in rabbit aqueous lies in the range of 190 to 750 pg/ml depending on animal age (Yanagawa et al., 1998). If this reflects the levels of VEGF present in rat aqueous and, assuming an immediate 10-fold dilution of injected material, our injection protocol would have elevated the concentration of VEGF in the anterior chamber over the range of 200- to 8000-fold. In experiments analyzing at least 10 PMs at each concentration, we observed a small but significant increase in the total number of capillary cells remaining at the highest concentration of VEGF used (Fig. 6A). This was not reflected as a quantifiable change in the percentage of cells undergoing apoptosis (Fig. 6B).

VEGF antagonists increase the number of vessels showing synchronous apoptosis

To test whether VEGF was necessary to suppress apoptosis, we injected soluble VEGF receptors of two types to inhibit the activity of VEGF in vivo. This strategy, using either soluble receptors (Ferrara et al., 1998) or anti-VEGF antibodies (Adamis et al., 1996) has proven effective in assessing the role of VEGF in vivo in other systems. Both KDR-(human Flk)-alkaline phosphatase and KDR-immunoglobulin (IgG) fusion proteins were obtained and injected into the circulation (85 μg per dose) of rat pups at either A8.5 or A9.5. 24 hours after injection, PMs were harvested and the number of capillaries undergoing synchronous apoptosis counted. Both the KDR-AP and KDR-IgG (Fig. 6C) fusion proteins gave a significantly increased number of capillaries showing a synchronous pattern of apoptosis when compared with controls. This response did not result in a readily observed precocious regression of the PM.

DISCUSSION

Previously, it has been proposed that, during programmed regression of the PM, there are two ways for capillary cells to die (Fig. 1). It has been shown that some capillary cells die as a consequence of macrophage action (Diez-Roux and Lang, 1997; Lang and Bishop, 1993) in what is termed initiating or isolated apoptosis (Lang et al., 1994). However, in addition, the junction-to-junction pattern of PCD that occurs as another step in regression (referred to as synchronous or secondary apoptosis; Lang et al., 1994) argued that flow stasis could also result in cell death. A correlation between flow stasis and synchronous apoptosis was observed using vital analysis (Meeson et al., 1996) and was consistent with earlier experimentation showing that an imposed block to plasma flow in vessels of the corpus luteum resulted in the PCD of endothelial cells (Azmi and O'Shae, 1984).

As a means of imposing cessation of flow in vessels of the PM, enucleated rat eyes were cultured. This resulted in all the capillary segments of the structure showing a synchronous pattern of apoptosis. Importantly, this demonstrated that flow stasis resulted in segmental apoptosis even though the aqueous environment was retained. This simple observation is consistent with an extension of the model (Fig. 1) where it was proposed that synchronous apoptosis might result from growth factor deprivation if a factor necessary for endothelial cell survival was circulating in the plasma (Meeson et al., 1996; Lang et al., 1994). Since a block at any point in a capillary lumen would result in absent flow throughout, this would explain why synchronous apoptosis occurred from junction to junction. In addition, it was possible that synchronous apoptosis was the result of flow-stasis-induced hypoxia in a capillary segment. In the present analysis, we have examined these possibilities more closely.

The absence of distinctly hypoxic capillary segments in normal PMs argues that oxygen deprivation is not the means by which synchronous apoptosis is induced, especially given the marked hypoxia that can be experimentally induced in all cells of the structure. However, these experiments cannot exclude the possibility that a change in oxygen tension throughout the aqueous might have a role in determining when PM regression begins. Indeed, assessment of premature infants (Hornblass, 1971) has suggested that hyperoxia might result in persistent PM. The ability of macrophages to respond to hypoxic conditions in a variety of ways (Melillo et al., 1995; Wilhelm et al., 1996; Flavin et al., 1997; Xiong et al., 1998; Harmey et al., 1998) and previous demonstrations that

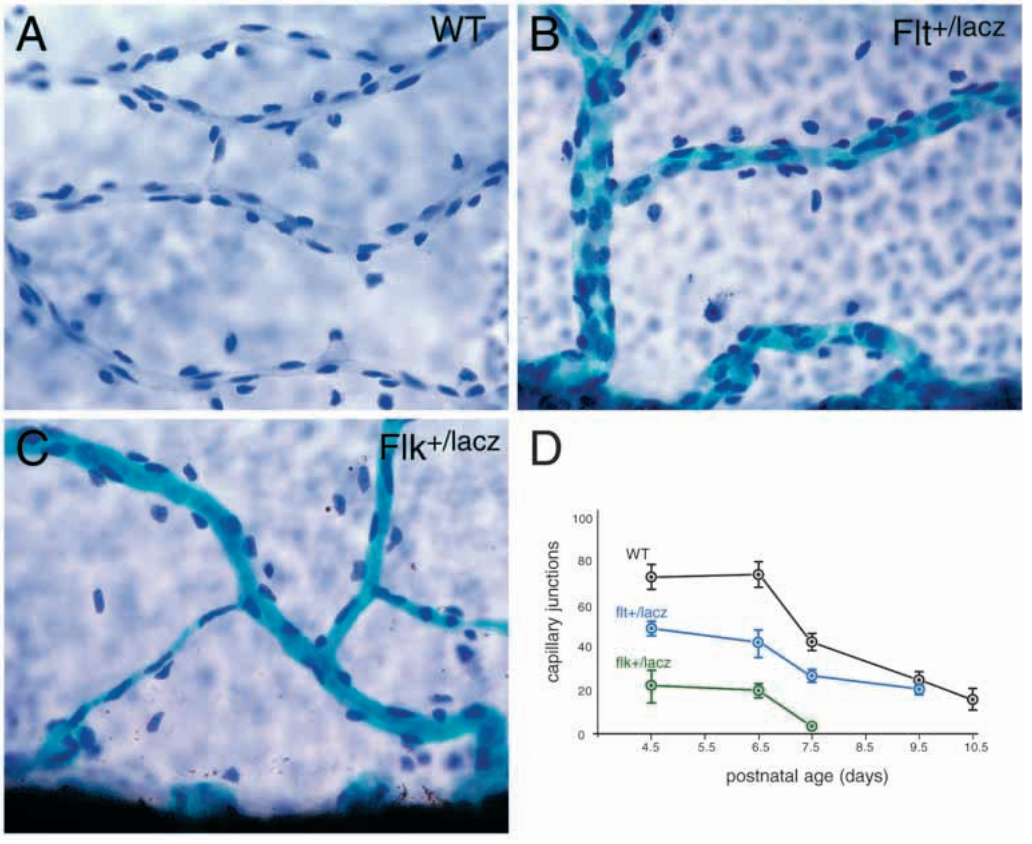

Fig. 5. Heterozygous Flt and Flk mice have a defect in PM development. (A) Micrograph of a wild-type PM. (B) Micrograph of an X-gal-stained PM from an $Flk^{+/lacz}$ mouse. Endothelial cell cytoplasm is stained blue. (C) An X-gal-labeled PM from an $Flt^{+/lacz}$ mouse. All membranes are from mice at A6.5 and are counterstained with hematoxylin. (D) Graph showing the number of capillary junctions in wild-type (black line), heterozygous Flt (blue line) and heterozygous Flk (green line) mice from 4.5 to 10.5 days after birth. Between 4 and 7 PMs were scored for each time point. The error bars represent standard errors.

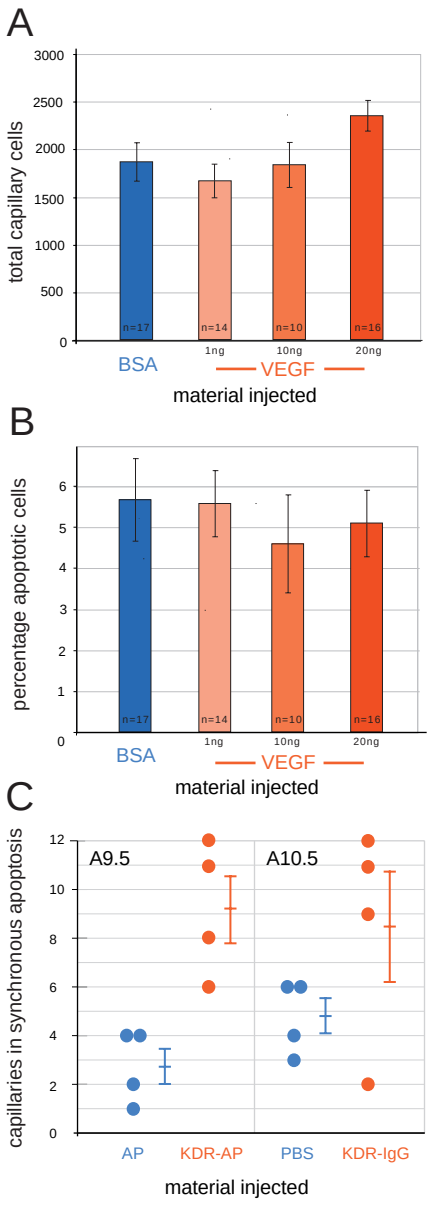

Fig. 6. VEGF is necessary, but not sufficient to suppress synchronous apoptosis. (A) A comparison of the effect of delivering bovine serum albumin (BSA) and VEGF to the anterior chamber via trans-corneal injection. The amount of VEGF injected is indicated below the bars and the total number of PMs scored indicated by the *n* on each bar. (B) The membranes were also scored for the percentage of apoptotic cells with either BSA or VEGF injection. (C) The effect of injecting a soluble human Flk-1/KDR fusion protein on the occurrence of synchronous apoptosis. PMs were scored at either A9.5 (left panel) or A10.5 (right panel), 48 hours after injection of soluble VEGF receptor. In each case, blue dots represent the number of capillaries in synchronous apoptosis for controls, the red dots, the number for experimental animals. For all panels, error bars represent standard errors of the mean.

macrophages induce capillary cell apoptosis (Diez-Roux and Lang, 1997; Lang and Bishop, 1993) may also suggest that oxygen tension-regulated regression is a possibility.

Several experimental results support the proposal that synchronous apoptosis results from deprivation of a circulating growth factor accompanying flow stasis. A recognized endothelial cell survival factor, VEGF, is found in the circulation in the plasma fraction and is active in stimulating endothelial cell survival according to antibody inhibition studies performed in culture. These observations, combined with previous vital cell analysis (Meeson et al., 1996) argue that endothelial cells would be denied a constant supply of VEGF as flow stasis is established and that this might well have an impact on cell survival. The presence of active VEGF in human plasma is consistent with our observations and argues that the VEGF released through platelet aggregation (Maloney et al., 1998) is not required if endothelial cells are to receive a VEGF signal.

Injection of excess VEGF into the aqueous has only a mild effect on regression and increases the total number of capillary cells present 4 days after the first of two VEGF injections. That this change cannot be discerned in the recorded percentage of cells undergoing apoptosis attests to the limited response. However, that there is a response at all is consistent with the argument that VEGF is a survival factor for cells of the PM and that its in vivo action is to suppress cell death.

The limited ability of VEGF to suppress regression in vivo contrasts with data from explantation analysis where VEGF clearly does have the ability to suppress PCD in a PM where flow has ceased. While there could be trivial explanations for this (such as the rapid clearance of injected VEGF from the aqueous), it is also possible that in vivo there may be negatively acting factors that contribute to the regulation of cell death and suppress the action of VEGF. In an explantation analysis, since the aqueous is washed away from the membrane, the influence of these factors would be removed. It has been shown that there are high levels of active TGFβ2 in the adult aqueous of many species (Cousins et al., 1991) and we have confirmed that it is also present at biologically significant levels at the time of PM regression (G. Diez-Roux and R. A. L., unpublished). Since TGFβs are pro-apoptotic and can antagonize the effects of growth and survival factors and in particular those of VEGF (Pepper et al., 1993; Mandriota et al., 1996), it is possible that the removal of this activity in the explant assays unveils the survival stimulating activity of VEGF. The potential role of TGFβ in regulating cell death during PM regression is currently under investigation.

Delivery of inhibitory soluble VEGF receptor fusion proteins into the circulation of neonatal rats has provided evidence that VEGF suppresses synchronous apoptosis; the number of capillary segments showing synchronous apoptosis was significantly increased 48 hours after a single 85 μg dose of Flk-1/KDR-fusion protein was administered. This argues strongly that VEGF is a necessary survival stimulus. However, the overall change in the PM after VEGF inhibition was not dramatic. There was not, for example, a readily observed precocious regression of the structure. The small but significant changes observed might be explained by one or more of the following. It is possible that injected soluble receptors may have a limited half-life and that, as a consequence, in vivo VEGF inhibition is transient. In addition, VEGF may be just one of several activities that normally suppress endothelial cell apoptosis and its inhibition may only minimally diminish the total endothelial cell survival activity. Finally, vital analysis has shown that the flow stasis that accompanies synchronous apoptosis results from the death of isolated capillary cells. If

the model that we propose is correct, then synchronous apoptosis will occur when a critical minimum threshold of survival activity is reached in a segment. Assuming that VEGF is one of several survival factors and that soluble receptors may not completely abrogate VEGF activity, it is most likely that, in these experiments, we have simply decreased the time it takes for the critical threshold to be reached in any given segment.

While the present analysis provides evidence that synchronous apoptosis is a simple response to growth factor deprivation, there is likely to be a complex relationship between flow cessation and the regulation of survival factor expression. Specifically, it has been shown that a number of growth factor genes are regulated by shear force (Malek et al., 1993; Resnick and Gimbrone, 1995) and this could have a direct impact on the survival of capillary cells in a segment that is haemodynamically disadvantaged. Additional studies will be required to understand the interplay between growth factor deprivation and shear-force-mediated gene regulation.

The presence of VEGF in both the plasma (Maloney et al., 1998) and the aqueous (Yanagawa et al., 1998) suggests that both these sources may contribute to an endothelial cell survival signal. Plasma VEGF clearly has direct access to the VEGF receptors Flt-1 and Flk-1 and provision of a survival signal would be consistent with many studies of VEGF activity in culture (Spyridopoulos et al., 1997; Gerber et al., 1998). In the developing retina, immature vessels appear to depend on VEGF for their survival until they become covered by pericytes (Benjamin et al., 1997) and it is proposed that, during the period of VEGF dependence, retinal vessels can be forced to regress when high oxygen tension suppresses the level of VEGF produced by retinal cells (Alon et al., 1995). Our current investigation argues that VEGF located in the plasma may also be important in suppressing the PCD associated with both normal and pathophysiological vessel remodeling. Whether VEGF is available from the aqueous to provide a survival signal is complicated by the presence of the basal lamina that surrounds capillaries. This may be a diffusion barrier, perhaps because it acts as a molecular sieve or alternatively may contain binding sites for some VEGF isoforms (Park et al., 1993; Poltorak et al., 1997) and therefore may regulate availability.

Diminished PM complexity in the $Flk^{+/lacz}$ and $Flt^{+/lacz}$ animals clearly shows that formation of the PM is dependent upon normal numbers of Flk and Flt receptors. Since $Flt^{+/lacz}$ and $Flk^{+/lacz}$ mice have 67% and 33%, respectively, of the normal complexity, it is also clear that Flk is the more important receptor for PM development. Short of showing that PM cells are responsive to VEGF signals, the PM deficiency in the $Flk^{+/lacz}$ and $Flt^{+/lacz}$ animals eliminates their value in assessing the role of VEGF signaling in regulating capillary regression. The observed phenotypes show that heterozygous $Flk^{+/lacz}$ and $Flt^{+/lacz}$ animals may be useful for some assessments of VEGF function, but should also serve as a cautionary note to those who would use these animals as a convenient endothelial cell marker line. Perhaps in the $Flk^{+/lacz}$ and $Flt^{+/lacz}$ mice, other microvascular networks will also show a subtle deficiency.

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