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REGULATION OF CELL GROWTH BY VITREOUS HUMOUR

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SUMMARY

Extracts of normal vitreous have been found to inhibit angiogenesis in two animal models: tumour-induced neovascularization in the rabbit corneal micropocket and retinal extract-induced angiogenesis in the chick chorioallantoic membrane assay. Using *in vitro* assays, we have found recently that an extract of bovine vitreous, free of hyaluronic acid, inhibits proliferation of cells in the aortic wall, i.e. endothelium and smooth muscle cells, as well as capillary and corneal endothelium. The inhibition is dose-dependent, as determined by either cell count or [^{3}H]thymidine incorporation, and not due to cytotoxicity, as demonstrated with a double-label thymidine assay. The inhibitor is trypsin-sensitive and heat-stable (95 °C for 10 min).

Conversely, proliferation of pericytes, lens epithelium and fibroblasts (dermal and corneal) was stimulated by the vitreous extract. This mitogenic activity was heat-labile. Growth of pigment epithelium and several tumour cell lines was unaffected.

The data demonstrate that normal vitreous contains a heat-stable growth inhibitor specific for endothelium and smooth muscle cells, and a non-specific heat-labile mitogen. The paradoxical effect of this antiangiogenic factor on arterial and capillary contractile cells, smooth muscle and pericytes, suggests a basic difference in the regulation of the two vasculatures. The results suggest that a substance in normal vitreous may be important in controlling neovascularization that results from diabetic and other retinopathies, and could be useful for inhibiting tumour-induced angiogenesis.

INTRODUCTION

Neovascularization (angiogenesis) represents one aspect of a tissue response to insult or injury. While it is considered a normal component of the wound-healing (Schoefl, 1963) and inflammatory processes (Dvorak, Mihm & Dvorak, 1976), the angiogenic response is central to the pathology of solid tumour growth (Folkman, 1972) and the proliferative retinopathies (Henkind, 1978). The potential usefulness of antiangiogenic therapies, proposed by Folkman (1972), in arresting pathological neovascularization has prompted considerable interest in discovering inhibitors of new vessel growth. Recently, Taylor & Folkman (1982) demonstrated the validity of this concept by discovering that protamine inhibition of neovascularization caused tumour remission in mice. Avascular tissues, such as cartilage or vitreous, are also logical candidates as endogenous sources of antiangiogenic factors.

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Cartilage, which is vascularized during embryogenesis, becomes avascular as development continues (Haraldsson, 1962). Neonatal rabbit cartilage was found to inhibit tumour or tumour extract-induced neovascularization occurring in the rabbit cornea or in the chicken chorioallantoic membrane (CAM) (Brem & Folkman, 1975; Langer *et al.* 1976). The most definitive *in vivo* study of cartilage as an antiangiogenic substance is that of Langer *et al.* (1980), who clearly demonstrated that infusion of a highly purified cartilage factor into rabbits resulted in the inhibition of tumour-induced neovascularization and subsequent tumour growth.

Vitreous is another tissue that becomes avascular during development (Jack, 1972) and appears to possess antiangiogenic properties. Brem *et al.* (1976) observed that tumours implanted within the vitreous as close as 0·1 mm from the retina failed to elicit an outgrowth of retinal vessels. Proliferation of retinal capillaries with subsequent tumour growth occurred only when the tumours were in direct contact with the retina. Based upon these observations, they suggested that vitreous contained an inhibitor of tumour-induced capillary proliferation. Subsequent tests using the rabbit corneal micropocket bioassay demonstrated that crude vitreal extracts from rabbit (Brem *et al.* 1977; Patz *et al.* 1978), bovine and human (Felton *et al.* 1979) sources could inhibit tumour-induced neovascularization. The antiangiogenic activity of vitreous has also been demonstrated using the chick chorioallantoic membrane (CAM) assay (Lutty *et al.* 1983). In this study bovine retinal factor, a mitogen for endothelial cells, was used to stimulate neovascularization, which vitreous extract inhibited dose-dependently. This work implied that retinal factor and vitreous inhibitor were putative natural antagonists.

Present use of *in vivo* bioassays has, in part, contributed to the difficulty in purifying and completely characterizing the cartilage and vitreous factors. Technical problems such as quantitation, low sample capacity, long assay duration, and whole animal variations inherent in many bioassays might be better managed or controlled using more defined *in vitro* cell culture systems. Correlation between the use of *in vitro* cell culture assays and *in vivo* bioassays is observed in the work of D'Amore, Glaser, Brunson & Fenselau (1981) and Glaser *et al.* (1980*a*,*b*), in which retinal extracts capable of inducing angiogenesis in the chick CAM bioassay also produced mitogenic and chemotactic effects when assayed on foetal bovine aortic endothelial cell cultures.

In this report we describe the effects of extracts of bovine vitreous on the *in vitro* proliferation of endothelial, smooth muscle, epithelial and fibroblastic cells.

MATERIALS AND METHODS

Maintenance and passage of cells in culture

Stock cultures, unless stated otherwise, were maintained on a standard growth medium (MEM_{10}) consisting of Minimum Essential Medium (MEM with Earle's salts, Gibco, Grand Island, N.Y.) supplemented with 10% foetal bovine serum (FBS, Sterile Systems, Logan, Ut.) L-glutamine (2 mM), and sodium bicarbonate (2·2 mg/ml). Cultures were incubated in 75 cm^2 flasks (Falcon Plastics, Oxnard, Calif.) in an atmosphere of 5% CO_2/95% air at 37 °C with 90% relative humidity (i.e. standard incubation conditions). The cells were subcultured at split ratios of 1:3 or 1:5 using

0·1 % (w/v) trypsin in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Gibco).

Foetal bovine aortic endothelial (FBAE) cells were isolated essentially as previously described (Fenselau & Mello, 1976) except that 0·1 % (w/v) trypsin (type III, bovine pancreatic, Sigma, St Louis, Mo.) was also present. Primary cultures were maintained on MEM_{10} with penicillin G (200 units/ml) and streptomycin sulphate (200 μg/ml). Identification as vascular endothelium was accomplished by their morphology at confluence and by Factor VIII as determined by immunofluorescence localization (Gitlin & D'Amore, 1983) using antiserum against bovine Factor VIII (provided by Dr E. Kirby, Temple University).

Rabbit (RCE) and human corneal endothelial (HCE) cell cultures were established by the following technique. Human eyes were provided by the Medical Eye Bank of Maryland, Inc. A corneal button was excised and a no. 10 trephine imbedded on the posterior side. The trephine was filled with 4 units (u) dispase/collagenase (Boehringer-Mannheim, Indianapolis, Ind.) per ml of serum-free MEM (MEM_0) for 1 h at 25 °C. The digest was centrifuged, and the cell pellet resuspended in MEM_{20} with antibiotics. After the first passage, the cells were maintained in MEM_{10} without antibiotics. Cells were identified as corneal endothelium by their morphological appearance at confluence.

Foetal bovine aortic smooth muscle (ASMC) cells were acquired by the technique of Ross (1971) (kindly provided by Dr Patricia D'Amore, Harvard School of Medicine). ASMC were maintained, passaged, and tested in the same manner as FBAE.

Bovine retinal capillary endothelium (BCE) and pericytes (BCP) were isolated by the method of Gitlin & D'Amore (1983). Both cell types were provided by and tests conducted by Dr Patricia D'Amore (Harvard School of Medicine). Both cell types were maintained on Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10 % FBS. BCE tests were conducted in the presence of $DMEM_{10}$, while pericyte tests were with $DMEM_5$. Because of slow growth the BCE tests lasted 7 days with a medium and sample change at day 3. BCP tests were 12 days long with medium and sample changes every 3 days.

Epithelial cell types included Nikano lens epithelium and human pigment epithelium. Nikano mouse lens epithelium (NAK) was provided by Dr Ted Reid (Yale School of Medicine). Human pigment epithelium (HPE) was provided by Dr Sampei Miyake (Nagoya School of Medicine, Nagoya, Japan) and Dr David Newsome (Johns Hopkins School of Medicine). HPE was identified by pigment production in early passage. These cells were maintained and tested in Coon's Medium F-12 (Irvine Scientific, Santa Ana, Calif.) with 5 % FBS, 2 mM-L-glutamine, 0·3 mM-L-ascorbic acid, and antibiotics.

Human skin fibroblasts (HSF) were kindly provided by Dr Allan Fenselau (Miami Heart Institute, Miami, Florida). Human corneal stroma (HCS) was obtained by trypsin/collagenase digestion of corneas after both epithelium and Descemet's membrane were peeled off the corneal button.

Tumour cell lines tested included a rat schwannoma cell line (kindly provided by Dr Ruben Adler, Johns Hopkins School of Medicine), Y-79 retinoblastoma, and K562 cells (kindly provided by Elaine Young, Johns Hopkins School of Medicine, Baltimore, Md). Y-79 and K562 (Lozzio & Lozzio, 1975) were grown and tested in static suspension cultures. The mouse schwannoma cells, a malignant strain of Schwann cells, were maintained in Dulbecco's Modified Eagle's (DME, Gibco, Grand Island, N.Y.) with 10 % FBS. These cells were passaged with 0·1 % (w/v) trypsin, 1 mM-EDTA (ethylenediamine tetraacetic acid, Sigma) in PBS, and tested in DME with 10 % or 1·5 % FBS.

Cell proliferation assays

Cells were plated in 24-well multiwell dishes (Falcon Plastics) at 20 000 cells/well. After 3–5 h the medium was removed and replaced with 1 ml/well of MEM containing 1·5 % FBS. Up to 100 μl/well of filter-sterilized (0·22 μm, Millipore Corp., Bedford, Ma.) test samples or control solutions were added directly to the medium. The cultures were then incubated under standard conditions for 48 h. Cell proliferation was determined either by cell counting or by the incorporation of [^{3}H]thymidine into acid-insoluble cellular material.

Cells were trypsinized (0·1 % (w/v) trypsin, 1 mM-EDTA, in PBS) and counted using a Coulter counter (Coulter Electronics, Hialeah, Fla.). Results are expressed as total cells per well. Inhibition

was quantitated by subtracting the cell number determined at $t = 0$ h from the total cell number at $t = 48$ h to obtain a net cell count. The data can be expressed either as a ratio of experimental net cell counts to control net cell counts or as % inhibition (non-inhibited controls = 0 % inhibition). All tests were performed in triplicate.

[^{3}H]thymidine incorporation was determined by the following technique. Test medium was removed and replaced with serum-free MEM containing 0·625 μCi/ml [*methyl*-^{3}H]thymidine ([^{3}H]dThd, Amersham, 6·7 Ci/mmol). After a 1 h incubation, the medium was removed and the cells rinsed twice with PBS at 4 °C. This was followed by a 5-min wash at room temperature with a 1:1 solution of PBS : acetic acid/ethanol (1 part glacial acetic acid plus 3 parts 80 % ethanol), then fixed with acetic acid/ethanol for 0·5 to 3 h at room temperature. Cellular, unincorporated [^{3}H]thymidine was then re-extracted with 0·3 N perchloric acid for 10 min. After an extensive water rinse, the fixed cells were dissolved with 0·4 ml/well of 0·25 M-sodium hydroxide for 20 min at room temperature. The cell hydrolysate was transferred to 4-ml minivials along with an additional 0·2-ml water rinse. Counting fluid (Budget-Solv, Research Products, Intl., Mount Prospect, Ill.), 3·5 ml/vial, was added, and the vials were counted in a Beckman LS-2800 liquid scintillation counter. Results are expressed either directly as counts per min per culture (c.p.m.), as the ratio of experimental ^{3}H c.p.m. to control ^{3}H c.p.m., or as % inhibition (100 % − (c.p.m. ratio × 100)). Assays were performed in triplicate. ID_{50} was determined for either cell count data or [^{3}H]thymidine incorporation by simply determining the dose of vitreous protein that yielded 50 % inhibition.

Static suspension cultures were assayed by cell counts only. After the 48-h assay, the contents of each well were aspirated and counted electronically.

Cytotoxicity assay

An assay to distinguish cytotoxicity from inhibition of proliferation was developed and used on cell types that exhibited apparent inhibition. Confluent monolayers of cells were trypsinized, and 4×10^5 cells were plated per 6-cm plastic dishes. After attachment for 3–5 h, the cells were rinsed once with PBS, then incubated with 5 ml of MEM containing 10 % FBS and 0·2 μCi/ml [2-^{14}C]thymidine ([^{14}C]dThd), 56 mCi/mmol (Amersham, Arlington Heights, Ill.). After 24–48 h the medium was removed, the cells were rinsed with PBS, and fresh unlabelled growth medium was added. Following incubation for an additional 24 h, the labelled cells were trypsinized and plated at 2×10^4 cells per well for use in the [^{3}H]thymidine cell proliferation assay (as described above).

To count ^{14}C and ^{3}H isotopes in the same sample, prior adjustments were made to the scintillation counter such that only 6 % of the ^{14}C c.p.m. would be detected by the ^{3}H channel, and none of the ^{3}H c.p.m. would be detected by the ^{14}C channel. Corrections for counting efficiency were then applied to the raw data to determine the actual level of ^{14}C and ^{3}H present in each sample.

Preparation of vitreous extract

Vitreous extract was prepared by a similar method to that reported previously (Lutty *et al.* 1983). Adult cow eyes were obtained from a local abattoir within 2 h of slaughter. The eyes were cut open and the vitreous was removed with forceps. After removing any adhering ocular tissue, the vitreous was homogenized and centrifuged at 40 000 ***g*** for 30 min at 4 °C. The supernatant was dialysed (Spectrapor 2, 12 000 to 14 000 molecular weight cut-off, Spectrum Medical Industries, Los Angeles, Calif.) at 4 °C for 48 h against distilled water, and then lyophilized to dryness. Dried vitreous was dissolved in 0·1 M-sodium acetate, 0·15 M-sodium chloride, pH 5·3 (3 mg vitreous/ml of buffer). Ten turbidity reducing units (TRU) of fungal hyaluronidase from *Streptomyces hyaluronlyticus* (Calbiochem, La Jolla, Calif.) were added per ml of buffered vitreous solution. This solution was dialysed against 1000 vol. of 0·1 M-sodium acetate, 0·15 M-sodium chloride for 18 h at 25 °C, and then dialysed against distilled water for 24 h at 4 °C. The dialysate was brought to 45 % saturation of ammonium sulphate, incubated for 20 min at 4 °C, then centrifuged at 40 000 ***g*** for 20 min at 4 °C. The resultant precipitate was resolubilized in water, dialysed at 4 °C for 48 h against distilled water, lyophilized to dryness, and stored desiccated at −20 °C until used.

Heat treatment of vitreous consisted of maintaining aqueous solutions of the extract at 95 °C in a water bath for 10 min. Soluble samples were electrophoresed on 10 % polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS/PAGE), essentially as previously described (Mello, Brown, Goldstein & Anderson, 1980).

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. Hexuronic acid content was measured by a minor modification of the carbazole method of Dische (1947) and Gregory (1960). We used 0·5-ml sample volumes, 3 ml of borate–sulphuric acid reagent, and 0·2 ml of 0·1 % (w/v) carbazole (recrystallized 3 times from ethanol) in absolute ethanol. The first heating was at 100 °C for 20 min; the second was at 100 °C for 10 min. D-glucuronic acid (Sigma) was used as standard.

RESULTS

The vitreous preparation used in this work has been partially characterized previously (Mello *et al.* 1982). More than 95 % of the hyaluronic acid is removed by digestion with fungal hyaluronidase, as determined by the carbazole reaction. From SDS/PAGE it was determined that the fungal hyaluronidase is eliminated in the supernatant of the 45 % ammonium sulphate precipitation. The inhibitor of endothelial cell proliferation is sensitive to trypsin and Pronase, although it is heat-stable (Mello *et al.* 1982).

The proliferation of FBAE, as measured by cell counts, was inhibited by both heated and non-heated samples in a dose-dependent manner (Fig. 1). The cells were far more sensitive to the heated sample as demonstrated by a lower plateau in the mean cell number curve, and an apparent arrest in cell proliferation at doses as low as 10 μg

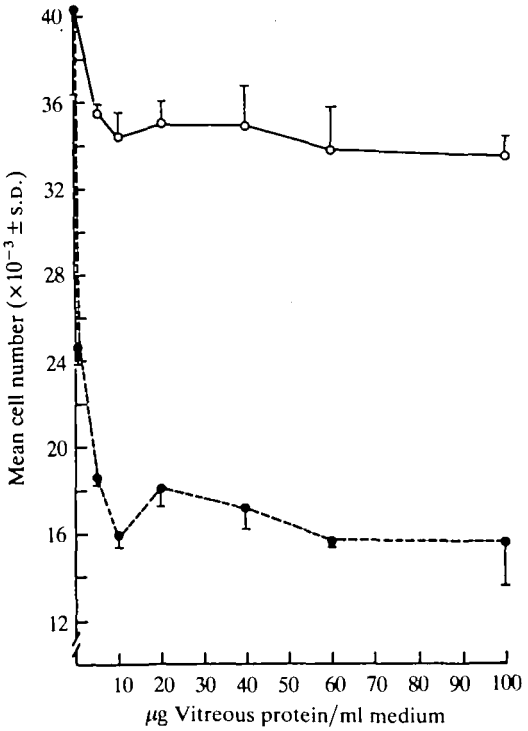

Fig. 1. The effect of bovine vitreous protein on foetal bovine aortic endothelium. Cells were plated ($2{\cdot}0 \times 10^4$ cells/well) in MEM_{10}, and after 4 h $1{\cdot}6 \times 10^4$ cells had attached per well. Test was run for 48 h in $MEM_{1{\cdot}5}$ with the indicated concentration of heated (●---●) or non-heated (○——○) vitreous proteins.

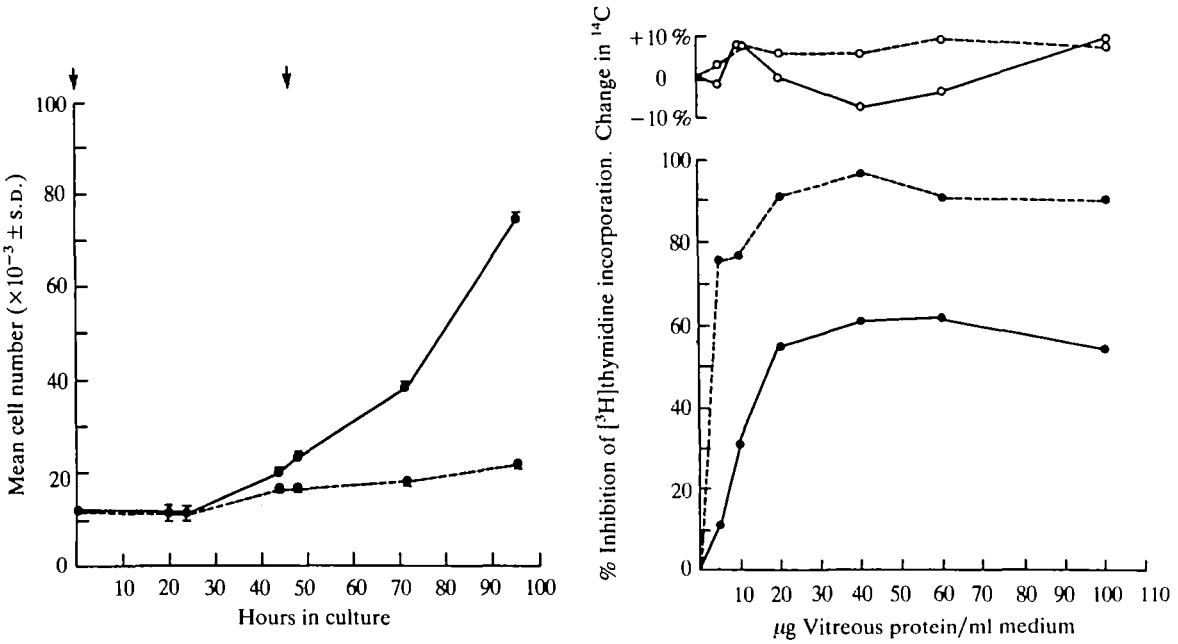

Fig. 2. Prolonged inhibition of FBAE proliferation; 100 μg heated (●---●) vitreous protein (in 100 μl/H_2O) and 1 ml of $MEM_{1\cdot5}$ were added per well at the times indicated (↓). Control wells (●——●) received additions of 100 μl H_2O.

Fig. 3. Cytotoxicity assay of FBAE cells with heated (----) or non-heated (——) vitreous samples. Cells were prelabelled for 24 h in MEM_{10} with [^{14}C]thymidine, then maintained on MEM_{10} without isotope for an additional 24 h. Prelabelled cells were trypsinized and plated ($2\cdot0 \times 10^4$ cells/well) in MEM_{10}. After 4 h, medium was replaced with $MEM_{1\cdot5}$ and the vitreous concentrations indicated were added. After 44 h, the wells were pulse-labelled with MEM_0 containing [^{3}H]thymidine for 1 h. Loss of ^{14}C c.p.m. (○) denotes toxicity, while decrease in ^{3}H c.p.m. (●) indicates inhibition of mitosis.

heated vitreous/ml of medium. In this experiment only 16 000 cells adhered per well in the 4-h attachment period after plating. The effects of heating were investigated because of evidence for heat-labile mitogens in bovine vitreous (Raymond & Jacobson, 1982). The arrest in proliferation can be continued for up to 96 h if vitreous sample and medium are changed after 48 h (Fig. 2). The inhibition of [^{3}H]thymidine incorporation parallels the cell count results (Fig. 3). Heated vitreous effects a 90 % inhibition in [^{3}H]dThd incorporation, whereas the unheated material is less effective. Both curves exhibit dose-dependence. Using this assay the unheated sample does achieve a 50 % inhibition in [^{3}H]dThd at 18 μg protein/ml (i.e. an ID_{50} of 18 μg/ml) but the heated sample resulted in an ID_{50} of $3\cdot5$ μg/ml. This inhibition is not due to cytotoxicity since the change in [^{14}C]dThd present never varied more than 10 % from control cultures (Fig. 3).

The heat-stabile inhibitor was also effective on another cell type from large vessels, bovine smooth muscle cells (ASMC). Analogous to FBAE response, heated vitreous yielded a more dramatic inhibition of proliferation (Fig. 4). The highest dose of

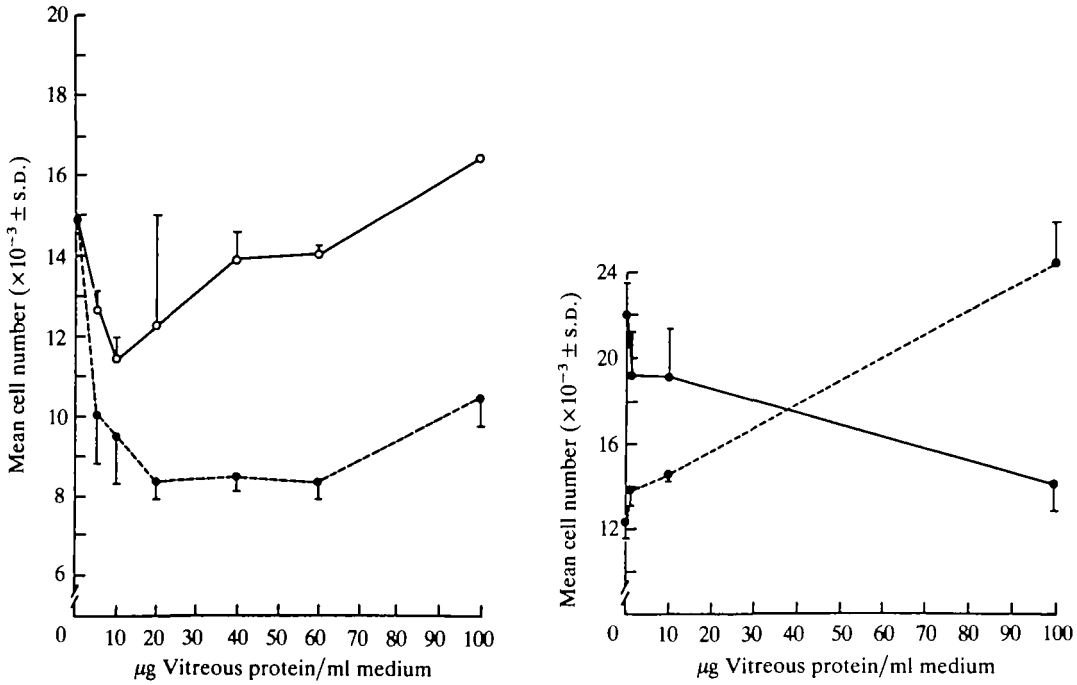

Fig. 4. Bovine aortic smooth muscle cells (ASMC) were plated at ($2{\cdot}0 \times 10^4$ cells/well) in MEM_{10}, and after 4 h 8×10^3 cells had attached. Assay was performed in the presence of $MEM_{1{\cdot}5}$ with doses of heated (●---●) or non-heated (○——○) vitreous proteins for 48 h.

Fig. 5. Paradoxical response of bovine retinal capillary endothelium (BCE) and pericytes (BCP) to heated vitreous samples. BCE (●——●) was maintained and tested on $DMEM_{10}$ over the course of 7 days, with a sample and medium change at 3 days. BCP (●---●) were maintained on $DMEM_{10}$ and tested on $DMEM_5$. The BCP test lasted 12 days, with medium and samples changed every third day.

unheated vitreous actually stimulated proliferation of these cells. Neither sample was cytotoxic as determined by the double-label assay (results not shown).

Unlike the aortic cell populations (Figs 1, 4), the two cell types from retinal capillaries had a paradoxical response (Fig. 5). Bovine retinal capillary endothelium (BCE) was inhibited by heated vitreous proteins, though the response was not as dramatic as the response by aortic cells. Bovine retinal pericytes, the contractile extramural cell in capillaries, were stimulated by heated vitreous, suggesting a major difference between the contractile cells in large and small vessels.

Other cell types that were inhibited included both species of corneal endothelium. Rabbit corneal endothelium (RCE) was very sensitive to this bovine vitreous inhibitor (Fig. 6B); but, as in the previous cell types, the unheated sample was less effective. Human corneal endothelium (HCE), however, was almost equally sensitive to both samples (Fig. 6A). The non-heated sample did have a higher ID_{50}, however. Vitreous was not cytotoxic to either corneal endothelium line.

The enhanced proliferation of pericytes in response to vitreous proteins was also

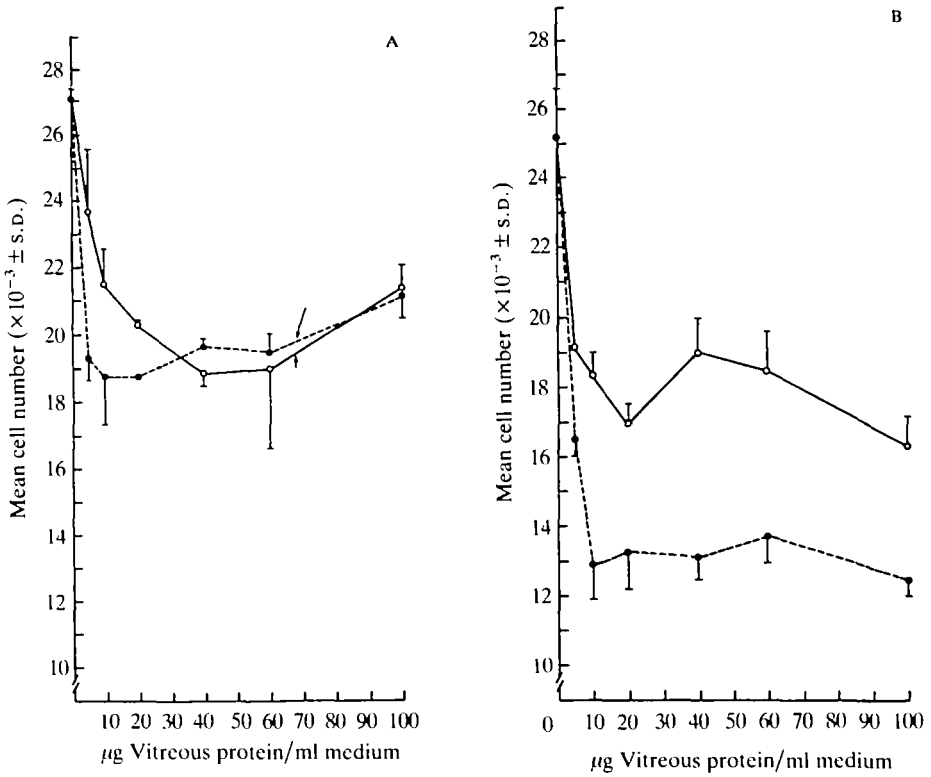

Fig. 6. A. Dose-dependent inhibition of human corneal endothelium (HCE) by heated (●---●) and non-heated (○——○) vitreous extract. HCE was maintained and tested on MEM_{10}. B. Rabbit corneal endothelium (RCE) dose-dependent response to vitreous protein. RCE was maintained on MEM_{10} and tested for 48 h on $MEM_{1\cdot5}$.

observed with the two fibroblastic cell lines tested. Human skin fibroblasts (HSF) were stimulated by both test materials in a dose-dependent manner (Fig. 7A). Heating the vitreous proteins, however, appeared to diminish the effect of a putative mitogen. The response of human corneal stromal (HCS) cells was less dramatic but similar to the HSF response (Fig. 7B). So the response of corneal connective tissue cells was, therefore, the antithesis of the corneal endothelial response.

The epithelial cells tested varied in their response to the vitreous preparation. Nikano mouse lens epithelium (NAK), like the fibroblastic cells, demonstrated enhanced proliferation in response to the unheated proteins, but the response does not appear to be dose-dependent. NAK was unaffected by the heated material (data not shown). The proliferation of human pigment epithelium (HPE), on the other hand, seemed unaffected by the presence of either vitreous sample. There was slight inhibition by the unheated material but most of the data points on the curve were not significant (data not shown).

The tumour cell lines tested gave a response that was similar to HPE; they seemed unaffected by either vitreous material. The proliferation of Y-79 retinoblastoma cells was not stimulated by the unheated material. The heated sample appeared to inhibit

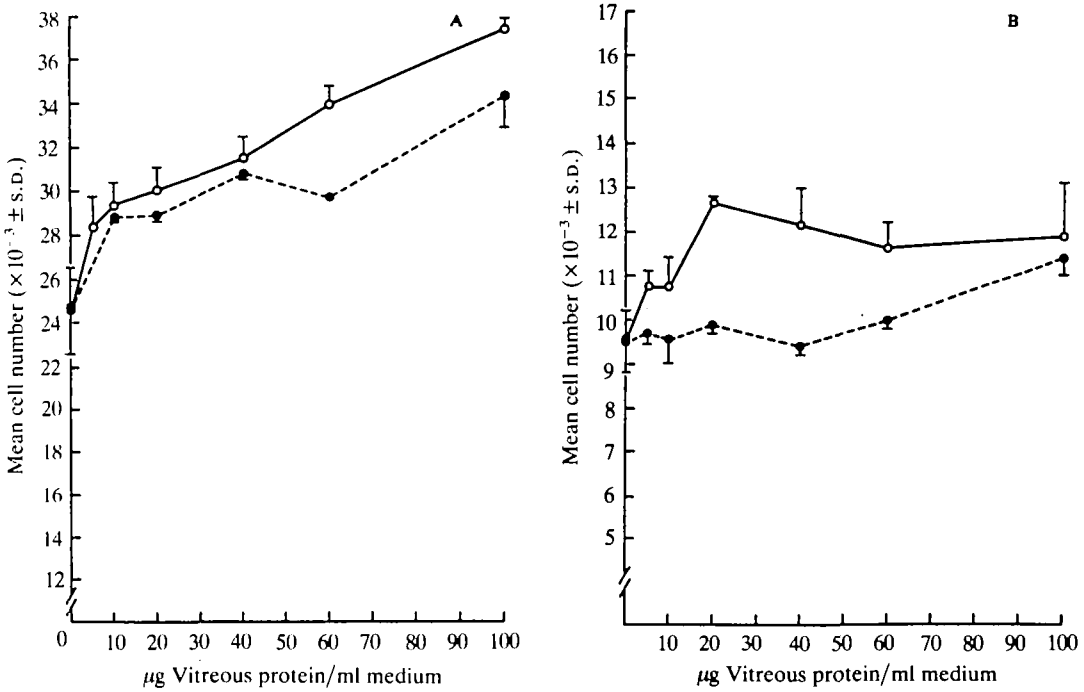

Fig. 7. Human skin fibroblasts (A) and human corneal stroma (B) responses to heated (●---●) and non-heated (○——○) vitreous extract. Both cell-types were maintained on MEM_{10} and tests conducted for 48 h on $MEM_{1\cdot5}$.

proliferation slightly but only one point on the curve was significant. The K562 and schwannoma cells responded similarly, being unaffected by either heated or non-heated samples (results not shown).

DISCUSSION

We have found in extracts of adult bovine vitreous a heat-stable growth factor that inhibits the proliferation of endothelial cells without being cytotoxic. The inhibitory factor is a protein greater than 12 000 M_r as determined by dialysis. In addition to the determination of bona fide inhibition of cell growth by the double-label assay, we have previously established the reversibility of the inhibition produced by vitreous. Removal of the inhibitor and its replacement with standard growth medium resulted in the return of the cells to a normal growth state (Mello, 1981). Such an effect was also observed by Sorgente & Dorey (1980) in their studies on the inhibition of endothelial cell growth by a cartilage-derived factor.

The vitreous inhibitor also worked effectively on aortic smooth muscle cells and, more importantly, on all endothelial cells tested, whether they were of aortic, capillary, or even corneal origin. Corneal and vascular endothelium have also been found to respond similarly to mitogens such as retinal-derived growth factor (Arruti & Courtois, 1982), but never for inhibitors of vascular proliferation. The use of cells

from several species demonstrates that the bovine inhibitor effectively crosses species barriers, since bovine, human and rabbit endothelium were inhibited.

The inhibition of aortic endothelial and smooth muscle cell proliferation by a vitreous protein has been previously demonstrated by Raymond & Jacobson (1982). It seems unlikely that this is the same substance since their inhibitor was heat-labile and 6200 M_r, while our activity was enhanced by heating and had an M_r greater than 12 000. Also, Raymond & Jacobson concluded on the basis of a ^{51}Cr release assay that their low M_r substance was cytotoxic at higher doses, whereas our higher M_r material was found to be non-toxic.

Both studies are in agreement on the presence of a mitogen in vitreous that is heat-labile. Raymond & Jacobson (1982) found this substance to be a protein of greater than 13 000 M_r, which was mitogenic for endothelium, smooth muscle and fibroblasts. We found the mitogen active on both fibroblast lines, lens epithelium and pericytes. Probably, the mitogen in our extract was also active on smooth muscle and endothelial cells since the inhibition of these cells increased substantially when the test material was heated; i.e. the inhibitory activity was masked by the presence of the heat-labile mitogen. Although no one has decisively demonstrated that retina-derived growth factor (RDGF) exists in normal vitreous, this factor is an obvious choice as the mitogen. Raymond & Jacobson (1982) reasoned that, even if RDGF is not normally present, it may leech out of ischemic retina into vitreous in the time between killing the animal and collection of vitreous. RDGF is a heat-labile protein recently demonstrated to have a molecular weight of 18 000 (D'Amore & Klagsbrun, 1984). This mitogen is active on aortic and capillary endothelium (D'Amore, 1982) and on corneal stromal cells (Glaser *et al.* 1980*a*). RDGF, however, does not stimulate the proliferation of smooth muscle cells (Glaser *et al.* 1980*a*) or pericytes (Gitlin & D'Amore, 1983), and our non-heated vitreous extract stimulated both of these cell types. Other ocular mitogens include VGF (vascular endothelial growth factor), which is found in vitreous, but this factor is specific for endothelial cells (Chen & Chen, 1982). Barritault, Arruti & Courtois (1981) suggest that there is an ubiquitous eye-derived growth factor (EDGF) that is produced in a broad spectrum of ocular tissues including retina and vitreous. It seems impossible to determine at this time if there is only one mitogen in our vitreous extract and certainly not possible to determine if it is one of the ocular growth factors previously characterized.

The role of the vitreous inhibitor may be twofold. First, this substance may be responsible during development for the regression of the hyaloid vasculature and the tunica vasculosa lentis. This regression is accompanied by a congregation of hyalocytes, the macrophage-like inhabitants of vitreous. Raymond & Jacobson (1982) suggest this in their work and further demonstrate that hyalocytes can produce such an inhibitory growth factor. Secondly, vitreous inhibitor may ensure that normal adult vitreous humour remains avascular. Only in the advanced stages of several retinopathies do retinal vessels emerge into the vitreous chamber and proliferate along the surface of the retina (Henkind, 1978). However, in retrolental fibroplasia (RLF), the oxygen-induced retinopathy of prematurity, the new vessels actually do grow into the vitreous body (Patz, 1969). These forms of ocular neovascularization may involve

an imbalance in mitogenic and inhibitory growth factors in vitreous and retina (Mello, 1981). If this assumption is true, increasing the concentration of inhibitor may be useful in controlling pathological neovascularization that is associated with RLF and many retinopathies.

Since vitreous inhibitor is truly an antiangiogenic substance (i.e. inhibits proliferation of vascular cells only), it could also be useful for inhibition of tumour-induced neovascularization, the role originally envisioned by Folkman (1972) for antiangiogenic factors. Recently, Folkman and co-workers have demonstrated that protamine (Taylor & Folkman, 1982) and heparin with cortisone (Folkman *et al.* 1983) inhibit tumour-induced neovascularization, resulting in tumour regression. Like these antiangiogenic factors, vitreous inhibitor does not inhibit tumour cell proliferation but rather endothelial cell proliferation.

Finally, the inhibitor effectively stops proliferation of arterial smooth muscle and endothelium, but does not inhibit pericytes, the extramural contractile element in capillaries. This antithetical response of the two contractile elements illuminates a basic difference between arteries and capillaries, which parallels the observation that most ocular angiogenic events originate from capillaries (or veins) and not arteries. Thus, in the ocular milieu where this antiangiogenic factor is present, proliferation from capillaries and veins would be more likely.

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