Journal of Cellular Biochemistry 49:66–73 (1992)

Growth Regulation of the Mammalian Ocular Lens by Vitreous Humor

Amit Banerjee, Julian Parafina, and Mihir Bagchi

Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Abstract Experiments were performed in our laboratory to study the effects of a mammalian 8 kD vitreous humor (VH) factor on the DNA synthesis and mitosis of the epithelial cells of organ cultured rabbit lens. The 8 kD polypeptide factor was purified from mature rabbit vitreous humor by liquid chromatography. Proliferative activities of the epithelial cells of organ cultured lenses were stimulated by 3% rabbit serum. The data from our experiments depicted that the 8 kD VH factor effectively inhibits DNA synthesis and mitosis by the epithelial cells of the organ cultured lens. Our experiments also showed that this 8 kD VH factor can maintain its growth inhibitory activity even when heated for 3 min at 95°C. The growth inhibitory effect of the 8 kD VH factor was dose dependent. Using iodinated vitreal proteins it was demonstrated that the VH proteins are able to enter or bind to lens epithelial cells. The growth inhibitory effect of the 8 kD VH factor was also tested on tissue cultured lens epithelial cells. These experiments showed that the 8 kD VH factor has no growth inhibitory effect on the tissue cultured lens epithelial cells. This experiment has been repeated many times using different concentrations of the factor. These observations suggest that the 8 kD VH factor may have receptors in the lens capsular material (extracellular matrix) and the factor-receptor binding is essential for the growth inhibitory effect.

Key words: vitreous humor, growth regulation, cell proliferation, DNA synthesis, mitosis, protein synthesis, ocular lens, epithelial cells

Reports from our laboratory have clearly demonstrated that the rate of protein synthesis by epithelial cells of lenses incubated in the presence of total vitreous humor or an 8 kD VH factor, is significantly decreased when compared with epithelial cells of lenses cultured without VH (Singh and Bagchi, 1989; Banerjee et al., 1991). It has also been reported that the early events of cell proliferation are dependent on continuous protein synthesis and that a 50–70% inhibition of protein synthesis effectively inhibits cell growth (Pardee, 1989). The VH has been shown to contain growth inhibitors for endothelial cells (Jacobson et al., 1984; Lisnayk, 1988). Thus far the effects of VH factor(s) on lens epithelial cell division have not been examined.

The maintenance of mitotic quiescence of lens epithelial cells is important for lens shape, size, and clarity (Naumann and Naumann, 1986). Why the majority of lens epithelial cells remain in the G_0 phase of the cell cycle, even in presence of a multitude of ocular fluid mitogens is not yet known. Do these cells lack receptors for growth factors? This is not likely, since any in vivo injury, caused either mechanically or chemically, induces an active proliferation of the epithelial cells of the intact lens (Harding et al., 1959; Weinsieder et al., 1975). Furthermore, serum or insulin stimulates cell division by the epithelial cells of the central and pre-equatorial region of organ cultured lenses (Harding et al., 1971). Thus it is possible that the mitogenic activity of ocular fluids is normally counteracted by some other factor(s) present in ocular fluids.

Experiments were performed in our laboratory to determine the effect of the 8 kD VH factor (Banerjee et al., 1991) as well as the total VH on the mitotic activity of the epithelial cells of the organ cultured lens.

MATERIALS AND METHODS

Lenses were obtained from freshly killed 1-month-old New Zealand white rabbits. The culture medium was TC-199, pH 7.4 with an osmolarity of 310 mOsm. All lenses were cultured without attached VH, unless otherwise stated. The effect of the 8 kD VH factor (100

Received December 2, 1991; accepted December 23, 1991.

ng/ml) was tested on lenses without attached VH.

Tissue Culture

Capsule/epithelium preparations were obtained from freshly sacrificed 1-month-old New Zealand white rabbits and cultured in DMEM containing 20% rabbit serum at 37°C with 5% CO_2 in a moist chamber. All experiments used nearly confluent tissue cultures from the 2nd passage. Before exposure to the VH factors, all tissue cultured (TC) cells were thoroughly washed with warm DMEM and further incubated in a serum free medium.

Analysis of DNA Synthesis and Mitosis

DNA synthesis. The control (8 kD VH treated) and experimental (without 8 kD VH) lenses were incubated in 3% serum containing TC-199 for 24, 48, and 72 h. The VH-treated (100 ng/ml) lenses were labelled normal because VH is normally required to maintain lens metabolic homeostasis (Chylack and Kinoshita, 1972). It is known that at least 5% rabbit serum is needed for moderate activation of mitosis by epithelial cells of organ cultured (OC) lenses with attached vitreous humor. A concentration of 3% rabbit serum in the TC-199 medium does not actively stimulate epithelial cell mitosis in lenses cultured with attached vitreous humor (personal observation).

The control (VH) and experimental (noVH) lenses were incubated in 3% serum containing TC-199 for 24, 48, and 72 h. During the last 6 h of incubation the lenses were exposed to ^{3}H-thymidine (Amersham, Arlington Heights, IL, 10 μCi/ml). At the termination of the experiment, all lenses were washed with cold phos-

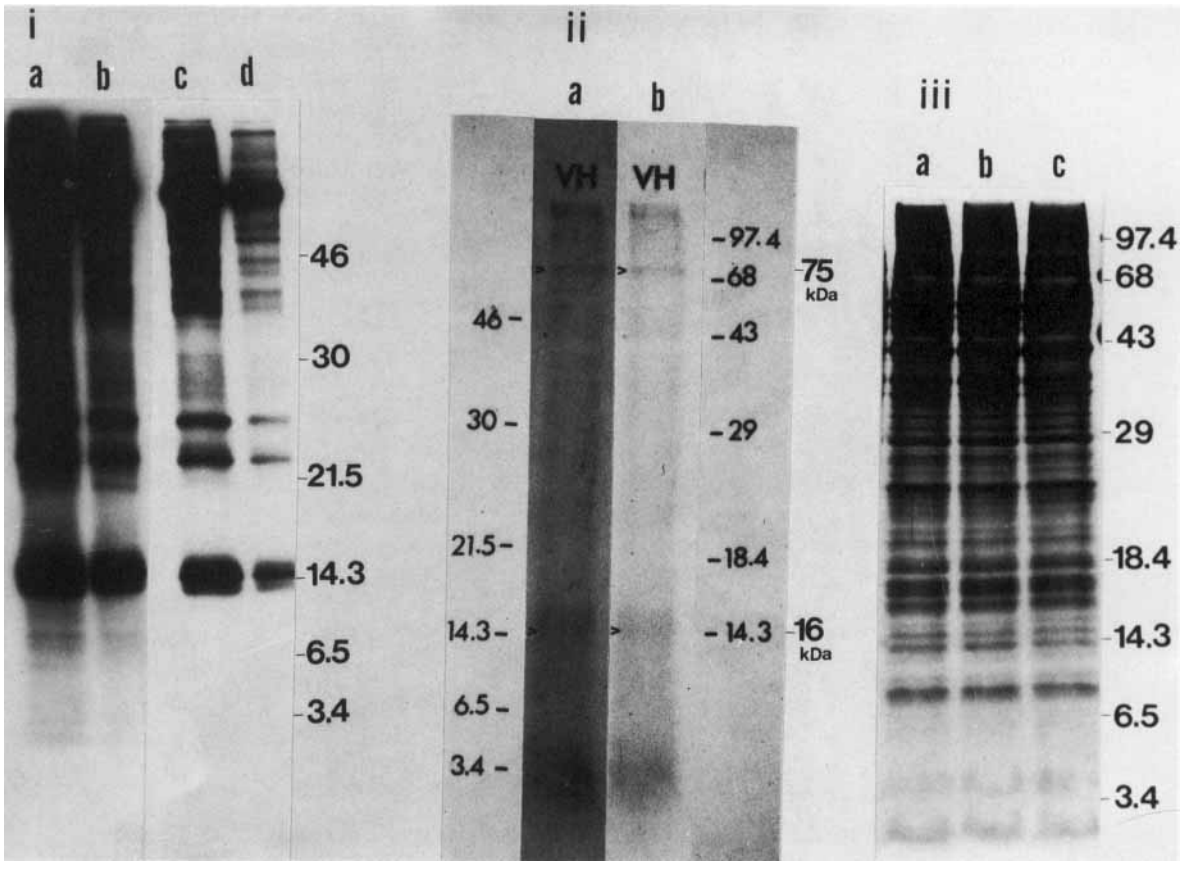

Fig. 1. (i) Autoradiogram of a 16% SDS gel showing iodinated vitreal proteins. a, b, c and d represent 100 μg, 50 μg, 25 μg and 10 μg protein. At higher load the low molecular weight polypeptides are visible. (ii) Tissue cultured lens epithelial cells were incubated with iodinated vitreal proteins for 24 h, washed with PBS, then solubilized and their proteins separated by 16% SDS-PAGE. The autoradiogram of these proteins show several VH proteins present in the cultured lens cells. Lane a was exposed twice as long as lane b for autoradiography. (iii) Coomassie blue stained SDS gel (16%) of tissue cultured lens epithelial cell proteins. a) epithelial cells exposed to ^{125}I VH; b) exposed to 100 μg/ml BSA; and c) control showing no difference in the banding pattern.

phate buffered saline and the capsule/epithelium preparation was isolated by dissection and stored frozen in liquid nitrogen.

The total amount of DNA per epithelium was measured by Hoechst 33258 dye binding, using the fluorimetric method. The amount of the ^{3}H-thymidine incorporation was determined by liquid scintillation spectrometry after the labelled cells were treated with 5% (W/V) trichloroacetic acid (twice for 1 h each) and then retained on GF/C (Whatman, England) filters for counting. The results are presented as CPM/unit Hoechst 33258 bound to DNA of the epithelial cells.

Mitosis. Freshly isolated ocular lenses were cultured with or without 8 kD VH (100 ng/ml) for 72 h in TC-199 containing 3% serum. At the end of the experiment, all lenses were fixed in Carnoy's solution. Whole mount preparation of lenses were prepared according to the technique of Harding et al. (1960). The epithelial preparation was stained with hematoxylin and examined by light microscopy.

Protein synthesis. Protein synthesis was monitored using ^{3}H-leucine (Amersham, IL) as a precursor amino acid. The lenses were incubated in RPM1-1640 without leucine, except for the last 6 h of culture, when they were exposed to ^{3}H-leucine (50 μCi/ml, Bagchi, 1982).

Isolation of VH protein. The 8 kD VH factor was isolated according to the technique of Banerjee et al. (1991). VH was dissected out from freshly isolated rabbit lenses and centrifuged for 3 h at 100,000*g*. The supernatant was freeze dried and fractionated by column chromatography on a Sephacryl S-100 (Pharmacia-LKB) column. The procedure yielded 7 major fractions, of which the last fraction had protein synthesis and growth inhibitory activity. The major polypeptide band (only band visible by Coomassie blue staining) in this fraction has a molecular weight of ca 8 kD, calculated from its electrophoretic mobility in SDS-PAGE.

Iodination of VH protein. Total VH was centrifuged for 3 hours at 100,000*g* and the supernatant was retained and iodinated with exogenous ^{125}I (NEN, DuPont, Wilmington, DE) using iodobeads (Pierce, Rockford, IL), according to the technique of Markwell (1982). Aliquots were solubilized in SDS-buffer and electrophoresed by SDS-PAGE (Laemmli, 1970) or used in binding experiments with cultured lens epithelial cells.

RESULTS

Figure 1(i) illustrates the autoradiogram of iodinated total VH protein. Four different amounts of iodinated VH protein were electrophoresed in the SDS-PAGE system to detect radioactive VH protein bands of higher and lower concentration. The autoradiogram clearly shows several prominent radioactive protein bands of molecular weight of 14.3 kD and higher. A few less prominent low molecular weight bands (3.4 kD to 14.3 kD) are also seen. These autoradiograms demonstrate that the VH contains proteins ranging from 3.4 to 100 kD. It is possible that some of the low molecular weight proteins are breakdown products of high molecular weight proteins.

Tissue cultured lens epithelial cells were incubated in serum free DMEM with (40 μg/ml) ^{125}I-VH proteins for 24 h. At the end of the experiment, cells were thoroughly washed with cold phosphate buffer, solubilized in SDS sample buffer, and electrophoresed (Laemmli, 1970). Figure 1(ii) shows the autoradiogram of epithelial cell proteins. It is evident from the autoradiogram that there are several vitreal proteins present in the cultured lens epithelial cell preparation. There is one prominent band at 75 kD range, which is possibly transferrin (Beebe et al., 1986), and three discernable bands between

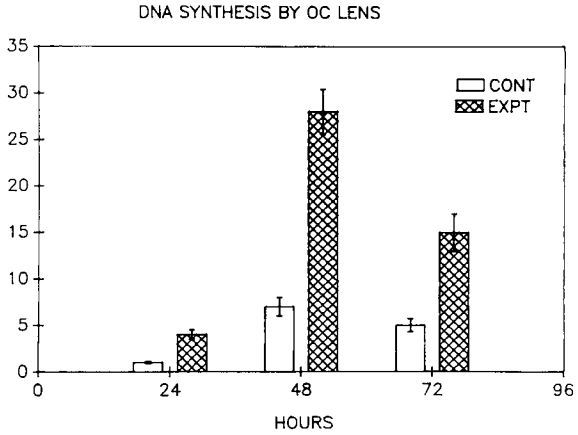

Fig. 2. Histogram of ^{3}H-thymidine incorporation by epithelial cells of control lenses (100 ng/ml 8 kD VH) and experimental lenses (noVH) cultured in 3% serum containing TC-199 medium. The amount of total DNA was measured by fluorimetry and radioactivity by liquid scintillation spectrometry. Data is presented as CPM/unit Hoechst 33258 bound to DNA showing the most pronounced depression of DNA synthesis by VH at 48 h of culture.

ca 3.4 kD to 16 kD. When the aforementioned gel was stained with Coomassie blue (Fig 1(iii)), the banding pattern of lens epithelial cells treated with iodinated VH, bovine serum albumin, or control (non-treated) showed no difference.

Figure 2 illustrates the DNA synthesis of epithelial cells of lenses cultured in 3% serum containing TC-199 medium. Control lenses were incubated with the 8 kD VH fraction (Banerjee et al., 1991). DNA synthesis of the experimental (no VH) epithelial cells of organ cultured lenses were considerably higher than control cells at all time periods examined, and this increase was most pronounced at 48 h of incubation.

Figure 3 illustrates whole mount preparations of epithelial cells of organ cultured lenses. It is clearly evident that the presence of the 8 kD VH factor significantly decreased the number of mitotic cells in the epithelium. Five contralateral sets of whole mount preparations of epithelial cells of lenses incubated with and without VH were examined. The epithelium of lenses cultured without VH had an average of 2987 ± 63 mitotic figures, whereas in the presence of the VH factor the number of mitotic figures in the epithelium was 576 ± 37. It is also evident from the whole mount preparation of epithelial cells of the experimental lenses (no VH) that

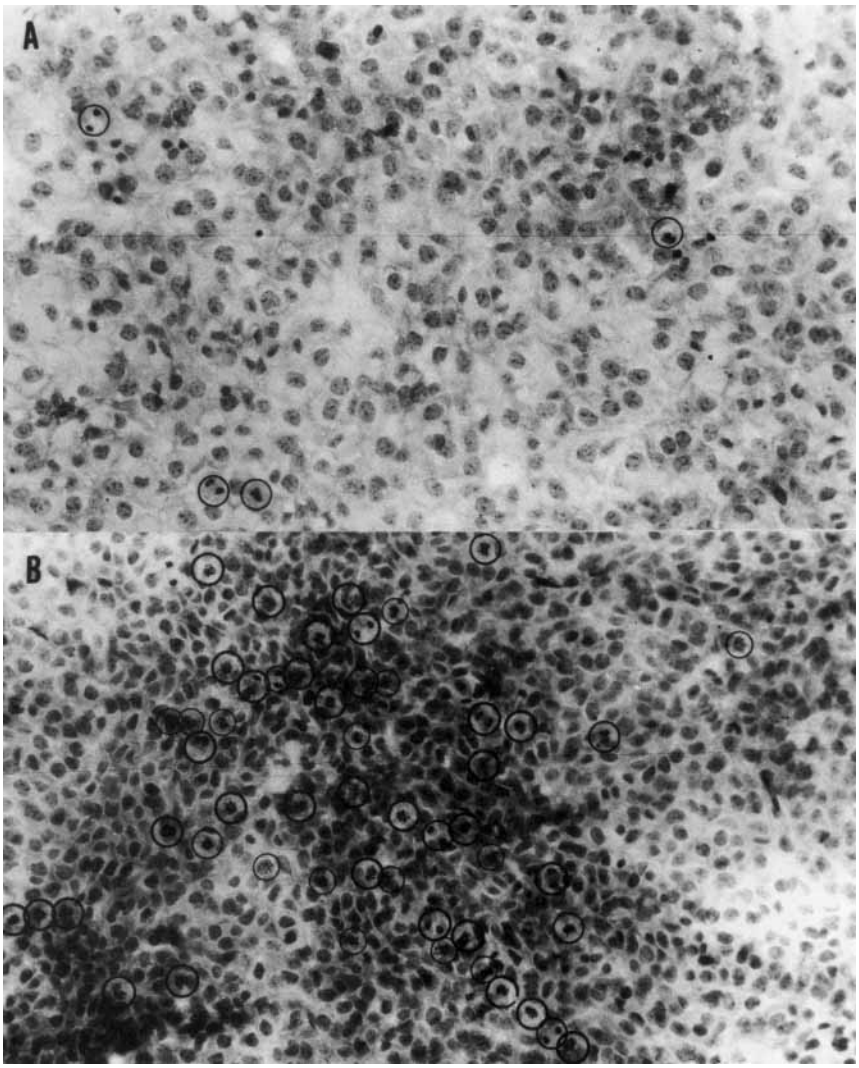

Fig. 3. Whole mount preparation (stained with hematoxylin) of the epithelial cells of lenses cultured with and without VH in presence of 3% rabbit serum in TC-199 for 72 h. **A:** Control lens (with 100 ng/ml 8 kD VH); **B:** experimental lens (no VH). Mitotic figures are circled. Apart from the increase in number of mitotic figures in the experimental lens, abundant prominent nuclei and crowding of the cells is seen.

there are abundant prominent nuclei and crowding of cells, indicative of cellular migration. A similar type of effect was also observed when lenses with attached VH were cultured with 20% rabbit serum (Harding et al., 1968).

Figure 4 shows the Coomassie blue R-250 stained gel pattern and corresponding fluorogram of proteins obtained from epithelial cells of lenses incubated in TC-199 medium for 24 h. The VH from all lenses was removed. The lenses were incubated with 8 kD VH at three concentrations (C = 100 ng/ml, B = 50 ng/ml, and D = 15 ng/ml). The Coomassie blue stained gel of all three epithelial cell preparations displays distinctly similar banding patterns and dye intensities. However, the fluorogram of these gels showed a significant decrease of protein synthesis by the 8 kD VH treated epithelial cells, and this inhibition was dose dependent.

Figure 5 demonstrates the effect of heat on the stability of 8 kD VH factor. The 8 kD VH factor was heated at 95°C for 3 min in boiling water, cooled, and tested for its effect on protein synthesis by epithelial cells of organ cultured lenses.

Lanes A and C represent total epithelial cell proteins of lenses cultured without VH, whereas lanes B and D display protein bands from epithelial cells of lenses incubated with heated 8 kD VH (B) and non-heated 8 kD VH (D). The Coomassie blue stained banding patterns of all lanes are similar. However, the fluorogram of these gels showed that both heated and non-heated 8 kD VH significantly inhibited protein synthesis. This result also shows that the 8 kD VH factor is heat stable.

The Coomassie blue stained gel and corresponding fluorogram in Figure 6 demonstrate the effect of VH on the protein synthesis of tissue cultured (TC) lens epithelial cells. TC cells were incubated with 100 ng/ml VH (c), 100 μg/ml BSA (b), and without any additive (a) in

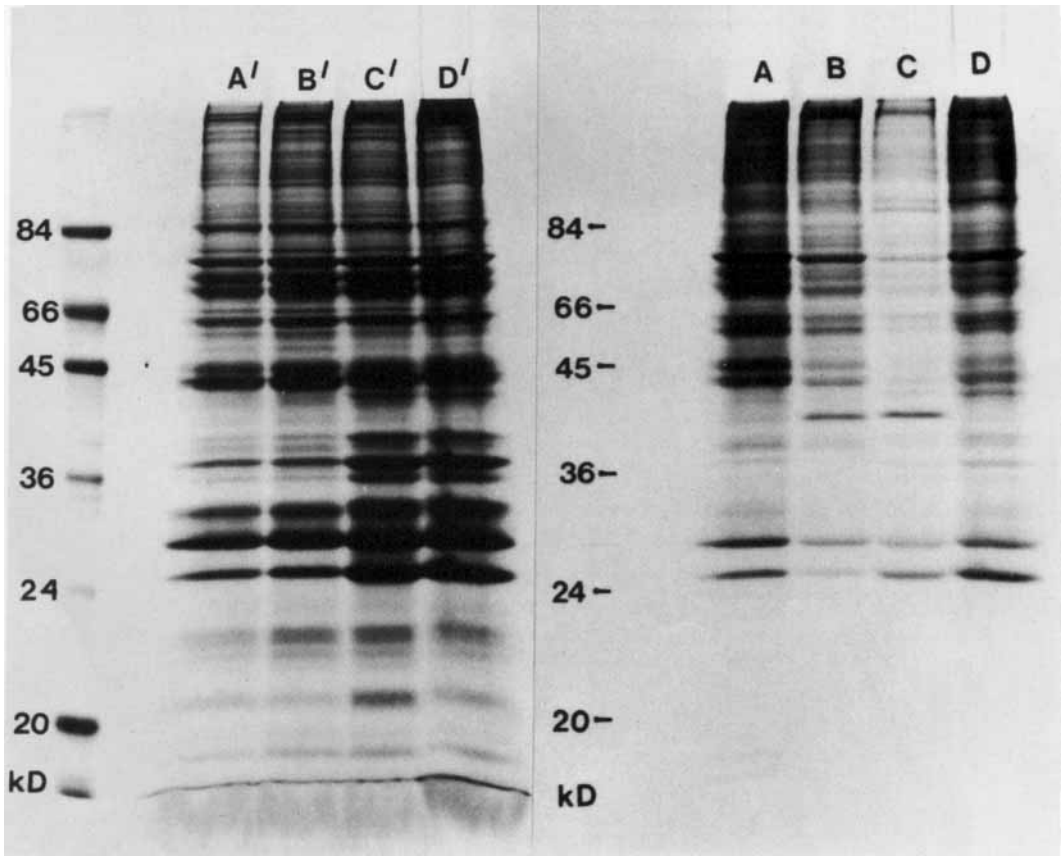

Fig. 4. Fluorogram and Coomassie blue stained gel pattern of epithelial cell proteins of organ cultured lenses exposed to 100 ng/ml, 50 ng/ml and 15 ng/ml of 8 kD VH factor showing a dose dependent inhibition of protein synthesis of lens cells by the VH factor. A, B, C, and D are fluorograms of A′, B′, C′, and D′ respectively. A′ represents lens without VH; B′, lens exposed to 50 ng/ml 8 kD VH; C′, lens exposed to 100 ng/ml 8 kD VH; D′, lens exposed to 15 ng/ml 8 kD VH. A 12% SDS gel was used for electrophoresis.

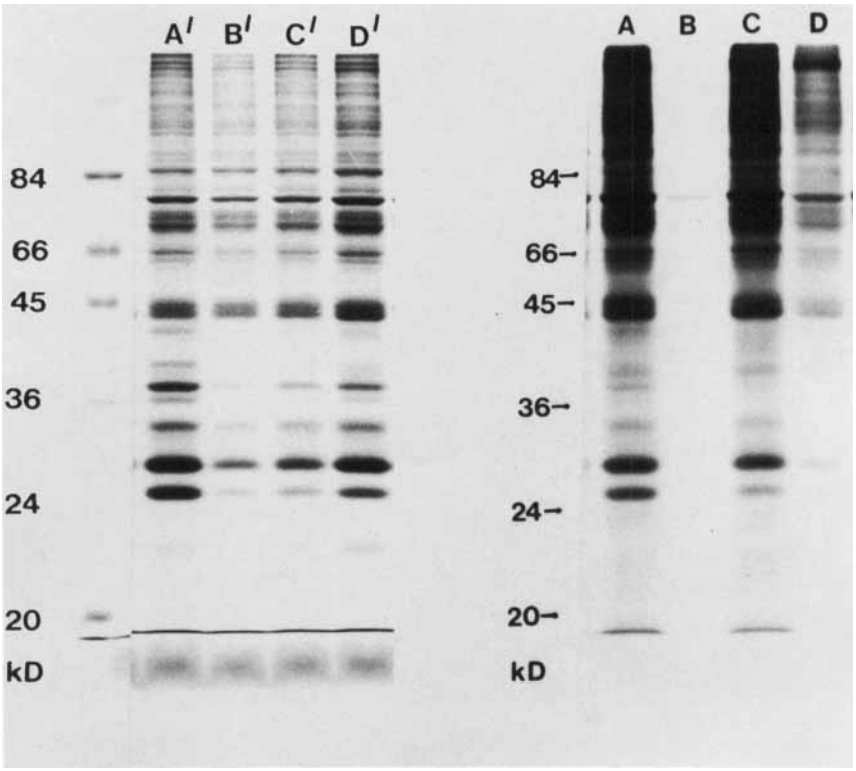

Fig. 5. The 8 kD VH factor was heated at 95°C for 3 min and then tested for its effect on protein synthesis by lens epithelial cells. A 12% SDS gel shows Coomassie blue stained bands and their fluorogram for proteins of epithelial cells of lenses exposed to heated and normal 8 kD VH. A′ and C′ represent proteins of epithelial cells of lenses incubated without VH; B′ represents proteins of epithelial cells of lenses exposed to heat treated 8 kD VH (100 ng/ml); D′ represents proteins of epithelial cells of lenses exposed to normal 8 kD VH (100 ng/ml). A, B, C, and D are fluorograms of A′, B′, C′, and D′ respectively. A, B and C, D were contralateral lenses. The fluorograms show that both heated and non-heated 8 kD VH significantly inhibit protein synthesis.

serum free medium for 24 h. During the last 6 h of culture, cells were pulsed with ^{3}H-leucine. At the end of the experiment all tissue cultured cells were thoroughly washed with cold phosphate buffer and dissolved in SDS sample buffer. The Coomassie blue stained lanes (a′, b′, c′) of proteins obtained from all three groups of epithelial cells display similar banding pattern and dye intensity. The fluorograms (a, b, and c) also show similar ^{3}H-leucine incorporation into the proteins of epithelial cells of all three groups. These results indicate that the 8 kD VH, at the given dosages, does not inhibit protein synthesis by the tissue cultured lens epithelial cells.

DISCUSSION

The experiments reported here clearly establish the growth inhibitory effect of rabbit vitreous humor. Our results demonstrate that the VH factor inhibits mitosis, as well as DNA and protein syntheses by the epithelial cells of the organ cultured lens. The use of iodinated vitreal proteins showed that some of the radioactive proteins are either able to enter the cytosol or bind to epithelial cells. Since the tissue preparations were thoroughly washed and only a few of the vitreal proteins were visible on the autoradiogram, it is presumed that the radioactive bands in the SDS-buffer soluble cell extract are not due to non-specific adhesion. Many prominent radioactive vitreal proteins were not visible in the epithelial cell preparations, except for the ca 16 kD VH protein. For non-specific adhesion one could expect many radioactive bands in the cell extract corresponding to prominent radioactively labelled bands of the VH protein. Lack of a sufficient amount of 8 kD VH protein prevented us from determining whether or not this protein binds directly to the epithelial cells.

The DNA synthesis results clearly demonstrate that the VH factor can effectively inhibit serum stimulated DNA synthesis by the epithe-

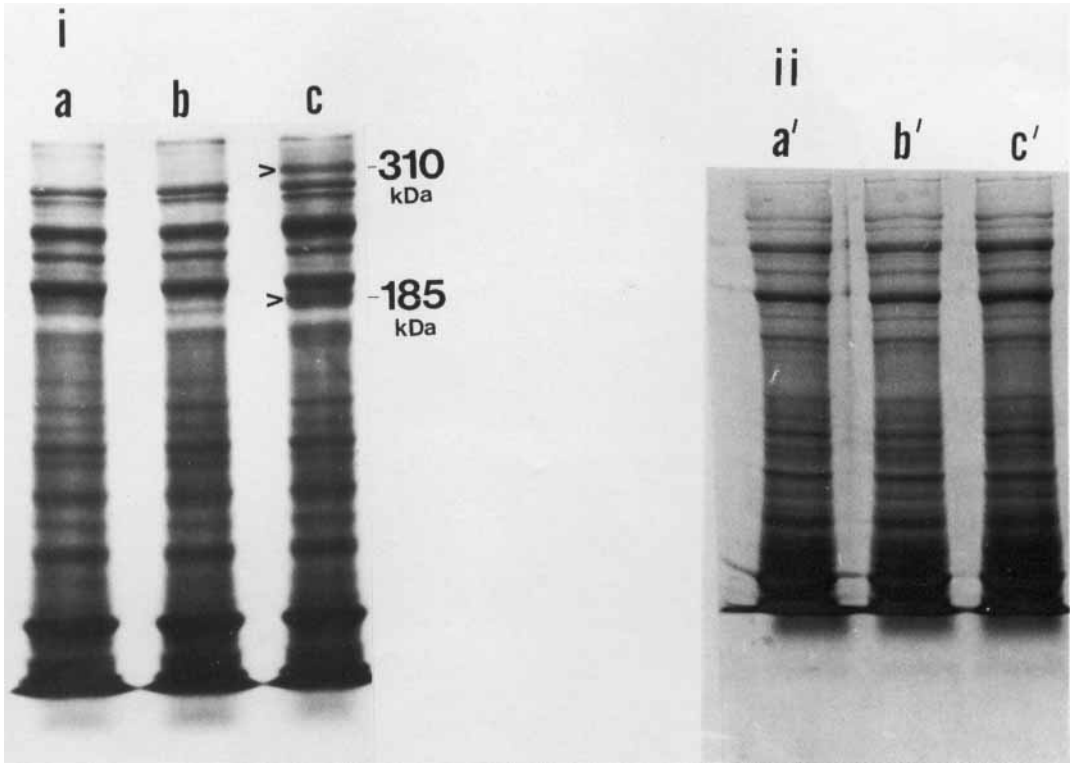

Fig. 6. Effect of the VH factor on protein synthesis by tissue cultured lens epithelial cells. Coomassie blue stained 6% SDS gel (a', b', c') of TC epithelial cell proteins and fluorogram (a, b, c) with similar banding pattern in the Coomassie blue stained gel. a: Control epithelial cells; b: epithelial cells incubated with 100 μg/ml BSA for 24 h; c: epithelial cells exposed to 100 ng/ml 8 kD VH for 24 h. The fluorograms show similar ^{3}H-leucine incorporation into the proteins of all three groups with no depression in protein synthesis.

lial cells of the organ cultured lens. This inhibitory activity was most pronounced at 48 h and the delayed effect is probably due to a net optimum VH concentration. Alternatively, it is possible that it takes more than 24 h for the lens epithelial cells to accumulate enough VH factor necessary for a significant inhibition of DNA synthesis.

Earlier, we had established (Bagchi et al., 1971; Harding et al., 1968) that those rabbit lens epithelial cells that have an "S" phase between 42–48 h will divide (M-phase) at 72 h. Whole mount preparations of epithelial cells of lenses incubated with or without VH protein in the presence of 3% serum displayed significant differences in the number of mitotic figures. The epithelial cells of lenses cultured with VH showed few mitotic figures, whereas epithelial cells of lenses without VH showed at least a fivefold increase in the number of mitotic figures. Both the DNA synthesis and mitosis data demonstrate that the 8 kD VH protein is growth inhibitory to the epithelial cells of the organ cultured lens.

It has also been demonstrated that the effect of the 8 kD VH factor on protein synthesis is dose dependent, an increased concentration of the factor results in an increased inhibition of protein synthesis. This result strongly implies that the effect of the factor is specific. The 8 kD VH factor is heat stable, as are many known growth regulator molecules (Massague, 1990).

Another important finding of this study is that the VH factor did not inhibit protein synthesis by tissue cultured lens epithelial cells. This experiment, which has been repeated more than 20 times using various concentrations of the VH factor, suggests that the effect of 8 kD VH factor on protein synthesis by the epithelial cells is mediated via some specific pathways. The basic difference between the 2nd passage lens epithelial cells and the epithelial cells of the whole lens is the presence of the capsule (extracellular matrix) in the whole lens.

Recent publications demonstrate the important role played by extracellular matrix (ECM) proteins in the regulation of cell division (Massague, 1990; Klagsburn, 1990). Many growth

stimulators or growth inhibitors bind with extracellular matrix proteins for their activity. It can be argued that the VH factor has a specific ECM receptor and without this receptor binding the VH factor is not biologically active. Experiments in our laboratory are in progress to examine this possibility. In preliminary experiments where commercially available ECM proteins such as matrigel- (Collaborative Research Inc., Medford, MA) and ECL- (Upstate Biotech, Lake Placid, NY) coated TC dishes were used to grow lens epithelial cells, the 8 kD VH factor did not inhibit protein synthesis. Further experimentation is needed to resolve these ambiguities. It is also possible that during tissue culture, the polarity of the epithelial cells were reversed and wrong sides of the cells were exposed to the factor, and thus there was no effect of the VH on protein synthesis. This possibility will be examined by growing epithelial cells on porous surfaces where both sides of the cells will be exposed to VH factors. The presence of two high molecular weight bands (185 kD and 310 kD) in the fluorogram of the SDS-PAGE of proteins of epithelial cells exposed to 8 kD VH but not in the untreated cells is intriguing. These two bands are not visible in the Coomassie stained gels, indicating a possible high turnover rate of these proteins. The identity and possible function of these two proteins are not yet known.

Many ocular tissues, such as ciliary body, iris, corneal endothelial cells, sclera, and retina are exposed to the VH, and if VH contains a nonspecific inhibitor, it might affect the stability of all ocular tissues. Thus, teleologically a specific receptor mediated inhibitory factor is preferable.

ACKNOWLEDGMENTS

Supported by a grant from the Michigan Eye Bank. The authors would like to thank Dr. H. Maisel for critical reading of the manuscript.

REFERENCES

Bagchi M, Harding C, Unakar N, Reddan J (1971): Experimental modification of the temporal aspects of the serum-induced cell cycle in the cultured adult rabbit lens. Ophthalmic Res 2:133–142.

Bagchi M (1982): Biosynthesis of crystallins by rabbit lens fiber cells. Ophthalmic Res 14:148–152.

Banerjee A, Richiert DM, Emanuel K, Singh AK, Bagchi M (1991): Studies on the possible role of vitreous humor on the protein synthesis and morphology of the organ cultured adult rabbit lens. II. Epithelial cells. Biochim Biophys Acta 1076:330–336.

Beebe DC, Latkar CH, Jebens HA, Johnson MC, Feagans DE, Feinberg RN (1986): Transport and steady state concentration of plasma proteins in the vitreous humor of the chicken embryo. Implications for the mechanisms of eye growth during early development. Dev Biol 114:361–368.

Chylack LT, Kinoshita JH (1972): The interaction of the lens and the vitreous. I. The high glucose cataract in a lens-vitreous preparation. Exp Eye Res 14:58–64.

Harding CV, Donn A, Srinivasan B (1959): Incorporation of thymidine by injured lens epithelium. Exp Cell Res 18:582–585.

Harding CV, Srinivasan B (1960): Stimulation of DNA synthesis and mitosis by injury. Ann N Y Acad Sci 90:610–615.

Harding CV, Wilson WL, Wilson J, Reddan JR, Reddy VN (1968): Triggering of the cell cycle in an organized tissue in vitro. J Cell Physiol 72:213–220.

Harding CV, Reddan JR, Unakar NJ, Bagchi M (1971): The control of cell division in the ocular lens. Int Rev Cytol 30:215–300.

Jacobson B, Basu PK, Hasany SM (1984): Vascular endothelial cell growth inhibitor of normal and pathologic human vitreous. Lab Sci 102:1543–1545.

Klagsburn M (1990): The affinity of fibroblast growth factors (FGFs) for heparin; FGF-heparan sulfate interactions in cells and extracellular matrix. Curr Opin Cell Biol 2:857–863.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685.

Lisnyak IA (1988): Isolation and properties of the neovascularization inhibitor from vitreous body. Vopr Med Sinsko Khimi 34:16–18.

Markwell MAK (1982): A new solid state reagent to iodinate proteins. 1. Conditions for the efficient labelling of antiserum. Anal Biochem 125:427–432.

Massague J (1990): The transforming growth factor family. Annu Rev Cell Biol 6:597–641.

Naumann GOH, Naumann LR (1986): The intraocular inflammations. Pathology of the Eye, New York: Springer-Verlag.

Pardee AB (1989): G_1 events and regulation of cell proliferation. Science 246:603–606.

Singh AK, Bagchi M (1989): Studies on the possible role of vitreous humor on the protein synthesis and clarity of the organ cultured ocular lens. Lens and Eye Toxicity Res 6:823–831.

Weinseider A, Briggs R, Reddan JR, Rothstein H, Wilson D, Harding CV (1975): Aqueous humor in lens repair and cell proliferation. Exp Eye Res 20:33–44.