Secondary Aqueous Humor Stimulates the Proliferation of Cultured Bovine Corneal Endothelial Cells

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Secondary aqueous humor (2°AH) is known to contain elevated levels of serum macromolecules and has been shown to stimulate the proliferation of lens epithelial cells both in vivo and in vitro as well as corneal endothelial cells in vitro. The purpose of this study was to characterize the response of bovine corneal endothelial cells to 2°AH from rabbits and to compare the effect when the cells were grown on plastic dishes covered with an extracellular matrix or on plastic alone. The addition of varying amounts of 2°AH protein (0.1 to 10 mg/ml) to bovine corneal endothelial cells (cultured in MEM plus 1% serum) resulted in a dose dependent proliferative response as measured by the incorporation of ^{3}H-thymidine into DNA. Except for a 2-hr lag phase, the proliferative response increased with increasing time of exposure (6–18 hrs) of the cells to 2°AH containing a constant amount (2.0 mg/ml) of protein. The generation time and final density of the cells, but not the plating efficiency, was significantly greater when the cells were grown in the presence of 2°AH protein on an extracellular matrix rather than plastic alone. Selective adsorption of prostaglandins and aromatic compounds from 2°AH reduced its ability to produce a proliferative response to control levels. These results indicate that 2°AH can alter or regulate events in the cell cycle of corneal endothelial cells. The responsible factor(s) could be involved in control of cellular regeneration in the eye following injury. Invest Ophthalmol Vis Sci 24:557–562, 1983

The composition of aqueous humor is altered as a result of ocular injury. These alterations include elevations in the level of serum macromolecules,[1] insulin,[2] and prostaglandins.[3] Such changes in the molecular composition of the aqueous humor may facilitate the repair of damaged ocular tissue because all of the above molecules have been shown to affect cell division.[4] In the rabbit, secondary aqueous humor is known to promote the proliferation of lens epithelial cells in vivo and in vitro,[5,6] as well as corneal endothelial cells in vitro.[7]

In this study we sought to characterize the response of cultured corneal endothelial cells to rabbit secondary aqueous (2°AH), occasioned by anterior chamber paracentesis, and to determine if the proliferative response was different when cells were maintained on either plastic or extracellular matrix. Evidence suggests tht interactions with the underlying extracellular matrix may alter the sensitivity of epithelial and endothelial cells to growth factors.[8,9]

Materials and Methods

Collection of Secondary Aqueous Humor

New Zealand white rabbits (4 to 5 kg) were anesthetized by intraperitoneal injection of sodium pentobarbital (1.0 gm/kg). Each eye received two drops of 0.4% Dorsacaine prior to removal of aqueous humor by insertion of a 26-gauge needle at limbus. Secondary aqueous humor (2°AH) was removed in a similar manner every 20–30 min over a 2-hr interval. Secondary aqueous humor obtained from three to five rabbits was pooled, allowed to clot at 4 C, and centrifuged to remove the clot. The supernatant was sterilized by filtration (0.22-μm pore). Sterile 2°AH was stored at −20 C for up to 3 weeks until use. The protein content of the pooled 2°AH was 30 mg/ml as determined by a modified Lowry assay[10] using bovine serum albumin as a standard.

Cell Culture Technique

Bovine corneal endothelial cells were established as primary cultures in 25 cm² flasks (Costar) as previously described.[9] Cells were grown in Minimal Essential Medium with Earle's salt mixture (MEM), 10% calf serum (CS), and 50 µg/ml garamycin sulfate. For the maintenance of postconfluent cell cultures, 38 µg/ml of sodium ascorbate was added to the medium. The cultures were gassed with 5% CO_2 in air to maintain a pH of 7.4 and incubated at 37 C.

Primary cell cultures were subcultured twice at 8-day intervals. Briefly, the cells were rinsed with serum-free MEM and then exposed to calcium-magnesium-free MEM containing 0.25% trypsin (1:250, GIBCO) and 5 mM Na_2EDTA, pH 7.5, for 3–4 min at 37 C. Cells were dislodged by repeatedly pipetting the enzyme solution across the flask bottom. Dissociated cells were added to an equal volume of serum containing medium. Following centrifugation, cells were resuspended in culture medium and dispensed into 25 cm² flasks. Cells from several flasks were pooled at the time of the second subculture and seeded into 25 cm² flasks at a density of 7×10^4 cells/cm² in order to provide a homogenous cell population for assessing the growth-promoting properties of 2°AH. Cells were incubated overnight in MEM containing 10% CS. The culture medium was then replaced with medium containing 1% CS and the cells incubated for 48 hrs. After this time the cells were defined as quiescent and were used for labeling studies. Cell counts were made using an automated cell counter (Coulter Model ZBI). Triplicate counts were performed on all samples.

Plastic dishes coated with an extracellular matrix (ECM) were obtained by maintaining confluent corneal endothelial cell cultures on plastic dishes for three weeks.[8] The ECM deposited over this period was isolated by solubilizing the endothelial cell monolayers in warm 0.5% sodium deoxycholate (DOC) in distilled water. The ECM was rinsed twice with 0.5% DOC, followed by at least three rinses with sterile double distilled deionized water. The ECM-coated dishes were stored at −80 C for up to 8 weeks.

Incorporation of ³H-Thymidine

The medium of quiescent cultures maintained in MEM containing 1% CS was replaced with MEM containing 1% CS and supplemented with varying amounts of 2°AH. The final concentration of 2°AH protein in the culture medium was adjusted to contain between 0.1–10 mg/ml. The control cultures contained medium supplemented with 1% CS alone. After the cells had been in contact with medium containing 2°AH for 24 hrs, the monolayers were rinsed

twice with MEM and pulse-labeled for 2 additional hours in culture medium supplemented with ³H-thymidine at a final activity of 1 µCi/ml ([³H-methyl]-thymidine, 20.0 Ci/mmole). During the periods when cells were exposed to 2°AH or ³H-thymidine, the culture medium consisted of MEM supplemented with 1% CS and garamycin (50 µg/ml) and was stabilized at pH 7.4 by addition of 15 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 10 mM N-tris [hydroxymethyl] methyl-2-amino-ethanesulfonic acid (TES), and 10 mM N,W-bis[2-hydroxy-ethyl]-2-amino-ethanesulfonic acid (BES).[11]

Following exposure to 2°AH and ³H-thymidine, culture flasks were rinsed three times with unlabeled medium, and the cells dislodged following incubation with the MEM trypsin-EDTA mixture previously described. One portion of the resultant cell suspension was used to determine cell number; the remaining cells were added to an equal volume of chilled 1.0 N perchloric acid (PCA). PCA precipitable proteins and nucleic acids were collected by centrifugation under the conditions of Davidson and Smellie.[12] Following solubilization of RNA in 0.3 N KOH, the DNA was solubilized for scintillation counting in 0.5 N PCA for 30 min at 70 C.[13] Samples were dissolved in Insta-Gel (Packard), counted in a liquid scintillation counter, and counting efficiency determined by external standard channels ratio.

Replicate, quiescent cultures were also exposed to medium containing 2.0 mg/ml 2°AH protein for increasing periods of time (0.5 to 18 hrs). Following exposure to 2°AH, cell cultures were rinsed three times with MEM and returned to culture medium lacking 2°AH for the duration of a 24-hr period. Control cultures were maintained for 24 hrs without exposure to 2°AH after the addition of fresh MEM containing 1% CS. Cells were subsequently pulse-labeled in culture medium containing ³H-thymidine for 2 hrs at 37 C and processed as above. Each experiment was repeated three times. The data presented here represents a single replicate experiment.

Growth rate was calculated by the equation 1:

$$\text{Growth rate } (\alpha) = \frac{\log_{10} N_2 - \log_{10} N_1}{.3101(T_2 - T_1)} \quad (1)$$

where N_1 and N_2 equal the number of cells per unit volume at times T_1 and T_2. The generation time then equals $1/\alpha$.

Plating efficiency was calculated by dividing the number of cells attached to the substratum after 24 hrs by number of cells seeded into the dish.

Selective Adsorption of 2°AH

Samples of primary and secondary rabbit aqueous humor were analyzed to determine the levels of 6-
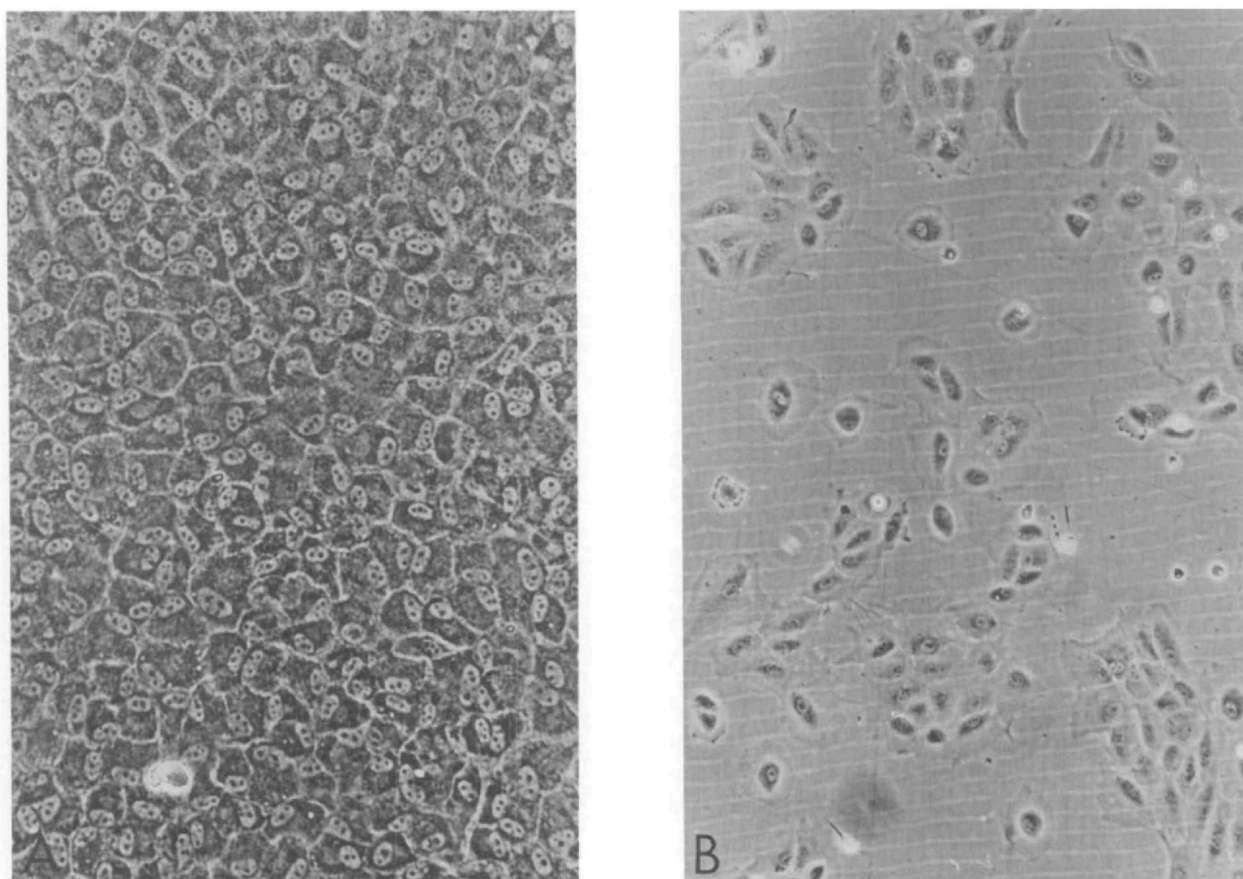
Figs. 1A–B. A, phase photomicrograph of a second passage endothelial cell culture, 1 week postconfluence (×435). B, phase photomicrograph of a preconfluent endothelial cell culture 72 hrs after subculture. Replicate cultures containing similar numbers of cells were used to assess the growth promoting properties of rabbit secondary aqueous humor (×340).

keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2(TXB_2) by radioimmunoassay (New England Nuclear). This 6-keto-derivative represents the stable metabolite of prostacyclin (PGI_2), while TXB_2 is the stable metabolite of thromboxane A_2. Prostaglandins, nucleotides, and unidentified aromatic compounds were selectively adsorbed from 2°AH with acid washed, activated charcoal Norit A and Aerosil 380 (Dr. W. Harris, New England Nuclear, personal communication). The recovery of 2°AH protein averaged 73%.

Results

Effects of 2°AH on ^{3}H-thymidine Incorporation

Confluent bovine corneal endothelial cells in vitro formed an organized, tightly packed cell monolayer of uniform appearance (Fig. 1A). Following the second subculture, quiescent preconfluent endothelial cells assumed a flattened polygonal or circular shape characteristic of low density endothelial cells (Fig. 1B). Stimulation of ^{3}H-thymidine incorporation into

DNA occurred in a dose-dependent manner when the culture medium of quiescent, preconfluent cell cultures was replaced by medium containing 1% CS and various concentrations (0.1–10 mg/ml) of 2°AH protein for 24 hrs (Fig. 2). Linear regression analysis of the dose response curve indicated that the correlation coefficient was 0.9768. Concentrations of 2°AH protein as low as 0.1 mg/ml resulted in a significant ($P < 0.01$) threefold increase in ^{3}H-thymidine incorporation into cellular DNA as compared to cultures receiving 1% calf serum alone.

Exposure of quiescent, preconfluent cultures to a constant amount of 2°AH protein (2.0 mg/ml) for increasing lengths of time (0.5 to 18 hrs) also resulted in increasing levels of incorporation of ^{3}H-thymidine into DNA (Fig. 3). A twofold stimulation of ^{3}H-thymidine incorporation above controls occurred (1% CS and no 2°AH protein or 1% CS and selectively adsorbed 2°AH protein) when cells were exposed to 2°AH for 0.5 or 2 hrs. A three-, five-, and 11-fold increase over controls was observed when cells were exposed to 2°AH for 6, 12, and 18 hrs respectively.
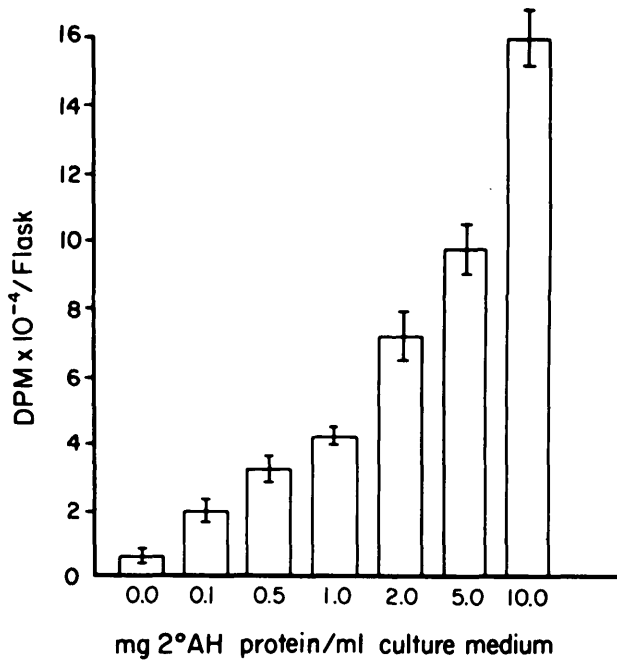
Fig. 2. Effect of increasing concentrations of secondary aqueous protein on ^{3}H-thymidine incorporation into DNA of cultured corneal endothelial cells. Each point represents the mean and standard deviation of five replicate flasks. Control cultures were supplemented with fresh medium containing 1% calf serum and are shown in the figure as containing 0.0 mg 2°AH protein. Forty-eight hours after addition of fresh medium, cells were pulse labeled for 2 hr with ^{3}H-thymidine 1 Ci/ml (20.0 Ci/mmole) in MEM supplemented with 1% CS. Cells were harvested, washed and the DNA isolated as described in Materials and Methods section.

Effects of Extracellular Matrix on Cell Proliferation

The generation time and final density, but not plating efficiency of corneal endothelial cells in vitro, was found to be influenced substantially by the type of substratum on which the cells were plated and maintained. When secondary cultures of endothelial cells were seeded onto plastic or ECM at a density of 7.0 × 10^4 cells/cm^2 in MEM containing 10% CS, and incubated overnight, the plating efficiency was the same for both groups. Approximately 40% of the cells in the suspension attached to each kind of substratum. The generation (doubling) time of log phase bovine corneal endothelial cells grown on plastic was about 20 hrs, while on ECM-coated plastic, it was 15 hrs. When equal numbers of corneal endothelial cells were plated in MEM containing 10% CS and maintained for 48 additional hrs in MEM containing 1% CS and organic buffers, the number of cells per flask (density) was significantly greater in cultures plated on ECM as compared to plastic (Fig. 4). During the 48-hr period following plating, cells plated on ECM and exposed to 2°AH had completed cell divisions, and, therefore, cell number was observed to increase.

The cells maintained on plastic had not completed as many divisions, and therefore cell number was not significantly increased over controls (Fig. 4). In contrast, incorporation of ^{3}H-thymidine into DNA of cells plated either on plastic or ECM was significantly increased in media containing 2.0 mg/ml of 2°AH protein, indicating a significant increase of S phase cells under both conditions (Fig. 5). Therefore, the absence of increased cell numbers in cultures plated on plastic and exposed to 2°AH may have been a result of either longer generation times or a higher percentage of cells arrested in G-2.

Effects of Selectively Adsorbed 2°AH on Cell Proliferation

Selective removal of prostaglandins and aromatic compounds from 2°AH was achieved by Norit A adsorption. The recovery of protein in adsorbed samples was 73% whereas all detectable 6-keto-PGF$_{1\alpha}$ was removed. Adsorbed aqueous humor samples appeared to contain no cross-reacting substances that interfered with the RIA assays.
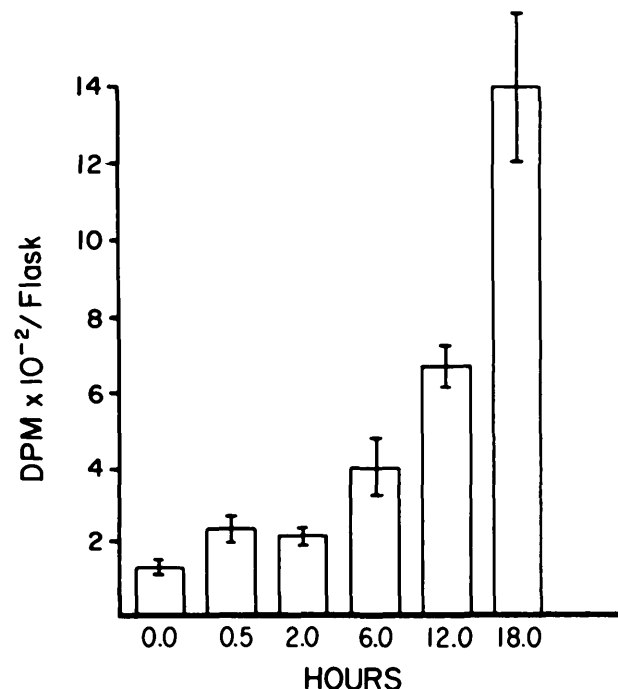

Fig. 3. Effect of time of exposure to a constant amount of secondary aqueous protein (2 mg/ml) on ^{3}H-thymidine incorporation into DNA of cultured corneal endothelial cells. Each point represents the mean and standard deviation of five replicate cultures. Control cultures were supplemented with 1% calf serum and are shown in the figure as 0.0 hours exposure to 2°AH. Quiescent cells were given fresh media containing 1% CS and 2°AH protein 2.0 mg/ml. The cells were incubated for the specified length of time and returned to media containing 1% CS for the duration of the 24-hr period. The cells were then pulse labeled with ^{3}H-thymidine as described in Figure 2.
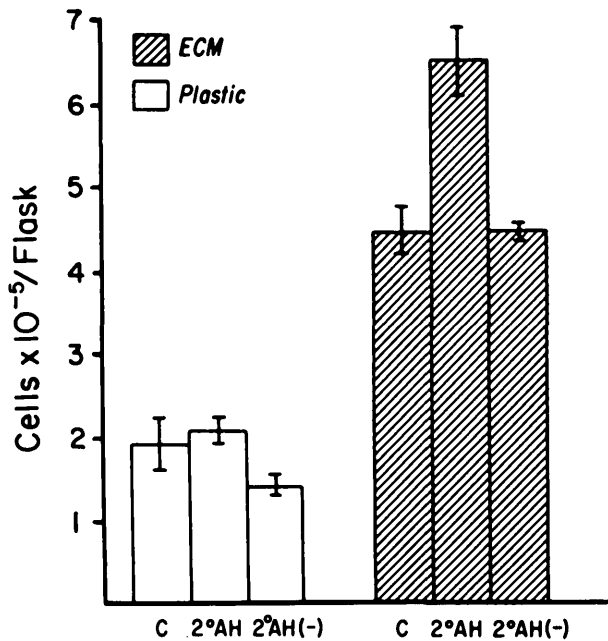
Fig. 4. Effect of secondary aqueous humor (2 mg/ml) on the final cell density of corneal endothelial cells cultured on plastic and ECM coated plastic. Each point represents the mean and standard deviation of five replicate cultures. C indicates control cultures with no aqueous protein; 2°AH indicates cultures containing 2 mg/ml secondary aqueous humor protein; 2°AH (−) indicates cultures containing 2 mg/ml secondary aqueous humor protein from 2°AH previously adsorbed with Norit A. Cells were seeded at a density of 7.0 × 10^4 cells per cm^2 in MEM containing 10% CS. After 24 hrs media was replaced with MEM containing 1% CS and 2°AH. Forty-eight hours later cells were harvested by trypsinization, washed and counted in a Coulter counter.

Cells were grown for 24 hrs in the presence of 2 mg/ml 2°AH from which prostaglandins and other unidentified aromatic compounds were removed by adsorption with acid washed Norit A (2°AH−). Primary aqueous humor contained less than the detectable limit of PGF$_{1\alpha}$ (<100 pg/ml) and 0.80 mg/ml protein. Secondary aqueous humor contained about 3160 pg/ml PGF$_{1\alpha}$ and 30 mg/ml protein. Selectively adsorbed aqueous humor contained no detectable PGF$_{1\alpha}$ and 22 mg/ml protein. The number of cells per flask under these conditions were not significantly different from controls when either ECM or plastic substratum were used (Fig. 4). The incorporation of ^{3}H-thymidine into DNA, however, was substantially reduced, but not eliminated, in cells exposed to selectively adsorbed 2°AH (Fig. 5).

Discussion

We have examined the ability of 2°AH to promote cell proliferation in preconfluent cultured bovine corneal endothelial cells in order to understand better the regeneration of corneal endothelial cells. The cell

culture system employed in this assay expresses morphologic (Fig. 1) and biochemical properties[9] typical of in vivo corneal endothelium and as such represents a useful in vitro model.

We have demonstrated that rabbit secondary aqueous humor stimulates the incorporation of ^{3}H-thymidine into DNA of cultured bovine corneal endothelial cells. The stimulatory effect was more pronounced with increasing concentrations of 2°AH, in a dose-dependent manner. We have also shown that there is about a 2-hr lag phase in thymidine incorporation when cells were exposed to a constant amount (2 mg/ml) of 2°AH for different lengths of time. The level of incorporation did not change significantly between 0.5 and 2.0 hrs of exposure to 2°AH; however, following 6, 12, 18, or 24 hrs of exposure, significantly different increases in tritium incorporation were recorded. The occurrence of a lag phase prior to the maximum response is not unexpected and is known to characterize the cellular response to mitogenic stimulation in a number of cell types including rabbit lens epithelial cells exposed to rabbit secondary aqueous humor.[6]

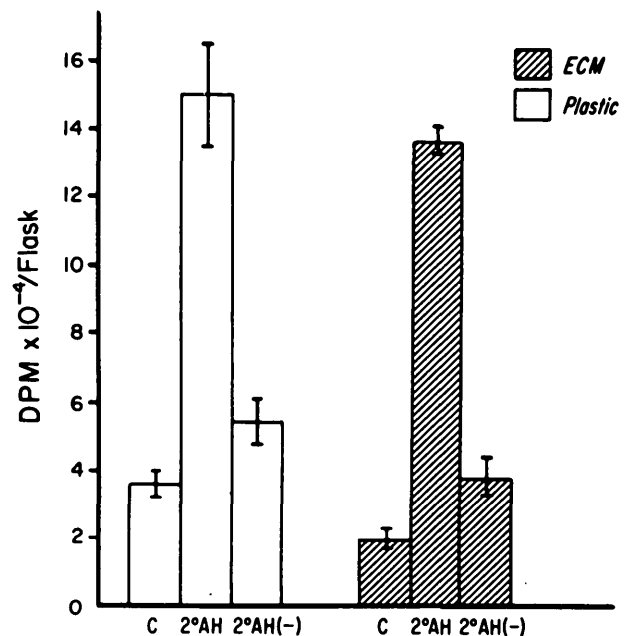The nature of the growth promoting properties of secondary aqueous are incompletely understood. It

Fig. 5. The effect of secondary aqueous humor on the incorporation of ^{3}H-thymidine into DNA of corneal endothelial cells maintained on either plastic or extracellular matrix. Each point represents the mean and standard deviation of five replicate cultures. C indicates control cultures with no 2°AH protein; 2°AH indicates cultures containing 2 mg/ml secondary aqueous humor protein; 2°AH− indicates cultures containing 2 mg/ml secondary aqueous humor protein which had been previously adsorbed with Norit A. Cells were grown as described in Figure 4 and pulse labeled for 2 hr with ^{3}H-thymidine as previously described.

is possible that 2°AH enhances the mitogenic response to serum or that 2°AH contains mitogens of its own. Secondary aqueous humor in our system was found to contain highly elevated levels of protein and 6-keto-prostaglandin $F_{1\alpha}$. The 2°AH used in these studies contained no measurable TXB_2, which suggests that the aqueous did not contain platelets or platelet derived factors such as platelet growth factor. Other investigators have reported increased levels of insulin[2] and E-type prostaglandins[3] in secondary aqueous. These compounds are known to be mitogenic to cultured cells.[4,5,14]

Other investigators have demonstrated that the underlying extracellular matrix can affect the responses of corneal endothelial cells to factors such as FGF and EGF. Our data clearly indicate that the presence of an extracellular matrix affects both the growth rate and final density of corneal endothelial cells. The factors in 2°AH that are responsible for the stimulation of ^{3}H-thymidine incorporation and cellular proliferation affect endothelial cells grown on plastic as well as cells grown on an extracellular matrix; however, due to the reduced generation time of cells plated on an ECM, the proliferative effects are apparent in a shorter time than when cells are plated on plastic.

The substances responsible for the proliferative stimulation reported here have not been identified. Selective adsorption of secondary aqueous by acid wash Norit A removed the mitogenic compounds from 2°AH. These data suggest that factors such as prostaglandins and compounds with highly aromatic structures may play a role in inducing a response. Other forms of injury such as X-radiation that do not produce high 2°AH protein concentrations have been observed to result in 2°AH which is mitogenic.[6] Further investigations will be required to determine if any proteins or peptides with aromatic character were selectively adsorbed during the adsorption.

Key words: corneal endothelium, cell culture, aqueous humor, proliferation, extracellular matrix, 6-keto-prostaglandin $F_{1\alpha}$, mitogenic

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