PROGRESS REPORT

CELLULAR PROLIFERATION AND REGENERATION

FOLLOWING TISSUE DAMAGE

Contract EY-76-S-02-2086-2401

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PROJECT ABSTRACT

This project involves a study of wound healing in tissues of the eye, particularly lens, cornea, and surrounding tissues. The reactions of these tissues to mechanical injuries, as well as injuries induced by chemotoxic agents are under study. It is felt that a better understanding of the basic reactions of the eye to injurious agents may be of importance in the evaluation of potential environmental hazards.

In order for a tissue to repair itself following injury, new cells must be produced, and considerable cellular migration and re-arrangement occur. We need a better understanding of the mechanisms which control cellular proliferation in normal, injured, and repairing tissues. One important question, therefore, is what reactions trigger the cell cycle following injury, and turn off the cell cycle at a certain time during healing. Another is what factors control the migration and re-arrangement of cells during wound healing.

The reactions which trigger the cell cycle (promote the $G-0 \rightarrow G-1$ transition) control the amount of cellular proliferation and tissue growth in normal and injured tissues. These reactions can be conveniently studied during the process of wound healing or exposure of the isolated whole rabbit lens to a serum mitogenic factor in organ culture. It has now been established in the lens, in tissue culture, that the triggering process can be effected by insulin in a completely defined medium. It is proposed to determine the components of the defined medium which are essential for the promotion of the $G-0 \rightarrow G-1$ transition, and to determine if various phases and subphases of the cell cycle can be

characterized by the requirement for specific components of the defined medium. It is also planned to continue studies on the correlation of changes in fine structure (e.g., the burst of ribosome production which characteristically follows exposure of the lens to rabbit serum) with changes in macromolecular synthesis (RNA, protein); to investigate, through the use of specific anti-metabolites, the possible role of increased ribosomal and non-ribosomal RNA production in the stimulation of cellular proliferation during wound healing.

It is also planned to study the lens epithelial cell surface as well as the topography of the underlying lens fibers by means of the techniques of electron microscopy and scanning electron microscopy, before and after the addition of mitogenic agents (rabbit serum, insulin) to the culture medium, and at various times after mechanical and chemical injury in vivo. On the premise that cellular inter-relationships (e.g., cell communication, or coupling) play an important role in the control of tissue growth, particular attention will be paid to changes in the junctional complexes, as seen in replicas of freeze-etched preparations and in thin sections for T.E.M. The techniques developed during the past year here in the Kresge Eye Institute for studying the internal structure of the ocular lens with scanning electron microscopy (S.E.M.) will also help to serve as a basis for this portion of the program. Also, studies which have been initiated on the ultrastructure of the corneal surface utilizing the S.E.M. and T.E.M., will be expanded to include an analysis of the changes which

occur on the epithelial and endothelial cell surfaces after injury in the regenerating cornea. In addition it is proposed to study the stimulation of growth of new vessels (neovascularization) which can then invade the cornea following chemical injury, and reduce the possibilities of subsequent successful corneal transplantation. Within the last several months we have developed a method of isolating and culturing rabbit retinal vessels and have been able to demonstrate DNA synthesis in these cultures. It would seem then that this procedure could serve as a model for the study of what causes vessels to proliferate and therefore what factors might be involved in neovascularization, in much the same way that the cultured lens has been used to study those factors that regulate cellular proliferation in the lens epithelium. It is also planned to study the growth of new vessels into the cornea with the use of plastic corrosion casts, which can then be studied in detail with scanning electron microscopy.

It is expected that the proposed program will lead to a better understanding of some of the initial structural and biochemical changes which are essential to the mechanisms which control cellular proliferation, migration and re-arrangement following injury. The new knowledge gained from this study may be of use in helping to prevent or ameliorate the effects of various chemical and physical injurious agents on the eye.

INTRODUCTION

The present proposal is based on findings obtained during the past several years. The results of previous experiments are included in publications, the reprints of which are attached. The ultimate goal of this program is a further elucidation of the mechanisms that underlie the control of cellular proliferation and cellular migration in the normal and injured tissues of the eye. This should, in turn, lead to a better understanding of those phenomena which are basically related to cellular proliferation and migration: differentiation, regeneration, growth, wound healing, and the response to injurious agents. From a practical point of view, it is felt that a better understanding of the basic reactions of the eye to injurious agents may be of importance in the evaluation of potential environmental hazards.

Earlier work has shown that the central epithelial cells of the adult rabbit lens normally become "blocked" in the G-1 phase of the cell cycle (see attached reprint of review for International Review of Cytology). For convenience, this state of blockade has been referred to as G-0-1 (Srinivasan and Harding¹, 1965. See reprint, C00-2401-2). Following mechanical injury, many of these cells are triggered to enter the cell cycle and undergo DNA synthesis and mitosis. Some of these cells may go through a second, or possibly a third, cell cycle. At the end of the wound healing process, however, the cells presumably once again become blocked in the G-1 phase (enter G-0-1). The results emphasize that the key to understanding the mechanism(s) which controls cell division and the

1 Invest. Ophthal. 4:452-470, 1965

consequent tissue growth related to cell division, lies in an understanding of the reactions which trigger the cell cycle. These reactions can be conveniently studied during the process of wound healing or exposure of the isolated whole lens to a serum mitogenic factor in organ culture.

Recent work on the E.R.D.A. Contract indicates that such reactions can now be studied in the isolated lens maintained in organ culture in a completely defined medium.

The relationship of the G-0 $\rightarrow$ G-1 transition to the cell cycle is summarized in the accompanying diagram (Fig. 1). Data on the various topics listed in the diagram are described in detail in the publications (derived from the E.R.D.A. contract) listed in the bibliography on pages V-1 - V-5. Photocopies of the majority of the publications (reprints or pre-prints) are also included. This also includes recent publications and manuscripts on the scanning electron microscopy of the ocular lens and the corneal surface, which we believe, will serve as a basis for studies of the effects of injurious agents on the lens, cornea, and surrounding tissues.

- (1) INJURY
SERUM FACTOR(S),
INSULIN
- (2) INITIAL CHANGE IN
TISSUE ORGANIZATION
(E.M. LEVEL)
- (3) RNA AND PROTEIN
SYNTHESIS INCREASES
RIBOSOMES INCREASE
- (4) CELLULAR REARRANGEMENT
EVIDENT AT HISTOLOGICAL LEVEL
- (5) SERUM DEPENDENCY ENDS
ACTINOMYCIN-D SENSITIVITY ENDS
- (6) POSSIBLE DIFFERENTIATION

FIG 1

BACKGROUND

Reaction to Injury

The ocular lens has proven uniquely suitable for studies on the initiation of cell division in an organized tissue (see attached reprints including review C00-2401-30. The epithelium which is situated as a monolayer on the anterior portion of the lens just subjacent to the lens capsule is very responsive to injury. A small mechanical wound to the anterior surface of the rabbit lens in vivo results in a wave of stimulation of DNA synthesis and mitosis that moves out from the wound site. X-irradiation of rabbit lenses two days after a mechanical injury can, within less than two hours, completely suppress the wave of mitosis, but allow DNA synthesis to continue. Additional studies on lenses x-irradiated at the time of mechanical injury suggest that radiation also tends to prevent the G-1 to S transition of the cell cycle. (For additional details, see Harding et al., International Review of Cytology, Reprint C00-2401-30 attached).

Initiation of DNA Synthesis and Mitosis in the Cultured Lens

The lens maintained in organ culture has also proven useful in studies on the control of cellular proliferation. The lens is avascular, lacks a nerve supply, and is entirely enclosed within a membrane, the lens capsule. Because of these and other characteristics, it is relatively easy to isolate and study in organ culture with a minimal change in tissue organization. It has been found that the establishment of the whole lens in organ culture under a variety of

conditions can result in marked changes in the rate of DNA synthesis and mitosis. By suitable changes in the culture medium, it is possible to obtain either a marked stimulation of DNA synthesis and mitosis or a maintenance of the epithelium in a normal quiescent state. Rabbit lenses cultured in a medium consisting of medium 199 containing 23% serum (M-199-S) undergo a series of characteristic changes in epithelial mitotic activity. During the first 9 hours mitosis practically ceases. It subsequently resumes, reaches a peak at 20 hours, and then once again decreases to a low level. The above changes, all of which occur in the periphery of the epithelial layer, herald the initiation at approximately 22-24 hours of a pronounced stimulation of DNA synthesis (thymidine incorporation) in the central region. At 44 hours, the number of cells in mitosis in the central region increases enormously. At this and later times the epithelium appears as a multilayer of cells which have lost their original organization. (For details of these experiments see reprints COO-2401-30 and COO-2401-8).

The marked stimulation of DNA synthesis and mitosis which characterizes lenses cultured in M-199-S for 48 hours or more does not occur in the lenses cultured in medium 199 containing 23% serum dialysate (M-199-SD). In lenses cultured in this latter medium, the central epithelium is maintained in a non-proliferating state. This state of non-proliferation can be terminated by transfer of the lens to M-199-S. Thus lenses which have been cultured in M-199-SD for 48 hours and subsequently in M-199-S for an additional 48 hours, show a marked stimulation of cellular proliferation in the central epithelium.

It appears, therefore, that cellular proliferation in the cultured lens can be triggered at will by a change in composition of the culture medium. (For details of these experiments, see Harding et al., reprint attached C00-2401-21). Some characteristic, or combination of characteristics, of the serum-containing medium appears to be responsible for the stimulation.

The above experiments indicate that the central epithelium of the cultured rabbit lens can be kept in the G-0-1 state, characteristic of this tissue in the intact living adult organism. The subsequent addition of serum to the culture medium can trigger these cells to enter the cell cycle and divide. Cultured lenses, triggered by exposure to M-199-S during the first 24 hours of culture and then switched to M-199-SD, show extensive DNA synthesis at the end of 48 hours of total culture time, and very little DNA synthesis at 72 hours. These results also show that these cells have once again become "blocked" at some point in the cell cycle. The results also show that the addition of serum to the medium at 72 hours can cause a re-stimulation of these cells to divide. The cultured lens can, therefore, serve as a model system for studies on the control of cell division in an organized tissue in which the central epithelial cells can be maintained in the G-0 state (similar to the normal epithelium in the intact animal), or triggered at will to enter the cell cycle. It now appears that following a marked stimulation, the G-0 state can be re-established by removal of whole serum from the medium; and that once this "blocked" state has been achieved, the

cells can be re-triggered to enter the cell cycle upon the second addition of serum to the culture medium.

Serum is not required throughout the whole period of culture in order to elicit a stimulation of DNA synthesis, but only during an initial period which ends prior to the onset of both DNA synthesis and mitosis. Some of the characteristics of the serum factors which promote these reactions and the conditions of culture which affect their activity have been reported (see attached reprints). More recently, freshly isolated rabbit serum was fractionated by DEAE-cellulose column chromatography according to the methods of Sober and Peterson¹(1958). This fractionation procedure yields four major fractions; the first fraction, which according to its electrophoretic mobility and analytical ultracentrifugal analysis was tentatively identified as containing gamma globulin, showed mitogenic activity. Fraction one does not show cellular disorganization and multilayering comparable to whole serum, although it does show some cellular disorganization. Mitogenic activity of the first fraction is significantly higher than that of whole serum, when activity is expressed on the basis of milligrams of protein per milliliter in the culture medium.

Recent experiments have shown that in a chemically-defined medium, KEI-4 as developed in Kinsey's laboratory (Compare with C00-2401-60), insulin can replace the serum mitogenic factor. This seems to depend on the integrity of the insulin molecule, since neither the α nor β chains evoked the mitotic response. Therefore, we have now reached the point where the cell cycle can be chemically triggered in an organized tissue maintained in a completely defined medium in organ culture.

1 Fed. Proc. 17(4), 1116-1126, 1958.

Period of Serum Dependency and Actinomycin D Sensitivity

Previous experiments have shown that in order to obtain a pronounced serum-induced stimulation of mitosis at a total of 48 hours of lens culture, it is not necessary to have the serum in the culture medium beyond the first 20 hours of culture. It is possible that this "period of serum-dependency" is a reflection of special requirements of the cells for a portion or all of the G-1 phase, since serum dependency ends just prior to the initiation of DNA synthesis. The need for specific serum components may change at this time (and, indeed, each phase of the cell cycle may eventually be characterized by its own set of specific requirements).

It appears, therefore, that there is a discrete and definable period of serum dependency, which may reflect specific requirements for the G-0 to G-1 transition, and perhaps for the G-1 phase itself. Serum may not be required during all portions of this 20 hour period. Lenses which have been cultured 24 hours in M-199-SD and which do not, therefore, show a stimulation, can be subsequently stimulated by the addition of whole serum to the medium (transfer to M-199-S).

Under these conditions, however, the time between addition of serum and the initiation of DNA synthesis is significantly reduced. One possible explanation for this effect is that the triggering of the cell cycle (G-0 $\rightarrow$ G-1 transition) and perhaps some of the initial reactions of the G-1 phase can occur in M-199-SD, (i.e., whole serum is not required during the beginning of the first 20 hours of culture).

This assumes that the pre-synthetic phase (the period between explanation and the initiation of DNA synthesis) can be divided into sub-phases, characterized by their dependence on the presence of specific components in the culture medium (see C00-2401-34 for a further discussion of these experiments). Another possible explanation is that the preincubated lenses have had time to "recuperate" at least partially from the trauma of isolation and establishment in culture. If this trauma delays the mitogenic action of serum, the shorter period of time required for the serum factor(s) to stimulate DNA synthesis in the preincubated lenses, which have had time to "recuperate", can be explained. Recent evidence, however, indicates that there is indeed an initial sub-phase which is serum-independent (C00-2401-49). The results of this investigation, indicate that approximately the first 4 hours of the 20 hour period are also serum-independent. It is of interest that the end of the period of serum dependency corresponds to the end of the time during which the mitotic-stimulatory mechanism is actinomycin D sensitive. Exposure of the freshly-isolated lens to actinomycin D as early as the first 4 hours of culture in M-199-S is sufficient to completely inhibit the DNA synthesis and mitosis which normally follows. However at intervals beyond 18 hours, actinomycin D (0.03 mg/ml) has little or no effect on the entrance of cells into DNA synthesis. It is apparent, therefore, that the initiation of the DNA synthetic phase is preceded by a phase characterized by the production of RNA which is necessary for the epithelial cells to subsequently undergo DNA synthesis and mitosis. (For additional details, see reprints C00-2401-27 and C00-2401-30).

Necessity of RNA Synthesis for Subsequent DNA Synthesis and Mitosis

There is a significant increase in RNA synthesis (as evidenced by the DNA-ase resistant incorporation of tritium-labeled uridine) which commences at 7 hours and remains at elevated levels from 7-25 hours. Moreover, the inhibition of DNA synthesis and mitosis which is brought about by exposure of the lenses to actinomycin D during the early hours of incubation (see C00-2401-27) results in a 20-50% reduction in the incorporation of tritium-labeled uridine. It is during this period of increased RNA production that the system is dependent upon the presence of the serum. As mentioned above, pre-incubation for 24 hours in the non-stimulatory medium M-199-SD, decreases the time required between the addition of serum and the initiation of DNA synthesis. Preincubation also shortens the time required for the serum-induced increase in RNA production and ribosome formation, (see C00-2401-34 for details of this experiment). Serum-induced ribosome formation in preincubated lenses is relatively intense (as compared with the freshly-isolated lens). The preincubated lens, therefore, appears to be an excellent system in which to study induced ribosome formation, which has been suggested by Yamada to characterize the G-0 $\rightarrow$ G-1 transition.

Changes in Ultrastructure

Other studies on the ultrastructure of the cultured and wounded lens showed a distinct pattern of changes, a detailed description of which appears in (C00-2401-28). Scanning electron microscope studies have been initiated to follow changes in the cell surface at various times after injury. Preliminary observations on the dogfish lens epithelium show a striking orientation of the cells toward the site

of injury within 24 hours after injury. Among the earliest changes which have been detected following injury in vivo or (exposure) of the lens in culture to mitogenic agents, are changes at the ultra-structural level and, in particular, changes in cell-to-cell relationship (COO-2401-30).

This is of interest since there is increasing evidence that alterations in cell-to-cell relationships may play an important role in the control of cellular activities, including cellular proliferation and tissue growth. In the lens correlations can be made between the distribution patterns of the stimulated cells and the observed changes in structural organization of the epithelium; that is, both tend to assume concentric patterns relative to the center of the epithelial layer. This correlation between proliferation and tissue structural organization is of particular interest in relation to the concepts of contact inhibition (Abercrombie,¹ 1970; Abercrombie and Ambrose,² 1962; Eagle,³ 1967) and cell coupling (Loewenstein,⁴ 1968a,b,1975)⁵ Observations on the growth of cultures of dissociated cells have shown that when the cultured cells come in contact there are distinct changes in cellular properties and activities. When a sufficient degree of contact is made among the originally dissociated cells, cellular motility is markedly reduced and mitosis decreased.

The observations of Loewenstein on cell coupling may be importantly related to contact inhibition. When dissociated cells in culture come in contact, the junctional membranes show a decrease in electrical

1 In Vitro 6:128-142, 1970.

2 Cancer Res. 22:525-548, 1962.

3 Growth Regulating Substances for Animal Cells in Culture, Philadelphia, Wistar Inst. Press, 1967.

4 Dev. Biol. 19, Suppl. 2:151-183 and Perspect. Biol. Med. 11:260-272, 1968.

5 Cell Membranes, New York, H.P. Publishing Co., Inc., 1975.

resistance and an increase in permeability to the extent that ions and relatively large molecules can diffuse freely from cell to cell. This relatively high permeability of junctional membranes has also been described for epithelial cells in their normal tissue organizations (Loewenstein,¹ 1968a,b; 1975²).

It has been suggested that this "communication" among the cells of a tissue affords a mechanism for the coordination of cellular activities, and it presumably may be through this mechanism that a tissue can control proliferative activity (Loewenstein,¹ 1968a,b, 1975²). The concepts of contact inhibition and cell coupling offer a highly attractive hypothesis to explain the relation between changes in structural organization in the lens epithelium and the triggering of the cell cycle.

At the ultrastructural level, the gap junctions, which make up part of the junctional complexes between cells, have been implicated as the structures responsible for cell coupling. Considerable experimental evidence indicates that at the gap junctions, there is relatively free diffusion of ions and molecules up to about 200 M.W., from cell to cell. If the gap junctions are the structures responsible for cell communication, and cell communication is involved as a primary mechanism in the control of cellular proliferation, there should be changes in the ultrastructure of the gap junctions following injury, that might be observed in replicas of freeze-fractured preparations.

1 Dev. Biol. 19, Suppl. 2:151-183 and Perspect. Biol. Med. 11:260-272, 1968.

2 Cell Membranes, New York, H.P. Publishing Co., Inc., 1975.

The Kresge Eye Institute will soon have a new Balzers 301 freeze-etch unit with all accessories (a gift of the Kresge Foundation). This will be installed in our cell biology research laboratory, close to the E.M. facilities. Also, newer techniques of examining the ultrastructure of the lens using a combination of scanning and transmission electron microscopy have been established in our laboratory during the past year. A preliminary report of this study is presented in reports COO-2401-68 and COO-2401-66. It is felt that the results presented in COO-2401-68 and COO-2401-66 can serve as a basis for further experimental studies at the ultrastructural level on wound healing, cataract formation, development and aging.

Studies on Other Tissues. Injury-induced Growth of Vascular Endothelium; Neovascularization.

While we plan to continue our studies on the control of cellular proliferation in the ocular lens, we would like to expand the program to studies on other ocular tissues. Neovascularization following injury or certain diseases occurs in the cornea and the retina and can lead to blindness.

In many cases, the loss of sight, due to a chemically-induced opaque cornea, can be corrected by means of corneal transplantation. However, as Duke-Elder has pointed out "...a deep injury involving the entire thickness of the cornea, particularly when the tissue is replaced by fibrous tissue and is heavily vascularized, provides a more difficult problem. Unfortunately, many chemical injuries belong to this category, and most authorities have found that such corneae provide unfavorable material for full-thickness grafting owing to the tendency for the transplant in its turn to become vascularized and opaque." (Duke-Elder, 1972)¹.

Therefore, a blind eye due to a heavily vascularized opaque cornea resulting from chemical injury has a much reduced chance of successful corneal transplantation due to the increased tendency of the transplant to become vascularized and opaque. Very little is known about why blood vessels do not grow into a normal cornea, but do grow into corneae following certain kinds of chemical injury. The growth of new vessels (neovascularization) is dependent in part upon the production of new cells that make up the vascular system.

1 System of Ophthalmology, Vol. XIV Injuries, Part II. Non-Mechanical Injuries, St. Louis, The C.V. Mosby Co., 1972, p. 1035.

We know very little about the factors which control the production of the vascular endothelium. A better knowledge of the factors which control neovascularization in the chemically-injured cornea should serve as a basis for the development of new procedures to control vascularization, and increase the probability of restoration of sight through successful corneal transplantation. In spite of the important and extensive investigations on neovascularization little is known about the initial events that trigger the growth of new vessels. It is generally assumed that a proliferation of vascular endothelial cells in one of the earlier events in neovascularization (Futtermann,¹1975). For example, it has been observed that "In human retrolental fibroplasia, where its early stages have been studied, multiplication of endothelial cells in patent superficial retinal capillaries is the earliest visible evidence of new vessel formation." (Wise, Dollery, and Henkind,²1971a,b)Very little, or virtually no cellular proliferation occurs in the retinal vessels of the normal adult. In certain disease states, however, vascular endothelial proliferation and neovascularization can be a complication leading to blindness. These include diabetes mellitus, sickle cell disease, and other disorders.

Compared with other tissues, we know relatively little about the factors which initiate the cell cycle in vascular endothelium, and the ultrastructural and molecular changes which accompany it. For example, does blood serum contain a mitogenic agent for vascular

1 Invest. Ophthal. 14:4-6, 1975.

2 The Retinal Circulation, New York, Harper & Row, 1971.

endothelium? Since vascular endothelium unlike most tissues is directly exposed to blood serum it might be assumed that (1) serum does not contain mitogenic activity for vascular endothelium or (2) the endothelial receptors for the serum mitogenic factor exist on the basal surface of the cell which is not directly exposed to the serum. If the latter is true then serum might show a strong mitogenic action on the cultured vasculature, since under these conditions the basal surfaces would be exposed to a higher concentration of the serum factor. If serum mitogenic receptor sites do exist on the basal surfaces of the endothelial cells then any high molecular weight serum mitogenic factor would not normally promote proliferation of the endothelium unless the permeability of the capillaries or vessels greatly increased. From that point of view it is interesting to note that neovascular vessels are characterized by a pronounced increase in permeability to fluorescein. "By light microscopy normal retinal capillary and neovascular walls resemble one another. They differ physiologically in the profuse leakage of fluorescein from even the smallest neovascular formations. Thus, fluorescein angiography provides an easy means of identifying early new vessel sites." (Wise, Dollery and Henkind,¹ 1971a,b). Another example related to this is seen in the cornea after mustard gas injury. As the new vessels invade the cornea the tips of the extending loops are preceded by hemorrhages. (Duke-Elder, 1972;² Mann, et. al., 1948)³. Another technique for identifying these sites is by injecting hemoprotein enzymes of relatively low molecular weight and following

1 The Retinal Circulation, New York, Harper & Row, 1971, p.271,273.

2 System of Ophthalmology, Vol. XIV Injuries, Part II. Non-Mechanical Injuries, St. Louis, The C.V. Mosby Co., 1972.

3 Brit. J. Ophthal. Suppl. 13, 1948.

their passage through leaky vessels. Enzymes such as peroxidase and myoglobin have been used to establish the ultrastructural basis of capillary permeability in various tissues and to detect alterations in the permeability of the capillary endothelium under abnormal conditions. The "leakiness" of neovascular formations is also of interest from the point of view of contact inhibition (Abercrombie,¹ 1970; Abercrombie and Ambrose², 1962 and Eagle³, 1967) and inter-cellular communication (Loewenstein⁴, 1968).

Tissue Culture of Retinal Vessels: The techniques do exist for a study of the effects of serum and other possible mitogens on the isolated retinal vessels in culture. The entire "retinal" vasculature of the young rabbit can be isolated and established in tissue culture (Ashton,⁵ 1966; Tripathi, Knight, and Ashton,⁶ 1974). During approximately the first three weeks after birth the "retinal vasculature" develops along the surface of the retina. Since during this period of time it does not become firmly attached to the retina, it can be dissected out essentially in its entirety and established in tissue culture. Ashton and his co-workers have utilized this in vitro system for studies on the direct effects of oxygen on the retinal vascular endothelium. Tripathi et al, 1974⁶ were able to maintain these cultures for over 90 days. Growth in these cultures is very complex, involving several different kinds of cells, macrophages, mesenchyme-like cells derived from endothelium and pericytes but "mature endothelial cells could also be seen proliferating...without passing through a mesenchymal phase" (Ashton,⁵ 1966; Tripathi, Knight, and Ashton,⁶ 1974).

1 In Vitro 6:128-142, 1970

2 Cancer Res. 22:525-548, 1962

3 Growth Regulating Substances for Animal Cells in Culture, Philadelphia, Wistar Inst. Press, 1967.

4. Dev. Biol. 19, Suppl. 2:151-183 and Perspect. Biol. Med. 11:260-272, 1968.

5. Am. J. Ophthal. 62:412-435, 1966.

6. Exptl. Eye Res. 19:449-475, 1974.

Within the last several months we have developed a method of isolating and culturing rabbit retinal vessels and have been able to demonstrate DNA synthesis in these cultures. It would seem then that this procedure could serve as a model for the study of what causes vessels to proliferate and therefore what factors might be involved in neovascularization. The studies on the cultured vessels which have been initiated could be also carried out on excised "buttons" of cornea which have undergone some neovascularization. Using these two systems much might be learned about this process.

In addition, preliminary experiments have shown that plastic corrosion casts can duplicate the vasculature of experimental animal eyes (e.g. rat, rabbit, dog). The procedures required for this study have been used extensively here at Wayne for studies on splenic vasculature. (Barnhart and Baechler¹, 1974). We feel that these corrosion casts, as observed with scanning electron microscopy, can be used for studies of neovascularization of the cornea following injury. Preliminary experiments, carried out in collaboration with Dr. Charles Baechler and Dr. Marion Barnhart of the Department of Physiology at Wayne State University, School of Medicine, have yielded replicas of the ocular vasculature.

1 Scanning Electron Microscopy, Part III, IITRI, 705-712, 1974.

Other Studies on the Cornea

Since the outer surface of the cornea is the most exposed part of the eye, it is most susceptible to physical and chemical injury. S.E.M. experiments in our laboratory have shown a variety of micro-structures on the corneal surface. Comparative studies of these structures have been fruitful in furthering our understanding of the relationships of their role in the human. What the meaning of these microprojections is and what effect toxic agents and physical injuries might be on them is as yet unknown. It is known, however, that in the rabbit the application of a local anesthetic, proparacaine hydrochloride, causes these microprojections to be greatly altered (see next page). Also, there is a strong indication that the pattern of the epithelial microprojections may react sensitively to environmental factors.

Legend for photographs on following page:

The scanning electron micrograph at the top is of a normal rabbit cornea (epithelial surface). Portions of the two cells with their numerous microprojections can be seen. 10,000 X.

The second micrograph is of a rabbit cornea 10 mins. after dropping a solution of proparacaine hydrochloride on the corneal surface. The microprojections are greatly altered. 5,000 X.

The main research accomplishments, with special reference to the originally stated objectives.

Summary of Results Obtained

The following is a summary of results obtained during the course of this program. The details of these results plus additional experimental data are given in the reprints which are included in this report. The numbers in parentheses refer to publications from this laboratory which have resulted from this project (see publication list, pgs V-1 - V-5).

In the publication list on pages V-1 - V-5, the last 15 publications (39-53) resulted from the current contract. Previous publications are included for background and continuity. In the statement of accomplishments below, the reference numbers which are underlined refer to publications from the current contract.

1. Mechanical and chemical injury to the rabbit lens, in vivo, initiates the cell cycle and is accompanied by propagated waves of DNA synthesis and mitosis that move out slowly from the site of injury. Ultra-structural and cytochemical changes which occur during this propagated injury response and wound healing have been studied (1,2,25,41,43,47,48).

2. A determination of the in vitro culture conditions which bring about a reproducible stimulation of DNA synthesis and mitosis in the cultured mammalian lens has been accomplished. It has been found that the lens in culture provides an excellent model system for studies on the response of tissue to injury under carefully controlled and defined conditions (3,6,9,10,12,26,32,34,36,40,44,49).

3. A system has been established in this laboratory for the procurement and processing of fresh rabbit serum with optimal mitogenic and migratory activities. (13,25,26).

4. A chronological pattern of RNA, DNA, protein synthesis and mitosis and the effect of selected antimetabolites on these processes has been characterized in lenses cultured in serum-containing media (4,6,7,8,17,23,31).

5. The mitogenic serum factor(s) has been partially characterized and the effect of various treatments on the mitotic-stimulating activity of serum has been determined (11,12,13,14,26,27).

6. Rabbit serum was fractionated on DEAE-cellulose. Preliminary experiments indicate that fraction one, which is primarily γ - globulin, triggers a central mitotic stimulation. Moreover, fraction one brings about mitosis without the pronounced cellular migration that typifies the response realized in the presence of whole rabbit serum. Fraction one is more mitogenic on a mg protein/ml of culture medium basis than whole rabbit serum. The second fraction, i.e., beta globulin, gives a limited mitotic response. Cohn fraction IV, a mixture of alpha and beta globulin, also evoked limited mitotic response.

7. Modification of the time required for the serum-induced metabolic and ultrastructural changes in lenses which are preincubated in a non-stimulating medium has been determined. The results of these experiments have provided evidence that the pre-synthetic period can be divided into three sub-phases (22,23,25,40).

8. Cell division can be initiated in the ocular lens in a completely defined medium. Insulin added to a specific formulation of medium 199 or to medium KEI-4 brings about a central mitotic stimulation. Recent findings suggest that KEI-4 alone may stimulate cell division at a low pH (15,16,19,20,33,37,44,49).

9. Chemical injury by the injection of methylene blue into the anterior chamber of the rabbit eye causes extensive stimulation of DNA synthesis and mitosis in the lens epithelium. This and other changes in ocular tissues during wound healing following exposure to chemotoxic agents have been reported in a recent publication (47).

10. Wound healing in a teleost ocular lens has been described. Seasonal variations in mitotic activity have also been described in this species (50).

11. Changes in tissue organization and cell structure which precede and accompany DNA synthesis and mitosis in the cultured rabbit lens have been partially characterized at the ultrastructural level (18,21).

12. A number of fine structural changes which precede and accompany tissue repair in the rabbit lens in vivo have been characterized (27,28,29,30,38,48).

13.. Scanning electron microscope observations of the corneal surface, have revealed intricate patterns of microprojections on the epithelial cell surface. The structural patterns of these microprojections (as seen with S.E.M.) react sensitively to topically applied chemicals; and appear to vary with cellular maturation and senescence. These patterns in certain fish might be a sensitive indicator of environmental changes in the aquatic environment. (39,42,45).

14. Techniques and procedures have been worked out to visualize the lens epithelium, capsule, and fibers using the scanning electron microscope (30,35,51,52,53).

15. A technique has been worked out in which lenses which have been fixed, dehydrated, and dried by a critical-point drying method, can be fractured through the cytoplasm of the differentiating lens fibers, exposing the cell nuclei. The fracture, under these conditions, causes a complete separation of the two membranes of the nuclear envelope from one another, thus exposing entire membrane surfaces (those which line the perinuclear space). These surfaces are not seen in their entirety in typical freeze-fracture or freeze-etch preparations, and consequently have not been described previously. The exposed membrane surfaces which line the perinuclear space have numerous convex structures of approximately 1,000 Å, and some larger more irregularly shaped structures. These appear to be fragments of the nuclear pore complexes. Differences in these structures between young fibers and those nearing completion of differentiation is suggested. The application of this technique to studies on changes in the nuclear membranes during wound healing, involving lens fiber cell regeneration is suggested. (52).

16. The scanning electron microscope has been applied to a study of a human disorder, which affects the lens capsule and other tissues as well (The Capsular [Iridocapsular] Exfoliation Syndrome: A Review) (51).

17. Spherical structures in the inner part of the lens have been discovered with scanning electron microscopy and described. This region is among the oldest parts of the lens, derived from early stages of development. Some of these structures, embedded and thin sectioned for transmission electron microscopy showed that they were made up of varying numbers of multiple membranes. One hypothesis is that they are fiber cell fragments, derived from an injury sustained during early development, and, because of the nature of development in the lens, were preserved.

18. Some basic tissue preparative techniques for the ocular lens have been developed, which allow a study of the lens fiber cells at various locations in the lens, with a combination of scanning and transmission electron microscopy. The structure of the rabbit lens cells at different stages of maturation is presented as a basis for experimental studies on wound healing, cataract formation, development and aging. (53).

NEW WORK COMPLETED DURING THIS FISCAL YEAR

In relation to the new emphasis on our energy proposal, progress has been made in establishing techniques that will be used in studying potential toxicity of compounds of importance in the energy field. Specially modified culture dishes have been tested and proven feasible for the growth of larger numbers of cultures to be used in the testing of potentially toxic compounds.

A summary of the work accomplished during the current fiscal year follows.

1. New born rabbit retinal vasculature in tissue culture

Techniques for the growth of new-born rabbit vasculature in tissue culture have been further worked out. Techniques that involve the use of collagen and coated cover-slips in addition to the use of plasma to maintain the vascular spread out in culture have been established. Time-lapse photography of the isolated vessels is now in routine use. Also a preliminary study of the incorporation of tritiated thymidine by the cells in these isolated vessels at various times after being established in tissue culture is well under-way. Thus far the incorporation of thymidine in vessel cells has been detected following periods of a week in culture. The effects of growth factors such as Volkman's tumor growth factor will now be studied.

2. Studies on human corneal endothelium in primary graft rejects

Eye bank corneas which were used for transplantation and then subsequently underwent primary graft failure were used in a study of the scanning electron microscopy of their endothelial surface. These human primary graft failures showed a variety of cellular forms and cellular interrelationships on their endothelial surfaces. The main results of this study were reported at the A.R.V.O. Spring meeting, 1977 and are being written up for full length publication.

3. Corneal neovascularization following injury

Mrs. Hedwig Murphy, a graduate student working toward her Ph.D. in our department, has been concentrating her efforts primarily on the study of neovascularization in the rabbit cornea following injury. At the present time, with partial support from our grant, she has

been carrying out experiments on injury induced neovascularization in the rabbit cornea in the laboratories of Drs. Claes Dohlman and Richard Thoft at Harvard Medical School. This investigation was planned and is being carried out in conjunction with our proposed study of induced vessel growth as outlined in the original proposal. Significant progress has been made in the study of injury induced thymidine incorporation in blood vessels of the limbal area and correlated permeability changes in these vessels about to undergo neovascularization.

4. Freeze-etch set up

The Balzers 301 freeze-etch unit with all ancillary equipment has been set up and is now in routine use. This major equipment purchase was made possible through funds provided by The Kresge Foundation. Freeze etch E.M. studies of lens epithelium and lens fiber cells have been initiated. - Gap junctions have been identified in both epithelium and lens fiber cells. Further analysis of junctional complexes in the normal lens are necessary before a study of possible changes in the junctional complexes during development, wound healing, and cataractogenesis are possible. The availability of this equipment is now making it possible for other studies on other ocular tissues such as in Dr. Essner's studies on the retinal pigment epithelium and outer segments.

5. Scanning electron microscopy

A study of the structure of the normal rabbit lens emphasizing the use of scanning electron microscopy has been completed and

published (Ophthal. Res. 8, 1976). This is to serve as the basis for further studies on wound healing and cataract development. The unique properties of the rabbit lens as outlined in that publication, make it particularly suitable for certain kinds of studies involving S.E.M.

6. Spherical structures or globules (Morgagnian bodies), found in the lens under certain abnormal circumstances including human cataracts

'Spherical structures', found in the nuclear region of some rabbit lenses have been observed with scanning electron microscopy (S.E.M.) and transmission electron microscopy (T.E.M.). Their surface structure, as seen with S.E.M., is similar to that of fiber cells normally seen in this region of the lens. However, T.E.M. reveals that multiple unit membranes (2-10) make up their surface. Evidence for the possible mode of origin of this membrane redundancy is presented and discussed. Comparisons and contrasts are made with globular structures (possibly Morgagnian bodies) seen in human cataractous lenses. Differences between these 'spherical structures', seen in the rabbit, and 'globular structures' (possibly Morgagnian bodies) seen in human cataractous lenses are discussed.

7. Human cataractous lenses

S.E.M. is also proving very useful in the study of human cataracts. The formation of spherical bodies or globules located within certain areas of human cataractous lenses may be the basis for some kinds of opacities. Other structures localized to certain regions of human cataractous lenses assume a crystal-like form. These findings which have heretofore not been reported are being written up for publication.

8. N.E.I. Cooperative Cataract Research Group

Our laboratory is now set up to obtain human cataractous lenses, which can be photographed within minutes after surgery, and then immediately prepared for one of a number of different kinds of study (e.g., fixing for electron microscopy; rapid freezing for biochemical analysis). These lenses will then be sent to other laboratories in other parts of the country for study, or utilized in our own laboratory.

Our closest collaboration in this program is with Dr. Venkat Reddy at the Institute of Biological Sciences, Oakland University, and Dr. Leo Chylack of Harvard Medical School. Dr. Chylack has visited our laboratory, and checked over our equipment so that our photographic standards will be comparable with those of the other eighteen laboratories involved in the N.E.I., Cooperative Cataract Research Group (CCRG).

Our own contribution to the CCRG program, in addition to supplying lenses classified under standard conditions, will be the study of human cataracts utilizing transmission and scanning electron microscopy in addition to freeze-etch electron microscopy. Further analysis of the spherical structures (Morgagnian bodies) and the crystal-like structures and junctional complexes will be carried out.

Plans for the continuation of present objectives and possible new objectives in consideration of past results.

During the past three years we have established an active electron microscope facility, with a Philips-301 T.E.M., and Philips-500 S.E.M. Due to the generosity of the Kresge Foundation, we have received funds for a Balzers 301 freeze-etch unit with ancillary equipment, that we propose to use for studies on changes in the junctional complexes of cells during wound healing, or following exposure to mitogenic and migratory factors in organ culture. It is also planned to use the techniques of transmission electron microscopy in conjunction with cytochemistry in the study of these junctional complexes. The Kresge Foundation also provided funds for the purchase of a goniometer stage for the Philips - 301 T.E.M., which we propose to use in studies on cell membrane changes following injury.

This, therefore, represents a new objective, which is based on our findings during the past few years, and which is made possible by the new equipment obtained with the Kresge Foundation funds.

Other new objectives include the use of scanning electron microscopy and E.M. cytochemistry in studies on the reactions of corneal epithelial and endothelial surfaces to chemical and physical injuries; the stimulation of corneal neovascularization following chemical injury; and the factors which control the proliferation of cells of the ocular vasculature.

This project involves a study of wound healing in tissues of the eye, particularly lens, cornea, and surrounding tissues. The reactions of these tissues to mechanical injuries, as well as injuries induced by chemotoxic agents are under study. It is felt that a better understanding of the basic reactions of the eye to injurious agents may be of importance in the evaluation of potential environmental hazards.

In order for a tissue to repair itself following injury, new cells must be produced, and considerable cellular migration and re-arrangement occur. We need a better understanding of the mechanisms which control cellular proliferation in normal, injured, and repairing tissues. One important question, therefore, is what reactions trigger the cell cycle following injury, and turn off the cell cycle at a certain time during healing. Another is what factors control the migration and re-arrangement of cells during wound healing.

The reactions which trigger the cell cycle (promote the G-0 $\rightarrow$ G-1 transition) control the amount of cellular proliferation and tissue growth in normal and injured tissues. These reactions can be conveniently studied during the process of wound healing or exposure of the isolated whole rabbit lens to a serum mitogenic factor in organ culture. It has now been established in the lens, in tissue culture, that the triggering process can be effected by insulin in a completely defined medium. It is proposed to determine the components of the defined medium which are essential for the promotion of the G-0 $\rightarrow$ G-1 transition, and to determine if various phases and subphases of the cell cycle can be characterized by the requirement for specific components of the defined medium. It is also planned to continue studies on the correlation of changes in fine structure (e.g., the burst of ribosome production which characteristically follows exposure of the lens to rabbit serum) with changes in macromolecular synthesis (RNA, protein); to investigate, through the use of specific antimetabolites, the possible role of increased ribosomal and non-ribosomal RNA production in the stimulation of cellular proliferation during wound healing.

It is also planned to study the lens epithelial cell surface as well as the topography of the underlying lens fibers by means of the techniques of electron microscopy and scanning electron microscopy, before and after the addition of mitogenic agents (rabbit serum, insulin) to the culture medium, and at various times after mechanical and chemical injury in vivo. On the premise that cellular interrelationships (e.g., cell communication, or coupling) play an important role in the control of tissue growth, particular attention will be paid to changes in the junctional complexes, as seen in replicas of freeze-etched preparations and in thin sections for T.E.M. The techniques developed during the past year here in the Kresge Eye Institute for studying the internal structure of the ocular lens with scanning electron microscopy (S.E.M.) will also help to serve as a basis for this portion of the program. Also, studies which have been initiated on the ultrastructure of the corneal surface utilizing the S.E.M. and T.E.M., will be expanded to include an analysis of the changes which occur on the epithelial and endothelial cell surfaces after injury in the regenerating cornea. In addition it is proposed to study the stimulation of growth of new vessels (neovascularization) which can then invade the cornea following chemical injury, and reduce the possibilities of subsequent successful corneal transplantation. Within the last several months we have developed a method of isolating and culturing rabbit retinal vessels and have been able to demonstrate DNA synthesis in these cultures. It would seem then that this procedure could serve as a model for the study of what causes vessels to proliferate and therefore what factors might be involved in neovascularization, in

much the same way that the cultured lens has been used to study those factors that regulate cellular proliferation in the lens epithelium. It is also planned to study the growth of new vessels into the cornea with the use of plastic corrosion casts, which can then be studied in detail with scanning electron microscopy.

It is expected that the proposed program will lead to a better understanding of some of the initial structural and biochemical changes which are essential to the mechanisms which control cellular proliferation, migration and re-arrangement following injury. The new knowledge gained from this study may be of use in helping to prevent or ameliorate the effects of various chemical and physical injurious agents on the eye.

Graduate students trained, degrees granted, and post-doctoral tenures completed.

The present program is playing an important role in the training, and preparation for training, of graduate students and post-doctoral fellows. With the fine cooperation of the Departments of Basic Medical Sciences the faculty of the Kresge Eye Institute can have graduate students, doing thesis research in the Kresge Eye Institute. It is planned to increase the emphasis on graduate training, as well as post-doctoral training in the Kresge Eye Institute. We believe that the central location of the Kresge Eye Institute within the new Detroit Medical Center should facilitate the interchange of ideas among students and faculty of the basic and clinical departments. The new Kresge Eye Institute is adjacent to the newly-constructed section of Harper Hospital; Grace Hospital (under new construction); Detroit General Hospital (under new construction); Children's Hospital (recently re-constructed), and Hutzel Hospital. At the current time, one student, Mrs. Hedwig Murphy, working for her Ph.D., is doing research related to the E.R.D.A. contract. During the tenure of the current contract and the two which preceded it, a number of pre- and post-doctoral trainees have worked in our laboratory on research related to the E.R.D.A. contract. The following is a list of some of these people and their current positions:

(Dr. Bagchi, the most recent post-doctoral fellow, was a Special N.I.H. fellow working in the Kresge Eye Institute).

FORMER TRAINEES

Dr. Carl Feldherr

Dr. Howard Rothstein

CURRENT POSITIONS

Prof. of Anat. and Path.,
Univ. of Florida, Gainesville

Prof. of Zoology, Univ. of Vermont

Dr. Dobli Srinivasan

Faculty Member, Ophthalmology
Columbia University

Dr. Laszlo Bito

Research Prof., Ophthalmology
Columbia University

Dr. Maynard Wheeler

Faculty Member, Ophthalmology
Univ. of Connecticut

Dr. Jane Kaltenbach Townsend
(when on sabattical)

Prof. of Biological Sciences
Mount Holyoke College

Dr. John Reddan

Prof. of Biological Sciences
Oakland University

Dr. Allan Weinsieder

Assist. Prof. Anatomy
Wayne State Univ.

Dr. Mihir Bagchi

Assist. Prof. Anatomy
Wayne State Univ.

Mrs. Hedwig Murphy

Current Graduate Student

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Your opinion as to the present state of knowledge in this area of research, its significance in the fields of biology and medicine and needed future investigations.

The control of cellular proliferation is a major bio-medical problem. It is basic to an understanding of normal tissue growth, as well as the process of wound healing. The key reaction(s) which triggers the $G-0 \rightarrow G-1$ transition, therefore, plays a dominant role in the control of tissue growth.

The usefulness of the cultured lens as a model system for studies on certain aspects of wound healing continues to expand, and this model system is now being used in several laboratories. It is proving particularly useful for studies on the mechanisms which control cellular proliferation in normal, injured, and repairing tissues, as well as the factors which control the migration and re-arrangement of cells during wound healing.

Blood serum contains both potent mitogenic and migratory factors. Most of the work on the ocular lens in culture has followed two lines of endeavor: 1) the nature of the mitogenic and migratory factors, and 2) the sequence of events [changes in ultrastructure, macromolecular synthesis (RNA, protein, DNA); mitosis, migration] which characterize the epithelial tissue between exposure of the lens to the serum factors and the initiation of cellular proliferation.

1) In terms of mitogenic agents, it has now been established in the lens in organ culture, that the reactions which trigger the cell cycle (promote the $G-0 \rightarrow G-1$ transition), and therefore control the amount of cellular proliferation, can be initiated by insulin in a completely defined medium.

This makes it possible to determine the specific components of the defined medium which are required at different times (i.e., for the 3 subphases) between explantation of the lens, and the initiation of DNA synthesis (see below).

2) The sequence of events

a. Subphases

Further work with the serum mitogenic factor has now shown definitely that at least three subphases exist between explantation and the initiation of DNA synthesis. These subphases are defined on the basis of their requirement for the serum factor. The first subphase is independent of the presence of serum, the second subphase is serum-dependent, and the third subphase is serum-independent.

These findings indicate that here-to-fore unknown subphases exist in cells between the time of 'injury' (explantation), and the initiation of DNA synthesis; and that these subphases may be characterized by their specific chemical requirements. The theoretical implications of these findings have been discussed (40).

Further investigation of these three (or more?) subphases which are prerequisite for the activation of DNA synthesis are needed.

b. Sequence of changes in ultrastructure and macromolecular synthesis.

Changes in ultrastructure, particularly changes at the cell surface and changes in cell-to-cell relationship, occur very early after explantation. These are followed by increased production of ribosomes, RNA, and protein, and subsequently the activation of DNA synthesis.

The fact that changes in the cell surface and in cell-to-cell relationships occur first in this sequence of events suggests that they may play a primary role in the G-0 $\rightarrow$ G-1 transition. This is of particular interest in view of the increasing evidence in some tissues that the cell membrane, cell coupling (cell communication), may play a role in regulating certain activities of cells, including cellular proliferation. The gap junctions appear to be the sites of free diffusion of ions and molecules up to about 200 M.W., and therefore appear to be the structures responsible for cell coupling. It is proposed, therefore, to study any changes which may occur in junctional complexes at different times after exposure of the lens in culture to mitogenic agents, or after injury in the intact animal.

For this purpose, investigations of replicas of freeze-etch specimens, to be examined with T.E.M., are needed. Also thin sections of specimens, in which tracer molecules such as peroxidase or myoglobin can be localized cytochemically at the E.M. level, are needed to further investigate changes in the junctional complexes.

Other areas needing investigation:

Also some of the procedures which have been developed for the preparation of tissues for electron microscopy (fracture procedures for S.E.M., 52, 53; and replica procedures for T.E.M.) should now be applied to studies of wound healing in the lens. A needed future investigation, for example, is a study of the healing process in the nucleated, differentiating lens fiber cells, as compared to the mature non-nucleated cells. This could serve as the basis for a better understanding of abnormalities of the lens due to injury or metabolic disorders.

The same procedures should be applied to further studies of the corneal surfaces (both epithelial and endothelial). The pattern of surface microprojections can be greatly altered after exposure to certain chemicals (e.g., page III-18 in proposal). The feasibility of examining such changes as a potentially sensitive indicator of chemical injury in mammals and fish should be investigated.

An examination of the endothelial surface of human corneas, which were rejected following transplantation, has indicated the need for further investigation of this surface, as it relates to the causes of graft rejection (page VI-18 in proposal).

Another area which needs investigation is the biological mechanism which controls vascular endothelial cell proliferation, a primary event in neovascularization. We feel that an investigation as suggested in answer to question 2 (p.8) and in the proposal is needed. A better understanding of the physical and chemical reactions underlying this primary event could serve as the basis for developing agents which can control the proliferation of new vessels, following injury or certain disease states, a condition which can lead to blindness.

The present division of federal support for your overall research program.

A grant request to the National Eye Institute, N.I.H., has recently been approved and should be activated in February. This grant is for a separate but related project. We also receive support from the Kresge Foundation and Research to Prevent Blindness, Inc.

PUBLICATIONS AND MANUSCRIPTS IN PREPARATION
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