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Cellular Proliferation and Regeneration Following Tissue Damage

Oakland University, Rochester, Michigan

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1. CELLULAR PROLIFERATION AND REGENERATION
FOLLOWING TISSUE DAMAGE

2. OAKLAND UNIVERSITY
DEPARTMENT OF BIOLOGICAL SCIENCES
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Introduction

The present report is based on findings obtained during the past seven years on A.E.C. Contract AT (11-1) 1427 at Oakland University. 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 lens. 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 such as radiation. With specific regard to the lens, it should increase our understanding of the mechanisms of cataract formation.

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 Harding, 1965. see reprint, p. 37). 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 consequent

tissue growth related to cell division, lies in an understanding of the reactions which trigger the cell cycle $G-0 \rightarrow G-1$.

The reactions which trigger the cell cycle 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.

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 A.E.C. contracts at Oakland) listed in the bibliography on pages 27, 28 and 29. Photocopies of the majority of the publications (reprints or pre-prints) are also included.

TRIGGER
MECHANISM
(G-0¹ G-1)

RESPONSE OF CELLS
ENTERING CELL CYCLE

- (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

Reaction to Injury

The ocular lens has proven uniquely suitable for studies on the initiation of cell division (see attached reprints and preprints including a review published in the International Review of Cytology, see pages 114-200.) 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. Small mechanical wounds to the anterior surface of the lens in vivo results in multiple waves of stimulation of DNA synthesis and mitosis that move out slowly 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., reprint, p. 30).

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 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.

It has been shown that 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. One of the first objectives of the present investigation was the establishment of a procedure which would yield reproducible and quantitative measurements of changes in DNA synthesis (thymidine incorporation) and mitosis. The culture technique of Merriam and Kinsey (1950) has been used. It has been found that 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 review pgs. 114-200, and Wilson, Harding and Wilson, 1967, reprint pgs. 59-67.)

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., 1968, reprint attached pgs. 68-75). 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 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.

(a) 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 have not, therefore, been triggered, can be subsequently triggered by the addition of 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 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 hrs. of culture). This assumes that the pre-synthetic phase (the period between explantation 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 Review pgs. 114-200, and reprint p. 225, 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.

It is of interest that the 20 hr. period of dependency on the presence of the serum factor(s) corresponds to 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 ug/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 Reddan, Crotty and Harding, 1970, reprint attached pgs. 78-91).

(b) Necessity of RNA Synthesis for Subsequent DNA Synthesis and Mitosis

There is a significant increase in RNA synthesis (as evidenced by the DNAase 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 pgs. 78-91) 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, preincubation for 24 hrs. 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 p. 225 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 (p. 171).

(c) Partial Characterization of the Serum Mitogenic Factor(s)

The factors in serum responsible for the stimulation of mitosis and cellular disorganization have been partially characterized. Some of the results obtained thus far can be summarized as follows (see pgs. 68-75 for details):

1. Effect of Age: The potency of the mitotic stimulating factor in rabbit serum decreases with the age of the serum. Lenses cultured in (M-199-S) containing serum aged 5 weeks, have very few mitotic figures compared to lenses cultured in M-199-S containing control serum. Although the serum aged for 5 weeks or more has lost its mitotic stimulating activity, it has not lost its ability to induce cellular disorganization.
2. Effect of Freezing and Thawing: Serum which has been frozen and thawed repeatedly or maintained in the frozen state for many months retains its mitotic stimulating activity.
3. Effects of Heating: Heating serum to 60° for 60 min. or 70° for 90 min., has little or no effect on its mitotic stimulating activity, and does not result in a decrease in the ability of the serum to cause disorganization. Heating serum to 85°C for 4 min., cooling and heating again to 85°C for 4 min., decreased the mitotic stimulating activity significantly.
4. Effect of Centrifugation: Centrifugation for 7 to 17 hours at 127,000 g results in a significant centrifugal displacement of mitotic stimulating factor.

5. Effect of Fractionation by Sephadex Gel Filtration: Serum was fractionated by gel filtration through Sephadex G-25 into two fractions, one fraction containing all serum components with a molecular weight exceeding 5,000, the other containing the remaining components. No central mitotic stimulating activity was observed with either the high or low molecular weight components. However, when these two fractions were recombined the resultant "reconstituted" serum contained high levels of mitotic stimulating activity. The so-called mitotic stimulating factor, therefore, apparently consists of at least two components, one or more with molecular weight less than 5,000 (H fraction) and one or more with molecular weight less than 5,000 (L fraction).
6. Effect of Fractionation by DEAE Cellulose Column Chromatography: Rabbit serum was dialyzed and subsequently fractionated on a DEAE cellulose column according to the techniques of Sober and Peterson (1958). The first fraction was predominantly gamma-globulin, the second beta globulin, the third albumin and the fourth alpha globulin.

Serum proteins were also precipitated with 40% ammonium sulfate. Electrophoretic analysis of the precipitate showed a preponderance of gamma globulin. Each fraction was dialyzed, lyophilized and total protein was determined by Lowry's technique. Rabbit lenses were cultured in media which contained the serum fractions dissolved in M-199 (Morgan, Morton and Parker, 1951). The concentration of

each fraction was adjusted to correspond to the concentration of the fraction in 23% serum. Lenses cultured in 23% whole rabbit serum in M-199, and in M-199 alone were used as controls. The lenses were cultured for various durations and were fixed in Carnoy's solution. Epithelial whole mounts were prepared and mitotic, migratory activity was noted. Each fraction contained less migratory activity than whole serum. However, dialyzed lyophilized serum showed the same extent of migratory and mitotic activity that was obtained with 23% whole rabbit serum. It would appear that the migratory activity was reduced in the process of fractionation or that two or more fractions are necessary for this reaction. The first fraction and the fraction which precipitated in 40% ammonium sulfate showed extensive mitosis. The mitotic figures were found throughout the central epithelium. The distribution of mitosis is similar, therefore to that noted in whole serum. Moreover, preliminary results suggest that fraction one is more mitotic on a mg/ml basis than whole rabbit serum. Furthermore fraction one yielded a single peak in the Model E analytical ultracentrifuge. The second fraction occasionally showed mitogenic activity. The third fraction which is predominately albumin appeared toxic, as indicated by cell death and pycnotic nuclei. Finally the fourth fraction, which is α -globulin, did not show a central mitotic stimulation. The time required for the serum fractions to initiate mitosis is about 24 hours greater than the time required for the media containing whole rabbit serum. However, when the first fraction was sup-

plemented with rabbit serum dialysate, it was possible to reduce the time required for mitosis to the comparable time required by whole rabbit serum, (Harding et al., 1968). A further purification of the mitogenic and migratory factor(s) and an analysis of their mode of action is in progress.

(d) Replacement of the Mitogenic Serum Factor(s) by Insulin:

Stimulation of Mitosis in a Completely Defined Medium

One of the long-range goals of the present investigation is to bring about a reproducible stimulation of cell division in the isolated lens maintained in organ culture in a completely defined media. We have attempted to define the culture conditions that are conducive to the triggering of mitosis in mitosis in the cultured lens. As pointed out above previous work in this laboratory indicated that both the high molecular weight and low molecular weight components of rabbit serum are essential for the mitotic response (Harding et al., 1968, reprint attached, p. 68-75.) Present experiments show that insulin can replace the high molecular weight serum components that are normally required for the mitotic response. Furthermore it has been shown that even when it constitutes part of a completely defined medium insulin can induce heavy mitotic activity in both rabbit and frog lenses.

The experiments were carried out using medium 199 of different formulations (i.e., M-199-S and M-199-B) (see Table VI pgs. 218-219 for formulation). It was found that variations in the composition of the culture medium had a profound effect on the mitogenic action of insulin. Lenses cultured in M-199-A alone exhibited a low level of mitotic activity which was confined to the

peripheral region of the lens. The non-stimulating M-199-A could support a central mitotic activation if both insulin and 23-46% serum dialysate were added to the medium. The chronology, magnitude, and pattern of DNA synthesis and mitosis has been quantitated in rabbit lenses cultured in M-199-A containing 23% rabbit serum dialysate and insulin. The results indicate that the temporal aspects of DNA synthesis and mitosis engendered by insulin are comparable to those obtained in lenses exposed to whole rabbit serum (Reddan, Crotty, and Harding, 1970a).

In additional experiments lenses were cultured in M-199-B and the mitotic response at 52 hours of culture was quantitated. Rabbit lenses cultured in M-199-B alone exhibited a substantial number of mitotic figures which were confined to the peripheral area of the whole-mount. The addition of 80 mU/ml of insulin to M-199-B alone, i.e., without the dialysate supplement, resulted in a central mitotic stimulation. Insulin can therefore initiate a central mitotic response in a completely defined medium.

The previous experiments were carried out using either Iletin or Protamine zinc insulin. Further studies were carried out on rabbit lenses cultured in M-199-B containing crystalline beef insulin. A central mitotic stimulation was obtained when crystalline insulin was present at concentrations of 80, 40, 20, 10, 1 and 0.1 mU/ml. Lenses cultured in M-199-B containing glucagon in concentrations equivalent to the level present in crystalline insulin (0.1% by weight) did not show a mitotic stimulation. In addition, neither the A and/or B chains of beef insulin evoked a mitotic response. The mitotic response, therefore, appears to be dependent on the integrity of the insulin molecule. A complete description of the aforementioned

experiments on insulin is given in the manuscript on p. 241. In subsequent preliminary studies, lenses were cultured in a slight variation of a synthetic media that had been formulated by Wachtl and Kinsey (1958) for use with cultured rabbit lenses. These authors reported that the mitotic index at 9, 18 and 24 hours of culture was comparable to that of control contralateral lenses which had been fixed immediately upon isolation. The media was modified (Kinsey and Reddy, 1965) and lenses could be cultured in KEI-4 medium for 24 hours without an appreciable change in the free amino acid level within the lens. In view of the wide use of this media for biochemical and physiological studies and in view of the fact that the media is completely defined we investigated the effect of insulin on cell division in KEI-4. Lenses were cultured in KEI-4 containing 80 mu/ml of insulin. Lenses were fixed at 9, 24 and 52 hours of culture and the number and distribution of mitotic figures was noted. The results clearly indicate that insulin in KEI-4 evokes a heavy mitotic response at 52 hours. The mitotic response is equal to or greater than that triggered by 23% whole rabbit serum. Control lenses, i.e., lenses cultured under identical conditions for 52 hours in KEI-4 without insulin, did not show a mitotic stimulation comparable to that noted in KEI-4 plus insulin. The mitotic response noted in medium KEI-4 alone may depend upon the hydrogen ion concentration of the medium. Recent preliminary data show that lenses cultured in KEI-4 alone at a pH of 7.0-7.1 may be triggered to undergo a central mitotic stimulation. Although this data is preliminary, its conformation and extension, would be conducive to probing the mechanism by which a change in a specific culture media could effect genetic expression. It would seem that the use of a medium of known chemical composition i.e.,

insulin in conjunction with either 199-B or KEI-4, should greatly enhance the possibility of probing in detail the control mechanism(s) of mitosis in the ocular lens.

(e) 1. Ultrastructural Changes Which Precede the Initiation
of DNA Synthesis and Mitosis in the Cultured Lens.

Additional studies were carried out in order to characterize the fine structural changes which precede and accompany the serum induced mitotic activation. Lenses which were isolated and cultured in a serum-containing medium exhibited several modifications in fine structure, as compared with lenses which were fixed immediately after isolation. Lenses fixed immediately after isolation showed the typical orientation of the lens capsule, epithelium and underlying lens fibers. These lenses showed numerous intercellular spaces, elaborate infoldings of the cell membranes, and some pinocytotic vesicles. These fine structural features are confined to the basal portion of the epithelial cells immediately subjacent to the lens capsule. Lenses fixed at 1 hour of culture were completely devoid of the intercellular spaces which typified the 0-hour preparations. However, with this notable exception, the fine structure of the lens cultured for one hour was ostensibly similar to the 0-hour preparation. Other changes in the basal portion of the epithelial cells were noted at three hours of culture and consisted of marked variations in the degree of infolding of the cell membranes. These interdigitations were much more pronounced in the peripheral region of the lens. At 7 hours of culture there was a pronounced increase in the number of ribosomes. This cytological change occurs at the time at which a significant increment in the incorporation of ^3H -uridine and ^3H -labelled protein hydrolysate has been detected (Reddan et al., 1970b , reprint attached).

The intercellular spaces which were noted at 0 hours were not present in lenses fixed at one, three or seven hours of culture. However, at 15 hours of culture, intercellular spaces similar in location and appearance to those detected at 0 hours were present. The spaces in lenses cultured 15 hours exhibit "periodic expansions" and, in this respect, are similar to those described by Cohen, (1965), in the human lens. In addition to the presence of the intercellular spaces, cells in the peripheral region of the 15 hour lenses were noticeably elongated and there was a dramatic change in the overall organization of the epithelium, including multi-layering of the cells in the peripheral regions. The cells in the central portion of the lens at this time exhibited the typical monolayered pattern which was similar to that noted at 0 hours.

Several of the changes in tissue organization were first noted in the peripheral region of the epithelium and, subsequently, throughout the central region. For example, the pronounced changes in surface membranes, as well as the marked cellular elongation and multilayering, were initially confined to the sections obtained from the periphery of the lens, but were later observed throughout the central region. It should be noted that the stimulation of DNA synthesis and mitosis is also first observed in the peripheral region of the epithelium and, subsequently, throughout the central region. The disappearance of the intercellular spaces (one hour), appearance of extensive invaginations of the surface membranes (three hours), increase in the number of free ribosomes (seven hours), the possible reappearance of the intercellular spaces and changes in overall organization (15 hours), all precede the triggering of DNA synthesis and mitosis which

commences at about 22 and 35 hours, respectively (Harding et al., 1968; Reddan et al., 1970). A detailed description of the fine structural changes which occur in the cultured rabbit lens is given in the manuscript on p. 92-111.

2. Fine Structure of Tissue Repair in Lens In Vivo

a. Scanning Microscopy of Normal and Injured Tissue:

As a result of injury the central lens epithelial cells, can be stimulated to undergo DNA synthesis and mitosis (see review p. 114). Studies using transmission and scanning electron microscopy were undertaken in order to characterize the change in fine structure that might herald the mitotic response and to characterize the process of tissue repair.

Scanning electron microscope studies were carried out at the Marine Biology Laboratory, Woods Hole, Mass. at the Howe Laboratories of Ophthalmology. The tissues used were from the toadfish (*Opsanus Tau*) the dogfish, and the rabbit. The main objective of the scanning electron microscopic studies was to visualize the apical surface of the lens epithelium, the lens capsule, and to follow changes in the cell surface, by this procedure, at various times after injury. In order to accomplish this the epithelium was carefully separated from the underlying lens fibers. After extensive trial runs, a procedure was established which involved fixing in neutral formalin, followed by osmium fixation, dehydration in alcohol, and air drying. With the above procedures it proved possible to visualize the apical cell surface as well as the nuclear surface in fragmented cells. Hexagonal patterns were found on the inner surface of the epithelium. The

hexagons correspond roughly in size to the epithelial cells. Structures were observed on the external surface of the capsule, some of which might correspond to the attachment sites of the zonule fibers. A number of these observations, which have not previously been made, require further interpretation. Preliminary (observations) on the dogfish lens epithelium show a striking orientation of the cells toward the site of injury within 24 hours after wounding. These studies, which open up some new and promising approaches will be extended to the normal and injured rabbit lens. For a more detailed description of the scanning microscopic studies see attached preliminary manuscript (p. 280).

3. Fine Structure of Tissue Repair in Lens In Vivo.

a. Transmission Electron Microscopy of Normal and Injured Tissue:

The fine structural changes which occur during injury repair in the rabbit lens has been characterized using the transmission electron microscope. Lenses were injured and prepared for the electron microscope according to previously described techniques, (Reddan et al., 1970). Concomitant studies were carried out at the light microscope level employing specific histochemical techniques. Capsular repair was also monitored by following the fate of labelled sulfate. Some of the results are summarized below (additional details will be found in a preprints (pgs. 247 and 278).

A definite change in tissue organization was evident 1 hour after injury. The capsule separated from the underlying epithelium, ruptured cells were found at the site of the wound and lens fibers were extruded through the

injury. Extensive changes in cell-to-cell relationships preceded the initiation of DNA synthesis which commenced at 12-14 hours. At 18 hours epithelial cells in the focus of the wound were elongated. Ribosomes and fine filaments were prominent characteristics of the elongated cells. The ribosomal increment noted in the injured tissue is similar to that obtained in lenses which were prompted to divide by exposure to rabbit serum (Reddan et al., 1970). The period of cellular elongation and changes in the ribosomal pattern was followed by the migration of various cell types into the wound. Non-epithelial cells were first observed in the wound at 12 hours. These cells were embedded in a fibrous matrix which covered the injury area. Many of the fibers in the "wound coagulum" exhibited a periodicity characteristic of fibrin. Cells that could be identified in the bionecrotic area of the wound included neutrophils, monocytes, and macrophages. Many of the cells contained lysosomes and gave a positive histochemical reaction for acid phosphatase (see preprint p. 278).

AT 24 and 48 hours the wound was filled in with aforementioned cell types all of which were embedded in an extensive fibrillar matrix. The epithelial cells were elongated and multilayered. The elongated cells exhibited prominent ribosomes, lipid droplets, areas of focal degeneration, and lysosomes. At 48 hours the epithelial cells showed, numerous mitochondria and extensive interdigitations between cells was also quite common. The fibrillar elements in the wound were extensive. In addition to epithelial cells, cells resembling immature fibroblasts, granular and agranular leukocytes, monocytes and macrophages, characterized the wound proper. Many of the cells appeared to be intermediate in morphology between the epithelial cells and the immature fibroblasts. At 72 hours many fibroblast-like cells were found lying above the lens capsule. These cells exhibited numerous cell processes, had a pronounced endoplasmic reticulum and possessed a well

developed Golgi complex. Tissue sections viewed at 7 and 14 days showed an increase in the number of cells lying above the capsule. However, cellular organelles were not pronounced in these cells.

Studies on the process of tissue repair were also carried out using histochemical techniques at the light microscope level. The studies of Dische and others indicate the presence of sulfated mucopolysaccharides in the lens capsule. Alcian blue at a pH of 0.5 is specific for sulfated mucopolysaccharides. Twenty-eight days after injury, material which stained intensely with Alcian blue appeared to join the edges of the broken capsule. One would predict that the areas of intensive Alcian blue staining would also incorporate radioactive sulfate. The data show an extensive incorporation in the region between the edges of the broken capsule. This data is taken to be indicative of capsular repair. At 42 days a discontinuity in the capsule was no longer evident in many lenses. In these cases the broken edges of the capsule were joined by material that stained intensely with P.A.S. and Alcian blue. Although the capsule was continuous, in many cases, cells were still found external to the capsule. These cells exhibited extensive cytoplasmic processes and at the fine structural level showed a paucity of cellular organelles. There is excellent overall agreement between the light and electron microscopic observations.

Additional fine structural studies i.e., at the transmission and scanning microscope level on the injured ocular lens under both in vivo and in vitro conditions will hopefully permit us to determine the fine structural changes which are essential pre-requisites to the mitosis and wound healing.

Summary of Results Obtained During the Last Seven Year Period

The following is a summary of some of the results obtained during the past seven year period. The details of these results plus additional experimental data are given in the preprints and reprints which are included in this report (pgs. 30). The numbers in parentheses refer to publications from this laboratory which have resulted from this project. (see Ref. pgs. 28, 29, 30).

1. Mechanical injury to the 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. The injury response, and the effect of x-irradiation on the propagated injury response and wound healing has been determined (1, 2, 9, 13, 26).

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, 10, 12, 26).

3. A system has been established for the procurement and processing of fresh rabbit serum in this laboratory.

4. The 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 a serum containing media (4, 6, 8, 18, 23, 26).

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 (12, 14, 26, 27).

6. Rabbit serum was fractionated on DEAE-cellulose. Preliminary experiments indicate that fraction one, which is primarily 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/ml basis than whole rabbit serum. The second factor i.e. beta globulin, gives a limited mitotic response. Cohn fraction IV-4, a mixture of alpha and beta globulin, also evoked a limited mitotic response (28).

7. Modification of the time required for the serum-induced metabolic and ultrastructural changes in lenses which are incubated in a non-stimulating medium has been determined (23, 24, 27).

8. Changes in tissue organization and cell structure which precede and accompany DNA synthesis and mitosis in the cultured rabbit lens have been characterized at the ultrastructural level (11, 15, 19, 22,).

9. A number of fine structural changes which precede and accompany tissue repqir in the rabbit lens in vivo have been characterized (26, 28, 29, 30, 26).

10. Techniques and procedures have been worked out to visualize the lens epithelium, capsule, and fibers using the scanning electron microscope (31).

11. 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. (16, 17, 20, 21, 26).

Publications and manuscripts in preparation
which are associated with the current contract

removed

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