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MATERIAL AND METHODS

In Vivo Studies

Subjects (studies I, II, and III)

Experiments were carried out on fourteen primates (Cynomolgus) with a weight range of 2.0-3.5 kg (studies I and II). Eighteen eyes received injections of a colloidal suspension of carbon microparticles into the vitreous (Batch C11/1431 a, Günther Wagner, Pelikan Werke, Hannover, Germany). The suspension contained approximately one million particles per μl in a 1% phenol solution. The particle size was 20-70 nm. The particle suspension was sterilized in an autoclave before use. Under general anesthesia (50 mg ketamine i.m.) and microscopic control, 100 μl of the suspension was injected with a 0.4 mm caliber needle one mm posterior to the limbus through the ciliary body into the center of the vitreous. Eyes were enucleated ten weeks later. To serve as controls five eyes were injected with 100 μl of 1% solution of phenol; another two eyes received 100 μl of BSS.

One primate (Cynomolgus) received repeated intravenous injections (0.6 ml/kg) of the colloidal suspension of carbon microparticles during a seven day period (study III). Three days later we applied seven laser burns in the posterior pole of the retina of each eye. An argon blue-green laser with principal outputs at 514 and 488 nm was employed via a slit lamp (Haag-Streit) and a Goldmann fundus contact lens. Burns were of sufficiently high intensity to cause focal rupture of Bruch's membrane without inducing severe choroidal hemorrhage. The spot diameter was 100 μm, duration 0.1 s, and power between 600 and 650 mW. The eyes were enucleated eight days later and all laser lesions were sectioned serially. Sections were alternatively thin (1 μm) and thick (10 μm). The specimens were then processed for light microscopy (LM) and transmission electron microscopy (TEM). Control specimens were taken from noncoagulated areas of the retina and the choroid distant from the laser lesions. A quantitative estimation of the various leukocytes in the systemic circulation (retinal and choroidal vessels) and at the site of laser lesions was performed. The cells were classified as mononuclear leukocytes, polymorphonuclear leukocytes, or carbon-laden mononuclear leukocytes, based on morphologic criteria.
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Light Microscopy (Studies I, II, and III)

Typical mammalian cells are 5-25 μm in diameter, colorless, translucent, and therefore not visible to the naked eye. Light microscopy (LM) cannot resolve details smaller than 0.3 μm, because radiation of a given wavelength cannot be used to probe structural details smaller than its own wavelength; details smaller are obscured by optical diffraction. The ultimate limit of resolution of LM is therefore set by the wavelength of visible light i.e. 0.4-0.7 μm. This resolution is sufficient for the study of many intracellular and extracellular structures including mitochondria, pigment granules, and collagen fibers or bundles. In the present works (studies I, II, and III), we have used traditional fixating, staining, and embedding methods. Enucleated eyes were fixed in 4% formaldehyde for a minimum of 24 hours. Specimens were dehydrated in graded concentrations of ethanol and embedded in paraffin. Sections were cut in an LKB microtome and stained with PAS, hematoxylin-eosin, and van Gieson dye.

Transmission Electron Microscopy (Studies I, II, and III)

The limit of resolution imposed by the wavelength of visible light can be reduced by using electrons instead of light, since electrons have a much shorter wavelength. With a high accelerating voltage, transmission electron microscopy (TEM) can give a practical resolving power about 100 times better than the resolution of light microscopy (LM). In order to minimize tissue distortion secondary to dehydration, we fixed our wet tissues (studies I, II and III) in a cold solution of 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 18 hours. A small incision was made through the ciliary body of the enucleated eye to facilitate the diffusion of fixative into the vitreous. Specimens were cut from the retina, vitreous, vitreal membranes, and ciliary body, and were immersed overnight at 4°C in cacodylate buffer. The specimens were postfixed in a 1% solution of OsO4, dehydrated in graded concentrations of ethanol, treated with propylenoxide, and then embedded in Agar 100. Sections (1-10 μm thick) were obtained with an ultramicrotome, and then stained with toluidine blue and surveyed by LM. Thin sections were obtained using a diamond knife in the ultramicrotome, stained with lead citrate and uranyl-acetate, and then examined by TEM.
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**In Vitro Studies**

*Retinal Pigment Epithelial Cell Cultures (Studies IV, V, VI, and VII)*

Retinal pigment epithelial (RPE) cells were isolated from human postmortem eyes obtained from the Lions Doheny eye bank (Table 3). Donor-age ranged from 57 to 78 years.

**Table 3. Inclusion and exclusion criteria of donor eyes.**

| INCLUSION                                      | EXCLUSION                                      |
|------------------------------------------------|------------------------------------------------|
| Delivery of enucleated eyes within 15 hrs postmortem time. | Donor history of eye disease (cataract and IOL-implant was accepted). |
| Eyes undamaged (removal of cornea for transplantation purposes was accepted) | History of cytotoxic agents, steroids, or other medications that could affect cellular metabolism. |
| Donor tested for HIV and hepatitis B virus. | History of type I Diabetes Mellitus (type II Diabetes was accepted) |
|                                                | Perforated eyes.                                |
|                                                | Eyes exhibiting vitreous bleeding or retinal damage. |

The donor eyes were cut circumferentially through the pars plana and the anterior segment was removed. The vitreous was then aspirated and the retina gently separated from the RPE cell layer with a fine forceps, after which the eye cup was washed with culture medium (Table 4, Eagle’s minimum essential medium (MEM), Irvine Scientific, Santa Ana, CA), trypsinized (0.25% trypsin/0.02% EDTA, Irvine Scientific), and incubated at 37°C for 45 minutes. Primary cultures were established by gently pipetting the cell suspension in the eye cup into a 75 ml tissue culture flask containing MEM supplemented with 20% fetal bovine serum (FBS, HyClone, Logan, UT) with addition of gentamycin, 40 ng/ml (Gentamicin-sulfate solution Irvine Scientific), and glutamine, 11 ml/1000 ml (L-Glutamine 200 mM, 29.2 mg/ml, Irvine Scientific). The cells were incubated at 37°C in 5% CO₂ / 95% air and passed by trypsinization when they reached confluency. The purity of cultures was visually
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checked under a phase contrast microscope. Only cultures that exhibited a homogeneous population of darkly pigmented, polygonal cells were used for further processing. Third passage RPE cells were used in all parts of the experiments (studies IV, V, VI, and VII). The culture medium used did not contain the semi-essential amino acid proline, because it would otherwise competitively retard the absorption of radiolabeled proline molecules added in a later phase of the experiment (studies V and VI).

Table 4. Composition of medium used for the cultivation of human RPE cells, human fibroblasts and human macrophages (studies IV, V, VI, and VII).

| AMINO ACIDS  | VITAMINS    | SALTS       | MISCELLANEOUS          |
|--------------|-------------|-------------|------------------------|
| Arginine     | Biotin      | NaCl        | *Fetal Bovine Serum    |
| Cystine      | Choline     | KCL         | **Glucose              |
| Glutamine    | Folate      | NaH2PO4     | Gentamicin-sulfate     |
| Histidine    | Nicotinamide| NaHCO3      | ***Phenol red          |
| Isoleucine   | Pantothenate| CaCl2       |                        |
| Leucine      | Pyridoxal   | MgCl2       |                        |
| Lysine       | Thiamine    |             |                        |
| Methionine   | Riboflavine |             |                        |
| Phenylalanine|             |             |                        |
| Threonine    |             |             |                        |
| Tryptophane  |             |             |                        |
| Tyrosin      |             |             |                        |
| Valine       |             |             |                        |

* Serum was used in concentration of 5% for the RPE and fibroblast cell cultures. Serum was not used in the cultures of macrophages to obtain macrophage-conditioned media (MCM).

** Glucose was used at a concentration of 5.5 mM.

*** Phenol red was used as a pH indicator dye whose color is monitored to assure a pH of 7.4. All cultures were kept in an incubator at 37°C in an atmosphere of 5% CO2 and 95% air.

Fibroblast Cultures (Study IV)

Primary cellular explants from donor eyes sometimes exhibited contaminating spindle shaped fibrocytes. If cells from such a mixed culture were seeded sparse, the individual cells grew out in well defined cell clusters that formed discrete islands of cloned colonies. The colony forming units of fusiform
cells grew faster and could easily be scraped off and seeded in separate flasks. Fourth passage fibroblasts were used in all parts of the experiments (study IV).

**Phase Contrast Microscopy (Studies IV, V, VI, and VII)**

In cell culturing, it is of crucial importance to ensure that the cells stay alive, grow, divide, and thrive in their environmental milieu. The purity of cultures, i.e. the possible contamination by bacteria, fungi, parasites, or non-requested cell-types also has to be determined. The best way to establish culture purity is to examine the living cell culture under a microscope without prior fixation or staining. The phase contrast microscope contains special optical devices designed to exploit the diffracting properties of cells, allowing many details of living cells to become clearly delineated. Intracytoplasmatic structures such as vacuoles, pigment granules, phagosomes, and extracellular matrices containing collagen fibers can thus be visualized.

Light is a system of electromagnetic wave pulses that propagate with a given frequency and amplitude. When light passes through an optically dense portion of the cell, such as the nucleus, the light is retarded and its phase consequently shifts relative to the light that has passed through an adjacent, less dense region of the cytoplasm. Interference occurs when the two sets of waves recombine.

**Vitreous Humor Collection (Studies IV and VI)**

Human donor eyes were frozen and the anterior segments were removed. The outer coats of the eye, including sclera, choroid and retina, were peeled off; the remaining vitreous body was thawed, homogenized with a Potter-Elvehjem tissue homogenizer, centrifuged at 10,000 × g for 30 min at low temperature, filter-sterilized and stored at -70°C until use. Donor-age ranged from 57 to 78 years.

**Macrophage-Conditioned Medium (Studies IV, VI, and VII)**

Human peritoneal macrophages were isolated from ascitic fluid of patients with chronic liver cirrhosis (Mantovani et al. 1979, 1980). To obtain macrophage-conditioned media (MCM), cells were cultured in serum-free MEM media on 35 mm Petri dishes for 24 hours. The supernatant was centrifuged and stored at -90°C until use. The purity of cultures was
estimated with anti-human macrophage specific monoclonal antibodies (anti-Leu-M5 [CD11C]), Becton Dickinson Inc., Mountain View, CA). With this antibody, 85% to 90% of the cells were stained. Macrophage viability, as evaluated by trypan blue exclusion test, was higher than 75%.

Cell Proliferation Assays (Studies IV, V, and VI)

RPE cells and fibroblasts were plated in either 12-well cluster plates (Corning Glass Works, Corning, NY), or 48- or 96-well cluster plates (Costar, Cambridge, MA). To exclude a possible interference with plating efficiency and cell attachment through the slightly more viscous vitreous, the media were replaced with experimental media 24 hours later. At this point, cell numbers were determined in at least 12 parallel wells to verify and assure equal spreading of cells per well at the start of each experiment. To establish dose response curves (study IV), vitreous and MCM were mixed in various final concentrations of 0, 10, 25, 50 and 95% respectively in the presence of 5% serum-media. Each experimental group was compared with an equal number of control wells in which the experimental substance was exchanged to the same percentage balanced salt solution, (BSS, Hanks balanced salt solution, Irvine Scientific). An additional group without serum containing 95% concentrations of vitreous, MCM and BSS was also prepared as extra controls. Each control and experimental group consisted of 8 or 16 parallel cultures. The same batches of vitreous and MCM were used for all cultures and cell types within the same trial. Cell numbers were determined three to five times in each well with a Coulter-counter (Coulter Electronics, Inc., Hialeah, FL) after 3-6 days of culturing. All experiments were repeated at least three times.

Radioactive Labeling (Studies V and VI)

RPE cells were plated at a density of 10,000 to 20,000 cells per well in 12-well tissue culture plates (diameter 22.1 mm/well, Corning Glass Works, Corning, Ny) using MEM supplemented with 10% FBS. To exclude possible interference with plating efficiency and cell attachment by the various experimental media, the same plating medium was used in all wells during the initial 24 hours of culture. At this point, cell numbers were determined in at least 12 parallel wells to verify cell count and to ensure equal spreading of cells in each well at the start of each experiment. At confluency, all experimental cultures as well as controls were treated with 25 μCi 3H-proline (L-3H-proline, Amersham, Arlington Heights, IL). The medium was removed after 72 hours and cultures were treated with 0.025 M NH₄OH to remove the cells while leaving
the extracellular matrices intact. At this stage, cell numbers were determined in parallel cultures as described below. After the NH$_4$OH treatment, the remaining extracellular matrices were treated sequentially for 3 hours with 20 µg of trypsin (bovine pancreas, A grade, Calbiochem, La Jolla, Ca), then 20 µg of elastase (type IV, Sigma Chemical Co), and finally 20 µg of protease-free collagenase (type VII, Sigma), in accordance with a method previously described (Jones et al. 1979). The radioactivity distributed within the different portions of the extracellular matrices was estimated by liquid scintillation using a Beckman LS 7500 spectrometer. All experiments were repeated at least three times using different batches of MCM (study V) or vitreous (study VI).

**Immunocytochemistry (Study VI and VII)**

RPE cells were cultured in 4-well Titer LabTek (VWR Scientific, Cerritos, CA) glass slides under the same experimental conditions described under section C.2.1. (study VI). After 14 days in culture, the cells were washed with phosphate buffered saline (PBS) and allowed to air dry. The slides were then incubated for 20 minutes with normal goat serum (NGS, Accurate Chemical & Scientific Corp. Westbury, NY), for 1 hour with primary rabbit anti-collagen polyclonal antisera (types I, II, III and IV, Chemicon International, Inc, El Segundo, CA) in three different PBS-dilutions for each collagen type, ranging from 1:10 to 1:500, and then for 1 hour in the dark with secondary goat anti-rabbit fluorescein isothiocyanate (FITC) linked antibodies (Chemicon International). The wells were washed three times with PBS (5 min/wash-cycle) between and after the treatments. Each group was run in quadruplicate and repeated four times. Negative controls were established in each group by replacing the primary antibody with normal rabbit serum (NRS, Accurate). In order to determine the level of background fluorescence, other control cultures were treated with FITC labeled antibodies only. The slides with cell layers were examined under a Zeiss photomicroscope equipped with epi-illumination. Evaluation and grading of staining were performed by two independent observers, who were presented slides masked as to the treatment and to the anti-sera used.

After 10 days of culturing (study VII), the tissue culture slides were washed two times with 0.05 M Tris-saline, pH 7.6. The slides were fixed for 10 minutes in acetone, and then washed two times for 7 minutes in tris-saline. The cells were blocked for 20 minutes with normal horse serum (NHS; Vector Laboratory, Burlingame, CA) diluted 1:4 in tris-saline containing BGEN (3% bovine serum albumin, 0.25% gelatin, 5 mM EDTA and 0.025% NP-40). Excess serum was blotted from the slides and 50 µl of a 1:5 dilution of a partially purified Moab (Kato 1988) against RPE cells was added and incubated overnight at 4°C in a moisture chamber. The slides were washed two times for 15 minutes in tris-saline, and incubated
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for 30 minutes with biotinylated horse anti-mouse IgG (Vector Lab). The slides were again washed two times for 8 minutes with tris-saline, then incubated for 10 minutes in 3% hydrogen peroxide in methanol and washed two times for 10 minutes with tris-saline. Slides were then incubated for 30 minutes with Vectastain ABC (avidin biotinylated peroxidase complex, Vector Lab) reagent (Hsu et al. 1981), after which they were washed for 40 minutes, dipped in 0.1 M acetate buffer (pH 5.2) for 1 minute, and incubated for 5 to 10 minutes in AEC (3-amino-9-ethyl-carbazole, Sigma, St Louis, MO) solution. The AEC solution was made from 3 ml of AEC (4 mg/ml) in N,N-dimethyl formamide (Sigma), 42 ml of acetate buffer, and 60 μl of 30% hydrogen peroxide. The slides were then washed in tap water for 10 minutes, mounted, and covergassed with a drop of warm glycerine jelly containing 8% gelatin and 50% glycerine. Each group was run in quadruplicate on different slides and repeated at least two times. Negative controls were established in each group, identical except for replaced of primary antibody with NHS.