A microculture technique for the evaluation of corneal cell metabolism in vitro

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A microculture technique for the evaluation of the metabolic activity of corneal cells is described and analyzed. The extent of DNA synthesis in microcultures with $10^4$ to $2.5 \times 10^3$ cells per well was initially low during day 1, increasing steadily thereafter. Higher initial concentration of $10^4$ to $2 \times 10^4$ cells per microculture demonstrated a high metabolic activity during days 1 and 2 in culture, followed by a rapid and marked decrease on days 3 and 4. The origin and concentration of serum in the system have been found to be crucial. Xenogeneic serum (fetal calf serum—FCS) had the most potent stimulatory effect on DNA and protein synthesis. Syngeneic serum (guinea pig serum, strain 13—SGpS) or allogeneic serum (guinea pig serum strain 2—AGpS) had a generally less stimulatory effect on the metabolic activity. However, both sera had a relatively much stronger effect on the protein synthesis.

Key words: microcultures, cornea, epithelium, endothelium, stroma, $^3$H-thymidine, $^3$H-leucine, DNA, protein, fetal calf serum, guinea pig serum.

The macrotechniques of corneal cell culture in vitro are awkward for a reliable evaluation of the metabolic activity of the cells tested. These methods need a large number of cells and a "mass" culture for a long period of time. Due to the need for many explants, a "contamination" of "pure cultures" by cells from other layers of the cornea could not possibly be avoided.

A microculture technique for obtaining reliable and reproducible information concerning the metabolism of corneal cells was needed and has been developed.

Materials and methods

Animals. Guinea pigs strain 13 or 2 were used. Young females 1 month old were bled to death by heart puncture and the eyes were removed immediately.

Preparation of corneal cultures

Macrocultures. Corneas are carefully removed, leaving a rim of corneal tissue toward the limbus, and transferred to a Petri dish (Falcon 3000) with Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.). Descemet's membrane and endothelium are stripped off gently with two fine jeweler's forceps. It was found that removal of a clean Descemet's membrane could be achieved only when the stripping off was started approximately 0.2 mm. inside the button and not at the edges. The removed Descemet's membrane and endothelium are transferred to a Petri dish (Falcon 3001, Falcon Plastics, Los Angeles, Calif.), endothelium up. The explant is split in two and one half is removed for microscopic examination. If on microscopic exami-
Fig. 1. Epithelial (a), endothelial (b), and stromal (c) outgrowth on day 8 of culture. Note the specific pattern of outgrowth demonstrated by the different layers of the cornea: epithelium outgrowth is progressing from the explant (Exp.) as a sheet of cells (a1, a3). Some of these are secretory cells (Sec.). Endothelial cells (b3, b4) outgrow individually and are larger cells. Note the transparency and folds of Descemet’s membrane (Desc.). Stromal outgrowth (c3, c4), although latest to be observed, is progressing very rapidly. Cells are moving out of the explant (Exp.) individually. The stromal explant exhibit few layers of cells. (Phase-contrast magnifications: a2, b2, c2, x325; a4, b4, c4, x650.)
explant is divided into two parts—one is used for microscopic examination of "purity" and the other is used as the original explant for primary culture.

All adequate explants are covered with a glass coverslip and a drop of culture medium is allowed to diffuse under it. Cultures are incubated overnight at 37°C in 100 percent humid atmosphere with a continuous flow of 5 percent CO₂ and 95 percent air. After 16 hr., 1 ml. of medium is added and the cultures are further incubated. On day 3 of culture the coverslips are removed, 0.5 ml. of fresh medium is added, and the cultures are further incubated as above.

On days 8 to 10 original cultures can be subjected to trypsinization and cells can be tested. Trypsinization is carried out after aspiration of medium, addition of 2 ml. of Dulbecco's PBS (without calcium or magnesium) containing 0.25 percent trypsin and 0.05 percent EDTA (NIH media unit), and incubation for 30 min. Trypsinized cells are harvested, washed with culture medium, counted, and transferred to microcultures. The changes in the metabolic activity of the different cells are also tested after various subcultures.

Microcultures. This report focuses on various parameters influencing the metabolic activity using, as a model, corneal epithelial cells from guinea pig strain 13. One original epithelial culture (with the original explant) was subjected to trypsinization and subcultured four times, yielding a homogeneous population of cells for all tests. The microcultures were carried out in microtest plates (Falcon 3040), 96 flat-bottom wells per plate.

Each culture is initiated in a volume of 0.2 ml. per well containing 5 x 10² to 2 x 10⁴ cells according to purpose. For the assessment of DNA synthesis, 1 μCi of tritiated thymidine (NEN, specific activity 6.7 Ci./mmol.) or 0.5 μCi of tritiated leucine (NEN, specific activity 5 mCi./mmol.) for the assessment of protein synthesis are added in a volume of 10 μl per culture 24 hr. before harvesting. All tests are carried out in three or six replicates. Protein or DNA synthesis is evaluated in the whole population of cells or in those attached to the bottom of the wells only. If only attached cells are tested, the metabolic activity using (according to purpose) with the multiple-automated sample harvester. Twenty-four wells are harvested on one running and the appropriate filters are transferred to scintillation bottles. Then 3 ml. of Aquasol (NEN, Universal L.S.C. Cocktail, NUF-934) are added and the radioactivity is evaluated by counting in a Beckman Scintillation Counter (Model LS-355; Beckman Instruments, Inc., Palo Alto, Calif.).

Culture medium. RPMI 1640 (Gibco) with glutamine 0.3 percent (NIH media unit), streptomycin 100 μg/ml., and 10 percent fetal calf serum (Microbiological Associates) was usually used (unless otherwise specified) for initiation of all original cultures. In microcultures different concentrations of fetal calf serum (FCS), syngeneic guinea pig serum strain 2 (SPS), or allogeneic guinea pig serum strain 13 (APPS) were tested.

Results

Primary outgrowths from original explants were observed in epithelial cultures after 24 to 30 hours. These progressed rapidly as a "sheet" (Fig. 1, a). In endothelial cultures, primary outgrowths were usually observed on day 3 and were formed by individual cells. Endothelial cells were large, became larger with time, and their number increased slowly (Fig. 1, b).
Fig. 3. Correlation between the number of cells per microculture and the $^3$H-thymidine uptake. Each point represents the mean uptake of three to six microcultures.

The microcultures were easily reproducible with a high degree of accuracy. The extent of $^3$H-thymidine or $^3$H-leucine uptake among replicates varied (in most cultures) within ±1 to 10 percent (Figs. 2 to 5). Concentrations of $10^5$ cells or $2.5 \times 10^3$ cells per culture demonstrate a low activity of DNA synthesis during the first 24 hr., increasing markedly thereafter. On day 4, the extent of DNA synthesis in microcultures with $10^3$ cells is nearly 50 times higher than that observed after day 1 (Fig. 2). In microcultures with $5 \times 10^3$ cells, the uptake of tritiated thymidine is high during day 1, increases during days 2 and 3, and decreases on day 4. Microcultures with $10^4$ or $2 \times 10^4$ cells demonstrate a very high uptake of tritiated thymidine during day 1, an increased activity on day 2, followed by a sharp decrease on days 3 and 4 (Fig. 2). From Fig. 3, it appears that on day 1 of culture, there is a linear correlation between the extent of DNA synthesis and the number of cells per culture. On day 2, the activity increases with the increase in the number of cells from $10^4$ to $10^5$ and reaches a plateau toward $2 \times 10^4$ cells per culture. On day 3, a decrease of $^3$H-thymidine uptake is observed, with $10^4$ cells per microculture as compared to $5 \times 10^3$ cells (Fig. 3).

Fig. 4. Effect of FCS concentration on the extent of $^3$H-thymidine uptake. Each point represents the mean uptake of three microcultures with $10^3$ cells per culture.

Stromal explants demonstrated an outgrowth of cells only on days 4 or 5 of culture. The stromal cells advanced from the explant as individual cells. These were smaller than the endothelial cells but their number increased rapidly (Fig. 1, c).

The effect of serum concentration on the metabolic activity is illustrated in Fig. 4.

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The extent of DNA synthesis is closely correlated to the serum concentration. Cells kept in culture medium without serum showed a very slight activity above background. Most of these cells attached to the bottom of the well and excluded trypan blue. Addition of 1 percent FCS boosted the incorporation of $^3$H-thymidine, which increased steadily during the period tested. Addition of 5 or 10 percent FCS to the culture medium multiplied the extent of DNA synthesis by two- to threefold and threefold to more than fourfold, respectively (Fig. 4). The effect of xenogeneic (FCS), allogeneic (AGpS), or syngeneic (SGpS) serum on DNA and protein synthesis is demonstrated in Fig. 5. The highest stimulatory effect is observed with the addition of FCS. However, a relatively higher protein synthesis as compared to DNA synthesis is observed in cultures with guinea pig sera. Syngeneic serum appears to be the least stimulatory among the sera tested (Fig. 5).

Discussion

The ability to carry out very adequate tests with a small number of cells may be useful in the study of human material when only a small piece of tissue is available through a biopsy. Microcultures with $10^3$ to $2.5 \times 10^3$ cells seem most adequate for the study of a slow in vitro influence, whereas higher concentrations of cells can be used for a more acute process (Figs. 2 and 3). It seems that the limiting factors are the nutrients available in the medium. However, an initial minimal concentration of cells is required for an adequate evaluation of the in vitro activity during the latent period. This minimal concentration is "the crucial concentration" and has been found to differ for the various layers of the cornea.

The serum, its concentration, and its origin play a major role in the metabolic activity of the tested cells. Xenogeneic serum has the most potent stimulatory effect. This effect appears to have a strong drive for DNA synthesis and multiplication of cells. Allogeneic or syngeneic sera have a less stimulatory effect on the synthesis of DNA and a relatively much stronger effect on the protein synthesis. Therefore, the serum origin should be carefully considered and adapted to the purpose of the study. The ease of the method as described, its high degree of accuracy, and its reproducibility enable adequate investigation of the ocular tissues at a cellular level.
REFERENCES


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