# SELECTIVE ELIMINATION OF FIBROBLASTS FROM CULTURES OF NORMAL HUMAN MELANOCYTES

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(Received April 18, 1984; editor A. B. Lerner)

## SUMMARY

The main obstacle to establishing pure normal human melanocytes in vitro is contamination of the cultures by fibroblasts. The obstacle can be overcome by selective destruction of fibroblasts with geneticin (G418 sulfate). Treatment of mixed cultures with this drug at a concentration of 100 μg/ml for two days results in pure cultures of normal human melanocytes.

*Key words:* human melanocytes; human fibroblasts; geneticin.

## INTRODUCTION

The discovery that TPA (4-0-methyl-12-tetradecanoly-phorbol-13-acetate) supported the proliferation of normal human melanocytes in culture, provided the first method of growing normal melanocytes routinely and in large quantities (1). Contaminating keratinocytes did not pose a problem because their attachment to the culture dish was inhibited by TPA. The keratinocytes that survived in the primary cultures disappeared after trypsinization and subculturing. Contamination with fibroblasts, however, remained a formidable obstacle to establishing pure melanocyte cultures. The fibroblasts proliferated vigorously despite the presence of cholera toxin, a second growth factor required for optimal proliferation of normal melanocytes in culture (1).

We describe here an easy method of producing pure melanocyte cultures by selectively killing fibroblasts with the antibiotic geneticin.

## MATERIALS, METHODS AND RESULTS

Normal human melanocytes were grown by a method (2) modified from that of Eisinger and Marko (1). Foreskins from newborns were cleaned of fat and incubated in 2.5 ml of Eagle's minimal essential medium without calcium (MEMS), supplemented with 0.25% trypsin, 200 unit/ml penicillin, 100 μg/ml streptomycin and 100 ng/ml TPA (Consolidated Midland Corporation, Brewster, NY) at 4° C, overnight. The epidermis was separated from the dermis and the tissues were shaken vigorously in melanocyte growth medium. Large pieces of tissue were discarded, and dissociated cells were plated in 25 $cm^2$ culture flasks (Falcon, Oxnard, CA). Melanocyte growth medium consisted of Ham's F-10 medium (Flow Laboratories, Inc., McLean, VA) supplemented with 10% Nu-serum (Collaborative Research, Inc.), 2% newborn calf serum (GIBCO Laboratories, Grand Island,

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NY), penicillin, streptomycin, $4.8 \times 10^{-8}$ *M* TPA, $2.5 \times 10^{-9}$ *M* cholera toxin (List Biological Laboratories, Inc., Campbell, CA) and $1 \times 10^{-4}$ *M* isobutylmethyl xanthine (IBMX) (Sigma Chemical Company, St. Louis, MO). Our experience showed that IBMX stimulated the rate of proliferation of melanocytes and reduced the requirement for cholera toxin, an expensive ingredient in the growth medium. The cells were kept in a humidified incubator with 5% $CO_2$ at 37° C.

We decided to test for selective killing of fibroblasts by geneticin (G418 sulfate, GIBCO Laboratories, Grand Island, NY) because of the known differential cytotoxic effects of this antibiotic on eukaryotic cells with different growth rates (3). Cytotoxicity is observed after 1–2 cell divisions, and the most rapidly growing cells are killed in the shortest intervals (3). We reasoned that the rapidly proliferating fibroblasts might die sooner than the slowly proliferating melanocytes.

Preliminary experiments with geneticin at concentrations ranging from 50 to 1000 μg/ml of active material indicated that both melanocytes and fibroblasts were killed at the higher concentrations. However, at low concentrations, such as 100 μg/ml of active material, fibroblasts died within 4–5 days, while melanocytes remained healthy. We therefore tested the effect of 2 days of treatment with geneticin at 100 μg/ml on fibroblasts and melanocytes, cultured from the same foreskin. We used cells that had been in culture for 1.5 months. During this period they were passaged at a ratio of 1:3 three times. Most of the melanocytes had been separated from the fibroblasts by differential detachment. That is, the cultures were incubated for 2–3 minutes with 0.25% trypsin, the detached melanocytes were re-

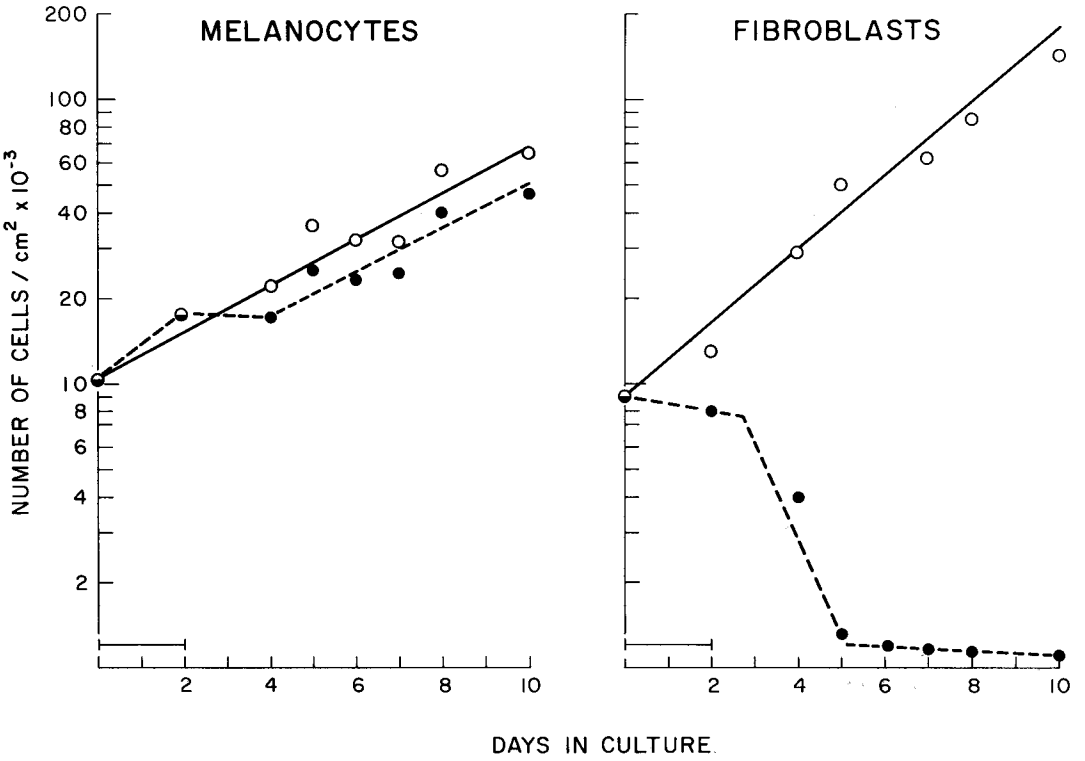


FIG. 1. Growth curves of melanocytes and fibroblasts illustrating the differential cytotoxic effect of geneticin on fibroblast cultures. The cells were detached from the wells by trypsin (0.25% plus 1 m*M* EDTA in MEMS medium) and counted in a Coulter counter. Each point is the average of duplicate cultures. Standard error did not exceed 10%. ○, Cells not exposed to geneticin; ●, cells treated with geneticin, 100 μg/ml for the two days indicated by the bar above the abscissa (|—|).

moved to a new flask, leaving behind the fibroblasts and a few attached melanocytes.

Both types of cell were subcultured into plastic cluster wells (4 $cm^2$ each) (Costar, Cambridge, MA). Geneticin (100 $\mu g$/ml of active material) in melanocyte growth medium was applied on the following day (day 0, Figure 1) for a duration of two days. The medium was then changed to regular melanocyte growth medium without geneticin, and this medium was renewed every 2-3 days.

The differential cytotoxic effect of this treatment on fibroblasts is illustrated in Figures 1 and 2. The rate of proliferation of melanocytes and fibroblasts during and after drug treatment is shown in Fig. 1. The proliferation of normal human melanocytes was inhibited for only two days beyond the exposure of the cultures to geneticin. Thereafter, the melanocytes resumed their normal rate of growth. Human fibroblasts proliferated vigorously in the presence of TPA, cholera toxin and IBMX but died shortly after exposure to geneticin.

The microscopic appearance of the cultures 4 days after termination of the antibiotic treatment is shown in Fig. 2. Cells in the fibroblast cultures that survived exposure to geneticin were mostly melanocytes.

The differential toxicity of geneticin may be due to the different rates of proliferation of the two types of cell. Population doubling times are approximately 2 and 3.3 days for the fibroblasts and melanocytes, respectively, as calculated from the slopes in Fig. 1. It is also possible that the two cell types differ in their ability to respond to the drug.

In summary, two days of treatment with 100 $\mu g$/ml geneticin kill most of the fibroblasts. Our routine method to establish pure melanocyte cultures whenever contaminating fibroblasts are present is to treat primary cultures with geneticin (100 $\mu g$/ml) for 2-4 days. In cases in which residual fibroblasts continue to proliferate, a second treatment is given. It is, therefore, possible to obtain fibroblast-free melanocyte cultures with minimum effort. In the absence of fibroblasts the

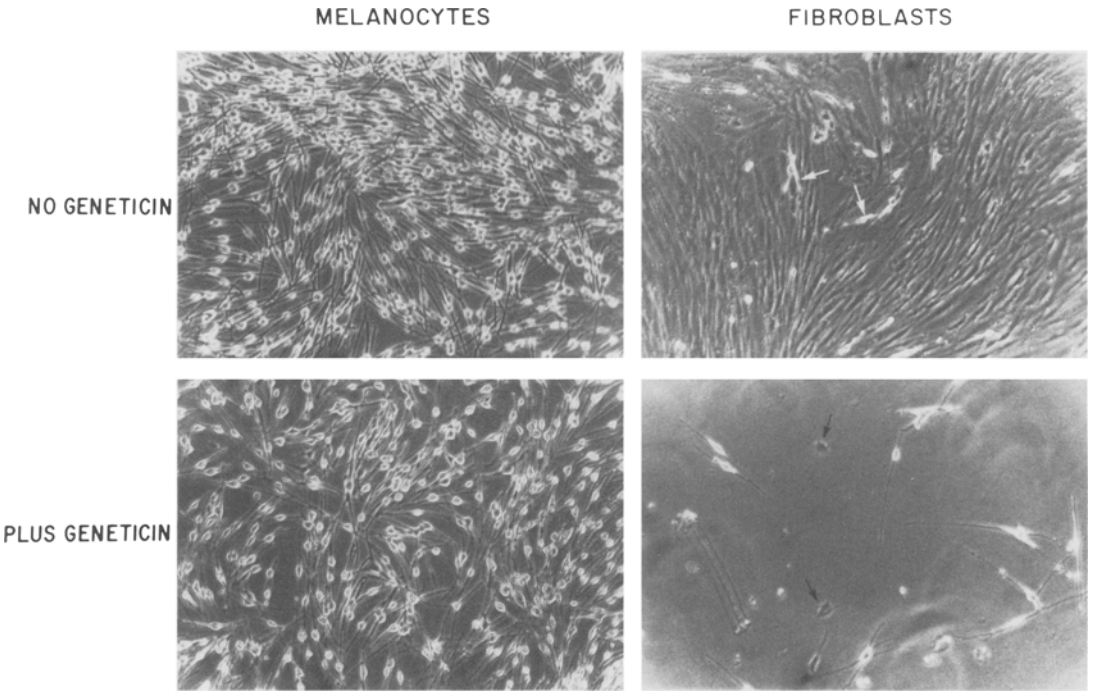


FIG. 2. Photomicrographs of the cultures of Fig. 1, taken on day 6. In the fibroblast cultures white arrows point to melanocytes, black arrows to fibroblasts ×191.

melanocytes grow faster. We have succeeded in growing approximately $1 \times 10^9$ cells from one foreskin in three months.

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This work was supported by National Institutes of Health Grant 5 RO1 CA04679, to A. B. Lerner. The authors are grateful to Dr. A. B. Lerner for his support, to Dr. G. Moellmann for editing and Mrs. C. Kocher for typing the manuscript.