Establishment of New Mouse and Human Pigmented Melanoma Lines in Tissue Culture

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INTRODUCTION

Few stably pigmented melanomas have been established as permanent lines in tissue culture, particularly from human sources. The availability of stably pigmented lines is a prerequisite for somatic cell hybridization studies to investigate the genetic control of pigment synthesis. Studies on the biochemistry of dopa oxidase would also be facilitated by the existence of additional permanent melanoma lines to provide a source of enzyme. Thus, it was decided to attempt to establish in tissue culture new melanomas from both human and murine sources.

MATERIALS AND METHODS

Establishment of human melanoma line RN in tissue culture. A pigmented melanoma which had been surgically removed from a male patient was obtained from Massachusetts General Hospital. The tumor was speckled in appearance and contained several black nodular areas, five of which were dissected from the remainder of the tumor. The nodules were washed once with 10 ml of McCoy's 5a medium without serum, and the tissue surrounding the nodules was removed. The nodules were minced to small fragments in several milliliters of McCoy's 5a medium without serum and inoculated directly into 35-mm Falcon plastic tissue-culture dishes (with coverslips) in McCoy's 5a medium and 10% fetal-calf serum. This medium will be referred to as M medium. The pigmented line which developed was called RN.

Several other treatments were tried, including trypsinization of the tumor and inoculation into various media with sera from different sources. These, however, either did not yield growing cultures or they produced cultures which rapidly lost their pigmented phenotype with continued passage.

Establishment of mouse melanoma line HP in tissue culture. A 4-week-old Harding-Passey melanoma, designated HP, was cut from a mouse immediately after sacrifice. After being rinsed in phosphate-buffered saline, the tumor was finely minced, and the pieces were inoculated into 60-mm Falcon plastic tissue-culture
dishes in Dulbecco's modified Eagle's medium with 10% fetal-calf serum. This medium will be referred to as D medium.

As for RN, other procedures were tried for HP. Again, either growing cultures were not obtained or those which were obtained rapidly lost their pigmented phenotype.

**Preparation of crude extracts for dopa-oxidase assays.** RN was grown in medium M and HP was grown in medium D. 10⁶ cells were inoculated into 100-mm Falcon plastic tissue-culture dishes and were collected at various times after inoculation with .05% trypsin and .02% EDTA. The cells were washed three times in 0.1 M sodium phosphate buffer, pH 6.8, and either stored frozen at -80°C or immediately resuspended in 1 ml of the same buffer and homogenized. Extracts were centrifuged for 5 min at 2000 g at 4°C. Supernatant fluids were assayed for dopa oxidase as previously described (1).

**Karyological techniques.** A modification of the air drying technique of Rothfels and Siminovitch (2) was employed to prepare the cells for karyological analysis. Cells were treated with Colcemid at a final concentration of 0.45 μg per ml for 2–3 hr, collected by trypsinization, treated with hypotonic solution, fixed in cold methanol–acetic acid, and dripped onto chilled slides. After the slides had dried at room temperature, they were stained with 4% Giemsa.

**RESULTS**

**Isolation of pigmented human melanoma line RN.** After 11 days, RN cells which had migrated from under the coverslip were treated with 0.05% trypsin and 0.02% EDTA, and the cells obtained were inoculated into 60-mm Falcon plastic tissue-culture dishes with medium M. Three weeks later, a focus of rounded pigmented cells developed in one of the inoculated dishes. The pigmented clump of cells was picked with a pipet and subcultured, giving rise to a pigmented culture which grew with a generation time of slightly less than 3 days. The pigmented line was called RN.

To confirm the human origin of the pigmented RN cells, the enzymes malate dehydrogenase and lactate dehydrogenase from RN were compared with the same enzymes from HeLa cells. Agarose electrophoresis was employed to separate the enzymes and the methods described by Ruddle and Nichols (3) were used for staining. Both enzymes from RN migrated to the same position as the corresponding enzymes from HeLa cells. Dopa oxidase from RN also migrated to a position characteristic of the human enzyme in isoelectric focusing (Burnett, Horn, and Davidson, unpublished).

By cloning under a variety of conditions, both pigmented and unpigmented subclones of RN were obtained. One pigmented subclone, RN-7 (Fig. 1), which grew with a generation time of 2 days in medium M was obtained as a well-isolated colony from a dish containing 50% M medium and 50% M medium which had been conditioned by the mass culture of RN. When RN-7 was itself subcloned, all of the more than 100 subclones observed were pigmented (Fig. 2), although the degree of pigmentation varied from subclone to subclone. The cloning efficiency was 6% in M medium. RN-7 was maintained in active growth for 6 months, during which time the culture remained pigmented.

RN-7 was also analyzed for karyotype. An analysis of 10 mitotic figures indicated a range of 67–84 chromosomes with an average of 76.6 per cell. A karyotype of RN-7 is presented in Fig. 3.
FIG. 1. Photomicrographs of RN-7 cells fixed with 100% methanol. Bright field illumination, $\times 60$. (A) The center of a colony; (B) the edge of a colony.

Isolation of pigmented mouse melanoma line HP. After several weeks, a pigmented culture was obtained from the minced pieces of the HP melanoma which had been inoculated directly into petri dishes. Several pigmented clones were ob-
Fig. 2. Photograph of a dish inoculated with 2500 RN-7 cells, fixed with 100% methanol after 3 weeks.

Fig. 3. Karyotype from a metaphase of an RN-7 cell.

tained from the pigmented culture by plating in medium D. One clone, HP-1 (Fig. 4), which grew with a generation time of 3 days, was itself subcloned and gave rise to all pigmented colonies, over 100 having been observed. The cloning
efficiency of HP-1 in D medium was 2%. HP-1 maintained its pigmented phenotype in tissue culture for the 6 months it was kept in active growth.

Analysis of 10 mitotic figures of HP-1 indicated a variation of 69–72 chromosomes with an average of 70.6 per cell. The number of submetacentric chromosomes varied from 13–16 with an average of 14.9 per cell. A karyotype of HP-1 is presented in Fig. 5.

**Dopa oxidase production in RN and HP.** RN was analyzed for dopa-oxidase activity at various times after inoculation as described in Materials and Methods. The results of two different experiments are presented in Fig. 6. No large changes in the activity were observed, although the enzyme activity did increase almost 2-fold (from 0.026 to 0.041 in one experiment and from 0.014 to 0.023 in the second experiment) late in the growth cycle. (Since RN yielded both pigmented and unpigmented subclones, it is possible that the pigmented subclones such as RN-7 would give different results with respect to dopa-oxidase activity). The relatively constant levels of dopa-oxidase activity throughout the growth cycle of RN contrast with the levels found for the Syrian hamster melanoma, 3460, for which the enzyme levels varied over 100-fold during the growth cycle (4). However, the peak specific activity of 3460 was 0.056, a value close to that for RN.
Fig. 5. Karyotype from a metaphase of an HP-1 cell.

Fig. 6. Dopa-oxidase activity during the growth cycle of RN. In experiment 1 (open circles), the cells were collected as described in Materials and Methods and assayed immediately for dopa oxidase. In experiment 2 (triangles), the cells were stored frozen at $-80^\circ$C and assayed for dopa oxidase at the end of the experiment. In both experiments, cells reached stationary phase at approximately 11 days. Specific activity is the change in optical density at 475 nm per min per mg protein.

HP-1 was also analyzed for dopa-oxidase activity 12 days after inoculation. The specific activity value obtained for HP-1 at this time was 0.0054, about 5- to 10-fold less than for RN.

Fusion properties of RN and HP. Both RN and HP-1 were treated in suspension with $\beta$-propiolactone-inactivated Sendai virus (5). Under the given conditions, over
52% of the HP-1 nuclei and over 43% of the RN nuclei were found in homokaryons. Thus, HP-1 and RN are promising candidates for use in hybridization experiments, since they fuse readily.

CONCLUSION

Two new stably pigmented melanoma lines have been established in tissue culture, one from a human and one from a murine source. Both lines should be useful for cell-hybridization studies on the control of pigment synthesis as well as providing a possible source of enzyme for studies on the biochemistry of dopa oxidase.

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