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Suppression of Malignancy and Differentiation in Melanotic Melanoma Cells*

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Abstract. Tumorigenicity of mouse melanoma cells is reduced or lost upon growth *in vitro* in 1 to 3 μg/ml of 5-bromodeoxyuridine (BUdR). The rate of growth is very little affected by these concentrations. The morphology of the cells is altered, such that the cells grow in a flattened, often fibroblastic monolayer, showing contact inhibition, instead of the rounded, multilayered mounds characteristic of the line. Melanotic cells lose their pigment-producing ability within one week. These effects are reversible and the inclusion of thymidine with BUdR prevents their occurrence. The reduction in tumorigenicity, the effects on cell morphology, and the suppression of pigmentation occur in clones derived from single cells growing in BUdR as well as in mass populations. These clones appear capable of indefinite growth *in vitro* in BUdR with altered morphology. The suppression of cytodifferentiation in these melanoma cells and in embryonic cells, coupled with the modification of malignancy, leads to the hypothesis that both differentiation and malignancy are regulated by similar cellular mechanisms. These effects of BUdR offer promise of a powerful tool to probe these regulatory mechanisms.

Cytodifferentiation in a variety of embryonic tissues is reversibly suppressed by low concentrations of 5-bromodeoxyuridine BUdR.[1–7] Pigmentation is among the differentiated functions suppressed, both in embryonic retina[3] and in iris epithelium (Fig. 3*A*). It was of interest to see whether similar effects would be obtainable in a mouse melanoma line that has been induced to grow *in vitro* with virtually every cell pigmented.[8] In addition, since malignancy can be viewed as an aberrant form of differentiation, it also seemed worthwhile to test the tumorigenicity of BUdR-treated cells.

Materials and Methods. The cell lines used were derived from a B16 mouse melanoma, and clonal derivatives were maintained in a fully pigmented state as described previously.[8] The amelanotic clone from which these lines were derived was also used. Methods of cell culture, cloning, and medium (Eagle's minimal medium with 10% fetal calf serum) used were the same as previously described.[8] BUdR (Nutritional Biochemicals, Cleveland, Ohio) was added to the medium at the final concentrations described.

Incorporation of ^{3}H-BUdR (0.5 μCi/ml, 11 Ci/m mole, New England Nuclear Corp., Boston, Mass.) was followed by autoradiography by using the procedures already described.[8] Cells on cover slips were grown in radioactive medium for 1, 5, and 24 hr. Some cover slips were immediately washed, fixed, and processed, others were washed with

nonradioactive medium and incubated for up to 23 hr in nonradioactive medium. All washes included an excess of nonradioactive BUdR.

For quantitative determination of incorporated radioactivity, cover slips containing cells grown in ^{3}H-BUdR for 1, 5, and 24 hr were washed as described, extracted twice with ice-cold 5% trichloroacetic acid, washed with ethanol:ether (3:1) and then with ether, and dried. They were placed in scintillation vials with 0.5 ml concentrated formic acid and scintillation fluid, and the radioactivity was determined with a Packard Tri-Carb liquid scintillation counter.

Protein was determined in replicate flasks at the time points shown in Figure 2 by the Oyama and Eagle[9] modification of the Lowry method. Melanin was extracted three times with ice-cold 5% trichloroacetic acid, twice with ethanol:ether (3:1), once with ether, and dried. The extract was dissolved in hot 0.85 *N* KOH, cooled, and the absorbance read at 420 mμ in a Beckman DU spectrophotometer.

Tumorigenicity was tested by injection of 10^6 viable cells (as determined by trypan blue exclusion) into 6-week-old C57BL/6J mice.

Results. Within the first week of treatment with BUdR (1–3 μg/ml), there is little or no effect on the growth of the cells (Figs. 1 and 2) but there is a marked effect on pigment production and cell morphology (Figs. 2–4). The cells grow without pigment and are flattened and largely contact-inhibited. This is in sharp contrast with the highly melanotic cells which pile up to form the dark mounds characteristic of the clonally derived melanoma line. The BUdR effects are reversible on growth in normal medium after up to 100 days on BUdR-containing medium (Figs. 5*C*, *D*, *G–I*). The effects can be prevented by the addition of equimolar quantities of thymidine (Fig. 3*C*), but reversibility is not improved by the addition of thymidine to normal medium.

As these cells continue to grow in BUdR-containing medium, they often form parallel rows of long narrow cells, similar to fibroblasts (Figs. 5*A*, *B*). Many are

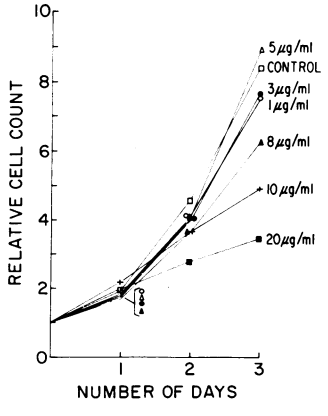

FIG. 1.—Growth curves of melanotic melanoma cells grown in medium supplemented by concentrations of BUdR ranging from 0 to 20 μg/ml.

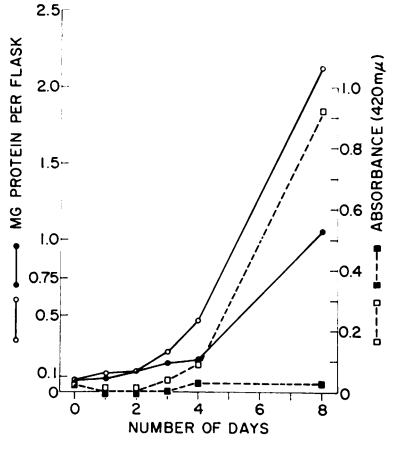

FIG. 2.—Comparison between growth (as determined by protein concentration) and melanin concentration, in melanotic melanoma cells grown in normal culture medium (*open circles* and *squares*) and in medium supplemented by 3.3×10^{-6} *M* BUdR (1 μg/ml) (*closed circles* and *squares*).

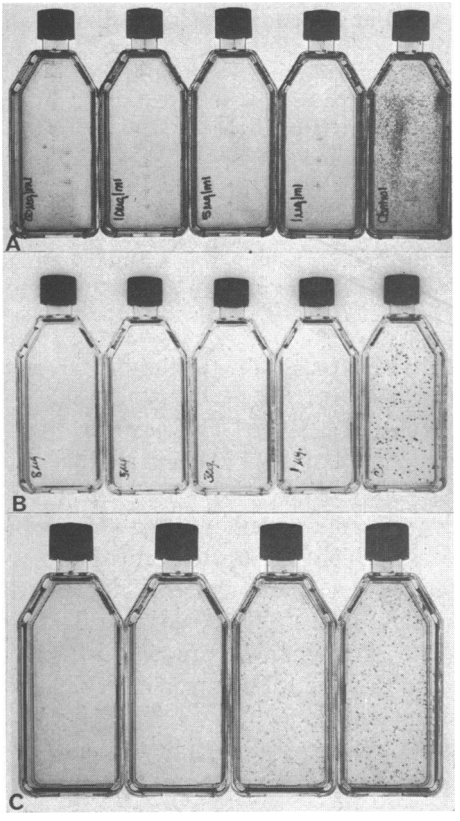

FIG. 3.—Effect of BUdR on pigment production in embryonic iris epithelium and melanotic melanoma. Only pigmented colonies are visible. Unpigmented cells cover almost the entire surface of the flasks containing BUdR.

(*A*) Pigmented iris epithelium from 15-day-old chick embryos. Flasks of sixth subculture photographed 9 days after beginning treatment with BUdR at the labeled concentrations. Each flask was seeded with 5×10^5 pigmented cells and allowed to grow in Eagle's minimal medium supplemented with 10% fetal calf serum and 1% chick embryo extract for 4 days before treatment began.

(*B*) Flasks of melanotic melanoma cells photographed 7 days after beginning treatment with BUdR at the concentrations per ml labeled. *C* = control. Each flask was seeded with 10^3 cells 4 days before treatment to insure that cells would be pigmented.

(*C*) Flasks of melanotic melanoma cells photographed 7 days after beginning treatment. Cells were seeded as described for (*B*). Treatment of flasks from left to right was as follows: 2 μg BUdR/ml; 2 μg BUdR/ml and 2 μg thymidine/ml; 2 μg BUdR/ml and 4 μg thymidine/ml; control—no treatment. (See Fig. 4 for microscopic view of cells on day 2.)

somewhat enlarged and vacuoles are common, both in the early and later stages. These are more prevalent in cells grown in 3 μg/ml of BUdR than in the lower concentrations (Figs. 5*E*, *F*). The growth rate decreases somewhat, but subculture is required as frequently as with the untreated cells—about once a week. Since there is contact inhibition of growth, the cells do not pile up and slough off the surface of the container, as do untreated cells if they are allowed to grow without subculture beyond a week or 10 days.

These cells seem capable of growing indefinitely in these low concentrations of BUdR. One line was maintained for 9 months in BUdR (1 μg/ml) before being frozen in liquid nitrogen. Viability of the lines ranged from 67 to 90%.

Clones derived from single cells isolated in each of these concentrations of BUdR grew like the mass populations described. The tumorigenicity of clones isolated in medium containing 1–2 μg BUdR/ml is much reduced from that of the original cell line, and tumorigenicity is completely abolished in the clones isolated in medium containing 3 μg BUdR/ml (Table 1, Fig. 6).

To test the required length of pretreatment with 3 μg/ml of BUdR before modification of tumorigenicity occurred, melanoma cells were grown in BUdR-containing medium (3 μg/ml) for 0 to 31 days *in vitro* before injection into C57BL mice. The results of such treatment for 0 to 7 days is given in Figure 7. Cells pretreated for 11 to 31 days formed one tumor in a total of 24 mice tested. All mice were kept under observation for at least 70 days after injection.

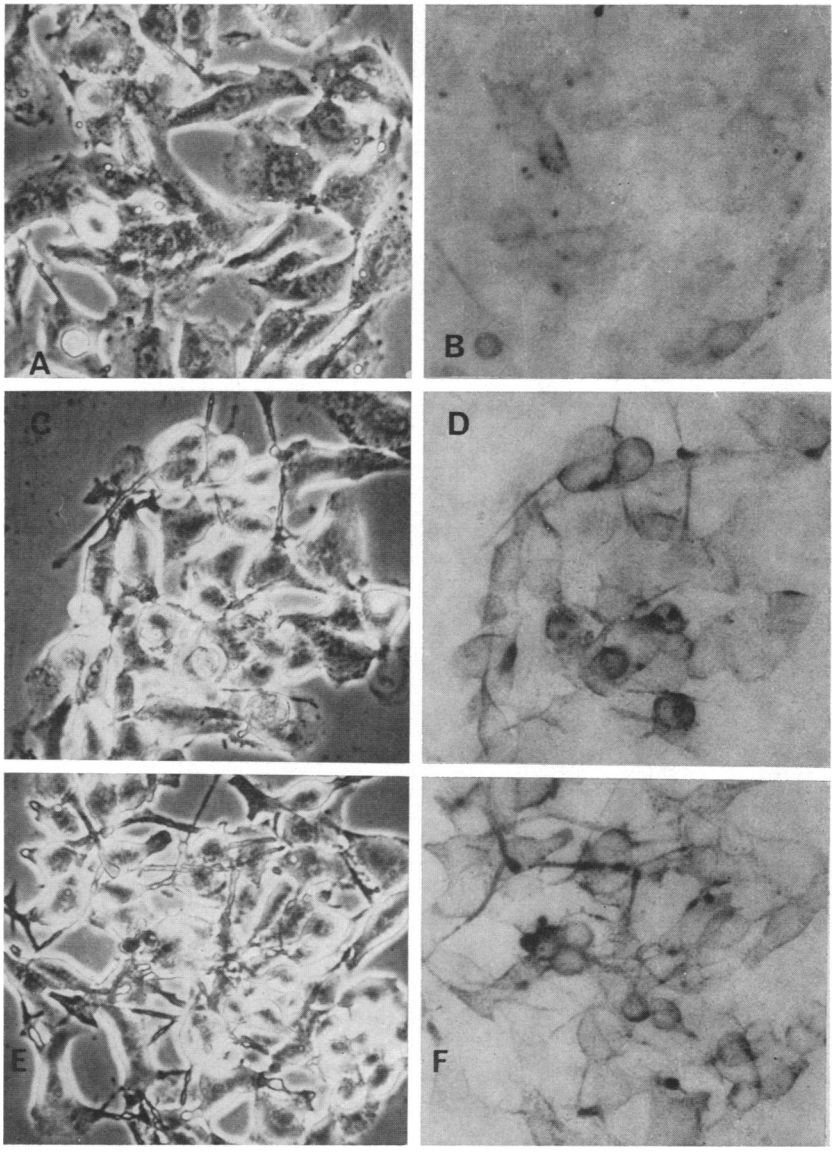

FIG. 4.—Living cells viewed with Wild inverted microscope 2 days after experiment described in Fig. 3(*C*) was begun. (*A*), (*C*), (*E*), phase contrast; (*B*), (*D*), (*F*), bright field with Koehler illumination, which permits only pigmented cells to be visible. (*A*), (*B*) Cells treated with 2 μg/ml of BUdR. (*C*), (*D*) Cells treated with 2 μg/ml of BUdR and 2 μg/ml of thymidine. (*E*), (*F*) Control Cells, no treatment. Note flattening of cells in (*A*). X310.

Clones derived from melanotic melanoma cells pigmented on agar and by growth in 1-β-D-arabinosylcytosine, as previously described,[8] were equally affected by BUdR, which indicates that the method used to evoke pigment production did not influence the BUdR effect.

Malignancy could also be modified by BUdR in a completely amelanotic

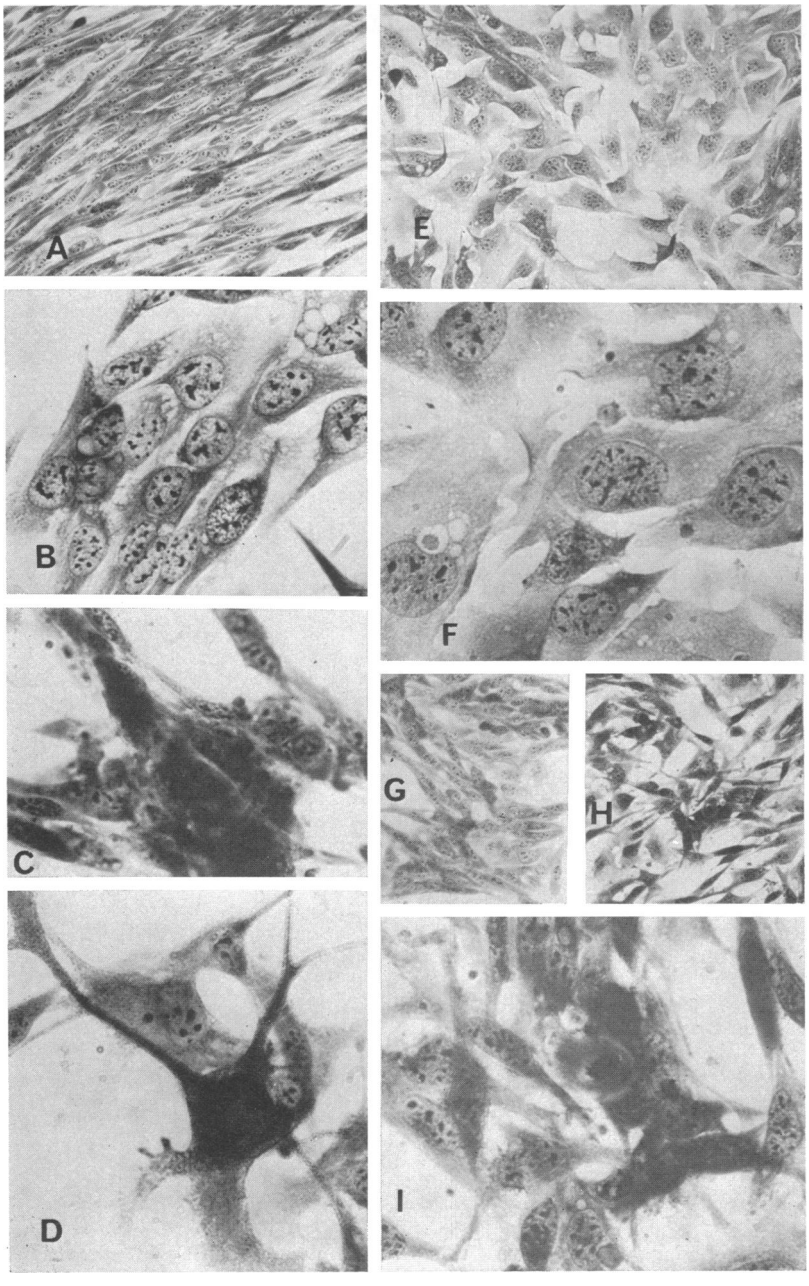

FIG. 5.—Effects of long-term treatment on cells grown in medium containing 1 μg/ml or 3 μg/ml of BUdR and reversal of BUdR effects after growth in normal medium without BUdR. May-Grunwald-Giemsa. (*A*) Growth after 109 days on 1 μg/ml of BUdR. Note fibroblast-like parallel growth. ×128. (*B*) Growth after 96 days on 1 μg/ml of BUdR. Note flattening of cells and presence of vacuoles. ×512. (*C*) Reversal after 57 days on 1 μg/ml of BUdR medium and 39 days on normal growth medium. Note lack of contact inhibition, with piling and rounding of cells. ×512. (*D*) Reversal after 57 days on 1 μg/ml of BUdR medium and 63 days on normal medium. Note pigment cell. ×512. (*E*) Growth after 112 days on 3 μg/ml of BUdR. Note flattening and enlargement of cells and presence of vacuoles. ×128. (*F*) Same as (*E*). ×512. (*G*) Reversal after 71 days on 3 μg/ml of BUdR and 62 days on normal medium. Note piling and rounding of cells. ×128. (*H*) Reversal after 71 days on 3 μg/ml of BUdR and 41 days on normal medium containing 4 μg/ml of thymidine. ×128. (*I*) Reversal. Same as (*H*). ×512.

TABLE 1. *Effect of BUdR on tumorigenesis of melanoma clones.*

Clone	BUdR (μg/ml)	No. days after injection	No. tumors/no. mice
B559	None	16–23*	28/28
a471	1 μg/ml	72†	7/10
b471	1 μg/ml	77	1/10
c471	1 μg/ml	75	1/10
c543	2 μg/ml	79	6/17
e543	2 μg/ml	79	5/19
f543	2 μg/ml	79	3/11
a726	3 μg/ml	77	0/10
b726	3 μg/ml	77	0/10
d726	3 μg/ml	72	0/10
f726	3 μg/ml	77	0/10

* Number of days after injection with untreated cells when tumor appeared.
† Number of days after injection with treated cells that mice were maintained. See Fig. 6 for day of appearance of tumors.

melanoma cell line of clonal origin. Whereas tumors grew in all 15 of the mice injected with untreated cells of this line by day 16, they grew in only 2 of the 10 mice injected with clonally derived cells isolated in 3 μg/ml BUdR on day 28. The mice were observed for 50 days.

Incorporation of ^{3}H-BUdR into acid-insoluble material in the nuclei of the melanoma cells was verified by both autoradiography and scintillation counting.

Discussion. Low concentrations of BUdR, a thymidine analog which is incorporated into DNA, simultaneously modify malignancy and a differentiated function (pigmentation) in melanotic melanoma cells without markedly affecting the growth of the cells. These two effects are apparently not dependent on each other, as shown by the fact that malignancy alone is modified by BUdR in amelanotic melanoma cells and pigmentation alone in nonmalignant em-

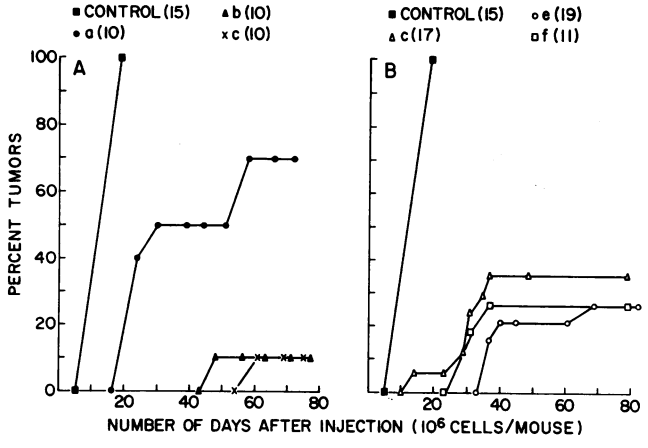

FIG. 6.—Time of appearance of tumors after subcutaneous injection of 10^6 viable clonally-derived cells into C57BL mice. Left-hand graph gives results with clones *a* 471, *b* 471, and *c* 471 isolated in BUdR medium (1 μg/ml). Right-hand graph gives results with clones *c* 543, *e* 543, and *f* 543 isolated in BUdR medium (2 μg/ml). Clones isolated in 3 μg/ml of BUdR medium produced no tumors. The numbers in parentheses indicate the number of mice injected.

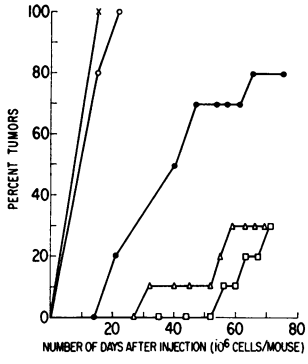

FIG. 7.—Effect of length of time of pretreatment with 3 μg BUdR/ml on tumorigenicity. Not shown on graph: 11 days—0/8 (0 tumors/8 mice); 16 days—1/10; 21 and 31 days—0/6. X, Control (0 days); O, 1 day; ●, 2 days; △, 4 days; □, 7 days.

bryonic iris epithelium. Since these changes were demonstrable in single-cell-derived clones isolated in BUdR, they were not due to selection of aberrant cells in the total population. Similar suppression of cytodifferentiation has been catalogued in a variety of other embryonic tissues, including chondrocytes and myoblasts.[1-7] Most studies with embryonic cells also note at least partial reversibility of the inhibition. Flattening of embryonic cells, similar to that described in this report, has also been observed.[1, 6]

The partial or complete loss of the ability of melanoma cells of clonal origin to form tumors, while still retaining the ability to grow *in vitro*, the acquisition of *in vitro* contact inhibition of growth, the suppression of the ability to form melanin, and the reversibility of the BUdR effects after growth in normal culture medium are all phenomena whose full significance remains to be explored. It should be noted that all functions affected by BUdR in the melanoma cells and in embryonic cells, i.e., malignancy and the various forms of cytodifferentiation, normally become fixed and heritable to their progeny cells.

The apparent universality of this suppression by BUdR of cytodifferentiation, and the concomitant suppression of malignancy lends support to the hypothesis set forth by Braun[10] and others[11, 12] that tumors are examples of anomalous differentiation, and that the cellular mechanisms that regulate differentiation should apply to tumors as well. The effects of BUdR herein described offer a tool to probe these regulatory mechanisms.

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