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Control of growth of benzo[*a*]pyrene-transformed 3T3 cells

(density-dependent regulation of growth/depletion of serum/fibroblast growth factor/initiation of DNA synthesis/serum factors)

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ABSTRACT The growth controls observed in benzo[*a*]pyrene-transformed 3T3 cells (BP3T3) are compared with those of virus-transformed and normal 3T3 cells. Superficially, the chemically transformed BP3T3 cells have the same behavior as virus-transformed SV3T3 cells. Both types of transformed cells have a low serum requirement. Both grow to high cell density in culture medium with 10% serum, both form colonies in Methocel, and both are tumorigenic. Closer examination, however, has disclosed that BP3T3 cells exhibit "normal" growth controls at low serum concentrations. In contrast to the behavior of SV3T3 cells, the initiation of DNA synthesis in BP3T3 cells is still dependent on a serum factor. If BP3T3 cells are grown in medium with 0.2% serum, the cells become quiescent, with growth arrested in the G_1 or G_0 phase of the cell cycle. The addition of serum or the fibroblast growth factor (FGF) to such quiescent cells leads to the initiation of DNA synthesis and the resumption of growth. As with normal 3T3 cells, if the growth rate of BP3T3 cells is limited by a suboptimal concentration of serum, the growth rate of the cells is increased by the addition of FGF. Also, BP3T3 cells show density-dependent regulation of growth, if the medium contains a low concentration of serum.

BP3T3 cells, therefore, have the behavior of "transformed" cells when cultured in medium with 10% serum, but behave as "normal" cells in medium with low serum. In comparison with normal 3T3 cells, the difference in growth behavior of BP3T3 cells appears to be due to a substantial decrease in the cells' requirement for a serum growth factor of the FGF type. Exploration of possible causes of this substantial decrease indicates that the primary cause is a lower rate of depletion of the serum growth factor from the culture medium by BP3T3 cells. The decrease in rate of depletion is sufficient to account for the uncontrolled growth of BP3T3 cells in medium with 10% serum. It is suggested that a decreased rate of depletion of a growth factor may contribute to tumorigenicity of cells *in vivo.*

The growth of mouse embryo 3T3 cells in cell culture is normally controlled by serum factors (1, 2). The requirement for serum factors is highest during the G_1 phase of the cell cycle, prior to the initiation of DNA synthesis (3). Once the cells are committed to initiate DNA synthesis, a much lower serum concentration is required and the lower concentration suffices for the completion of the cell cycle.

For 3T3 cells, the serum factors that are needed during G_1 to control the initiation of DNA synthesis have been studied extensively (4–7). The serum factors can be replaced, to a large extent, by known materials (4): insulin, a glucocorticoid, and the fibroblast growth factor (FGF) of Gospodarowicz (6).

Transformation of 3T3 cells to tumorigenic cells by simian virus 40 (SV40) decreases the serum requirement of the cells (8, 9). A low requirement for serum remains for survival of SV3T3 cells (10) and a somewhat higher serum concentration (approximately 0.8%) is required for maximum growth rate. However, requirements for the serum factors that control the initiation of DNA synthesis in 3T3 cells appear to be lost completely in highly transformed SV3T3 cells (8).

Abbreviations: BP3T3, benzo[*a*]pyrene-transformed 3T3 cells; SV3T3, simian-virus-40-transformed 3T3 cells; FGF, fibroblast growth factor

The present paper describes some characteristics of a benzo[*a*]pyrene-transformed 3T3 cell (BP3T3). This transformed, tumorigenic cell, obtained from Dr. Joseph DiPaolo, has a low serum requirement (11). Growth in culture medium with 2% serum is comparable to that of SV3T3 cells (11). However, as described in the present paper, the low serum requirement of BP3T3 cells, in contrast to that of SV3T3 cells, still functions in the control of the initiation of DNA synthesis. When growth is limited by inadequate serum, the growth of BP3T3 cells is arrested in the G_1 or G_0 phase of the cell cycle. The addition of serum or FGF to the quiescent cells leads to the initiation of DNA synthesis.

Since BP3T3 cells retain a requirement for a growth factor required by normal 3T3 cells, these transformed, tumorigenic cells can be used to explore some of the possible causes of a decrease in the quantitative requirement for a growth factor. The role of such a decrease in the development of tumorigenicity is also considered.

MATERIALS AND METHODS

Cell Lines. The original culture of benzo[*a*]pyrene-transformed Balb 3T3 cells was obtained from Joseph DiPaolo of the National Cancer Institute. The cells were grown in Dulbecco–Vogt modified Eagle's medium (12) with 10% calf serum in a CO_2 incubator. Flow microfluorometric analysis of the cells indicated the presence of a mixture of approximately equal numbers of cells with two different contents of DNA. Clones of each DNA content were isolated. The clone used in the experiments described in this paper (BP3T3 clone 7) had approximately the same DNA content as normal 3T3 cells. The second clone (clone 2) had approximately 1.6 times this amount of DNA. Clone 2 was indistinguishable from clone 7 in its growth properties and response to FGF. Frozen stock cells were thawed at approximately 8-week intervals. Autoradiographic analysis after [*methyl*-^{3}H]-thymidine incorporation indicated the cells were free of mycoplasma. The tumorigenicity of the cells was confirmed (11).

Normal Balb 3T3 cells were obtained from Walter Eckhart and from Joy Hochstadt. Cells were cultured in Dulbecco–Vogt modified Eagle's medium with 10% calf serum.

Autoradiography. This was carried out as described (4).

Flow Microfluorometric Analyses. The cells were stained by the mithramycin procedure (13). The stained cells were analyzed in a Los Alamos design microfluorometer (14) with an argon laser at 457 nm. The photograph obtained from the storage oscilloscope was analyzed graphically (15).

RESULTS

Serum requirement of BP3T3 cells

Oshiro and DiPaolo (11) have reported that benzo[*a*]pyrene-transformed 3T3 cells grow to a much higher cell density than normal 3T3 cells, in medium with either 2 or 10% calf serum, indicating that the transformed cells have a low serum re-

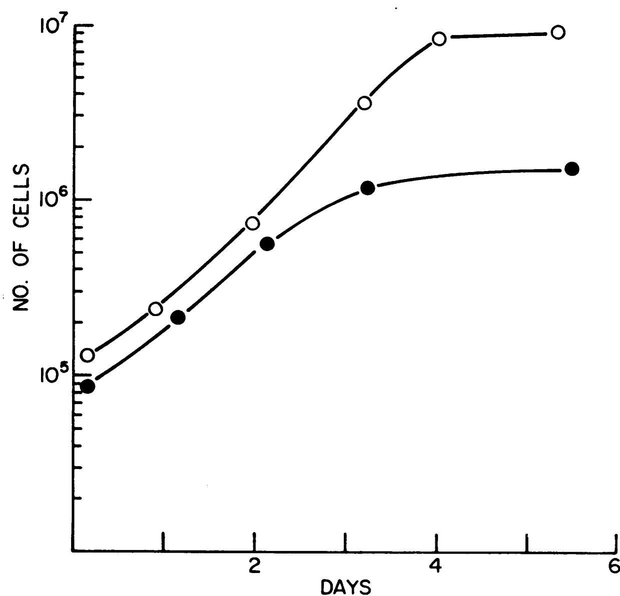

FIG. 1. Growth curves of BP3T3 (O) and 3T3 (●) cells in 5 cm plates with 5 ml of Dulbecco–Vogt modified Eagle's medium containing 10% calf serum.

quirement. We have confirmed their findings, using two clones of BP3T3 cells isolated from a culture of benzo[*a*]pyrene-transformed cells obtained from J. DiPaolo.

Data on the serum requirement of BP3T3 cells are presented in Figs. 1 and 2. Fig. 1 shows typical growth curves of BP3T3 cells and 3T3 cells in medium with 10% serum. BP3T3 cells grow to approximately six times the cell density attained by 3T3 cells. Fig. 2 shows the relationship between the initial amount of calf serum in the medium and the number of cells obtained after 5 days. The transformed BP3T3 cells have a markedly lower serum requirement than normal 3T3 cells. A similar serum requirement was observed for SV3T3 cells (also see ref. 8).

Depletion of serum factors by growth of 3T3 cells

Though both BP3T3 and SV3T3 cells have low serum requirements, the specific serum factors required by the two cell types have been found to differ. Culture medium after it has supported the growth of 3T3 cells is largely depleted of serum growth activity for 3T3 cells. This can be shown in growth assays with 3T3 cells in 0.4% serum, comparing the growth obtained after the addition of 1 or 2 ml of depleted medium with

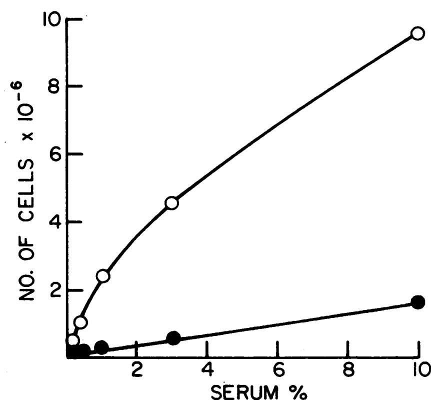

FIG. 2. Number of cells obtained from 10^5 cells in 5 cm plates after 5 days' growth in medium with various concentrations of calf serum: O, BP3T3 cells; ●, 3T3 cells.

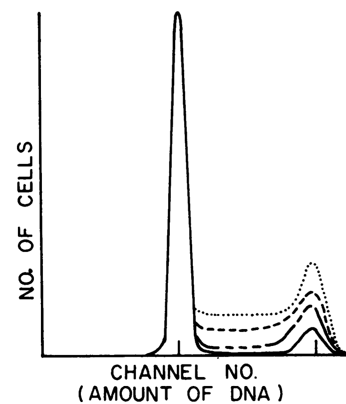

FIG. 3. Flow microfluorometric analyses on successive days of BP3T3 cells plated at 10^5 cells per 5 cm plate in Dulbecco–Vogt modified Eagle's medium with 0.2% serum and supplemented with 2% of a concentrate of additional constituents of F12 and McCoy's media (4): · · · ·, starting cells; - - -, after 24 hr; ----, after 48 hr; ——, after 72 hr. DNA content corresponds to G_1 phase (left peak), S phase (between the peaks), and G_2 phase (right peak).

the growth given by the addition of 1 or 2 ml of medium with various concentrations of fresh serum. In such an assay, medium with 10% serum, after depletion by growth of 3T3 cells, showed growth activity for 3T3 cells approximately equivalent to medium with 2% fresh serum. When the same depleted medium was used in growth assays with BP3T3 and SV3T3 cells in low serum, the depleted medium had the activity of 2% serum with BP3T3 cells but assayed as 10% serum with SV3T3 cells. This indicates that a serum factor that is depleted by the growth of 3T3 cells is required by BP3T3 cells but is not required by SV3T3 cells.

Arrest of growth of BP3T3 cells in medium with low serum

Further evidence that BP3T3 cells differ from SV3T3 cells comes from flow microfluorometric studies of the control of the cell cycle in the two types of cells. Normal 3T3 cells arrest in the G_1 or G_0 phase of the cell cycle as the growth rate is slowed by inadequate serum factors (3, 16). In contrast, SV3T3 cells remain distributed throughout the cell cycle, with approximately 50% of the cells in the S phase, even when the growth rate is slowed by a deficiency in serum factors (16). As shown in Fig. 3, BP3T3 cells show "normal" arrest of growth. They are found almost completely in the G_1 or G_0 phase of the cell cycle after 3 days of growth in medium with 0.2% serum.

Initiation of DNA synthesis in quiescent BP3T3 cells

Quiescent BP3T3 cells, obtained by culture in 0.2% serum, as in Fig. 3, initiate DNA synthesis in response to the addition of serum or FGF. This is shown in Fig. 4. Initiation of DNA synthesis is more complete and more synchronous if serum is added, but approximately 40% of the cells initiate DNA synthesis in 18 hr in response to FGF alone. Initiation is somewhat greater if a heated ammonium sulfate fraction, which potentiates DNA initiation by FGF in 3T3 cells (4), is also added. The addition of insulin and/or dexamethasone had no effect on the initiation of DNA synthesis in quiescent BP3T3 cells, though these hormones potentiate the action of FGF on quiescent 3T3 cells (4).

Addition of FGF alone, or FGF plus the heated ammonium sulfate fraction, to quiescent BP3T3 cells is not sufficient to give sustained growth of the cells, but does give approximately a doubling of the cell number.

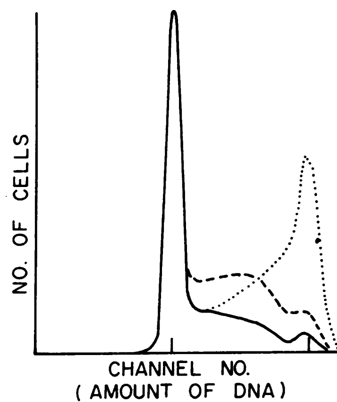

FIG. 4. Flow microfluorometric analyses of BP3T3 cells 18 hr after additions to quiescent cells (72 hr) in Fig. 3: ——, 50 ng/ml of FGF; - - -, FGF plus heated ammonium sulfate fraction (4); · · · ·, 2% calf serum.

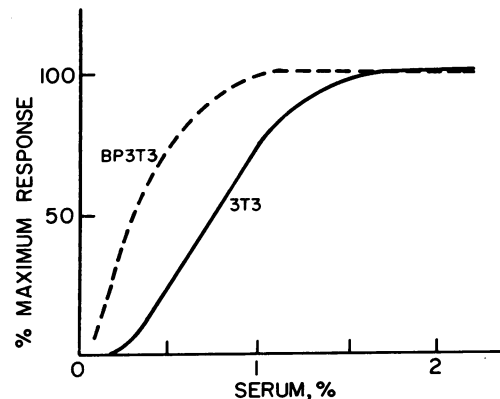

FIG. 5. Initiation of DNA synthesis in quiescent BP3T3 (- - -) and 3T3 (——) cells by various concentrations of calf serum. The quiescent BP3T3 cells were obtained as in Fig. 3. The preparation of quiescent 3T3 cells and the scintillation counting procedure were as previously described (4). Similar results were obtained, using autoradiographic procedures (4), starting with quiescent BP3T3 cells prepared as in Fig. 3 and quiescent 3T3 cells obtained after culture of 10^5 cells for 4 days in 5 ml medium with 0.5% serum.

Serum concentration required to give a doubling time of 24 hr with 3T3 and BP3T3 cells

Table 1 indicates that there is approximately a 7-fold difference between the serum concentrations required by the two cell types to give a doubling time of 24 hr. The culture medium was changed daily in these experiments, but depletion of serum factors is probably still significant (see below). Under the conditions of Table 1, FGF, added at 10 ng/ml, increased the growth rates of both cell types.

Concentrations of serum and FGF required to initiate DNA synthesis in 3T3 and BP3T3 cells

Fig. 5 compares the serum concentrations required to initiate DNA synthesis in sparse quiescent 3T3 and BP3T3 cells. In this experiment, DNA synthesis was initiated in quiescent BP3T3 cells at approximately one-third to one-half the serum concentration required by 3T3 cells. The exact serum concentration required to initiate DNA synthesis in each cell type varies with the cell density and with the previous culture history. Therefore, it is difficult to be sure that quiescent cultures of the two types are comparable. However, several experiments, under various conditions, indicate that there is a 2- to 3-fold difference between the serum concentrations required for the initiation of DNA synthesis in the two cell lines.

With FGF, the concentration required for initiation of DNA synthesis also varies with the cell density and the culture history. With relatively sparse cells, in one experiment, a half-maximal response was given by both BP3T3 and 3T3 cells at approximately 2 ng of FGF per ml. However, the "sparse" quiescent BP3T3 cell cultures were more crowded than the "sparse" quiescent 3T3 cell cultures. Quiescent 3T3 cell cultures at a higher cell density, the same cell density as the "sparse" quiescent BP3T3 cell cultures, require a fewfold higher FGF concentration. Again, the results indicate that 3T3 cells require a slightly higher concentration of growth factor to initiate DNA synthesis.

Table 1. Serum concentration required for a cell doubling time of 24 hr

Cell line	Serum alone, %	Serum plus FGF, %
BP3T3	0.2	0.1
3T3	1.5	0.6

The medium of growing cells at 10^5 cells per 5 cm dish was changed daily and the cells were counted daily for 2 days. FGF was added at 10 ng/ml of medium.

Rates of depletion of serum factors by 3T3 cells and BP3T3 cells

Table 2 summarizes data on the depletion of serum factor activity during growth of 3T3 cells and BP3T3 cells. Depletion of serum factor activity by relatively sparse, growing 3T3 cells appears to be approximately 10-fold greater than depletion by growing BP3T3 cells, for a given amount of cell growth. Because of a large difference in rates of depletion and the smaller difference in serum concentration required to initiate DNA synthesis, it seems likely that differences in rates of depletion of serum activity play a role in the results shown in Table 1.

Density-dependent regulation of growth

By two criteria, BP3T3 cells show "density-dependent regulation of growth."

First, as shown by autoradiography, quiescent BP3T3 cells, grown to confluence in 0.4% serum, without medium change, show labeling of nuclei along the edge of a "wound" in a "wound healing" type of experiment (1), when [*methyl*-^{3}H]

Table 2. Depletion of serum activity during growth of 3T3 and BP3T3 cells in medium with 1% serum.

Cell line	Serum activity of depleted medium (%)	(a) Loss of serum activity (%)	(b) Increase in no. of cells $\times 10^{-5}$	(b)/(a)
3T3	0.3	0.7	0.7	1
BP3T3	0.5	0.5	5.6	11

Medium with 1% serum was placed on growing cultures of approximately 2×10^5 cells in 5 cm dishes for 24 hr. The serum activity that remained in the depleted medium was determined in a DNA initiation assay (4) using quiescent 3T3 cells, replacing 3.5 ml of the 0.2% serum medium with 3.5 ml of depleted medium and comparing the response with that given by medium with fresh serum at various concentrations. The loss of serum activity (a) was calculated by subtracting the serum activity of the depleted medium from the original activity, 1%. Column (b) is the increase in number of cells that took place during the depletion period.

thymidine is added 20 hr after "wounding." The "wound healing" phenomenon appears to be similar to that in 3T3 cells (17), since serum factors are required.

Second, if the cells are cultured under "steady-state" conditions, in which the cells are restricted to a small coverslip placed in medium in a large culture dish and the medium is changed daily (8), BP3T3 cells become quiescent at a density of 0.85×10^5 cells per cm^2 in 0.2% serum and at a density of 1.6×10^5 cells per cm^2 in 0.4% serum.

Growth in Methocel

The colony-forming ability of BP3T3 cells in 1.2% Methocel (18) with 10% serum was approximately 20%. Under comparable conditions the colony-forming ability of SV3T3 cells was approximately 10% and of 3T3 cells was 0%.

DISCUSSION

The results indicate that chemically transformed BP3T3 cells have many of the properties of highly transformed cells. The cells show uncontrolled growth in medium with 10% serum. The cells grow at low serum concentrations and they form colonies in Methocel, a property of transformed cells that correlates with tumorigenicity (18, 19). Nevertheless, the cells retain some "normal" growth controls. In contrast to highly transformed cells such as SV3T3 cells, BP3T3 cells arrest in the G_1 or G_0 phase of the cell cycle when growth slows. In this respect BP3T3 cells behave like normal cells.

The responses of BP3T3 cells and normal 3T3 cells to serum have other similarities. In a low concentration of serum, the growth rates of both BP3T3 and 3T3 cells are increased by addition of FGF, indicating that a serum factor of the FGF type is limiting growth of both cell types. This serum factor is depleted from the medium by both 3T3 and BP3T3 cells. If the factor becomes limiting for growth, both cell types arrest in the G_1 or G_0 phase of the cell cycle. Quiescent BP3T3 and 3T3 cells, obtained by serum limitation, both reinitiate DNA synthesis in response to FGF. Both cell types show density-dependent regulation of growth, that is, both cell types show an increasing requirement for serum for growth as the cell density increases.

Though there are parallels in the way BP3T3 and 3T3 cells respond to serum factors, there is a major difference between their quantitative requirements for serum. A given amount of serum supports the growth of six to ten times as many BP3T3 cells as 3T3 cells.

The major cause of this quantitative difference seems to be a difference in the rate at which the two cell types deplete serum factors from the medium. As is shown in Table 2, there can be approximately a 10-fold difference in this rate. This difference is sufficient to account for the growth of BP3T3 cells to very high density in medium with 10% serum.

Also favoring growth of BP3T3 cells relative to 3T3 cells is the slightly lower serum concentration required to initiate DNA synthesis in BP3T3 cells. This accounts for a difference in growth behavior of the two cell types in medium with low serum, but under normal culture conditions, in medium with 10% serum, BP3T3 cell growth is largely complete before the culture medium is depleted enough to limit the growth of either cell type. Therefore, the difference in growth behavior observed in medium with 10% serum must be due primarily to differences in the rate at which the two cell types deplete the serum of growth activity rather than to the ability of BP3T3 cells to grow at lower serum concentrations.

An intriguing question is whether a lower rate of depletion of growth factors contributes significantly to the tumorigenicity of cells *in vivo*. The question cannot be answered at this time, but the present work indicates that this type of change can lead to a decrease in growth control in culture, and it seems reasonable to suggest that it could contribute to the development of tumorigenicity in some situations *in vivo*.

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