Density-Dependent Growth Adaptation Kinetics in 3T3 Cell Populations Following Sudden [Ca^{2+}] and Temperature Changes. A Comparison with SV40-3T3 Cells

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Lowered extracellular [Ca^{2+}] causes low growth rates and low stationary cell densities in 3T3 cell cultures as compared to physiological [Ca^{2+}]. Under otherwise constant conditions the extracellular [Ca^{2+}] determines a stable stationary cell density, which can be reached by increase of net cell number or decrease of net cell number, depending on cell density at the time of [Ca^{2+}] adjustment. SV40-3T3 cells do not show this [Ca^{2+}] dependency. At 39 °C 3T3 and SV40-3T3 cell populations show an increased growth rate at low cell densities as compared to cell populations at 35 °C. Approaching the stationary density the growth rate of both cell sorts is reduced faster at 39 °C than at 35 °C, leading to lower stationary cell densities at 39 °C than at 35 °C. A temperature change from 39 °C to 35 °C or in the opposite direction can affect the stationary cell density of 3T3 cell populations only if applied before reduction of growth rate by density-dependent growth-inhibiting principles has taken place.

The control of the cell number in multicellular organisms is evidently of extreme importance for their survival. This is clearly demonstrated by the fatal consequences of malign tumors, the cells of which do not respond to growth-modulating signals in the same way as do normal cells. Cell culture systems offer the possibility to study the responses of normal and tumorgenic cell populations to growth-modulating changes of environmental parameters. In earlier work [1] we reported on the occurrence of density-dependent cell death in mouse 3T3 cell cultures following sudden reductions of serum concentration in the culture medium. SV40-virus-transformed 3T3 cells did not show such a stringent control of population cell number.

In the present article we want to describe the density-dependent responses of 3T3 and SV40-3T3 cell populations to changes of environmental parameters, which are easier to control than serum. We choose to vary extracellular [Ca^{2+}] and temperature, since the quantity of these variables does not depend on cellular consumption and modification as may be the case for certain serum components. In addition, Ca^{2+} [4–15] as well as temperature [2, 3] are well known for their effects on cellular proliferation. However, to our knowledge no reports have appeared concerning the effect of cell density on the response of a cell population to variations of these parameters. It is the aim of the present work to show such interrelationships and to point to similarities and differences in this respect between 3T3 and SV40-3T3 cell populations.

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Materials and Methods

Swiss 3T3 and SV40-3T3 cells were from stocks originally supplied by Dr. M. M. Burger, Basel. They were routinely passaged 2 to 3 times weekly. 3T3 cells were always passaged at low cell densities, not exceeding 30–40% of the saturation density. Stem cultures were kept free of antibiotics. Dulbecco's Modification of Eagle's Minimal Essential Medium (Flow Lab) supplemented with various concentrations of new-born calf serum (Gibco) has been used as culture medium. Cultures were kept in incubators at 37 °C in a moistened atmosphere of 10% CO_2 in the air.

For experiments cells were seeded from stirred suspensions by means of a hand-driven gently working dispensor in order to ensure equal and uniform cell density in all culture dishes at seeding time.

Ca^{2+} concentrations were adjusted by adding appropriate amounts of ethyleneglycol-bis (aminoethyl)-tetraacetic acid (EGTA) (Sigma) to the culture medium, the [Ca^{2+}] of which had been determined by photometric titration with EGTA in the presence of murexide as described by Flaschka and Ganchoff [20] at pH 8.5 to 9.0.

Temperature experiments were performed in two identical cell culture incubators (type B5060 EC,

Heraeus), the temperature of which could be adjusted to 35 °C and 39 °C so that fluctuations in dependence of time and differences between different places in one incubator did not exceed 0.2 °C.

Cell counting was performed with a Coulter Counter as described earlier [1].

Results

[Ca^{2+}] effects

In order to document the different [Ca^{2+}] sensitivity of these two cell lines growth curves of 3T3 and SV40-3T3 cell populations are shown in Fig. 1

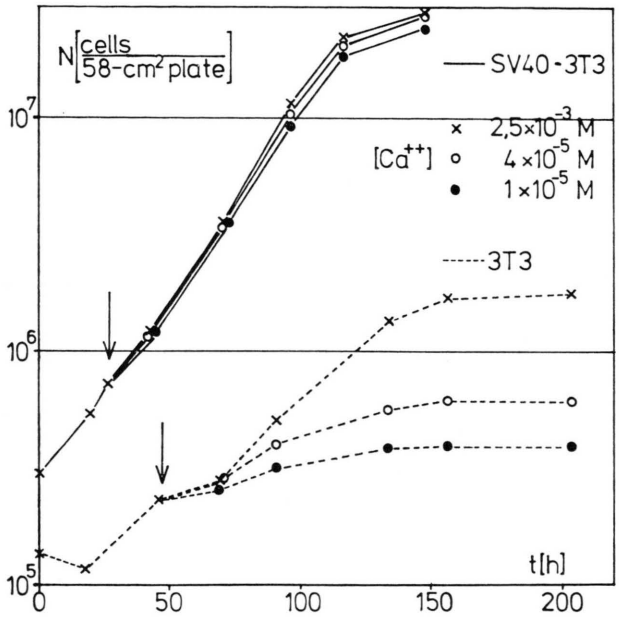

Fig. 1. Growth of 3T3 and SV40-3T3 cells at various Ca^{2+} concentrations. Cells were seeded at zero time in 10 ml medium per plate containing 10% new-born calf serum and 2.5×10^{-3} M Ca^{2+}. At the times indicated by arrows all cultures received a medium renewal with 25 ml per plate and [Ca^{2+}] adjustments as indicated.

at Ca^{2+} concentrations ranging from 2×10^{-3} M to 10^{-5} M. In this range the growth of SV40-3T3 cells does not show a significant dependence on [Ca^{2+}], whereas growth rates and saturation densities of 3T3 cells are strongly reduced at low [Ca^{2+}]. A similar difference has been reported for Balb-3T3 cells and their SV40-transformed counterparts [8].

In Fig. 2 the population development in 3T3 cell cultures has been recorded following [Ca^{2+}] changes at different cell densities. This is shown for cell populations at 10% and 2.5% serum. At fixed serum concentration the stationary cell densities are determined in these experiments largely by [Ca^{2+}]. This is obvious especially at 10% serum, whereas cell density adjustment after [Ca^{2+}] reduction at 2.5% serum is very slow, so that a new stationary state is not reached by the end of the experiment. The stationary cell density at 3×10^{-5} M Ca^{2+} is reached by net cell number increase (cell division) or net cell number decrease (cell death), depending on the cell density at which this low [Ca^{2+}] has been adjusted. Cells grown at low [Ca^{2+}] are viable. This is demonstrated by the cell number increase following a [Ca^{2+}] step up.

In the experiment shown in Fig. 3 serum concentration changes have been performed in stationary 3T3-cell cultures at high (2×10^{-3} M) and low (3×10^{-5} ff) [Ca^{2+}]. Here, the stationary cell density at fixed [Ca^{2+}] is largely determined by the serum concentration. This experiment again demonstrates that 3T3 cells at low [Ca^{2+}] remain viable and are able to respond to changes of environmental parameters (like serum concentration) by the same reactions (cell division and cell death) as at physiological [Ca^{2+}].

An important feature of the experiments shown in Fig. 2 and 3 is the fact that in every case low growth rates are coupled with low stationary cell densities and high growth rates are coupled with high sta-

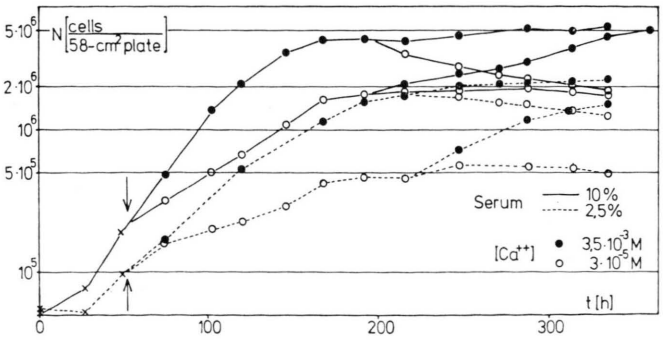

Fig. 2. Population development in 3T3 cell cultures following sudden [Ca^{2+}] changes at fixed serum concentrations. Cells were seeded at zero time in 15 ml medium per plate containing 10% or 2.5% new-born calf serum and 1.8×10^{-3} M Ca^{2+} (crosses). All cultures received daily medium changes with 15 ml per plate. At arrows [Ca^{2+}] changes were performed as indicated. After reaching stationary cell densities further [Ca^{2+}] changes were applied at both serum concentrations.

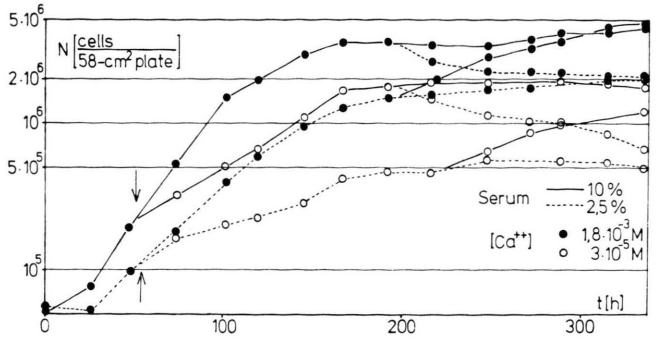

Fig. 3. Population development in 3T3 cell cultures following sudden serum-concentration changes at fixed Ca^{2+} concentrations. The experiment was performed with cultures from the same seeding as used for the experiment shown in Fig. 2. At arrows [Ca^{2+}] was reduced in part of the cultures as indicated. Serum-concentration changes were performed after stationary cell densities had been reached at both Ca^{2+} concentrations. All cultures received daily medium renewals.

tionary cell densities. In the following section it is shown that this coupling of growth rate and stationary cell density is not a stringent one.

Temperature effects

In contrast to the different sensitivity towards [Ca^{2+}] reductions (Fig. 1) a striking similarity has been observed between 3T3 and SV40-3T3 cells concerning the temperature dependency of their population kinetics. This is shown in Fig. 4. At low cell densities populations of both cell sorts grow faster at 39 °C (squares) than at 35 °C (circles). Approaching the stationary density inverse temperature effects are observed: the 39 °C curve and the 35 °C curve cross each other and so the stationary density is lower in cultures grown at 39 °C than in cultures grown at 35 °C. The serum concentration does not influence the characteristics of this temperature dependency, as can be seen by comparing the solid with the dashed curves.

Unfortunately, SV40-3T3 cells approaching the stationary state get easily detached from the culture plate. For this reason it was not possible to perform temperature-change experiments with SV40-3T3 cell cultures at higher densities. We, therefore, report in the following on temperature-change experiments with 3T3 cells, only.

During logarithmic growth a temperature increase (35 °C → 39 °C) is followed immeditely by an increase of growth rate and a temperature decrease by a decrease of growth rate (Fig. 5a and Fig. 6 first arow). The changes of growth rate are such that 1) the population experiencing a temperature drop grows slower than the control population at low temperature at equal cell density, and 2) the population experiencing a temperature rise grows faster than the control population at high temperature. If temperature changes are applied to populations in the stationary phase, no reversion occurs of stationary densities once attained at 35 °C and 39 °C (Fig. 5b, dashed curves). The same density-dependent effects of temperature changes are observed in cultures grown under large medium volume without medium renewal (Fig. 5) and in cultures receiving medium renewals every second day (Fig. 6).

The results reported here have been reproduced in several similar experiments. Quantitative variations were observed between different experiments leaving unimpaired the qualitative results stated here.

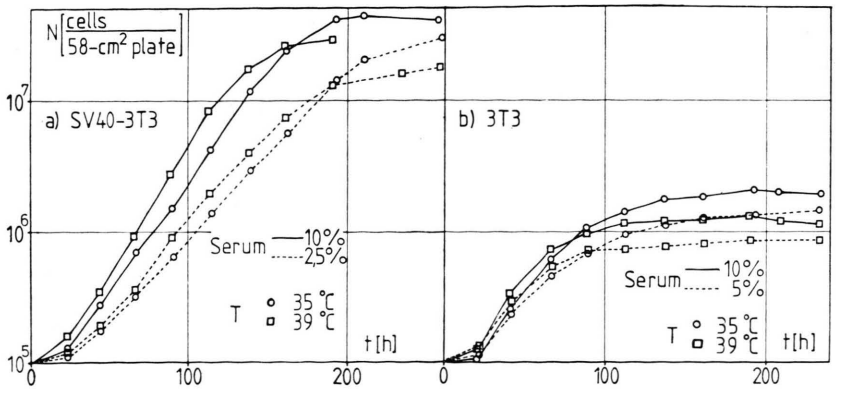

Fig. 4. Growth of SV40-3T3 cells (a) and 3T3 cells (b) at 35 °C and 39 °C at various serum concentrations. Cells were seeded at zero time in 25 ml medium per plate and grown at 35 °C (circles) and 39 °C (squares) without medium renewal.

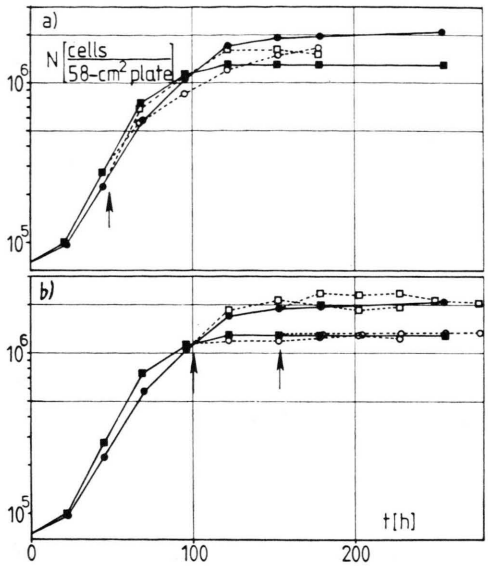

Fig. 5. Population development in 3T3 cell cultures following sudden temperature changes from 35 °C to 39 °C and vice versa. At zero time, 3T3 cells were seeded in 25 ml medium per plate and grown at 35 °C (circles) and 39 °C (squares) without medium renewal. Serum concentration: 10%. Temperature shifts (arrows) were performed from 39 °C to 35 °C and in the opposite direction during logarithmic growth (a) and in the stationary phase (b). Dashed lines: growth after temperature shifts. Squares: 39 °C. Circles: 35 °C.

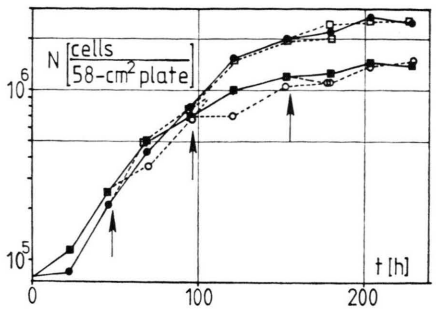

Fig. 6. Essentially the same experiment as shown in Fig. 5, but all cultures received medium renwals every second day (10 ml/plate). Arrows indicate temperature shifts.

Discussion

The regulation of stationary cell densities in cell culture probably is of considerable interest with respect to cell number control *in vivo*. The present results suggest in accordance with earlier work [1] that in stationary 3T3 cell populations cell division and cell death occur in response to changes of some environmental parameters like serum concentration and [Ca^{2+}]. These processes seem to be activated in order to adjust the stationary cell density according to the changed environment. SV40-3T3 cell populations do not display such environmental sensitivity.

In contrast temperature changes between 35 °C and 39 °C in 3T3 cell populations do not lead to a readjustment of stationary cell densities once reached. However, quite different stationary cell densities are adjusted at 35 °C and 39 °C if the growing cell populations are exposed to these temperatures before reaching a stationary state. Thus, mechanisms participating in the processes of growth and inhibition of growth seem to be influenced by temperature, whereas cells in the stationary state are not affected.

Comparing the temperature dependencies of 3T3 and SV40-3T3 cell growth (Fig. 3a, b) in the light of this reasoning suggests that during the phase of growth reduction similar temperature-dependent processes are at work in both cell sorts. The main difference seems to be the cell density at which these processes are switched on.

A far-reaching speculation concerning possible mechanisms of Ca^{2+} and temperature action does not seem appropriate here. But the reader is referred to the literature dealing with the possible involvement of cyclic nucleotides [18, 19], of Mg^{2+}-Ca^{2+} interrelationships and their effects on cellular metabolism [12, 13, 17] as well as of cell membrane permeabilities for inorganic cations and of their intracellular concentrations [14 – 16, 18].

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