Biophys Struct Mech (1982) 9:75–82

Biophysics of Structure and Mechanism

Analysis of Cellular Interactions in Density-Dependent Inhibition of 3T3 Cell Proliferation*

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Abstract. Subpopulations of different proliferative status are determined during cell-density dependent proliferation of 3T3 cells. From these data the probability of conversion of proliferative to quiescent cells is derived and found to correlate well with published data on binding of growth-inhibiting factors secreted from growth-inhibited cells.

Key words: Cellular population dynamics – Intercellular interaction – Inhibition of proliferation – 3T3 cells

Introduction

The mechanisms of cell density dependent regulation of normal cell growth and the defects of growth regulation in transformed cells, have been investigated intensively in past years but have not yet been elucidated. The most detailed studies on these problems have been done on fibroblast cell cultures in vitro, in particular on 3T3 mouse cells and their DNA-virus-transformed derivatives. The prevailing view on the nature of cell density dependent growth inhibition was put forward most clearly by Holley (1975, 1980), stating that the final cell density is determined by the availability of humoral growth-stimulating factors in the culture medium, which may be inactivated by the cells, this occurring more rapidly at high cell densities. However, there are results of fairly detailed recent studies, which are inconsistent with this notion (Vogel et al. 1980; Lieberman and Glaser 1981; Steck et al. 1982).

The major impediments of progress in this field appear to stem from the following aspects:
i) the elusive nature of the cell-derived growth-inhibitory factors,
ii) the assumption of homogeneity within the cell population with regard to the

* Based on material presented at the Symposium "Intercellular Communication" Stuttgart, September 16–17, 1982

0340-1057/82/0009/0075/$ 01.60

proliferative status, resulting in the neglect of a possible existence of subpopulations of different proliferative capacity,
iii) the failure to take into account quantitatively the ultimate effect of the multiple interactions between cells, leading to non-linear dynamics of the developing cell population.

Accordingly, closure of the existing hiatus between biochemical characterization of intercellular interactions and their ultimate consequences in the proliferation behaviour of the cells would require the characterization of the proliferative status of individual cells and the quantitative analysis of the dynamics of the resulting subpopulations of different proliferative status. The population dynamical analysis either might be based on the cellular interaction characteristics derived by biochemical investigation or conversely be applied to derive the cellular interaction characteristics from proliferative behaviour of in vitro cell preparations. Since biochemical analyses of cell derived growth effectors have been available only very recently and furthermore, are not yet very detailed quantitatively, we have used the latter approach.

We have followed the proliferative capacity of single cells during cell-density dependent inhibition of 3T3 cells, by using clonal growth analysis or flow-cytometric analysis. From the temporal evolution of the resulting subpopulations we have derived quantitatively the cellular interaction characteristics determining the conversion from proliferative to quiescent cells. These interaction characteristics correspond closely to those evaluated biochemically by work of other authors (Steck et al. 1982; Voss et al. 1982).

Materials and Methods

Swiss 3T3 cells were obtained from Flow Laboratories, Bonn. Stem cultures were grown as described in detail elsewhere (Adam and Schumann 1981). Cell density dependent inhibition of proliferation was analysed on cell populations grown under daily renewal of Dulbecco Eagle Medium, containing 5% newborn calf serum (Gibco-Biocult). In order to characterize the proliferative capacity of the cells from this "primary" population, parallel plates were growth-stimulated by replating to lower cell densities into fresh medium and thereafter, the proliferative response of the cells was determined by the following two independent methods.

For method 1) cells were reseeded to clonal cell density (about 25 cm^{-2}) on plates with an engraved 8×8 grid of squares 2×2 mm each. Under microscopical observation, the position of each cell and of its possible descendants could be identified each day after reseeding. In a histogram, the fraction of cells completing their first division after reseeding was plotted in daily time intervals. The number of cells observed in a reseeding amounts to about 100.

For method 2) cells are reseeded to cell density of about $1-2 \times 10^3$ cm^{-2} into a medium containing colchicin of a concentration (0.5 μM) arresting all dividing cells at metaphase of cell cycle. Growth-inhibited cells retain a G_1-equivalent amount of DNA, whereas the cells entering the cell cycle will eventually be

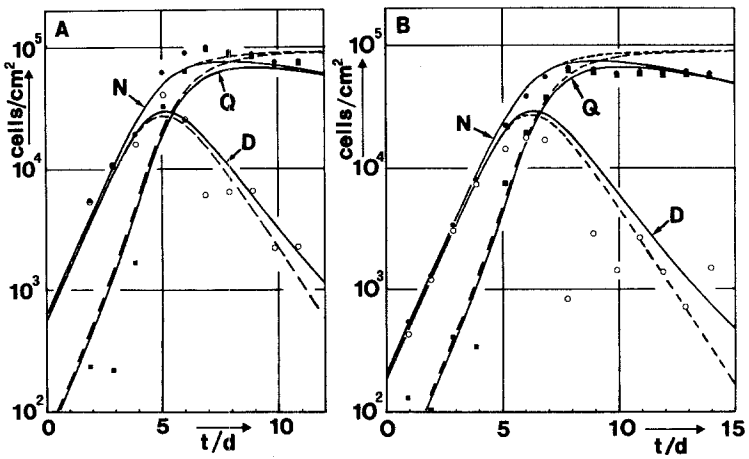

Fig. 1. Densities of cellular subpopulations versus time t of growth of 3T3 cells in medium containing 5% newborn calf serum with daily medium renewals. A and B represent two independent experiments performed more than a year apart. N is total cell density of primary population (full circles), D (open circles) and Q (full squares) are cell densities of subpopulations of proliferative and growth-inhibited cells as evaluated by microscopical analysis of the proliferative activity of 100–150 single cells reseeded from the primary population, as given in Fig. 2 for the experiment A. Full curves are drawn according to Eqs. (1), (2) and (6) as described in the text

stopped by the mitotic inhibitor with an amount of DNA equivalent to G_2-phase (stathmokinetic analysis). Histograms of cells with different amounts of DNA were measured in a flow-cytofluorometer (ICP 22 from Biophysics/Phywe), after labelling cells with the DNA-specific dye Hoechst 33258. In this method of stathmokinetic analysis several thousand cells were measured for each DNA-histogram.

Results

In Fig. 1, cell density dependent growth inhibition of the primary seeding is shown in two independent experiments A and B (full circles). At every day of primary growth, parallel plates were reseeded to low cell densities and the fraction of cells dividing on consecutive days after replating were observed microscopically. The results of this examination of proliferative response of cells from the primary population of experiment A of Fig. 1 is shown in Fig. 2. During exponential growth of the primary culture (Day 2–4), cells divide within 1 day after reseeding. However, cells taken at later times of primary growth, exhibit a progressive delay of first division after reseeding, which amounts typically to 3 days, but may be as large as 6 days. This data clearly show the emergence of long-term growth inhibited subpopulations in addition to the dividing cellular subpopulation. This result can be described quantitatively in a most simple manner, if we combine all cells requiring more than 1 day for their first division after reseeding into the fraction of "long-term growth-inhibited cells".

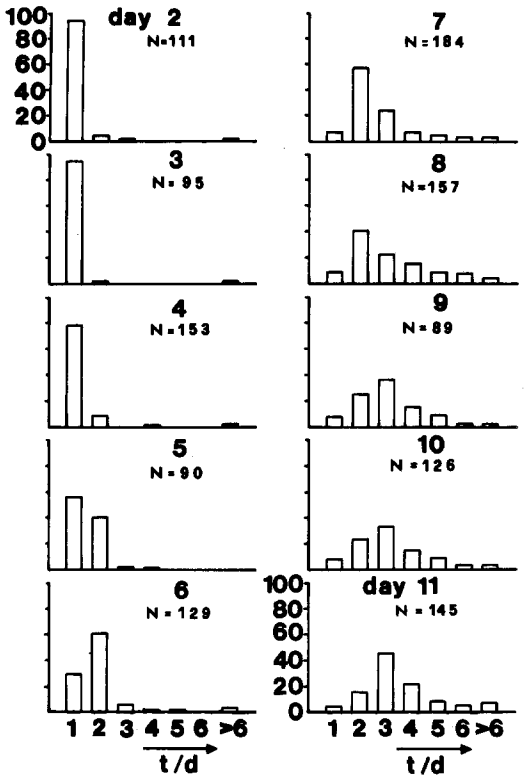

Fig. 2. Microscopical analysis of proliferative status of the primary population from Fig. 1, A: Histogram of percentage of cells completing their first division after reseeding. Subdiagrams are labelled with the time of growth in days of the primary population before reseeding. N gives the number of single cells observed in each reseeding. Abscissa is time in days after reseeding. Columns at > 6 days indicate percentage of cells which have not divided after 6 days

Multiplying this fraction with the total density N of cells yields the density Q of the long-term growth-inhibited (quiescent) cellular subpopulation (full squares in Fig. 1). Similarly, multiplying the fraction of cells performing their first division within 1 day after reseeding (i.e., within the average time of cell division in exponential growth of the primary population) with the total cell density N, we obtain the density D of the "proliferative" or "dividing" cellular subpopulation (open circles in Fig. 1).

The result of another independent experiment, using the same procedure, is shown in Fig. 1B. In this experiment, a parallel stathmokinetic analysis of the fractions of cells remaining in G_1-phase and of cells traversing the cell cycle to metaphase arrest within 1 day after reseeding has been performed on the same primary population (Steiner et al. to be published). The resulting dynamics of densities of subpopulations D and Q from this parallel experiment (not shown), does not deviate beyond statistical error from the result of the microscopical analysis, shown in Fig. 1B.

Derivation of Cellular Interaction Characteristic

The dynamics of cellular subpopulation densities D and Q are described by the following straight-forward phenomenological equations:

$$\frac{dD}{dt} = R(1 - 2g)D\,, \tag{1}$$

$$\frac{dQ}{dt} = R2gD - SQ\,, \tag{2}$$

where $R = k_b - k_d$ is the rate-constant of proliferation of D cells, given as the difference of rate-constants of cell birth k_b and cell death k_d. The rate-constant S describes random death of Q-cells. The essential term in Eqs. (1) and (2) is the rate of transition $R2gD$ of cells from the D- to the Q-subpopulation. In the "rate-constant" $R2g$ of this transition, the factor g is allowed to depend on cell density. This dependence of g on cell density will be evaluated from the experiments as follows. From Eqs. (1) and (2) we have for total cell density N

$$\frac{dN}{dt} = RD - SQ \tag{3}$$

combining Eqs. (2) and (3) results in:

$$2g = \frac{dQ}{dN}\left(1 - \frac{SQ}{RD}\right) + \frac{SQ}{RD}\,. \tag{4}$$

Up to about saturation density, cell death SQ is negligible compared to RD so that we can use as a good approximation for the rising part of the growth curve

$$2g = \frac{dQ}{dN} \cong \frac{\Delta Q}{\Delta N}\,. \tag{5}$$

Thus, we have evaluated the quotient $\Delta Q/\Delta N$ for consecutive daily time intervals, according to Eq. (5) and correlated with the logarithmic averages $Q = (Q_t \times Q_{t+1d})^{1/2}$ and $N = (N_t \times N_{t+1d})^{1/2}$. The result of this procedure applied to the two experiments of Fig. 1 is shown in Fig. 3 (open circles and squares). Here we have chosen to plot the cellular interaction function g versus the logarithmic averages Q of the "quiescent" subpopulation.

In order to test for the effect of cell death as indicated by the slow decline of N and Q at saturation densities (Fig. 1), we have used $R = 1.15\ \mathrm{d}^{-1}$ and $S = 0.15\ \mathrm{d}^{-1}$, as taken from the early part of the D-cell and the late part of the Q-cell populations, respectively (Fig. 1). Using Eq. (4), these figures on R and S and the data for D, Q and dQ/dN, the corrections in g due to cell death may be evaluated and are found to be minor in the range of cell densities used for derivation of g (Fig. 3, vertical crosses). The results on $g(Q)$, shown in Fig. 3, within experimental error are described by:

$$g(Q) = Z + (1 - Z)\frac{LQ}{1 + LQ}\,, \tag{6}$$

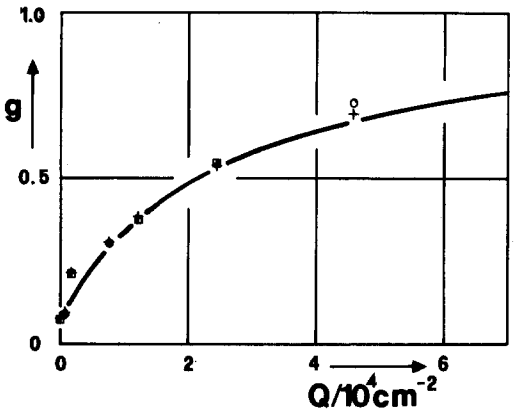

Fig. 3. Plot of cellular interaction function g versus density Q of growth inhibited cells, evaluated from experimental data shown in Fig. 1, as described in text. Open circles refer to data from Fig. 1A, open squares to data from Fig. 1B. Vertical crosses represent figures for g corrected for death of growth-inhibited cells, as described in the text. The curve is computed according to the relation $g = Z + (1 - Z)LQ/(1 + LQ)$ with $Z = 0.075$ and $L = 4 \times 10^{-5}$ cm^2 [see Eq. (6) of text]

where $Z = 0.075$ and $L = 4 \times 10^{-5}$ cm^2 (see curve in Fig. 3). As the range of cell densities used for evaluation of g was limited, it appeared of interest to check whether the functional dependence extrapolated beyond this range is able to consistently describe the entire experimental population dynamics. We have used therefore Eqs. (1), (2) and (6) (neglecting cell death SQ) with the parameters $R = 1.15$ d^{-1}, $Z = 0.075$ and $L = 4 \times 10^{-5}$ cm^2 and the initial condition $t = 0$: $Q/D = 0.1$ to describe the time course of N, D and Q in the experiments A and B (Fig. 1, broken curves). The effect of cell death SQ was determined using $S = 0.15$ d^{-1} and found noticeable albeit limited at higher cell densities (Fig. 1, full curves).

Discussion

According to the results presented, the interaction function $g(Q)$ derived from the experiments allows for a fairly detailed phenomenological description of the dynamics of subpopulations in 3T3 mouse cells. Some shortcomings, e.g. the failure of adequate description of the sharpness of attainment of saturation density, might be attributed to the neglect of any time delays, which may be expected to be required in any realistic account of the finer kinetic details.

Basically, the functional characteristic derived for g, represents a phenomenological description of cellular interactions, leading to transitions between the proliferative and the growth-inhibited cellular subpopulations. It is fitted well by the functional relation of Eq. (6). As Z is found to be very small, the essential parameter determining cellular saturation density is L.

The results of our population dynamical analysis may be compared with biochemical studies on cellular interaction during growth inhibition. Voss et al. (1982) have isolated from conditioned medium of density-inhibited 3T3 cells an inhibiting activity of molar mass of about 10^4 g/mol, which binds to 3T3 cells according to a Mechaelis-Menten-kinetics with a binding constant of $K_M = 5.8 \times 10^7$ M^{-1} and a number of binding sites of $n = 4 \times 10^5$ per cell.

According to these findings, the interaction characteristic g as derived above, may be interpreted according to the following model. Growth-inhibited (Q) cells are postulated to secrete or shed a growth-inhibiting factor at a constant rate of p molecules per day and cell. This factor accumulates during the day between two medium renewals; its concentration thus increases linearly with time t since last medium renewal:

$$c = (pQ/V)t\,, \tag{7}$$

where Q/V is the medium volume per Q-cell. The secreted factors are bound to D-cells, according to a Michealis-Menten formulation and lead to their conversion into Q-cells. Equating the binding characteristic according to this model with that resulting from the biochemical study yields

$$\frac{K_M\bar{c}}{1 + K_M\bar{c}} = \frac{LQ}{1 + LQ}\,, \tag{8}$$

where $\bar{c}$ is the effective factor concentration approximated according to Eq. (7) as:

$$\bar{c} = pQ\Delta t/(2V)\,. \tag{9}$$

From Eqs. (8) and (9) we can evaluate the rate of production p of the inhibiting factor as

$$p = \frac{2VL}{K_M\Delta t}\,. \tag{10}$$

With the parameters known for 3T3 cells, i.e., $K_M = 5.8 \times 10^7\ \mathrm{M}^{-1}$ (Voss et al. 1982), $V = 10$ ml/58 cm^2 = 0.17 cm, $L = 4 \times 10^{-5}$ cm^2, $t = 1d$ (this work), our model thus predicts a secretion rate of $p = 1.4 \times 10^8$ inhibitor molecules per day per Q-cell. This rate has been measured directly as 1 pg per 15 h per cell (Steck et al. 1982), yielding $p = 1.0 \times 10^8$ molecules per cell per day, if a molar mass of 10^4 g/mol is used. The agreement between both figures for p is startling and supports the validity of the approach followed in the present analysis.

It was thus of considerable interested to apply this procedure to cells grown in different concentrations of calf serum, which are known to lead to different saturation cell densities. The proliferative analysis resulted in the parameter L as decreasing with increasing serum concentration (Adam et al. to be published), which again is consistent with the result of inhibition by calf serum of binding of the isolated inhibiting factor(s), (Voss et al. 1982).

In SV40-virus-transformed 3T3 cells we could not detect any occurrence of long-term growth inhibited cells (Steiner et al. to be published). According to our present population dynamical model, this requires for the parameters of this cell system:
$Z \lesssim 0.01$ and $L \lesssim 10^{-5}$ cm^2, i.e., figures, that are at least smaller by a factor of $\frac{1}{5}$ than those of normal 3T3 cells. There are different possibilities as to how this

defect mya be effected. With regard to the parameter L, this could be due to deficient binding of the inhibiting factor to, and/or deficient secretion of the factor from, the transformed cells. Further work is needed to answer these questions.

Acknowledgements. We wish to thank Mrs. A. Kesper for her dedicated and skilful technical assistence. This work was supported by grants from Deutsche Forschungsgemeinschaft to Sonderforschungsbereich 138 and from Stiftung Volkswagenwerk (Schwerpunkt Synergetik).

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Accepted October 8, 1982