Cell multiplication in metazoans: Evidence for negative control of initiation in rat fibroblasts

(inhibitory factors/carcinogenesis/plating efficiency)

CARLOS SONNENSCHN AND ANA M. SOTO

Tufts University School of Medicine, Department of Anatomy and Cellular Biology, 136 Harrison Avenue, Boston, Massachusetts 02111

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ABSTRACT Experiments were performed to clarify the circumstances under which a rat fibroblast-like cell line proliferated in culture or when injected into syngeneic hosts. Rat FR3T3 and SV₄₀FR3T3 cells in culture multiply exponentially at comparable rates regardless of the horse serum concentration in the culture medium. The concentration of horse serum in the medium determines the length of the exponential phase of cell multiplication. Heat inactivation of the serum for periods as long as 24 hr does not impair the ability of the serum to sustain maximal cell multiplication rates. At low serum concentrations the exponential phase may be prolonged by refeeding the cultures daily with fresh medium. In medium supplemented with 50% fresh rat serum, FR3T3 and SV₄₀FR3T3 cells are prevented from multiplying by yet undefined component(s) in this serum. This correlates well with the lack of tumor formation by these cells when inoculated into syngeneic hosts. The evidence obtained in culture strongly suggests that the multiplication ability of established cells in culture is a dominant, constitutive character of these cells that is expressed whenever they are provided with optimal supply of nutrients and when no specific inhibitory conditions are present. Based on the data presented, we postulate that cell multiplication is a repressible function. These data also are compatible with a negative control mechanism for cell multiplication.

Cell multiplication is basic to understanding the growth of normal and malignant tissues (1, 2). Although many related cellular structures and organelles are affected during cell multiplication, it is generally accepted that the triggering of DNA synthesis is the key process in cell multiplication. In vertebrates, the most frequent models used to explore the subject have been liver regeneration, wound healing, and cell culture.

Recently, we have demonstrated that a plasma protein, α -fetoprotein, is able to prevent the multiplication of rat estrogen-sensitive cells both *in vivo* and in cell culture. These experiments indicate a definite target cell specificity even though these target cells may be placed in different organs (pituitary, mammary glands, uterus) (3-5). These results provided us with experimental bases to postulate that, in this case, cell multiplication may be regulated by means of extracellular specific inhibitors (6, 7).

Most of the experiments designed to study cell multiplication in culture use cells rendered quiescent by means of serum starvation. Clearly, these are not physiological conditions present at any stage of development in vertebrates (1, 2, 6). Even though this approach provided useful information regarding serum and plasma components, it can hardly be considered pertinent to the question of whether the initiation of cell multiplication is under a positive or negative control.

We have begun to explore the subject by using a somatic cell genetic approach. We use cells derived from an inbred strain

of rats; here, we compare their growth properties when challenged to grow (i) at different concentrations of heterologous and homologous sera and (ii) in sera of syngeneic hosts. Our interpretation of these results represents a departure from generally accepted ideas regarding cell multiplication in normal and malignant cells.

MATERIAL AND METHODS

Cells and Culture Conditions. FR3T3 cells were established from F₃₄₄ rat embryos (8). They are cloned derivatives that have a short population doubling time. They were generously supplied to us by C. D. Scher (Sidney Farber Cancer Institute, Boston, MA). Also, we used FR3T3 cells that were transformed with simian virus 40 (SV₄₀FR3T3), generously supplied by Robert Martin (National Institutes of Health, Bethesda, MD) (9). FR3T3 and SV₄₀FR3T3 cells were maintained routinely by subculturing with trypsin/EDTA solution (GIBCO) and were grown in Dulbecco's modified Eagle's medium (DME medium) plus 10% heat-inactivated horse serum [Flow Laboratories (McLean, VA) or Sterile Systems (Logan, UT)]. Fresh horse blood, graciously supplied by the Massachusetts Department of Public Health and the Boston Police Department (Boston, MA), was drawn under sterile conditions and the serum was removed 4 hr after the blood had clotted. After clearing by centrifugation and filtration through a Nalgene filter (0.45- μ m pores), the fresh horse serum was stored at -20°C. This serum was added to DME medium in various concentrations to test its nutritive value. We have also used nonfiltered fresh horse serum. Heat inactivation of these sera was at 60°C for 0.5-24 hr.

Fresh rat serum was obtained from 200- to 300-g F₃₄₄ male rats (Charles River Breeding Laboratories). Bleeding was done by inserting a capillary tube into the retroorbital venous plexus under aseptic conditions. Serum was recovered after clotting by successive centrifugations until erythrocytes were no longer apparent under the microscope. Heat inactivation was by keeping sera for 15-30 min at 60°C or 70°C.

Experimental Procedures. To test whether the exponential growth phase of these cells was being affected by the serum concentrations routinely used with DME medium, cells growing in commercially obtained 10% horse serum were trypsinized and aliquots were seeded on Costar Multiplate 3506 dishes in DME medium supplemented with 2%, 10%, 25%, and 50% horse serum. Similar experiments were done using the freshly drawn horse serum described above. We also tested the plating efficiency of FR3T3 cells previously grown in medium supplemented with different concentrations of serum (2%, 10%, and

Abbreviation: DME medium, Dulbecco's modified Eagle's minimal essential medium.

* Dr. Schmidt died in April 1981; the authors mourn the passing of an admirable man.

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50% horse serum for the week preceding plating in Costar Multiplate 3506 dishes). Colonies were scored by staining with 3% crystal violet after fixation with 10% formaldehyde and counting under a magnifying glass; an estimate of the cell number per well was obtained by measuring the DNA content per well in wells from duplicate plates.

These FR3T3 and SV₄₀FR3T3 established derivatives multiply in culture in an exponential pattern. Cell multiplication rate was determined by measuring the cell number achieved at different intervals after seeding. As expected, the pattern fitted well the equation $C_t = C_0 e^{\alpha t}$, in which C_t is the cell number at a given interval t , C_0 is cell number at the beginning of the experiment, and α is the instantaneous growth rate constant (6). DNA was measured by a fluorometric method (10).

All experiments designed to measure growth rate were initiated by plating cells in medium containing 2% horse serum; 24 hr later, the medium was replaced by the appropriate experimental one. This was considered day 0 of the experiment; 24 or 48 hr later, harvesting of the different groups began.

FR3T3 and SV₄₀FR3T3 were inoculated subcutaneously into syngeneic F₃₄₄ rats (10^6 and 10^7 cells per animal) to test tumorigenicity.

RESULTS

Heterologous Serum Concentration-Dependent Growth Pattern of FR3T3 Cells. Fig. 1A shows the growth pattern of FR3T3 cells in media supplemented with different concentrations of heat-inactivated horse serum. The lengths of the exponential portion of the growth curve, the period during which cell multiplication-related features should be measured, indicate that the replication rate was similar at the different serum concentrations tested. It is the serum concentration, however, that determines the duration of the exponential phase. Unless the measurements are made in the earlier stages of the experiments when low serum concentrations (2% or 10%) are tested, the full length of the exponential phase of cell multiplication

may be missed. Similar results were obtained in experiments comparing the cell multiplication pattern of these cells at different serum concentrations with and without daily changes of fresh medium (Fig. 1B). When FR3T3 cells were subjected to daily changes of fresh medium, the exponential phase of growth lasted longer than in similar cultures grown in the same medium from the beginning to the end of the experiment. This effect was more evident at the lower serum concentrations. These results suggest that substances present in horse serum-supplemented medium are being consumed proportionally to time and cell number in the dishes and that they seem to be the only factors that affect the length of the exponential phase. It is also apparent that the cell density achieved at the plateau stage is a function of serum concentration supplied in the medium (Fig. 1A and B; refs. 11 and 12).

We tested the effect of fresh horse serum compared to heat-inactivated commercially available horse serum. The results were similar whether the horse serum was heat-inactivated or freshly drawn [not heated or filtered (not shown)]. These results are interpreted as indicating that heat inactivation does not significantly affect the nutritive properties of the horse serum components essential for maximal exponential multiplication of these cells.

The lack of a significant inhibitory effect of increased concentrations of heterologous sera on the growth rate of the cell populations tested may be an indication that putative inhibitors of cell multiplication are species specific. Their heterologous origin would preclude a biological response in cells not belonging to the same species as the serum donor. This possibility has been tested (see below).

Plating Efficiency of FR3T3 Cells in Different Concentrations of Heterologous Serum. The effect of different concentrations of horse serum on colony size was explored. Cells exponentially growing in 2%, 10%, or 50% horse serum were plated in dishes in which the serum concentration was 2%, 10%, or 50%. Although the size of the colonies (measured as DNA

FIG. 1. (A) Growth curve of FR3T3 cells in medium supplemented with horse serum at 2% HS (▲), 10% (■), 25% (●), or 50% (◆). (B) Growth curve of FR3T3 cells in medium supplemented with horse serum at 2%, 10%, and 50% without daily changes (symbols as in A) or with daily changes (symbols as in A but open). (C) Growth curve of FR3T3 cells in medium supplemented with 20% horse serum heat inactivated for 0.5 hr (▲---▲), 3 hr (◇---◇), 15 hr (○---○), or 24 hr (△---△). (D) Growth curve of SV₄₀FR3T3 cells grown in media similar to those in A.

FIG. 2. Colony formation by FR3T3 cells. (A) Cells grown in 2% horse serum for 1 week preceding start of this experiment; (B) cells grown for 1 week in 10% horse serum; (C) cells grown for 1 week in 50% horse serum. 1, 4, and 7, seeded in 2% horse serum; 2, 5, and 8, seeded in 10% horse serum; 3, 6, and 9, seeded in 50% horse serum.

per well) varied in proportion to the serum concentration in the DME medium, the number of colonies did not vary significantly (Fig. 2). Similarly, there was a significant change in the size of the colonies depending on the serum concentration in which the FR3T3 cells were grown previous to the plating efficiency test: when cells were plated from cultures previously grown in 2% horse serum, colony sizes were smaller than when from cultures previously grown in 10% and 50% horse serum. In short, the more serum, the more cells per colony. Moreover, these results suggest that the cells grown at higher serum concentrations retain in their cytoplasm a pool of nutritive substances that they will utilize to further their multiplication capabilities when the nutrients present in the environment become exhausted. Alternatively, the cells grown at low serum concentrations are partially depleted of nutrients and they have a longer lag period of replenishment before initiating exponential growth after plating (1).

Cell Multiplication of FR3T3 Cells in Medium Supplemented with Horse Serum Heated at 60°C for Different Time Intervals. Horse serum was heat-inactivated at 60°C for 0.5, 3, 15, and 24 hr and then added to DME medium at a final concentration of 10%. When FR3T3 cells were seeded in wells containing these media the cells multiplied at similar rates regardless of the length of the heat-inactivation process (Fig. 1C). This experiment strongly suggests that the substances necessary for maximal exponential cell multiplication are thermostable. Similar experiments with 20% horse serum gave comparable results (not shown).

Cell Multiplication of SV₄₀FR3T3 Cells in Medium Supplemented with Different Concentrations of Horse Serum. As with FR3T3 cells, the growth rate of SV₄₀FR3T3 also was dependent on the serum concentration in the culture media. No differences in the slope of the exponentially growing phase could be seen while the nutritive content of the medium was sufficient to supply all cells potentially able to multiply (Fig. 1D). No significant difference in pattern was apparent when FR3T3 and SV₄₀FR3T3 cells were compared. Experiments in which the plating efficiency of these cells was tested in similar fashion as with FR3T3 cells gave comparable results (not shown).

Tumor Development After FR3T3 and SV₄₀FR3T3 Inoculation into F₃₄₄ Rats. When 10^6 and 10^7 cells of each type were injected subcutaneously into 10 adult male and female Fisher rats, no tumor had developed at the site of inoculation after 6 months of observation.

Cell Multiplication of FR3T3 and SV₄₀FR3T3 Cells in Rat Serum-Supplemented Media. Fig. 3 shows the result of ex-

FIG. 3. Inhibition of the growth of FR3T3 (A) and SV₄₀FR3T3 (B) cells by homologous serum. $\diamond$ — $\diamond$, 50% horse serum; $\bullet$ — $\bullet$, 10% horse serum; $\circ$ — $\circ$, 10% fresh F₃₄₄ rat serum; $\blacktriangle$ — $\blacktriangle$, 50% fresh rat serum; $\blacksquare$ — $\blacksquare$, 50% rat serum inactivated at 60°C; $\times$ — $\times$, 50% rat serum inactivated at 70°C; $\circ$ — $\circ$, 50% fresh rat serum for 5 days and then 50% horse serum.

periments in which fresh F₃₄₄ rat serum at 50% and 10% was used to determine the multiplication properties of FR3T3 and SV₄₀FR3T3 cells. In all experiments, a clear inhibitory effect was apparent when cells were challenged in medium supplemented with 50% rat serum. In medium supplemented with 10% rat serum, cells multiplied in a fashion not significantly different from that with 10% horse serum. When the effect of the 50% rat serum was eliminated by two successive washes with DME medium and the medium was changed to 50% horse serum, the viable cells began multiplying at a rate similar to that observed in cells grown originally in 50% horse serum. The inhibitory effect of 50% fresh rat serum was partially eliminated by heat treatment of the serum (60°C and 70°C). This pattern of increased but still limited cell multiplication compared to heterologous serum suggests that the inhibitor(s) is partially inactivated by heat. This is also the case with α -fetoprotein, an inhibitor of cell multiplication for E-sensitive cells (5). Alternatively, the inhibitory properties of the serum may be caused by the cumulative effect of several inhibitors having different physicochemical properties.

DISCUSSION

The experimental results described herein are compatible with the hypothesis that cell multiplication is a constitutive property of cells in culture and that elements in the plasma are able to regulate this constitutive cell multiplication ability (6). This negative control is substantially different from the one involving starvation due to depletion of nutrients in serum-supplemented medium or serum-free medium (1, 2). Although the chemical nature of the substances that prevent multiplication of FR3T3 cells in the animals is not yet known, it is plausible to postulate their existence because the evidence in Figs. 1 and 2 strongly suggests that these cells can multiply at a high rate in so-called optimal conditions of growth (horse serum at any of the concentrations tested). These conditions are emphasized by the apparent species-specific influences that may prevent the growth of these cells. In animals, these cells do not proliferate despite the availability of circulating nutrients (6). Moreover,

the fact that comparable mouse 3T3 cells remain viable at the site of inoculation but do not multiply in athymic mice strongly suggests that this phenomenon does not involve an obvious immunological rejection (13). Recently published data on the growth of chicken mesenchymal cells (14) as well as our previous work on the control of estrogen-sensitive cell multiplication (6, 7) favors the universality of the negative control of cell proliferation in metazoans.

In recent experiments, recloned FR3T3 cells "spontaneously" acquired, in culture, the ability to multiply when injected into F₃₄₄ rats and to form a tumor at the site of inoculation. No obvious phenotypic changes in culture have been recorded between cells that develop into a tumor and those that do not. When the cells derived from clones that produced tumors were tested for their multiplication ability in 50% rat serum they multiplied exponentially, as did those in comparable concentrations of horse serum (i.e., maximally). We cautiously interpret these results as evidence for the existence of recognition phenomena between putative serum-borne circulating inhibitors of cell multiplication and their targets in culture and animal experiments.

Arguments suggesting that the use of high concentrations of serum (>30%) in addition to generally available components (vitamins, amino acids, and salts) is toxic due to the increased concentration of ill-defined factors in such sera should now be considered cautiously. Although it may be true that some cells have difficulty in attaching to the growth surface and subsequently multiplying when the concentration of supplemental serum is >30%, this limiting physical property of the medium may be overcome by plating cells in medium containing the routinely used 10% serum-supplemented medium; once these cells are attached, switching to the higher serum concentration does not affect their viability [unpublished observations using C₂9RAP cells (15)]. These observations also cast doubts on the reliability of using plating-efficiency tests to investigate the growth-promoting properties of serum-supplemented media. It is more likely that plating-efficiency tests are useful to determine the toxicity of a given batch of heterologous serum (16). Our interpretation of events differs from that of others (17, 18) regarding the use of clonal efficiency as the best control for cell multiplication capacity.

The data presented in Figs. 1 and 2 indicate that cells will multiply as fast and as long as conditions in themselves and in their immediate environment secure the completion of a new cell cycle. In the absence of specific inhibitors, the availability of nutrients in the medium determines the length of the exponential phase whereas the growth rate remains maximal. Toward the end of a steady exponential growth period, at the time nutrients are no longer available in amount sufficient for the needs of all the cells capable of multiplying, the population doubling time will become longer. This does not necessarily mean that all cells are dividing at a slower pace; some may be multiplying at the fastest expected rate but an increasing proportion of cells are not multiplying. Alternatively, once they initiate the

multiplication process, they may fail to complete it because of lack of the necessary nutrients (1). Although cells made quiescent by nutrient deprivation seem to be stopped before entering the G₁ phase of the cell cycle ["restriction point" (19)] more data are necessary to determine whether cells will accumulate all nutrients necessary for a successful cycle traverse before the triggering of the cell multiplication process is decided or whether cells keep taking up these nutrients as the cycle proceeds (1, 2).

The cumulative data presented herein are compatible with our previously advanced postulates (6) that (i) cell multiplication is a constitutive character of cells, (ii) this character can be manipulated only through a negative control that involves the presence of specific inhibitors that are recognized by these cells in question, and (iii) the recognition process implies as well the possibility that the cells may not recognize available inhibitors. Despite the evidence presented in this paper, the putative fibroblast-specific inhibitors are yet to be identified, purified, and retested in a suitable culture system, as has been done with other inhibitors (5). This ultimately will shed more light on the nature of the control of multiplication of normal cells and the unregulated multiplication of tumor cells.

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