Proliferation of Rous sarcoma virus-infected, but not of normal, chicken fibroblasts in a medium of reduced calcium and magnesium concentration

(Samuel D. Balk*, Philip I. Polimeni†, Baldev Singh Hoon*, Dana N. LeStourgeon*, and Richard S. Mitchell*)

Departments of *Pathology and †Physiology, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada R3E 0W3

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ABSTRACT Both normal and Rous sarcoma virus-infected chicken fibroblasts proliferate actively in a culture medium containing physiological concentrations of calcium (1.2 mM) and magnesium (0.7 mM). In the presence of a physiological concentration of magnesium, reduction of the calcium concentration to 0.125 mM resulted in a significant decrease in the proliferation of the normal, but not of the neoplastic, fibroblasts. Reduction of the magnesium concentration to 0.05 mM in the presence of a physiological concentration of calcium had a similar effect. In a culture medium containing reduced concentrations of both calcium (0.20 mM) and magnesium (0.05 mM), the normal fibroblasts were maintained without proliferation, whereas the Rous sarcoma virus-infected fibroblasts continued to proliferate actively. The cytosol concentrations of ionized calcium and magnesium are known to be regulated by a balance between net passive influx and active extrusion and sequestration. On the basis of this consideration and the findings described above it can be hypothesized that: (i) Fibroblast replication is initiated when cytosolic concentrations of calcium, magnesium, or both rise above a critical level. (ii) Autonomous initiation of replication of neoplastic fibroblasts is a result of failure of cytoplasmic divalent cation homeostasis; alternatively, sarcoma virus infection may endow cells with a divalent cation-independent mechanism that bypasses an initiation mechanism that is, normally, divalent cation-dependent. (iii) Proliferation of normal fibroblasts is controlled by extracellular matrix components that interact with cell surfaces in a manner that limits the permeability of plasma membranes to divalent cations or otherwise functions to lower cytosol divalent cation concentrations.

We have argued (1-4) that the essence and defining characteristic of neoplasia is autonomous cell proliferation—i.e., proliferation that is to some degree independent of the organismal influences that control the proliferation of normal cells. While normal cells proliferate as a result of the presence of external stimuli or the absence of normal inhibitors, neoplastic cells replicate as a result of an innate and heritable change (4, 5). No differences have been found in the mechanism by which, once the process has been initiated, neoplastic and normal cells replicate (6). The replication of neoplastic cells is not, therefore, abnormal; it is the initiation of this process that occurs abnormally, inappropriately, and persistently. The phenomenon of autonomous cell proliferation that defines neoplasia is, consequently, described most exactly as follows (4): As a result of failure of cellular regulatory mechanism(s), the initiation of replication of neoplastic cells occurs with some degree of independence of the organismal controls that govern the initiation of replication of normal cells. In order, then, to understand the phenomenon of neoplasia, it is necessary to (i) identify the mechanism that initiates cell replication, and (ii) identify the failure in cellular regulatory mechanism(s) that results in initiation of replication of neoplastic cells in the absence of external stimuli (4). Invasiveness and propensity to metastasize, although clinically important, can be considered to represent secondary aspects of neoplasia occurring, as these phenomena do, with normal or hyperplastic as well as neoplastic tissues (2, 4).

We have already demonstrated that normal chicken fibroblasts are maintained in the G0 state, while Rous sarcoma virus-infected fibroblasts proliferate rapidly, in a defibrino- genated plasma-containing medium in which the ionized calcium was lowered below 10 μM with the specific calcium chelator ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (1-3). On the basis of these observations we have suggested that elevation of cytosolic ionized calcium may be involved in the initiation of fibroblast replication and that failure of cytosolic calcium homeostasis (e.g., due to leaky plasma membranes or defective mitochondrial calcium sequestration) may contribute to the autonomous initiation of replication of neoplastic fibroblasts. Our original observations have been confirmed in a number of systems of normal and neoplastic cells (7, 8); in all of these confirmatory studies calcium concentrations of 10 μM, or lower, were required to selectively arrest the proliferation of normal cells.

The extracellular ionized calcium concentration in higher animals is approximately 1000 μM, while the cytosolic ionized calcium concentration is known to be maintained by cellular extrusion and sequestration mechanisms in the range of 0.1-1.0 μM. Consideration of this substantial concentration difference makes it intuitively obvious that calcium ions have a strong tendency to enter cells passively down an electrochemical gradient. This strong tendency of calcium to move into cells forms the basis of the ion's role in excitation-response coupling, and makes the ion a legitimate candidate for a role in initiation of cell replication.

Assuming that an ion is passively distributed across the plasma membrane under the influence of the membrane potential (E_m), the ratio of cytosolic ([C_i]) to extracellular ([C_o]) concentrations of that ion is predicted by the Nernst equation,

\[ E_m = \frac{RT}{2F} \ln \frac{[C_i]}{[C_o]^z} \]

in which R is the universal gas constant, T is the absolute tem-
perature, $z$ refers to the ionic valency, $F$ is Faraday’s constant, and the activity coefficients of intracellular and extracellular ions are the same. $[\text{Mg}^{2+}]_i$ is known to be approximately 0.6 mM, while the $E_m$ of cultured diploid fibroblasts is known to be approximately $-75$ mV (9). Inserting these values into the Nernst equation reveals that $[\text{Mg}^{2+}]_j$ would exceed 300 mM were magnesium ion passively distributed. Estimates of $[\text{Mg}^{2+}]_i$ on the other hand, range from 0.1 to 3.0 mM (10, 11), indicating that a large, inwardly directed, electrochemical gradient exists for $\text{Mg}^{2+}$, as it does for $\text{Ca}^{2+}$. The presence of such a gradient implies that $[\text{Mg}^{2+}]_i$, like $[\text{Ca}^{2+}]_i$, tends to move passively into cells in response to increases in membrane permeability (12).

The fact that magnesium, like calcium, is poised to move into cells down an electrochemical gradient suggested to us that magnesium, like calcium, might have a role in the initiation of cell replication. In addition, we felt that simultaneous lowering of both calcium and magnesium in the culture medium might result in selective arrest of the proliferation of normal fibroblasts without the radical calcium lowering that is required when this ion alone is manipulated; this was desirable because lowering of external calcium below 0.1 mM is known to cause ‘leakiness’ of plasma membranes (13, 14). We present, herein, the results of these experiments.

MATERIALS AND METHODS

The basic materials and methods used in the experiments reported have been detailed previously (1-4, 15, 16). The synthetic medium (15) contained folic acid at 0.20 mg/liter. Stock cultures of normal and Rous sarcoma virus-infected chicken fibroblasts were prepared in synthetic medium plus heat-defibrinogenated chicken plasma at 5%. Cultures were incubated at 41.9°C in a humidified air/5% CO$_2$ atmosphere.

Synthetic medium plus heparin-treated chicken plasma at 5% was used for the seeding of experimental culture dishes and the performance of experiments. Heparin-treated plasma, rather than heat-defibrinogenated plasma, was used in experiments to rigorously exclude any cryptic release of putative platelet mitogens (1, 2, 17) during blood collection or any generation of thrombin, another putative mitogen (18), in the experimental culture media. Heparin-treated plasma was prepared by meticulous collection (1-4, 15, 16) of 10-ml quantities of cockerel blood into chilled, sterile, silicone-treated tubes containing 0.1 ml of heparin at 2000 units/ml. This blood (hematocrit 45-50%), with a final heparin concentration of 20 units/ml, was centrifuged by our standard method to yield plasma free of formed elements, with a final heparin concentration of approximately 40 units/ml; the heparin-treated plasma was not heat defibrinogenated. Because the complete culture medium used for experiments was 95% synthetic medium/5% plasma with heparin at 40 units/ml, the final heparin concentration in the complete medium was 2 units/ml. This final concentration of heparin was twice that required to prevent clotting of the plasma in the complete medium and half that which caused the slightest inhibition of cell proliferation; heparin, at 2 units/ml, will not complex any significant concentration of calcium (19).

The calcium concentration of the heparin-treated plasma was 2.5 mM, as we had found previously (2, 3); the magnesium concentration was 1.0 mM. A base medium for experiments was prepared by combining 95 parts of synthetic medium, without calcium or magnesium, with 5 parts of heparin-treated plasma; this yielded heparin-treated plasma-containing base medium with a calcium concentration of 0.125 mM and a magnesium concentration of 0.05 mM. CaCl$_2$ was added from 20 or 100 mM concentrates, as was MgCl$_2$.

Replicate quaternary cultures of normal and Rous sarcoma virus-infected fibroblasts for experiments were prepared by seeding, respectively, 60,000 and 40,000 cells per 35-mm culture dish. Heparin-treated plasma-containing medium with a calcium concentration of 0.25 mM and a magnesium concentration of 0.05 mM was used for seeding experimental cultures. On the day after seeding, experimental cultures were changed to test media of different calcium and magnesium concentrations. Experimental cell proliferation curves were determined over 4- or 6-day periods; culture media were changed on day 2 and on each day thereafter. Cell counts were done with a Coulter electronic cell counter; each experimental point represents the mean ± SEM of two culture dishes (Fig. 1) or of four cultures dishes (Fig. 2).

RESULTS

In a heparin-treated plasma-containing medium of physiological calcium (1.2 mM) and magnesium (0.7 mM) concentration, both normal and Rous sarcoma virus-infected fibroblasts proliferated rapidly and extensively (Fig. 1, no. 16). In medium containing 0.125 mM calcium (i.e., only the calcium included

![Graph](image-url)
in the plasma component) and a physiological concentration of magnesium, the normal fibroblasts showed a significant decrease in proliferative activity, while the neoplastic fibroblasts did not (Fig. 1, no. 13). Similarly, in a medium containing 0.05 mM magnesium (i.e., only the magnesium included in the plasma component) and a physiological concentration of calcium, proliferation of the normal fibroblasts was significantly diminished while that of the neoplastic fibroblasts was not (Fig. 1, no. 4). In medium containing 0.125 mM calcium and 0.05 mM magnesium, the normal fibroblasts underwent attrition while the neoplastic fibroblasts showed considerable proliferative activity (Fig. 1, no. 1).

In media containing intermediate concentrations of calcium and magnesium (0.125 mM ≤ Ca2+ ≤ 1.2 mM, 0.05 mM ≤ Mg2+ ≤ 0.7 mM; Fig. 1, nos. 2–15), the normal fibroblasts showed proportional decreases in proliferative rates when compared to the physiological divalent cation control (Fig. 1, no. 16); the Rous sarcoma virus-infected fibroblasts, on the other hand, showed only slight decreases in proliferative rates. In general, combinations of reduced calcium and low magnesium (e.g., Fig. 1, no. 3: 0.5 mM Ca2+, 0.05 mM Mg2+) slowed the proliferation of normal fibroblasts to the same extent as did combinations of reduced magnesium and low calcium (e.g., Fig. 1, no. 9: 0.2 mM Mg2+, 0.125 mM Ca2+); again, as noted above, the proliferation of the neoplastic fibroblasts was little affected. In particular, in medium of magnesium concentration 0.05 mM and calcium concentration 0.25 mM (Fig. 1, no. 2), the normal fibroblasts appeared to approach quiescence while the neo-plastic fibroblasts proliferated rapidly and extensively. In media of magnesium concentration 0.10 mM and calcium concentrations 0.125 mM and 0.25 mM (Fig. 1, nos. 5, and 6), the normal fibroblasts proliferated at an appreciable, albeit slow, rate; it would appear, therefore, that selective arrest of proliferation of the normal fibroblasts can be achieved by systematic calcium titration in the presence of 0.05 mM magnesium.

The results of a systematic calcium titration, in the presence of 0.05 mM magnesium, are presented in Fig. 2. It can be seen that normal fibroblasts were maintained in a quiescent state, while Rous sarcoma virus-infected fibroblasts proliferated rapidly and extensively, in a medium of calcium concentration 0.20 mM and magnesium concentration 0.05 mM; in this medium the quiescent normal fibroblasts appeared healthy and well attached to the culture surface. Lowering of the calcium concentration to 0.15 mM resulted in gradual attrition of the normal fibroblasts, whereas raising of the calcium concentration to 0.25 mM resulted in sluggish proliferative activity.

**DISCUSSION**

We have demonstrated that normal chicken fibroblasts are maintained in a quiescent state, whereas Rous sarcoma virus-infected fibroblasts proliferate rapidly and extensively, in a culture medium of reduced calcium (0.20 mM) and magnesium (0.05 mM) concentrations. Because the cytosol concentrations of both calcium and magnesium ions are known to be regulated by a balance between net passive influx and active extrusion and sequestration, our observation suggests the following hypotheses:

(i) *Fibroblast replication is initiated when cytosolic concentrations of ionized calcium, magnesium, or both rise above a critical level.* Our observation that appreciable proliferation of the normal fibroblasts occurs in physiological-calcium (1.2 mM)/low-magnesium (0.05 mM) medium or in physiological-magnesium (0.70 mM)/low-calcium (0.125 mM) medium suggests that these two ions may function via a common mechanism in the initiation of cell replication, rather than by each ion activating distinct and complementary mechanisms. In view of the existence of biological systems in which one of these divalent cations modulates a regulatory activity of the other (20, 21), the possibility can be entertained that such a relationship exists in our system. Within the framework of the present hypothesis, extracellular agents that control cell replication can be thought to do so by influencing the cytoplasmic ion activities of calcium, magnesium, or both; e.g., a mitogenic hormone might function by increasing the permeability of the plasma membrane to divalent cations ("opening channels").

(ii) *The autonomous initiation of replication of neoplastic fibroblasts is the result of a failure of cellular divalent cation homeostasis or of the presence in neoplastic cells of a divalent cation-independent initiation mechanism that is divalent cation-dependent in normal cells.* Cytosolic concentrations of divalent cations might inappropriately and persistently rise above critical levels in neoplastic cells because of abnormal permeability of plasma membranes, failure in active extrusion, or failure of intracellular sequestration (mitochondrial or endoplasmic reticulum) or binding mechanisms. An increased magnitude of the membrane potential (Em) of neoplastic cells, as compared to normal cells, would, as predicted by the Nernst equation, steepen the electrochemical gradients that are responsible for divalent cation entry. An increase in intracellular sodium ion concentration, due either to abnormal permeability of the plasma membrane to sodium or to failure of the plasma membrane Na+,K+-ATPase, might elevate the cytosolic calcium ion concentration by compromising the Ca2+/Na+ exchange mechanism (22) that extrudes Ca2+ from cells by using energy provided by the transmembrane Na+ gradient.

![Figure 2](image-url)
Alternatively, the possibility can be considered that neoplastic cells are endowed with a divalent cation-independent initiation mechanism that is divalent cation-dependent in normal cells; initiation of replication of neoplastic cells would, thereby, occur in spite of maintenance of normal cytosolic divalent cation concentrations.

We have observed that normal fibroblasts undergo attrition, while Rous sarcoma virus-infected fibroblasts proliferate appreciably, in medium containing the lowest calcium and magnesium levels (0.125 mM Ca\(^{2+}\); 0.05 mM Mg\(^{2+}\)). This observation lends support to the concept that failed cellular divalent cation homeostasis is responsible for autonomy, rather than bypass of a normally divalent cation-dependent mechanism. We so reason because the latter formulation predicts that manipulation of external divalent cation concentrations would have similar effects on cytosolic divalent cation concentrations in both normal and neoplastic cells; a combination of external calcium and magnesium concentrations low enough to cause attrition of the normal fibroblasts would, therefore, be expected to have a similar effect on the Rous sarcoma virus-infected fibroblasts. Nonetheless, it can be speculated that an avian sarcoma virus-coded, transformation-specific protein, such as the src protein pp 60\(^{src}\) (23), might represent a divalent cation-independent substitute for a normal cell protein, such as the src protein pp 60\(^{src}\) (23), whose activity might be divalent cation dependent.

(iii) Proliferation of normal cells is controlled at the tissue level by interactions of cells with interstitial matrix components that limit the permeability of plasma membranes to divalent cations or act by otherwise lowering cytosolic divalent cation activities. Such matrix components, be they soluble or insoluble, might thus function as negative feedback inhibitors of cell proliferation. The possibility must be entertained, however, that the reduction of external calcium and magnesium that selectively arrests the proliferation of normal fibroblasts in vitro merely functions to compensate for an increased plasma membrane permeability that is an artefact of the cell culture system. Within the framework of this latter possibility, we would argue that infection with Rous sarcoma virus may cause a further increase in membrane permeability, above and beyond any permeability increase imposed by artificial culture conditions.

It can be noted that the calcium concentration (0.20 mM) of the reduced divalent cation medium that we have used to selectively arrest the proliferation of normal fibroblasts exceeds that calcium concentration (0.1 mM) below which plasma membranes are known to become "leaky." It should also be stressed that the quiescence of normal fibroblasts in the reduced divalent cation medium does not involve the phenomenon (24) of "density-dependent inhibition," because these cultures are maintained at low cell densities and the medium is changed daily. Similarly, the divalent cation-independent proliferation of the sarcoma virus-infected fibroblasts does not involve any of the phenomena (2, 25, 26) referred to as "transformation." Serious questions can, in fact, be raised concerning the relevance of the phenomena of "density-dependent inhibition" and "transformation" to an understanding of the essential qualities of the neoplastic state (2, 25–27).

It has been argued that intracellular magnesium ion concentration is the sole factor responsible for the coordinate control of cell growth and that the effects of experimental calcium manipulation on cell growth are exerted via magnesium and are biologically insignificant (28). The work on which this argument is based, however, was done with normal chicken fibroblasts only, rather than both normal and neoplastic fibroblasts, and was done with a limiting (1%), rather than a sufficient, concentration of chicken serum. There is, as yet, no definitive evidence as to whether calcium and magnesium ions have equally important roles in the initiation of cell replication or whether one or the other ion has a dominant role. We feel, nonetheless, that our observations strongly suggest that divalent cations have a significant role in the initiation of cell replication and that failure of cellular divalent cation homeostasis or bypass of a divalent cation-dependent initiation mechanism is responsible for the neoplastic state.

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