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Observations of Self-induced Mitosis and Autosynchrony in Sarcoma Cell Networks

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SUMMARY

Observations of groups of mouse sarcoma cells (L-929 clone) *in vitro* by means of time-lapse cinephotography using micro lagoons have revealed that the cells become linked into syncytial networks by intercellular bridges. These bridges are formed both by incomplete cytokinesis of dividing cells and by external merger of surface pseudopodia. In such networks the division of any one cell was seen to initiate a mitotic stimulus which traveled outward via the bridge connections to neighboring cells and induced them to divide. A chain reaction of self-induced mitoses spreading sequentially outward from the initiating cell was thus established. Proof of the cytoplasmic continuity of the bridges was demonstrated by direct hypotonic coalescence of connected cells through the bridge channel. Although insufficient data are presently available regarding the actual involvement of such bridges in malignant tumors, the observed process of mitotic induction through cell bridges clearly constitutes, in principle, a potential mechanism for cancerous proliferation *in vivo*. Cited observations on the natural and induced formation of giant cells by coalescence of connected cells in malignant cultures also offer a possible explanation of the source of giant cells characteristically found in malignant tissues.

INTRODUCTION

The occurrence of intercellular bridges or cytoplasmic connections between cells has been observed previously in a number of biologic systems. By virtue of these bridges, the connected cells form an extended syncytium in which the member cells retain their basic individuality, yet act together synchronously in such processes as metabolic syntheses and differentiation. A significant example of such syncytia is found in animal spermiogenesis, as described in detail by Fawcett *et al.* (5). It was found that in such widely different systems as the hydra, fruit fly, pigeon, rat, and man, all spermatids deriving from the same spermatogonium remain connected by cytoplasmic bridges through all stages of development, and through these bridges each step in the differentiation into mature sperm is precisely synchronized. The same process was also observed in the development of ectodermal cnidoblasts arising from interstitial cells in hydra (5). In both these cases, bridge formation resulted from incomplete cytokinesis of daughter cells caused by failure of the mitotic spindle to completely dissolve until some time after telophase.

The present paper reports a series of observations on the formation of similar intercellular bridges and syncytia among mouse sarcoma cells *in vitro*. In the case of these malignant cells, however, time-lapse film studies to be described have revealed that not only do very large groups of cells become connected by bridges, but also that division of just one member-cell acts to induce division of the entire syncytium through the apparent spread of some type of mitotic stimulus throughout the network. In this way groups of connected cells become mitotically autosynchronized. Bridge formation between these cells has been observed to arise from external merger of pseudopods of neighboring cells, as well as by incomplete cytokinesis.

In initial observations (2) such stable bridge formations were found to occur in malignant-cell cultures but not in cultures of normal cells. Bendich *et al.* (1) have likewise found that stable intercellular bridges are a characteristic of virus- and carcinogen-transformed cells. These findings, of course, raise the question of the possible involvement of intercellular bridges in the processes of malignancy. Although few specific data presently exist regarding the occurrence of cytoplasmic bridges in malignant tumors *in vivo*, it is suggested herein how such connections could, at least in principle, constitute a basic mechanism of cancerous proliferation in the animal body. Likewise, it is suggested that such cytoplasmic bridges can offer a possible explanation, in light of the observations of Fawcett *et al.* (5), as to the origin and mechanism of formation of the macronucleate and multinucleate giant cells so characteristic of many malignant tissues.

MATERIALS AND METHODS

The study consisted primarily of observations by time-lapse cinephotography of malignant mouse fibroblasts in culture. For all film studies, the cells (L-929 clone) were maintained in micro lagoons formed on the inner surface of a Rose-type perfusion chamber. Details of the author's micro lagoon technic for cell culture and its use in cinephotography are given in Ref. 4. The method consists in the formation of tiny ponds or lagoons of micro dimensions within a thin layer of grease deposited on the surface of the culture chamber and in the inoculation of these lagoons with the proper density of cells. Such a lagoon is illustrated in Fig. 1. The initial cell density was in most cases from 5 to 8 cells per lagoon. The lagoon walls serve to confine the movement of test cells to the time-lapse field and

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hence allow uninterrupted observations of the same cells for long periods of time. Time-lapse observations were made with both phase and brightfield microscopy using a Zeiss WL Standard microscope in combination with a Sage Series-100, Model-088 time-lapse unit. Most film records were made at one frame per minute, a rate quite adequate to record all cell movements in fine detail.

Subsequent analysis of the films was made using an LW Flickerless Photoanalyzer Projector, which allows a wide range of control over the time-scale of the projected cell motion, and a Vanguard Motion Analyzer, which allows precise measurement of cell dimensions and motion velocities.

RESULTS

Cultures of L-line mouse sarcoma cells are generally characterized by extensive networks of connecting processes (Fig. 2). Cell fields having an initially sparse number density can be followed easily by time-lapse photography, and the formation, movements, and interactions of individual processes and cells can be observed in detail as the culture proliferates. The following observations were made using such technics with L-cell populations confined to micro lagoons. Thirty separate lagoon fields were filmed and analyzed in the course of this study. All these fields demonstrated essentially the same behavior in regard to bridge formation and mitotic synchrony.

Intercellular Bridge Formation. Bridge formation between L-cells in relatively sparse fields was seen to take place by two different mechanisms. In the first type, bridges arose as a result of incomplete cytokinesis following mitosis. In the present observations, all mitoses observed in sparse fields terminated in incomplete cytokinesis, although an occasional fusion by teloreduplication of the type described by Moorhead and Hsu (6) was observed. Subsequent movement of the daughter cells tightly stretched the resulting bridge, which in some cases ultimately snapped (Fig. 3). In small lagoons where cell movement was restricted, however, the bridges usually remained intact, and chains of connected cells were formed by subsequent divisions.

In the second mode of formation, isolated cells were observed to acquire long, thin processes which arose from an initial attachment of a small pseudopod to a point on the culturing surface. The tips of such processes were extremely active, with rapid extension and withdrawal of many micropseudopodia. When these active tips encountered either the body surface or pseudopod of another cell, an apparent merger of the contact surfaces often occurred and a strong bridge was formed. From observations to be discussed, the bridges resulting from both mechanisms of formation appeared to be continuous, open cytoplasmic channels connecting the cells. By means of such bridges, appreciable numbers of cells became interconnected into syncytial networks as the population proliferated (Fig. 4).

Mitotic Induction and Autosynchrony. Mitoses in newly prepared, initially sparse fields of unconnected cells were random in occurrence. However, when bridges began to appear as a result of incomplete cytokinesis, it was observed that mitosis of one cell of a connected pair was followed, at a time interval essentially proportional in all cases to the length of the connecting bridge, by mitosis of the second cell. This phenomenon was observed in dozens of cell pairs and suggested that perhaps division of the second cell was being triggered by a stimulus from the first, transmitted via the bridge.

As larger numbers of cells (5 to 10) became interconnected, it was observed that entry into mitosis of any one cell in the network was followed in rapid succession by all other cells of the group, with mitoses always spreading *sequentially* outward from the initially dividing cell. Cells which were not connected with the dividing group did not divide, in general, when the group divided. This precise sequential spread of mitoses again strongly suggested that a mitotic stimulus was being transmitted through the network via the connecting bridges. Fig. 4 shows a typical autosynchronously dividing cell group in a micro lagoon; the time-lapse sequence illustrated is representative of all 23 synchronous group divisions filmed. The direct sequential relationship of mitoses was made especially apparent by the accelerated time-scale of time-lapse projection.

That some type of stimulus was indeed involved in the observed chain-reaction of mitoses in such networks was subsequently clearly indicated by the fact that when individual cells which had not divided for a time equal to one or more cycle periods became incorporated into a mitotically active syncytium of other cells, each divided in its proper sequential order according to the position it had assumed in the network relative to the "initiator" cell.

Tune-lapse measurements of 32 bridge connections revealed that the mitotic stimulus traveled at approximately 6 microns per minute for long, thin bridges; this is the average speed based on bridge length and elapsed time between the full roundup of the first dividing cell and the development of the first onset of mitotic roundup in the second. This speed suggests that the stimulus is possibly transmitted by electrodiffusional processes. Measurements of interior electrical potential of connected cell pairs using a microelectrode revealed that the potential was always the same in both cells prior to roundup, but that an appreciable potential gradient developed in the bridge when one cell of a connected pair suddenly rounded up for division.

Another pertinent set of observations clearly indicating the existence of a positive stimulus propagation through bridges involved the stimulation by dividing cells of distant masses of enucleate cytoplasm. In several cases, a cell within a connected group was seen to be linked by a long brige to an enucleate mass of cytoplasm. When the cell rounded up for division due to the usual sequential mitotic stimulation within the syncytium, the cytoplasmic mass was observed shortly after to fully round up in a manner almost identical to a true cell entering division, even though the mass was totally enucleate and only about half the size of a normal cell. These observations again clearly imply the existence of an appreciable stimulus, since it is scarcely to be expected that an enucleate mass of isolated cytoplasm would round up for "mitosis" without substantial external influence.

In addition to this direct evidence, it should be noted that the statistical probability of observing sequential mitotic events of the type seen within connected cell groups without the existence of a propagating stimulus is vanishingly small. For example,

assuming that mitoses of the seven cells in Fig. 4 are entirely independent, even though they are connected by bridges, there are 7! or 5,040 possible and equally probable divisional sequences. Assuming that a stimulus is active, however, and assigning the order of mitosis according to the total bridge length of each cell relative to the initially dividing cell (Cell 1), there is only one possible sequence once Cell 1 has been designated as the initiating cell, namely, the sequence actually observed. The probability that this particular sequence would have occurred without the presence of a mitotic stimulus is only one in 2,520 or 0.00036. In reality, this is a maximum probability; if all factors involved (such as time intervals of sequential divisions and sequences involving many more cells) are considered, the probability that the actually observed sequences would occur without a stimulus mechanism becomes vanishingly small indeed.

DISCUSSION

Bridge Formation Mechanics. In view of Fawcett's observations of bridge ultrastructure in sperm (5), it might be expected that the intercellular bridges formed by incomplete cytokinesis in L-cell cultures are also continuous cytoplasmic channels; this is indeed now known to be the case even though specific electron microscope demonstrations of this fact are not yet available. It seems equally likely from their basic morphologic similarity and stimulus-transmission effectiveness that bridges formed by external merger are also open channels and not merely extensions terminating in a close membrane abutment. The fact that the bridges are open channels has been demonstrated by the present author in the following way. Bridge-connected pairs of cells in culture were subjected to a medium with rapidly increasing hypotonicity in a perfusion chamber by injection of water, and the subsequent cell swelling was recorded by time-lapse (Fig. 5). Upon reaching a large distention where the bridge was reduced to essentially zero length, the cells suddenly coalesced into a single large binucleate cell by expansion of the remaining bridge segment, thus showing the bridge tubules to be continuous and open. In many cases, the swelling in one cell occurred more rapidly than in the other, and cytoplasmic material was clearly seen to flow through the bridge interior from the larger cell into the smaller one. Using a micropipet for local hypotonic swelling, whole chains of cells have been successfully coalesced into a single multinucleate giant cell.

Regarding external bridge formation, a quite plausible mechanism is suggested by the well-established ontogeny of phagocytosis. As illustrated in Chart 1, the membrane merger processes involved in a classical phagocytosis provide precisely the mechanisms required for open-tubule formation between cells. The plausibility of this suggested mechanism is made even stronger by the fact that most malignant cells in culture are exceptionally phagocytotically active (7). This activity is no doubt associated with the fact that the surfaces of malignant cells are highly aberrant and may indicate the absence of the stabilizing carbohydrate polymer coat thought to cover the cell membrane of normal cells.

Self-induced Mitosis and Autosynchrony. It is only to be

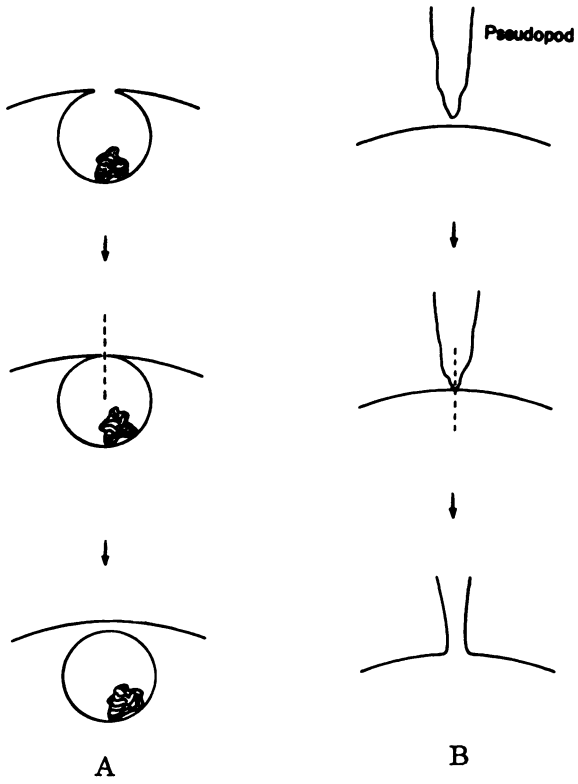

Chart 1. A possible mechanism of external bridge formation. (*a*) Membrane dynamics in phagocytosis. (*b*) Comparative hypothetical action in pseudopod merger with cell surface.

expected that cells connected by true cytoplasmic bridges will exhibit the observed properties of self-induced mitosis and autosynchrony described above for L-cells. For, if connected cells for some reason depart from a common equilibrium state, the resulting molecular diffusion gradients in the bridges will result in metabolite flows which will tend to restore all cells to the same metabolic state. In particular, synthesis precursors for DNA and other mitotic preparatory activities will become equalized in concentration throughout the syncytium. When one cell subsequently enters mitosis, the influence of the initiating conditions will be propagated to the second cell and activate it mitotically. Extended syncytia which form by cytokinesis will automatically maintain metabolic equilibrium by this means at all times. Cells which become connected by external merger may initially be out of metabolic synchrony but will tend to become synchronized by the above processes provided they are not too grossly out of equilibrium initially. It is possible, of course, that only cells in approximately the same metabolic state will be successful in forming external bridges.

The rates at which diffusional flows between cells take place depend upon the length and cross-sectional area of the bridge. Longer bridges require longer times for transmission of mitotic influences by diffusion. The significance of this effect is clearly demonstrated by L-cell groups possessing relatively long bridge connections. Here mitosis is observed to be parasynchronous due to the measurable time lag between onset of mitosis in one

cell and initiation in the second. However, in large groups of connected cells, many such "initial" divisions occur at random throughout the group, and this effect acts to compensate for the stimulus propagation lag and to ensure a more truly synchronous division of the group.

These effects are characteristic only of groups in which all cells are interconnected by a common bridge network. In very large cell populations, there are many such "local" syncytia, each dividing synchronously within itself, but randomly out of phase with one another; the proliferation of the population as a whole consequently appears to be quite asynchronous. This condition is graphically illustrated in large cultures of L-cells wherein the cell number growth in small areas of the culture as determined by time-lapse records takes place in discrete step-increases or bursts, while the growth function for the whole population follows a smoothly continuous logarithmic curve.

Intercellular Bridges as a Potential Mechanism of Cancer. The fact that the observed phenomenon of mitotic-induction was discovered in a cell line which is highly malignant *in vivo*, of course, raises the important question of its possible significance as a mechanism of cancerous proliferation in tumors. While few useful data presently exist for validating either the presence or absence of intercellular bridges in actual malignant tumors, it is clear that the self-exciting mitotic action associated with such bridges does constitute, in principle, an effective potential mechanism of cancerous growth. Bridge networks ensure the division of large numbers of cells as a result of one cell entering mitosis spontaneously, and the probability that at least one cell in a large group will divide spontaneously is also large. Thus, if large groups of networks were maintained in a tumor mass, the continued forced division of massive numbers of cells would be guaranteed.

A cancer mechanism such as this, it should be noted, is fundamentally different from that implied in the usual definition of the cancer cell as one which has escaped from or lost its capacity to respond individually to mitotic regulatory controls. In contrast, bridge networks are highly organized and act to regulate and to ensure division in a positive fashion as a result of mass propagation of the mitotic stimulus from a few randomly dividing cells.

While Bendich *et al.* (1) were unable to discern any bridge structures in a histologic section of a hamster tumor arising from a malignant transplant, the present author has obtained positive identifications of bridges in two living-cell specimens of human carcinoma (4). In both these specimens (an adenocarcinoma of the colon and a squamous-cell carcinoma of the external ear), hypotonic swelling by the technics described herein for L-cells revealed the presence of many cytoplasmic connections in masses of living cells. In view of the present observations of mitotic stimulation through intercellular bridges and the suggested potential of this process for support of malignant proliferation *in vivo*, it would appear highly desirable to clearly ascertain by a much more intensive study whether such bridges exist generally in any forms of human cancer. Also in this regard, it is of interest to note the macro- and multinucleate characteristic presence of giant cells in both cultures and tumors of malignant cells (Fig. 6). Considering the observations of Fawcett *et al.* (5) on the formation of giant cells by coalescence and the present observations and production of similar giants in L-cell cultures, the presence of numerous giant cells in actual tumors clearly suggests the possible involvement of intercellular bridges in malignant proliferation *in vivo*.

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Fig. 1. Typical micro lagoon field of silicone grease. Calibration: 60 microns.

Fig. 2. A network of mouse sarcoma cells (L-929 clone) showing cytoplasmic bridges.

Fig. 3. Bridge connections formed between cells by incomplete cytokinesis. The bridge of the bottom cell pair has just broken.

Fig. 4. The sequential development of mitosis in bridge-connected L-cells in a micro lagoon. Reproduced from a time-lapse movie film. The symbol t denotes the elapsed times in minutes between each individual frame and the initial frame ($t = 0$). Cells 1 through 5 divided almost simultaneously, while Cells 6 and 7, having longer bridge connections with Cell 1, required correspondingly longer times.

Fig. 5. Sequences from time-lapse film illustrating coalescence of connected cells through bridge channels by hypotonic swelling.

Fig. 6. A typical multinucleate giant cell occurring in a culture of L-929 clone fibroblasts.