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Cell-Cell Contact and Growth Regulation of Pinocytosis in 3T3 Cells

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In sub-confluent cultures of Balb/c-3T3 cells, pinocytosis rates were increased after exposure to specific growth factors (serum; platelet-derived growth factor, PDGF; epidermal growth factor, EGF). Conversely, as cells became growth-inhibited with increasing culture density, there was a corresponding decline in pinocytosis rate per cell. In order to test whether density-inhibition of pinocytosis was influenced either by the growth cycle or by cell contact independently of growth, cells were induced into a quiescent state at a range of subconfluent and confluent densities. Under such conditions, cell density did not significantly inhibit pinocytosis rate. When confluent quiescent cultures in 2.5% serum were exposed to 10% serum, the resulting round of DNA synthesis was accompanied by enhanced pinocytosis per cell, even though the cells were in contact with one another. Furthermore, in a SV40-viral transformed 3T3 cell line, both the growth fraction and the pinocytosis rate per cell remained unchanged over a wide range of culture densities. These studies indicate that density-dependent inhibition of pinocytosis in 3T3 cells appears to be secondary to growth-inhibition rather than to any direct physical effects of cell–cell contact.

Key words: pinocytosis, cell density, growth control, growth factors

The rate of fluid endocytosis (fluid pinocytosis) in a number of non-transformed and transformed cells in culture is related to the growth cycle [1–5]. We are particularly interested in vascular endothelium, where endocytosis is associated with transendothelial transport of molecules [6, 7] and where focal areas of arterial endothelial cell proliferation have been detected in vivo [8, 9]. Such areas are associated with increased permeability to a number of macromolecules [10, 11] and may be sites that are predisposed to atherogenesis. Recently, density-inhibition of arterial endothelial cell growth was demonstrated to correlate with decreased rates of pinocytosis in vitro [1, 4, 15]. In the case of density-inhibition of a membrane-associated event such as pinocytosis, however, the question arises as to whether cell–cell contact exerts direct influence upon pinocytosis independently of cell growth. This may be of importance with respect to endothelium, because in vitro removal of single cells results in spreading of adjacent cells to fill the "wound" without a round of

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cell division [12]. Thus, cell–cell contact is reestablished without growth. It is therefore of interest to test whether pinocytosis is stimulated by loss of cell–cell contact independently of growth. Unfortunately endothelial cells cannot be induced into a quiescent state (arrested in G_0/G_1) in the absence of known growth factors at sparse densities. The hypothesis that cell contact in the absence of growth can mediate pinocytosis was therefore tested in Balb/c-3T3 cells, a monolayer-forming, density inhibited cell line that, unlike endothelium, is responsive to growth factors. These experiments, conducted with quiescent serum-stimulated and SV40 viral transformed 3T3 cells, have led to the conclusions that cell contact in growth-arrested cells does not influence pinocytosis rate and that differences in pinocytosis rates at various cell densities are secondary to density-dependent inhibition of growth.

MATERIALS AND METHODS

Cell Culture

Mouse Balb/3T3, clone A31 (Balb/c) were obtained from the American Type Culture Collection and were maintained by serial passage. The culture medium was Dulbecco-Vogt's modification of Eagle's basal medium supplemented with 10% calf serum. For the studies with quiescent cells, cultures were trypsinised and plated at appropriate densities in 5% calf serum. After the cells were adherent to the plastic dish, the medium was replaced with 5% plasma-derived serum (PDS) prepared as described by Vogel et al [16]. The attainment of a quiescent state was determined by daily measurement of cell numbers (Coulter Electronics Inc., Hialeah, FL) and by autoradiographic labeling of cell nuclei with ^{3}H-thymidine. In quiescent cultures there were very few labeled nuclei (thymidine index range 0.03–0.08) after 48 h in the presence of PDS.

Quiescent cells were stimulated to divide either by addition of platelet-derived growth factor (PDGF, generously supplied by Dr. Ross and partially purified as previously described [16]), or by the addition of calf serum or, in some experiments, epidermal growth factor (EGF, Collaborative Research, Waltham, MA).

Pinocytosis Rate

Fluid phase pinocytosis was measured as the cellular uptake of (U-^{14}C)-sucrose (673 Ci/mole, New England Nuclear, Boston, MA) from the tissue culture medium by the method reported previously [1]. Radiolabeled sucrose was added in a small volume of BSS to a final activity of between 10 and 20 μCi per ml of tissue culture medium, depending upon the cell density. Following incubation at 4°C (control to inhibit endocytosis) or at 37°C, the cells were washed 5 times with ice-cold BSS containing 0.2% bovine serum albumen. Each culture was then rinsed twice with BSS, and the cells were dissolved in 1 ml 0.1% (aq) sodium dodecylsulphate (SDS, electrophoresis grade; Biorad, NY). The SDS lysate was transferred to a scintillation vial, 10 ml Ultrafluor cocktail (National Diagnostics, Somerville, NJ) was added, and the ^{14}C activities (dpm) were determined in a Beckman LS345 liquid scintillation counter. Counting efficiencies were determined from external standard ratios by reference to quenched (^{14}C)-toluene standards (Beckman). Rate of fluid endocytosis was expressed as the volume (nl) of fluid endocytosed per 10^6 cells per unit time [1, 17].

^{14}C-sucrose meets the criteria for a tracer of fluid phase endocytosis [18]. It is impermeable to the plasma membrane, does not significantly bind to the cell surface, and its uptake rate is directly proportional to its concentration in the culture medium. The rate

of uptake of ^{14}C-sucrose remained constant for 18 h and essentially represented a cumulative measurement because of the absence of an intracellular degradative enzyme [19] and a slow rate of exocytosis ($t_{1/2} > 40$ h at 37°C) (Davies, unpublished).

Autoradiography

Cells were labeled for 24 h by addition of 0.3 μCi ^{3}H-thymidine per ml of medium. After washing with BSS, cultures were fixed in ethanol-acetic acid, further rinsed, then air dried. The dish bottom was cut out, mounted in halves on a glass slide with polyvinylpyrrolidone, sealed with lacquer and the slides dipped in Kodak NTB2 emulsion. Following exposure for 10 days, the autoradiographs were developed, stained with haematoxylin-eosin, and % labeled nuclei determined.

RESULTS

3T3 cells were maintained in a quiescent state of growth at a range of cell densities by the use of plasma-derived serum (PDS), from which platelets and platelet-derived growth factors (PDGF) had been removed. The cells were plated at various densities in 10% serum until adherent, at which time the medium was replaced by 10% PDS. Under these conditions the cells were limited to a single round of division before accumulation in a G_0/G_1 phase of the cell cycle [13, 14, 20]. When either serum or partially purified PDGF was added back to the medium, there was a stimulation of DNA synthesis dependent upon the coordinate control of growth factor and plasma components, as fully documented elsewhere [16, 21]. In 10% PDS, the effects of increasing concentrations of PDGF upon DNA synthesis and pinocytosis rates are as shown in Table I at subconfluent and confluent cell densities. As Vogel et al [16] have recently demonstrated, 3T3 cells require increasing concentrations of PDGF to elicit equal mitogenic response with increasing cell density. Table I shows that the rate of pinocytosis in sparse cultures is significantly enhanced in the presence of a lower concentration of PDGF than is the case for confluent cultures.

When 3T3 cultures became growth-inhibited in 10% serum, the rate of pinocytosis per cell declined to a basal level (Table II). In light of the data presented in Table I, the findings of Vogel et al [16], and previous studies relating pinocytosis to the growth cycle [1–5], it was hypothesised that the decline in pinocytosis rate was related to the attainment of quiescence. At confluence, however, there was considerable cell–cell contact,

TABLE I. Stimulation of DNA Synthesis and Pinocytosis in 3T3 Cells in Relation to Cell Density*

	7×10^3 cells/cm^2					3.6×10^4 cells/cm^2					
μg PDGF/ml:	0	5	10	15	25	0	5	10	15	25	50
Fraction labeled nuclei:	0.04	0.80	0.74	0.91	0.82	0.03	0.21	0.59	0.74	0.73	0.81
Pinocytosis rate at 24 h: (nl fluid/10^6 cells/h)	47	101	1111	151	147	44	48	86	71	79	136

*Cells were plated at nominal densities of 4×10^3/cm^2 and 2×10^4/cm^2 in 10% calf serum. The medium was changed to 10% PDS 6 h after plating, and the cells were maintained in this medium for 48 h. PDGF was then added at various doses with 0.3 μCi ^{3}H-thymidine/ml medium. Twenty hours later ^{14}C-sucrose was introduced into some dishes for measurement of pinocytosis rate. Finally, after 24 h continuous exposure to PDGF, all dishes were harvested and assayed for autoradiography and pinocytosis rates. Cell densities in the table were measured by electronic counting at 24 h.

TABLE II. Density-Inhibition of 3T3 Cell Pinocytosis in 10% Calf Serum*

Cell density ($\times 10^{-4}/cm^2$)	0.48	1.4	3.1	4.0
Rate of pinocytosis (nl fluid/10^6 cells/h)	163	119	61	72
Fraction labeled nuclei	0.91	0.87	0.14	0.09

*Cells plated at a density of $4 \times 10^3/cm^2$ in 10% calf serum. As cell numbers increased, rates of pinocytosis were measured during a 2 h period at each of the cell densities. Cultures were also incubated with ^{3}H-thymidine for 12 h before and 10 h after the period of measurement of pinocytosis rates. Thus the fraction of labeled nuclei represents an average around the cell density indicated.

which might directly influence plasma membrane flow and invagination by the imposition of direct physical restraints upon membrane mobility. In an attempt to separate the growth component from other aspects of this system, two approaches were used. First, 3T3 cells were made quiescent in PDS at a range of subconfluent and confluent densities, so that different degrees of cell contact occurred in the absence of growth. Second, in order to maintain the growth fraction maximal despite increasing cell–cell contact, a SV40-viral transformed 3T3 cell line was used. Figure 1 demonstrates that, over a range of cell densities, the rate of pinocytosis per cell remained unchanged in quiescent 3T3 cells (fraction of ^{3}H-thymidine-labeled nuclei, 0.03–0.06). In SV40/3T3 under conditions of constant, maximal growth (fraction of ^{3}H-thymidine-labeled nuclei effectively 1.0 at all cell densities), the pinocytosis rate also remained unchanged over a 20-fold range of cell densities. When quiescent 3T3 cells at different densities were exposed to serum, however, the pinocytosis rate was more greatly stimulated at lower cell densities than in confluent cultures (Fig. 1). These results indicate that growth, and not cell–cell contact by itself, mediates pinocytosis rates. Supporting this conclusion are the data in Figure 2, which demonstrate that confluent, growth-inhibited 3T3 cells in 2.5% serum responded to 10% serum by an enhanced rate of pinocytosis together with a round of DNA synthesis.

DISCUSSION

The rates of fluid pinocytosis in 3T3 cells [1], vascular smooth muscle cells [1, 5], endothelium [4], S774 macrophages [3], and hepatoma cells [1] appear to be related to the cell cycle. Variations of pinocytosis rates of up to 10-fold have been reported. The methods employed to synchronize cultures in such studies have included colcemid block [2], density-inhibition [1, 4], and arrest of the cell cycle at G_0/G_1 in growth-factor-depleted medium [1, 5]. Coincident with density-dependent inhibition of growth in vascular endothelium and 3T3 cells, however, is the establishment of significant contact between cells. Such contact has been proposed to inhibit the lateral mobility of ligand–receptor complexes in the plane of the plasma membrane of cultured endothelium [15]. The formation of interdigitated gap junctions in many cultured cell lines may also directly inhibit membrane-mediated events such as pinocytosis. Thus it was of interest to determine the relative contributions made by cell contact and cell growth to the inhibition of pinocytosis in confluent 3T3 cultures.

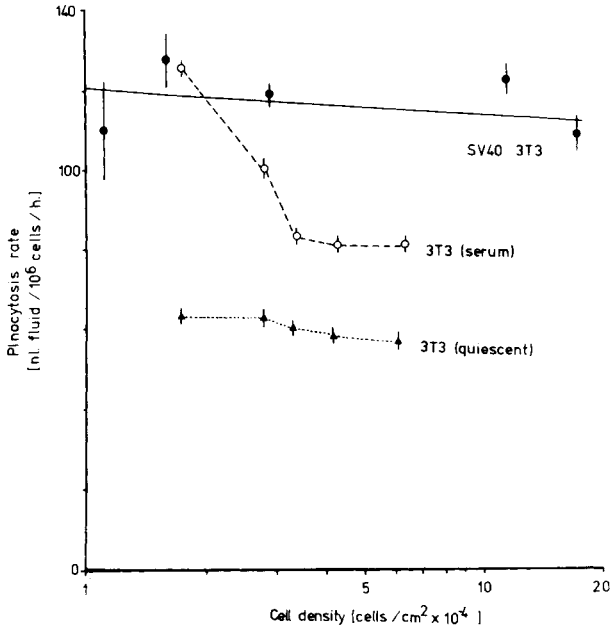

Fig. 1. Pinocytosis rate plotted against cell density for nontransformed Balb/c-3T3 cells and SV40 viral transformed 3T3 cells. Cells were plated at a range of densities in 10% calf serum After the nontransformed cells were adherent to the culture dish, the medium was changed to 10% PDS for 48 h. The 3T3 cells became quiescent at a range of cell densities. To half of the quiescent cultures was added 10% serum; the remainder received fresh 10% PDS. Twenty-four hours later, pinocytosis rate was measured during a 2 h period in the quiescent (▲) and serum-stimulated (○) 3T3 cells. Pinocytosis rates were also measured in the SV40/3T3 cells (●) at the various cell densities. Each point represents the mean of 4 determinations ± SD. The fraction ^{3}H-thymidine-labeled cells by autoradiography at lowest and highest density in each group was as follows: quiescent 3T3: 0.06 (low) 0.05 (high); serum stimulated 3T3: 0.86 (low) 0.11 (high); SV-40-3T3: 0.98 (low) 1.00 (high).

Plasma-derived serum (PDS), consisting of recalcified plasma from which platelets had been removed, was used to induce cells into a quiescent state of growth [13, 16, 22] at a range of cell densities. This occurs because platelets are removed from the plasma without degranulation and release of mitogenic platelet-derived growth factor [13]. Addition of purified growth factor to PDS results in growth responses that are comparable to serum. The measurements of pinocytosis rates in quiescent cells at different cell densities demonstrated that cell–cell contact in the absence of cell growth has no significant effect upon pinocytosis rates, suggesting that growth is the dominant mediator of density-dependent pinocytosis. This conclusion is supported by the observation that, in maximally growing transformed cells, the formation of a confluent monolayer, and even multilayers, did not significantly inhibit pinocytosis. The studies therefore suggest that the growth status of the cell most greatly influences pinocytosis rates at different cell densities, and that density inhibition of pinocytosis occurs secondary to density inhibition of growth.

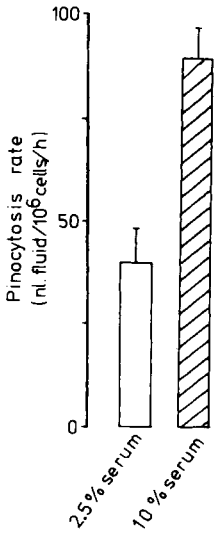

Fig. 2. Serum stimulation of pinocytosis in confluent 3T3 cells. 3T3 cells were plated in 2.5% calf serum at a density of $10^4/cm^2$. The cells grew to confluence (cell density $4 \times 10^4/cm^2$) and became quiescent. The medium was then changed in some dishes to 10% serum, and pinocytosis rate was measured over a 2 h period 22 h after serum-stimulation. Each column represents the mean of 4 determinations ± SD.

A number of mechanisms have been proposed for density-dependent inhibition of cell growth [23–25]. Most pertinent to the experiments described here are the recent studies of Vogel et al [26], who have provided evidence that a major regulator of density-dependent inhibition of growth in 3T3 cells is an increasing requirement for PDGF. Since we have previously demonstrated a close correlation between pinocytosis rate and entry into the cell cycle induced by specific growth factors, including PDGF, the present data are also consistent with the findings of Vogel et al.

Measurements of fluid pinocytosis rates indirectly reflect the internalization rate of plasma membrane, including ligand–receptor complexes at the cell surface. Since many such complexes are able to move laterally in the plane of the membrane, leading to patching, clustering, and capping [27–29], there may be a high degree of independence between fluid pinocytosis and ligand–receptor endocytosis, perhaps by the existence of separate populations of vesicles. Several studies, however, have provided evidence that the uptake of low-density lipoproteins (LDL) may be influenced by cell density in a pattern similar to that observed in the studies of pinocytosis. Stein and Stein [30] reported that uptake of LDL by cultured smooth muscle cells varied inversely with cell density, and recently Kruth et al [31] have demonstrated relationships between LDL receptor expression, lipid internalization, and fibroblast culture density. Their results were consistent with the possibility that LDL receptor-mediated uptake can be regulated via cell density. It will be of interest to apply methods of growth control such as used in the present study to investigate the specific endocytosis of macromolecules.

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