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Topical Review

Density-Dependent Regulation of Cell Growth: An Example of a Cell-Cell Recognition Phenomenon

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Summary. Cell-to-cell contact can result in a variety of changes in the cell's physiology. For different cell types, this may include both the initiation as well as the cessation of cell growth and changes in the state of differentiation. This review examines in detail one such phenomenon, density-dependent inhibition of growth, which is observed with many fibroblasts in culture. Data are summarized which demonstrate that the cessation of growth at high cell density is in part a consequence of cell-to-cell contact. An approach to the study of the molecular basis of this phenomenon is presented based on the demonstration that plasma membranes, when bound to sparse growing cells, mimic contact inhibition of growth. The present status of attempts to purify plasma membrane proteins responsible for this effect are summarized, and the properties of these membrane proteins are compared to those of previously described "soluble" proteins that inhibit cellular growth.

Key Words: Membranes, growth factors, growth control

During the past few years it has become clear that recognition components on the cell surface are important determinants in the normal patterns of growth and development of various species. These recognition events can be placed into a number of rather broad categories. The first is the signals which dictate cellular location, and may be mediated by cell attachment *per se*; examples are cellular migration or the formation of synaptic connections. The second category is that of cellular growth control in which contact events may be the initial trigger for an array of cellular responses, which lead to the initiation or cessation of cellular growth. The third category is systems in which cell contact is a necessary signal for the initiation of cellular differentiation.

Examples of systems in the first category include, among others, studies on the embryonic nervous system, aggregation of sponge cells, fertilization, and the aggregation steps involved in slime mold differentiation. Studies in these systems have demonstrated the following:

1) A possible biochemical basis for the site specific neuronal connections between the retina and optic tectum in developing chick embryos [1–3, 31].

2) The isolation and characterization of species-specific aggregation factors from sponges, as well as identification of possible receptors for these factors [9, 12, 35, 40].

3) A molecular mechanism can now be proposed to explain the species specific process of sea urchin egg fertilization [5, 25, 26, 75, 90, 91].

4) The identification and isolation of adhesive molecules, including lectin-like molecules, from the cellular slime molds [67, 68].

Examples of systems in the other categories will be discussed later in the review.

Cell recognition in higher eukaryotes has been studied by a number of techniques that are variations on the measurement of the cell's ability to adhere to other cells. Such investigations have been most fruitful in the study of cell recognition in dissociated embryonic cells, since in this case one can make certain predictions regarding the biological appropriateness of the observed specificity. It is fair to say that the precise molecular basis of cell adhesion is not well understood in most of these systems (for review *see* refs. 22, 29). A few general conclusions about cell recognition or adhesion in such systems can be stated as follows.

Cells may simultaneously express several adhesive specificities. A limited number of adhesive components may, by arrangement in various combinations, lead to a great deal of surface diversity. The adhesive molecules expressed by cells may change as a function of development [30, 49].

In many cases where the kinetics of adhesion have been examined carefully, adhesion has been found

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to be a multistep process [51, 73, 89]. Intuitively, one may expect some editing and multiple steps to be involved in the more precise form of cellular adhesion (recognition); i.e., cells must be able to recognize more appropriate from less appropriate adhesive events. "Appropriate" in this context implies either a higher density of adhesive molecules on one cell than another or the presence of molecules with higher affinity on one cell compared to another.

Long term cell adhesion may require an intracellular response; that is, that for two cells to remain together the initial binding must elicit a response, perhaps the synthesis or surface expression of a protein. The absence of this secondary response will ultimately result in the loss of cell-to-cell adhesion, an event that may be crucial for the establishment of optimal cell-to-cell contacts.

Over the last few years we have concentrated on cell recognition events that lead to an intracellular response. We have selected this approach for two reasons. First, this response can be used as a marker for a meaningful cell-to-cell adhesion as compared to a physiologically irrelevant cell-to-cell binding. Second, the physiological response of cell-to-cell binding can be used as a simple model of "differentiation", particularly for situations where appropriate cell-to-cell contact initiates the progression of a cell along a developmental pathway.

A large body of evidence has accumulated in the last 10 or 15 years that focuses attention on the cell surface as an important component of cellular growth control mechanisms. These related observations include the presence on the cell surface of receptors for a variety of growth factors (primarily polypeptide hormones such as insulin and epidermal growth factor (EG7) [11, 85], the observed interaction between cell surface molecules and the cytoskeleton [23, 24, 48], the effects of the extracellular matrix on cellular growth, and, finally, the effect of cell-cell interactions mediated by cell contact on growth control [8]. Cell-to-cell contact has been shown to provide a negative signal for cell growth in the case of density-dependent inhibition of growth observed with fibroblasts[1] [8], and a positive signal such as the stimulation of Schwann cell proliferation by neurites [8]. We will concentrate in this review on density dependent inhibition of growth, and attempt to focus attention on the evidence that supports the role of cell-to-cell contact in the phenomenon. We will then summarize recent efforts to purify the cell surface molecules presumed to be involved in contact inhibition of growth.

Density-dependent inhibition of growth was described over seventeen years ago with 3T3 cells [87]. These cells reach a limiting density which reflects the concentration of serum (or of defined mitogens) in the medium, and is attained even if the growth medium is changed at frequent intervals [36, 39]. The cells arrested at high cell density are arrested early in the G_1 portion of the cell cycle, a point sometimes designated G_0 [57, 59]. Within limits, the final cell density is a function of the serum concentration [36, 39], or the concentration of mitogens, such as the platelet-derived growth factors [74]. The possibility has to be considered that arrest of cell growth does not reflect cell contact, but either the depletion by the cells of medium components or the restriction of access of these components to the cell as a consequence of cell crowding [19, 82, 84]. More recently, changes in cell shape have been invoked as a possible factor responsible for density-dependent inhibition of growth [20]. Schultz and Mora [77], however, demonstrated in 1968 that cell contact is likely to be involved in density-dependent regulation of cell growth. This point will be further discussed below.

Density-dependent inhibition of growth is a characteristic of "normal" fibroblast-like cells and is not observed with malignant cells such as those that can be derived by viral transformation of these "normal" cells [36, 59].

I. Possible Causes of Density-Dependent Inhibition of Growth

We will consider each of the possible causes of density-dependent growth control in turn.

a) Is density-dependent inhibition of growth due to depletion of medium components?

In principle, this question can be answered by removing medium in equilibrium with cells arrested at high density and determining whether it will support the growth of cells at low density [8]. A much more elegant experiment was carried out by Dulbecco [17] who showed that if a wound is made in a confluent monolayer, cells will grow into this cell-free area. This growth in the wound area is supported by the same medium which does not support additional cell growth in the confluent areas of the same

[1] In this review we will use the word fibroblast to designate cells in culture which are usually considered to be of fibroblasta origin. The precise origin of these cells is not always clear (*see*, e.g., ref. 52), and therefore comparison of the behavior of these cells with the behavior of corresponding cells in the intact animal is often difficult. In many cases different clones of a certain cell, maintained in different laboratorie for long periods of time, may differ in their properties. We use the designation of mitogen for all polypeptides that are required for cell growth. In different assays some of these polypeptides would be designated as hormones.

dish. In addition, these experiments showed that the concentration of serum required to support growth was higher for cells in the monolayer (in contact with each other) than for cells at the wound, suggesting that the concentration of one or more serum components must be higher to initiate growth in cells in the monolayer than for isolated cells. This effect was most pronounced for 3T3 cells, but similar observations were made with other fibroblastic cell lines.

The major serum component which allows 3T3 cells to exit from G_0 and proceed into the cell cycle is the platelet-derived growth factor [74], a polypeptide with an approximate molecular weight of 35,000. This protein is released from platelets during coagulation and is therefore absent from plasma derived serum, in which the platelets have been removed before clotting has taken place. Recent experiments [92] have shown quite conclusively that the concentration of platelet-derived growth factor (PDGF) required to support the growth of sparse 3T3 cells is much less than the concentration required to initiate growth of confluent 3T3 cells; we will return to this observation later in this review. Additional experiments showed that cell arrest at high cell density can be observed under conditions where depletion of mitogenic factors (specifically PDGF) does not take place. Thus, as cells reach high density their ability to respond to a given concentration of mitogen is decreased. Similar observations have been made with EGF [6, 7] (epidermal growth factor), which is a less potent mitogen for 3T3 cells than PDGF.

It is unfortunate that at the present time the major mitogenic compounds present in such ill-defined mixtures as serum, embryo extract, etc., are not well characterized, and that epidermal growth factor remains the major mitogenic component readily available in large quantity as a pure substance. Whether results obtained with EGF can safely be extrapolated to other mitogens remains to be determined. A simple calculation will show that the restricted mitogenic response of confluent 3T3 cells to EGF, compared to sparse cells, cannot be due to depletion of EGF. In a typical experiment, sparse or confluent 3T3 cells arrested in G_0 by incubation in plasma-derived serum are incubated with 60 ng/ml of EGF at a ratio of 0.3 ml of medium per cm^2 of cells. The confluent cells (which respond poorly) have a density of 5×10^4 cells/cm^2, and contain 10^5 sites for EGF/cell (5×10^9 total sites/cm^2) which are rapidly down regulated to 2×10^4 sites/cell (1×10^9 sites/cm^2). The medium contains 1.8×10^{12} molecules of EGF per 0.3 ml (or cm^2 of surface). Assuming a turnover rate of cell surface receptor of 20 min, which is fast, then in 20 hr, at which time the rate of DNA synthesis is maximal, these cells would utilize 6×10^{10} molecules of EGF (or only 3.3% of the total). Since commitment of entry of cells into the cell cycle by EGF occurs in less than 20 hr [11, 69], the decreased effectiveness of EGF as a mitogen for confluent *vs.* sparse cells cannot be explained by depletion of EGF from the medium.

The relationship between the availability of medium components and cell contact is complex. Experiments were carried out by two different groups in which the ratio of media volume to available growth surface area was varied [18, 86]. One group found that when the medium to cell ratio was high the limiting factor for growth was the amount of available surface area [86]. When the medium to growth surface ratio was low, however, growth was primarily limited by medium components. These results are in contrast to those obtained by the second group, who only observed a medium limitation of the extent of growth irrespective of the ratio of medium volume to growth surface [18]. Both groups used the same clone of cells, so the reasons for the different results is not clear. It was suggested that the differences were related to the growth media.

b) Is density-dependent inhibition of growth a consequence of a restriction of access of mitogens to the cell surface at high cell density?

The observations described above do not rule out the possibility that at high cell density access of mitogens to the cell surface may be rate limiting [19]. The initial observations that suggested such a possibility were based on the following observations.

If a miniature pump was placed on the dish to circulate medium from a wound area to the monolayer, then cell growth (incorporation of [^{3}H]dThd into DNA) could be noted along the fluid path from the pump [82]. If dishes containing a confluent monolayer were shaken rapidly, some cells in the confluent monolayer started to grow [84]. The interpretation of these observations was that a diffusion barrier existed around cells, through which mitogenic compounds had to diffuse, and that these compounds were rapidly utilized by the cells. Mitogenic components in serum diffuse to confluent cells from a smaller cross section of medium (due to less surface area being exposed to the medium) than do sparse cells; hence the rate of delivery of mitogenic compounds to these cells becomes too slow to support cell growth.

If the rate of delivery of mitogens to the cell surface is a crucial event in regulating cell growth, then increasing the medium viscosity, which decreases the rate of diffusion, should further limit either the rate or the extent of cell growth under conditions where

the final density is a function of the serum concentration. When this experiment was done, neither effect was observed [97][2]; hence the diffusion limitation of cell growth does not appear to be a tenable theory for growth limitation of 3T3 cells at high cell density. It seems likely that the effect of the mechanical pump was to alter the relationship of the cells in the culture to each other or to the substratum.

Using different methodology, Westermark [95] has carefully examined the basis for the lack of response of normal human glial cells to EGF at high density. He has concluded that at confluence the access of EGF to the surface of glial cells is not restricted and that the affinity of the EGF receptor for EGF is unaltered. The maximum response for growth for both confluent and sparse cells is obtained at 1 ng/ml of EGF but is 30 times lower for confluent than for sparse cells

c) Is the cessation of cell growth at high cell density caused by changes in cell shape?

Cells that show contact inhibition of growth, like most normal cells in culture, do not grow in suspension or in agar, and will cease to grow rapidly if they are deprived of a suitable solid substratum (*see*, e.g., ref. 83)[3]. Recent work [20] has shown that cells grown on a plastic surface coated with poly (2 hydroxyethyl methacrylate) (poly HEMA) grow more slowly. As the concentration of poly HEMA is raised, the cells become more and more rounded and grow slower and slower. Measurements of cell height were carried out on cells growing on tissue-culture plastic. As the cells approached confluency, they were reported to be more rounded and an altered adhesion to substratum was postulated as the cause of density-dependent inhibition of cell growth.

Several arguments can be raised against this simple explanation, the most compelling of which is a 3T3 mutant isolated by Pouyssegur and Pastan defective in glucosamine 6P-acetyl-CoA acetyl transferase [63, 64]. These cells, at low density, are quite round; they flatten out at high cell density, yet these mutant cells grow at normal rates and reach a normal saturation density. Seher and Adam [78] did not find changes in cell height associated with approach to confluency in experiments similar to those of Folkman and Moscona [20]. Finally, it is hard to understand the concentration dependence of the poly HEMA effect, since covering the tissue-culture plastic with a continuous monolayer of poly HEMA should be sufficient for a maximal effect. This raises a concern regarding the chemical nature and the homogeneity of the substratum used in these experiments. Although substratum availability clearly provides an upper limit for the growth of normal fibroblasts, it seems very unlikely that this represents a major component in density-dependent inhibition of growth.

Fibroblasts at high cell density may synthesize a different extracellular matrix than at low cell density. The role of the extracellular matrix in modulating cellular growth and the interaction of cells with other mitogenic components is clearly an important aspect of the regulation of cell growth. At the moment there is very limited information regarding this aspect of growth control of fibroblasts compared to other cells such as endothelial cells [27, 28]. It is clear that matrix components such as fibronectin are important for the attachment of fibroblast to tissue-culture dishes, but they do not seem to affect the growth of cells in a manner more classically associated with mitogens or hormones [101].

II. Use of Membranes as Tools for the Study of Density-Dependent Inhibition of Growth

At high cell density the media components are destroyed at a faster rate than at low cell concentrations. Thus, it is sometimes difficult to separate the phenomena of cell contact and media depletion under normal culture conditions. We have utilized a system in which cells are always examined at low densities, such that media depletion does not play a role, to examine the role of intercellular contacts in 3T3 growth control. This system is described below. Our working hypothesis is that intercellular contacts generate intra-

[2] It is important to carry out such experiments under conditions where the measured quantity is the rate of entry of cells into *S* and not the final cell density reached by the culture. If a mitogen or any other component in the culture which is utilized by the cells is made limiting, then the final density will be a function of the total amount of that component and not its concentration. Thus, as an example, the cell yield per mole of leucine in the medium at infinite time is dependent on the total mass of leucine and not its concentration. Different interpretations of the viscosity experiment have been presented in the "Matters Arising" section of *Nature* (*London*) (**274**:722, 1978) by H.G. Maroudas, B. Whittenberger, and L. Glaser, as well as another view (*Nature* (*London*), **278**:283, 1979) presented by R.W. Holley and J.H. Baldwin, with reply by M. Lieberman, D. Raben, B. Whittenberger, and L. Glaser.

[3] In some instances measurements have been made of the relation of cell growth to cell surface exposed to growth medium in cells in suspension *vs.* attached cells [54]. The relation of such measurements to growth control is questionable for cells that show anchorage dependence of growth. Thus, for 3T3 cells and primary mouse embryo fibroblasts, less than 10% of growing cells put in suspension go through more than one round of division and at most 70% complete even one round of division after release from the substratum [54, 56]. For purposes of the present review we will consider that cells like 3T3 cells only grow on a suitable solid substratum. Measurements in cells detached from the substratum are designed to study the steps involved in the cessation of cell growth, due to anchorage dependence, but are not relevant to the study of density dependence of cell growth.

cellular "negative" signals for growth. External factors, such as mitogens, present "positive" growth signals. The cell in some manner integrates these signals and a decision is reached as to whether to undergo another round of division or to enter a quiescent state. Other factors, such as limitation of essential nutrients, or cell shape, would also input data to the system so that the cell can examine a variety of parameters before becoming committed to another round of division (Fig. 1).

Thus, under a given set of conditions, various factors will be required to regulate cell growth. When these conditions are altered, however, an entirely different set of factors may be required for regulation of cell growth (*see* ref. 45*a* for an example in which release from cell contact was not sufficient to initiate cell growth due to a lack of essential serum proteins).

Given the fact that all explanations for density-dependent inhibition of growth that do not include cell-to-cell contact have associated with them some conceptual or experimental difficulties (*see* above), we would like to consider experiments which strongly suggest that cell-to-cell contact influences cell function at high cell density. We will, however, explicitly leave out any discussion of cell motility from this review.

Whittenberger et al. [96, 98] examined the effect of adding partially purified membrane fractions to growing cells. The results of these experiments provide direct evidence for the role of intercellular contact in 3T3 cell growth control. After addition of plasma membrane fraction to sparse 3T3 cells, 50% of the cycling cells became arrested in G_0 during each cell cycle. The membrane-induced cell arrest was not due to depletion of essential nutrients by the membranes, as control experiments indicated that when medium was pre-incubated with membranes and the membranes were then removed by centrifugation, the resulting medium could support the growth of 3T3 cells as well as fresh medium. The membrane arrest was shown to be reversible through the use of two techniques. The first consisted of trypsinization and replating of membrane-arrested cells. The same efficiency of plating was found for cells treated with membranes as with control cells never treated with membranes. The second method consisted of adding serum to the membrane-treated cells. Upon raising the serum concentration, the arrested cells were able to re-enter the cell cycle and undergo another round of division. Serum effectively reversed the membrane induced cell arrest. These results indicate that membrane-induced arrest of DNA synthesis is not a consequence of a nonspecific toxic effect. The location of the cell block was shown to occur in early G_1, at a point identical to or very close to the site of cell

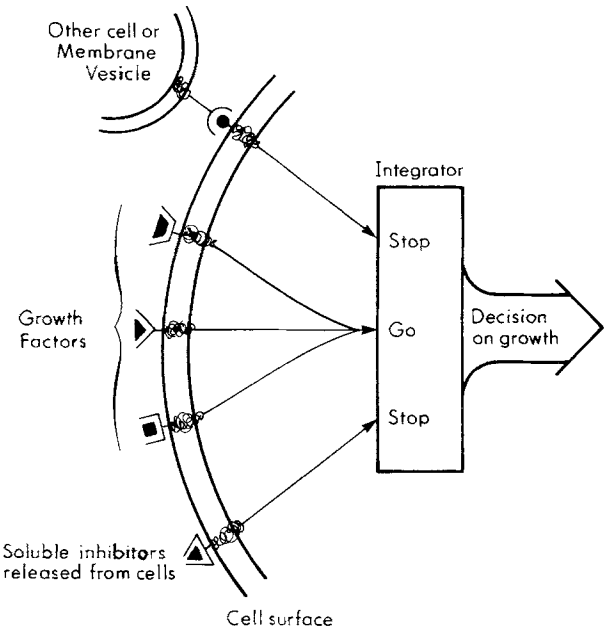

Fig. 1. A schematic diagram of cell surface events related to growth of fibroblasts. The intensity of each signal can vary according to the number and type of factors bound to the appropriate cell surface receptors. Thus, a low amount of inhibitory signals can be overcome by a larger amount of growth signals. The steps that occur in the integrator, as well as the output, are unknown at present. Antagonists to growth factors may also be important. Attachment to the substratum, an essential requirement for the growth of "normal" fibroblast, is assumed, but has been omitted from the drawing

arrest due to serum deprivation or high cell density (G_0). Membranes prepared from SV40 transformed 3T3 (SV-3T3) cells contained some inhibitory activity when assayed on 3T3 target cells, but 3T3 and SV3T3 membranes had no effect on the growth of SV3T3 cells. This finding suggests that the transformed cells can synthesize and express the molecules responsible for inhibitory signal but have lost the ability to respond to this signal. Membranes prepared from growing, low-density 3T3 cells contained less inhibitory activity than did membranes prepared from high-density quiescent cells. The reasons for this are not yet clear, but may indicate one or more of the following: the synthesis and/or insertion of the active components into the membrane are regulated during growth, or the appropriate factors are always present in the membrane but are regulated by covalent modifications as a way to generate a rapid transition between active and inactive forms. Overall, as outlined in Table 1, the inhibition of growth of 3T3 cells by plasma membrane enriched fractions of 3T3 cells is quite similar to the cellular arrest brought about at high cell density.

Utilizing membranes to arrest cell growth has enabled us to demonstrate that the transport of both

Table 1. Comparison of the inhibition of growth of 3T3 cells by high cell density and plasma membranes

Function	Cell growth arrested by	
	Plasma membranes	High cell density
Cells arrested early in G_1 portion of cell cycle	+	+
Maximum fraction of cells arrested per cycle	50%	Possible 50%
Reversibility:		
By trypsinization and replating	+	+
By increase in the concentration of defined mitogens such as PDGF or by addition of serum	+	+
Decrease in the rate of uptake of		
α-aminoisobutyric acid	+	+
Uridine	+	+
Glucose	−	+
Phosphate	−	+

For details, *see* text and refs. 8, 44, 96–99

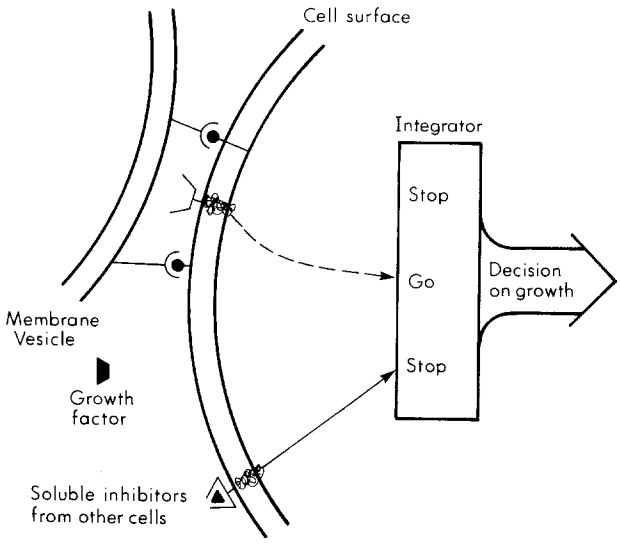

Fig. 2. An alternative model for membrane-induced cell event. The binding of membranes to cell may block the binding of growth factor to the cell surface. These membranes would not directly generate a signal to stop cell growth. Experiments that rule out this model [45] are discussed in the text

Table 2. Biological activity of octylglucoside extract of 3T3 plasma membranes

1) Inhibits DNA synthesis in 3T3 cells is a concentration-dependent manner to a maximum of 50%
2) Inhibition is reversible.
3) Inhibition of 50% is due to a steady state of cells becoming inhibited and escaping from inhibition.
4) Inhibition can be blocked by high concentrations of serum and other mitogens.
5) Inhibitory activity is heat labile.
6) Inhibits α-aminoisobutyric acid transport.

For details, *see* text and refs. 8, 65, 99.

glucose and phosphate, which are normally reduced 60% when the cells become quiescent, were not decreased in membrane arrested cells [44]. This provided further evidence that neither the glucose nor phosphate transport systems were causally related to G_0 arrest, as had been originally shown by Cunningham and coworkers [4].

The molecule(s) responsible for the growth inhibitory activity of the membranes appear to be associated with the cell surface, since during membrane isolation the growth inhibitory activity of various membrane fractions parallels the activity of phosphodiesterase, a plasma membrane marker. In addition, the activity appears to reside in an intrinsic membrane protein since it cannot be removed from the membrane by techniques which typically remove extrinsic proteins. It can be solubilized in active form by the detergent octylglucoside [99]. A careful study of the solubilized factor has shown that most of the properties observed for the membrane preparations are also observed with the extract (*see* Table 2).

One trivial explanation for the behavior of membranes is that they coat the cell surface such that the required growth factors cannot bind to their appropriate receptors (Fig. 2). Membranes have been shown to block the mitogenic response of EGF, on serum-starved cells, by greater than 60%. In that study both the binding of EGF, as well as the EGF induced down regulation process, were examined for both membrane-treated and control cells [45]. There was no difference in the binding of EGF to either sample, nor was there any effect on down regulation. Thus, the membranes are not blocking access of EGF to its receptor, and a different explanation is required for the extreme block of the mitogenic response [see also 21].

An important finding, and this is true for both the membrane fractions and the solubilized factor, is that the inhibitory activity expressed by the factor is dependent upon the serum (or purified mitogen) concentration in the medium. Thus, a fixed quantity of membranes will exhibit less inhibitory activity as the serum (or amount of purified mitogen) concentration is raised. These data suggest that membranes, upon contact with the cell, initiate events which lead to signals inside the cell which are antagonistic to the mitogenic signals generated by growth factors. This is shown diagrammatically in Fig. 1.

If cell contact events play an important role in cellular growth control, as they appear to do for Swiss 3T3 cells, then the surface molecules involved in this

process may have been conserved through the evolutionary process. We have found this to be the case, as IMR91 cells (a normal human fibroblast line which undergoes senesence after about 60 population doublings) have been shown to contain growth inhibitory proteins on the cell surface which can interact with those on the 3T3 cell surfaces [43]. As far as has been examined, the factors as found on IMR91 cells have similar properties to those associated with 3T3 cells, namely, reversibility by serum, sensitivity to heat, location in the plasma membrane, and successful solubilization of the factor by octylglucoside.

Other laboratories have reported the presence of growth inhibitory factors for 3T3 cells. These factors all appear to be distinct from the growth inhibitory protein (GIP) described above, and their properties are discussed below. The first report was a factor described by Yeh and Fisher [102] in 1969. This factor was released from 3T3 cells and appeared to reduce the rate of RNA synthesis in neighboring cells, which effectively will keep cells in a quiescent state. This factor was shown to be a heat stable, low molecular weight component. Pariser and Cunningham [60] further studied this factor and demonstrated that it acted by reducing the rate of transport of both uridine and phosphate. This accounted for the apparent reduction of RNA synthesis. This substance is therefore a transport regulatory factor and its relation to growth control is at the moment not clear.

Another growth regulatory factor, which is also released into the medium by 3T3 cells, was described by Harel et al. [33] and further elaborated on by Steck et al. [81]. This factor reversibly arrested growing cells such that DNA synthesis was inhibited (although the location of the block relative to the cell cycle has yet to be determined). The factor, however, does have a requirement for a minimum target cell density, below which the factor has no effect on the cells (the critical density is 5×10^3 cells/cm^2). It is also not known if this factor, which is released by the cells, is normally a membrane-bound factor or a soluble one, and if membrane bound, the nature of the signal that causes it to be released into the medium is not known. This soluble factor described by Harel et al. [33] and Steck et al. [81] may be the same as the one described by Yeh and Fisher [102]; the former factor is low molecular weight (less than 10,000 daltons), but its heat sensitivity was not examined. GIP is clearly different from these factors; GIP is heat sensitive, undialyzable (10,000 mol wt cut-off), is not released into medium in active form (D. Raben, *unpublished observations*), and has no requirement for a minimum target density (*see* ref. 50 and D. Raben, *unpublished observations*). The relationship, if any, between these factors is unknown.

A third set of inhibitory growth factors are the factor(s) described by Natraj and Datta [14, 53]. This factor, which is isolated from Balb/c 3T3 cells by urea extraction of washed cells, has been shown to reversibly arrest the cells in the early G_1 phase of the cell cycle. This factor appears to be membrane bound (FGRF, fibroblast regulatory growth factor) and also has the unique property of being regulated by the state of glycosylation. Preincubation of quiescent cells with UDP-N-acetyl-D-glucosamine yields extracts with no DNA synthesis inhibitory activity. Incubation of such an inactive extract with purified N-acetyl-β-D-glucosaminidase restored activity to the extract. This factor differs from GIP by the following criteria. First, GIP is trypsin insensitive, FGRF activity is destroyed upon incubation with trypsin. Second, FGRF is regulated by glycosylation, whereas GIP does not appear to be regulated in such a manner [8]. Carbohydrate residues may be important for GIP activity, however, as periodate treatment of membranes greatly reduces the GIP activity [65]. One important similarity between GIP and FGRF is that both are found on the surfaces of SV-3T3 cells, yet neither can arrest the growth of SV-3T3 cells. This again suggests that transformed cells contain these factors but cannot repond to them because they lack (in functional form) the appropriate receptors for these growth regulatory factors.

It appears that a number of potential growth regulatory molecules have been identified and characterized to varying extents. They fall into two classes; membrane bound and soluble. There are at least two distinct entities in the membrane bound class: the GIP described by Bunge et al. [8] and the FGRF described by Natraj and Datta [14, 53]. The data concerning the soluble factors suggest at least two classes for those molecules, one being the transport regulatory factors described by Yeh and Fisher [102] and Pariser and Cunningham [60], and the other being the growth inhibitory factors described by Harel et al. [33] and Steck et al. [81]. If all these factors survive the test of time and are shown to be truly independent entities, then their existence suggests that cells can respond to a number of different signals for inhibition of cell growth, and all may be utilized during the normal growth of a cell. The relationships and regulation of expression of these various factors is not known.

III. Density-Dependent Arrest of Cell Growth is Distinct from Arrest by Mitogen Deprivation

Since cells at high density are arrested in G_0 it is appropriate to ask whether cells arrested in G_0 by other means (deprivation of mitogenic compounds) show similar or different characteristics than cells ar-

rested at high cell density. If the latter is the case, then this provides direct evidence that a unique event is taking place at high cell density. We will review briefly several examples of such events, not all equally compelling, and many of them obtained with different cell types.

As mentioned above, the rate of uptake of a number of small molecules such as α-aminoisobutyric acid or glucose is decreased when cells are arrested in G_0, and also at high cell density. Moya and Glaser [50] have shown that in the case of 3T3 cells, a density effect on the rate of α-aminoisobutyric acid transport is observed even for cells already arrested in G_0. The addition of membranes to sparse cells already arrested at G_0 further decreased the rate of transport, thus leading to the conclusion that arrest of cells at G_0 by cell density can act independently of the arrest of cells by mitogen deprivation in controlling the rate of α-aminoisobutyric transport via a Na^+-dependent carrier (the A system of amino acid transport). Similarly, the rate of uptake of 2-deoxyglucose by sparse cells arrested in G_0 were as high as that of growing cells at the same density[4].

In yet another approach, there have been a number of recent reports indicating that colchicine would enhance the cell's mitogenic response to EGF (or other mitogens) when high density, serum starved, 3T3 cells were examined [23, 24, 55]. McClain and Edelman [48] recently examined the effects of cell density on this synergistic process and found that the synergistic effect, on 3T3 cells, was strongly dependent on cell density. When the cells were at low densities, colchicine could not stimulate EGF mitogenesis, but at high cell densities (when extensive intercellular contacts were established), the synergistic response was observed.

The same observations were made with other fibroblasts such as primary chick embryo fibroblasts [47, 48]. These results have many interesting implications. The first is supportive of the data of Moya and Glaser [50] and suggests that nutrient-deprived cells are not in the same biochemical state as cells arrested at high cell density, even though they may both be arrested at the same point in the cell cycle [58]. Both sets of data indicate that cells deprived of hormones or mitogenic compound behave differently than cells in contact with each other and deprived of the same hormones or mitogenic compounds. McClain and Edelman [48] postulate that the inhibitory signals generated by cell-cell contact are mediated or regulated through the cytoskeletal apparatus. The disruption of the microtubules by colchicine, therefore, would alter the transmission of the inhibitory signal. Numerically, colchicine alters the dense cells such that the frequency of initiation of growth in the culture is the same as in sparse cultures, but never higher. Thus, the dense cells, under these conditions, are acting like sparse cells, and colchicine would induce in dense cells a cytoskeletal alteration that allows them to respond maximally to EGF.

There have been a number of recent reports concerning the effects of cell density on hormone binding and mitogenicity. We would like to review these reports in light of the scheme shown in Fig. 1 and show how these data fit to a cell-contact theory of growth control.

Vogel et al. [92] have examined the role of the platelet-derived growth factor (PDGF) in the initiation of DNA synthesis in quiescent 3T3 cells as a function of the cell density. They found that as the culture density increased more PDGF was required per cell to initiate DNA synthesis. Depletion of the PDGF by high cell densities is unlikely, as placing a cover slip of sparse cells in a dish containing a confluent monlayer of cells still allowed for normal growth of the cells on the cover slip even though the confluent monlayer was slowly degrading and reducing the supply of PDGF. In light of the scheme shown in Fig. 1, we interpret these results as follows at confluency the cells are receiving more "negative" signals for growth via cell-cell contact and, perhaps, by diffusible factors, than the cells when they are at low cell density. Thus, a larger "positive" signal (more bound PDGF) is required to overcome the cell contact phenomena. It has not yet been demonstrated that spare receptors exist for PDGF, but for EGF only 10% of the sites need to be occupied for the maximal mitogenic effect to be seen in sparse cultures. Thus, it is possible to generate additional "positive" signals by binding more hormone. Our model, therefore, predicts that PDGF will also exhibit spare receptors.

Similar data and conclusions were obtained by Brown et al. [6, 7] using the mitogen EGF. Using Balb/c 3T3 cells, these investigators found that at high cell densities more EGF per cell was required to elicit a mitogenic response than was required at low cell elicit a mitogenic response than was required at low cell densities. In addition, confluent cells expressed more EGF receptors per cell than did sparse cells. Because of this, confluent cells should bind more EGF than sparse cells at low EGF concentrations (due to the two- to threefold difference in receptor numbers), and yet the confluent cells still required more EGF per cell to initiate another round of division. Brown et al. [6, 7] attributed this finding to

[4] In contrast to these observations, fluid pinocytosis, which decreases at high cell density in both smooth muscle and 3T3 cells [15, 16], is directly related to cell growth and independent of cell density effects.

either of two phenomena. The first is that the necessary "cofactors" for 3T3 competence and progression [69, 74] through the cell cycle were in limiting supply due to the high cell density. This explanation appears to be unlikely in light of the experiments by Todaro et al. [88] and Vogel et al. [92] (described above) in which a cover slip, containing cells seeded at low density, was placed in a dish containing a confluent, quiescent monolayer. The cells on the cover slip still grew at normal rates, indicating that the necessary "cofactors" were still present in the conditioned media. The amount per cell may have been low for dense cultures, but that is a contact effect, altering cellular requirements for growth factors.

The second explanation given to explain the data was that "increasing cell density gives rise to some inherent change within the cells that increases their EGF requirement." We are suggesting that the change is brought about by intercellular contacts, which lead to intracellular signals which arrest cell growth. In order to overcome the growth arresting signals, a larger dose of growth promoting signals must be provided. In line with this idea are data by Lieberman et al. [42] which shows that cells arrested in G_0 have two- to fourfold more receptors for EGF than do exponentially growing cells. This is true for G_0 arrest brought about by either high cell density, starvation at low cell density, or by adding membranes to growing sparse cells, thereby arresting a certain percentage of the cells at low density. We believe that the up-regulation of receptors is a compensatory response to growth arrest, as increasing the EGF receptor number would enable the cell to bind more EGF at low EGF concentrations. This could result in mitogenesis if the number of EGF-receptor complexes on the cell surface is sufficient to trigger that response. As modulation of the EGF receptor appears to be linked to modulation of the receptors for fibroblast growth factor (FGF) and PDGF (21,100), it would be of great interest to examine the effects of growth arrest on PDGE binding, as PDGF appears to be the most potent and major mitogen in serum for 3T3 cell growth. Once such assays become technically feasible, it would be possible to determine if the growth-arrest modulation of receptors is specific for EGF and insulin [85] or if the PDGF receptor is also regulated.

Virtually all of the experiments cited thus far utilized the 3T3 cell system and all present arguments for the involvement of intercellular contacts in the normal growth regulatory process of the 3T3 cell line. Data of a similar nature have also been obtained with other cell lines and types, and a few of these experiments are described below.

Holley et al. [37, 38] have studied the growth regulation of BSC-1 cells. One of their findings was that confluent cells contained one-tenth the number of receptors for EGF than did growing cells (this is the converse of all other cases examined, where confluent cells up-regulated their mitogen receptors). In addition, a higher concentration of EGF was required to initiate DNA synthesis in crowded cells than in sparse cells. The interpretation given was that the drop in EGF receptor levels was a primary cause of the density-dependent growth regulation of BSC-1 cells. They have since shown that the growth of BSC-1 cells is also exquisitely sensitive to the concentrations of nutrients in the media [37]. Thus, either a reduced nutrient concentration in the media, or the cells developing an increased requirement for EGF, would be enough to inhibit cell growth. We would like to suggest that the formation of cell-cell contacts is the event which leads to the eventual decrease in cell surface receptors for EGF in BSC-1 cells. The signal which is generated by cell-cell contact, however, remains to be elucidated.

Westermark [34, 94, 95] has examined growth control in cultured human glial cells. He has found that EGF is a potent mitogen for these cells at low densities, but that the effect of EGF on confluent cultures is greatly diminished. It was shown that dense cells had more receptors for EGF per cell than sparse cells and that the receptors on both sparse and dense cells had the same affinity for EGF. Westermark concluded, as mentioned above, that local starvation for EGF could not be the cause of growth arrest, but rather that some negative signals generated by the extensive cell-cell contacts formed inhibited the onset of mitogenesis usually brought about by EGF.

We have focused our attention thus far on the role of cell contact in the cessation of cell growth. We have not, however, addressed the question of possible mechanisms whereby cell contact can bring about this result. Experiments to answer this problem have not yet been attempted, but experiments dealing with possible second messengers for growth stimulation have been done. Possible candidates include alterations in ion fluxes [40*a*, 68*a*, 68*b*), (*see*, however, 21*a*) phosphorylation of membrane proteins, in particular at tyrosine residues (12*a*, 89*a*) and intercellular communication through gap junctions [45*b*]. The manner in which cell-cell contact interferes with these signals would be speculative at this point; these events could occur at the integrator (as shown in Fig. 1), in which case the second messenger would never be delivered, or cell contact could interfere with the magnitude of the second message. In either case, the pathways for growth stimulation and growth cessation must intersect, and more work needs to be done to determine that point.

In addition to the examples listed above, which are mostly related to growth control in fibroblastic cells in culture, there are a few other examples of homologous or heterologous cell-to-cell contact with profound physiological effects on the cells which should be mentioned briefly, since they support the notion that cell-cell contact has important physiological consequences on cells. These include the mitogenic effect of neurites on Schwann cells [70–72], the "differentiation" of C-6 glioma cells resulting in the synthesis of S-100 protein [32, 41, 62], the induction of muscle-specific proteins in BC_3Hl cells [10, 61, 76], the transport of amino acids in the Shionogi 115 carcinoma cells [66], and the induction of choline acetyl transferase in PC-12 cells [46], and no doubt many others. Recent experiments in these systems are mentioned only to indicate that cell-to-cell contact can have a variety of physiological effects, some of which are potentially less complex and may therefore be more amenable to detailed study than cellular growth.

Horizons

It is clear that the role of cell-cell contact in growth regulation is still a controversial topic. Alternative explanations for virtually all of the experiments cited in this article are possible; however, looking at the data as a whole, a strong case is being developed for an important role of cell-cell contact in the growth regulatory process. Purification and characterization of the various cell surface molecules involved in the process is essential. Techniques now in use with peptide hormones could be utilized to identify the appropriate receptors and to follow the fate of the receptors after binding to the solubilized factor. The ultimate aim is to identify the signal that cell-cell contact brings about. Intertwined with this problem is the unraveling of the cellular signal generated by mitogenic peptides. The two signals need to be integrated at some point, and the recent approaches being taken in the laboratories of Das [13] and Herschman [79, 80] appear promising in solving this question. Novel techniques will clearly be required to identify the intracellular mechanism by which contact inhibition of cell growth is brought about. However, currently available techniques seem adequate to allow the identification and isolation of the cell surface molecules responsible for contact inhibition of growth.

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