THE EFFECT OF CELL POPULATION DENSITY ON NUTRIENT UPTAKE AND CELL METABOLISM: A COMPARATIVE STUDY OF HUMAN DIPLOID AND HETEROPOID CELL LINES

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SUMMARY
The possibility that contact inhibition of growth in cultures of human diploid cells is influenced by the effects of cell crowding on nutrient uptake by the cells was investigated. Two human lung cell lines were compared, the diploid line MRC-5 and the heteroploid line L-132. In pre-confluent cultures the ability of these 2 cell types to accumulate amino acids was very similar. Post-confluent L-132 cells showed very little change from the pre-confluent cultures but the ability of MRC-5 cells in post-confluent cultures was greatly reduced. The intracellular concentrations of various amino acids necessary to achieve the maximum rate of protein synthesis were found. These values were identical for sparse and crowded cultures but due to the reduced uptake ability of crowded MRC-5 cells a far higher external amino acid concentration was required in post-confluent cultures. This meant that although amino acids did not become growth-limiting until over 80 % utilized in pre-confluent cultures, in post-confluent cultures they became growth-limiting when only 50 % utilized.

Although protein synthesis was significantly affected by extracellular amino acid concentration and cell crowding, thus contributing towards the effect of contact inhibition of growth, DNA synthesis was shown to be the major metabolic function in contact inhibition. Increased cell density had a very inhibitory effect on DNA synthesis in MRC-5 cultures, but not in L-132 cultures, and this was unaffected by extracellular amino acid and glucose concentration.

INTRODUCTION
Contact inhibition of growth is associated with those normal cells which characteristically form monolayers in culture. Altered or cancer cells, which form multilayers, are assumed to have escaped from the regulatory effects of contact inhibition. The possibility that cancer cells can grow in this uninhibited manner because the permeability properties of their membranes have changed has been discussed for a long time. Le Breton & Moulé (1961) produced evidence for permeability differences between normal and cancer cells and Pardee (1964) conjectured that growth-regulating substances were unable to enter the cancer cell. Sanford et al. (1967) also suggested that contact inhibition of mitosis was possibly a result of the effects of cell crowding on the exchange of nutrients and waste products. The involvement of cell membrane function in contact inhibition is also suggested by the consistent differences found in the membrane structure between normal and altered cells (Abercrombie & Ambrose, 1962; Burger & Noonan, 1970).
The possibility that contact inhibition of growth in cultures of human diploid cells (HDC) is closely correlated with the permeability of the cell membrane gained credence during a study of the nutritional state of contact-inhibited cultures (Griffiths, 1970a, b). It was found that Eagle's minimal essential medium supported higher yields of HDC than Eagle's basal medium even though no amino acid was more than 55% utilized in the latter medium (Griffiths, 1970a). Daily medium changes (Griffiths, 1971) and medium-perfusion cultures (Kruse & Miedema, 1965; Griffiths, in preparation) both provided a rich nutritional environment and allowed HDC to grow in multilayers despite an excess of all nutrients in the replaced medium. The hypothesis was proposed that confluent cultures of HDC required higher concentrations of certain nutrients than both pre-confluent cultures of HDC and cultures of altered cells. The results of experiments carried out to test this hypothesis are presented in this paper and they indicate how important the concentration of certain nutrients in the medium is for contact inhibition of growth.

MATERIALS AND METHODS

Cells

The human diploid cell line, MRC-5, was derived from foetal lung tissue and supplied by the National Institute for Medical Research (Hampstead Laboratories). It was cultured by the procedure of Hayflick & Moorhead (1961) and only cells between passages 18 and 29 were used. The cells were at least 98% diploid as revealed by chromosome analysis. The epithelial, heteroploid L-132 cell line was also derived from human foetal lung tissue (Davies & Bolin, 1960).

Culture procedure

Eagle's minimal essential medium (MEM) (Eagle, 1959) supplemented with 10% foetal calf serum (Flow Laboratories) was used. Experimental cultures were grown in 5-cm plastic dishes with 5 ml medium inoculated at 5 x 10^5 cells/culture. Growth was measured by the trypan blue viable count method. Cultures were found to be confluent when the cell density reached 1 x 10^6 cells/cm^2 (MRC-5) or 1.3 x 10^6 cells/cm^2 (L-132 cells).

Isotope labelling procedures

The following isotopes were obtained from the Radiochemical Centre, Amersham: a mixture of uniformly labelled ^14C-amino acids as precursors for protein synthesis, 1 μCi/culture; thymidine-6-T(n) (2 Ci/mM) as a precursor for DNA synthesis, 10 μCi/culture; uridine-5-T (5 mCi/mM) as a precursor for RNA synthesis, 10 μCi/culture; l-arginine-14C (U) (96 mCi/mM), l-leucine-14C (U) (10 mCi/mM), l-methionine (methyl-14C) (60 mCi/mM), L-valine-14C (U) (10 mCi/mM), 2-amino-isobutyric acid-1-14C (58 mCi/mM), and D-glucose-14C (U) (30 mCi/mol) were used to measure the intracellular concentration of these nutrients.

The measurement of protein, RNA and DNA synthesis has been described previously (Griffiths, 1970b). To measure the intracellular concentration of nutrients a concentration range of the isotope was made and after a 4-h pulse the cells were washed twice with ice-cold buffer and the radioactivity of an ice-cold trichloroacetic acid extract was measured and compared with a similar extract from the pulsing medium. This result, when adjusted for cell volume, gave a ratio of intracellular to extracellular concentration from which the intracellular concentration could be calculated.
RESULTS

The effect of cell density on amino acid uptake

The ability of human diploid and heteroploid cells to accumulate the essential amino acids arginine, leucine and valine and the non-metabolic amino acid aminoisobutyric acid was measured. Amino acid uptake was compared in cultures approximately 60% confluent with cultures approximately 130% confluent. The results are shown in Fig. 1. The intracellular concentration of the amino acids showed a great similarity in pre-confluent cultures indicating that before confluency the 2 types of cell had the same potential for amino acid uptake. However, when the cultures became crowded it was apparent that the heteroploid cell line was able to maintain higher intracellular concentrations than the diploid cell line. At the highest external amino acid concentration the intracellular level of arginine in confluent MRC-5 cultures was only 35% of the pre-confluent concentration compared to 65% in...
L-132 cultures. Similarly the post-confluent concentrations of leucine, valine and aminoisobutyric acid were 42, 68 and 75% respectively of the pre-confluent concentration in MRC-5 cultures compared to 87, 96 and 88% in the L-132 cultures.

**The effect of cell density on protein synthesis**

The rates of protein synthesis in MRC-5 and L-132 cells at various concentrations of arginine, leucine, valine and methionine (MRC-5 cells only) were measured in pre- and post-confluent cultures. The results are shown in Fig. 2. A higher concentration of extracellular amino acids was needed in post-confluent MRC-5 cultures than in pre-confluent cultures for the maximum rate of protein synthesis to be attained. The concentrations of arginine, leucine, valine and methionine needed for maximum protein synthesis in pre-confluent cultures were 10, 10, 12 and 6 µg/ml respectively, but in post-confluent cultures 16, 25, 24 and 12 µg/ml were required. This quantitative change in amino acid requirement meant that a medium would become growth-limiting far sooner in a confluent culture – for instance, leucine would be growth-limiting when 80% utilized and valine when 74% utilized in a sparse culture but
after confluency leucine and valine would become limiting when 52 and 48% utilized, respectively. The overall rate of protein synthesis was affected by cell density as the post-confluent rates were between 75 and 85% of the pre-confluent rate. Cultures of L-132 cells on the other hand showed no change in their quantitative amino acid requirements with variations in cell density and there was also a smaller difference in the overall rate of protein synthesis, the post-confluent rate varied between 90 and 110% of the pre-confluent rate.

The intracellular amino acid concentration is given in Fig. 2 at the point at which the maximum rate of protein synthesis was attained. These data were derived from Fig. 1 and show that protein synthesis did not reach its maximum rate until a certain intracellular concentration was reached. This concentration was the same for both pre- and post-confluent MRC-5 cultures but was reached at different extracellular concentrations due to the differences in amino acid uptake caused by cell crowding. There was no shift in amino acid concentration in L-132 cultures and the intracellular concentrations were approximately the same when protein synthesis was at the maximum rate. However, the intracellular concentrations in L-132 cells were much lower than in MRC-5 cells indicating the presence of a threshold concentration effect.

The effect of cell density on DNA and RNA synthesis

The rates of DNA and RNA synthesis in MRC-5 and L-132 cells at various concentrations of arginine and leucine were measured in pre- and post-confluent cultures. The results, shown in Fig. 3, are given as the percentage rate of synthesis in

![Fig. 3. The effect of cell density and extracellular amino acid concentration on the rates of DNA and RNA synthesis. L-132 cells, open symbols; MRC-5 cells, closed symbols. Arginine concentration range, unbroken line; leucine concentration range, dotted line. O, , DNA synthesis; A, A, RNA synthesis. Rates of DNA and RNA synthesis are given for post-confluent cultures as a percentage of the rates in pre-confluent cultures. Arginine and leucine concentrations as in Fig. 1.](image)
post-confluent cultures compared to the rates in the pre-confluent cultures, as the
effect of nutrient concentration in these 2 types of culture showed the same qualitative
pattern. The data in Fig. 3 show that DNA synthesis was greatly inhibited in post-
confluent cultures of MRC-5 cells (9–20% of the pre-confluent level) but not in
L-132 cultures. RNA synthesis, however, was considerably inhibited in post-
confluent cultures of both L-132 and MRC-5 cells but there was very little difference
in the response of the 2 cell types (the rate was depressed to 45% of the pre-confluent
level in L-132 cells and 35% in MRC-5 cells). External nutrient concentration did
not appear to have very much effect on the rates of DNA and RNA synthesis apart
from some small initial rises between 0 and 20% nutrient concentration.

The effect of glucose concentration and cell density on glucose uptake and cell metabolism

The effect of glucose concentration on the intracellular level of glucose and cell
metabolism was studied in pre- and post-confluent cultures of MRC-5 and L-132
cells (Fig. 4). The uptake of glucose showed a similar pattern to that of the amino

![Graph](image)

**Fig. 4.** The effect of cell density and glucose concentration on (A) the intracellular
concentration of glucose and the rates of (B) protein, (C) DNA and (D) RNA
synthesis. See Fig. 1 for explanation of symbols.

acids (see Fig. 1). Cell density had a significant effect in MRC-5 cultures (the post-
confluent intracellular concentration was 50% of the pre-confluent) but only a
limited effect in L-132 cultures (the post-confluent intracellular concentration was
75% of the pre-confluent). Cell density had very little effect on the rate of protein
synthesis in both L-132 and MRC-5 cultures (the post-confluent rates were 85 and
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76% respectively of the pre-confluent). External glucose concentration had an identical effect on protein synthesis in sparse and crowded MRC-5 cultures (maximum rate at 200 μg/ml) and a similar effect in L-132 cultures (maximum rate at 100 and 200 μg/ml respectively for pre- and post-confluent cultures). Cultures of MRC-5 cells had only about a half of the biosynthetic activity of L-132 cells.

The patterns of DNA and RNA synthesis were almost identical. The post-confluent rate of synthesis in MRC-5 cells was only 7–8% of the pre-confluent rate, and the pre-confluent rate was about 80% of the L-132 values. The rates of synthesis were completely unaffected by the density of L-132 cells.

The effect of nutrient concentration on cell growth

Yields of MRC-5 and L-132 cells at various concentrations of arginine, valine and leucine after 168 h growth are shown in Fig. 5. A consistent difference between the 2 cell types was that the yields of L-132 cells increased rapidly from 0% to about 10%

![Fig. 5. The effect of amino acid concentration on yields of MRC-5 and L-132 cells after 168 h growth. MRC-5 cells, open symbols; L-132 cells, closed symbols and dotted line. O, ●, arginine; Δ, ▲, valine; □, leucine. Amino acid concentrations given in Fig. 1.](image)

amino acid concentration, at which point a plateau was reached, whereas MRC-5 cells showed no increase in yield between 0 and 10% but a rapid rise from 10 to 30% before the plateau was attained. These data on cell growth patterns are consistent with the differences in rates of protein synthesis between L-132 and MRC-5 cells (Fig. 2).
This work was carried out to determine whether contact inhibition of growth in HDC was influenced by the effects of cell crowding on nutrient uptake by the cells. For this purpose an epithelial, heteroploid human lung cell (L-132) which did not exhibit contact inhibition of growth was compared with the human diploid fibroblastic lung cell MRC-5. The ability of these 2 cell lines to accumulate various amino acids intracellularly against an external concentration gradient of that amino acid was measured. In a pre-confluent culture the ability of the 2 cell types to incorporate nutrients was almost identical but a large difference showed up in post-confluent cultures (Fig. 1). The L-132 cell was able to accumulate nutrients far more readily than the MRC-5 cell, although the post-confluent level was slightly lower than the pre-confluent level. This result showed that a difference does exist between the 2 types of cells in their ability to take up nutrients and as the difference only showed up in crowded cultures it was indicative that this was influencing contact inhibition of growth.

Attempts were then made to relate this difference in nutrient uptake to cell growth and metabolism as a possible prelude to understanding the mechanism of contact inhibition. The effects of nutrient concentration on protein synthesis in both pre- and post-confluent cultures were studied (Fig. 2). In post-confluent MRC-5 cultures the external concentration of arginine, leucine, valine and methionine required to achieve the maximum rate of protein synthesis was higher than in pre-confluent cultures. However, calculation of the intracellular level of these amino acids at the point at which the maximum rate of protein synthesis was attained showed identical or similar values in both pre- and post-confluent cultures. Protein synthesis, therefore, was being controlled by the intracellular amino acid concentration which in turn was being controlled by both the extracellular concentration and cell density. L-132 cells showed no quantitative change in the amino acid concentration needed for protein synthesis with cell density, and this was a reflection of the relatively small differences in nutrient uptake and the lower concentrations of the amino acids required for maximum protein synthesis. These results are consistent with the concept that cells require a threshold concentration of amino acids for protein synthesis (Eagle, Piez & Levy, 1961). If cell density reduces the ability of the cell to reach this concentration then the concentration at which an amino acid can become growth-limiting will vary with the cell count. Thus leucine in the cultures described in Figs. 1 and 2 would be growth-limiting when 80% utilized in a pre-confluent culture but in a post-confluent culture when only 52% utilized. The data also showed that arginine and leucine were affected more than valine and much more than aminoisobutyric acid. This reflects previously found differences in the relative importance of these amino acids (Griffiths, 1970a).

A major contributory factor in contact inhibition of growth is inhibition of DNA synthesis (Rhode & Ellem, 1968; Schutz & Mora, 1968; Griffiths, 1971). This was confirmed by the data in Figs. 1–3 which showed the depression of macromolecular synthesis in post-confluent cultures. In MRC-5 cells protein synthesis was 75–85%
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of the pre-confluent level, RNA synthesis 35% and DNA synthesis only 9-20%. In L-132 cells DNA synthesis was only depressed to 85% of the pre-confluent level whilst protein synthesis was 100% and RNA synthesis 40%. The major difference between these 2 cell types in their response to cell crowding, therefore, was the far greater inhibition of DNA synthesis in MRC-5 cultures.

Glucose is more directly concerned in DNA synthesis than the amino acids and it must also be considered a possible growth-limiting nutrient (Griffiths, 1971). The pattern of glucose uptake was similar to that found for the amino acids (Fig. 4). Increased cell density had more effect on MRC-5 than L-132 cells and also MRC-5 cells became saturated at 500 μg/ml glucose whereas L-132 cells continued to accumulate glucose up to an external concentration of 1000 μg/ml. The effect which glucose concentration had on the rate of protein synthesis was identical for both cell types in both pre- and post-confluent cultures although the overall rate of synthesis of L-132 cells was twice that of MRC-5 cells. The patterns of DNA and RNA synthesis were identical. The rates were almost identical in pre-confluent cultures of both cells and post-confluent cultures of L-132 cells. In post-confluent MRC-5 cultures, however, both RNA and DNA synthesis were almost completely inhibited. High glucose concentrations therefore were unable to stimulate post-confluent DNA synthesis and glucose-limitation was not an important factor in the onset of contact inhibition of growth.

The results in this paper show quite clearly that nutrient uptake was significantly depressed by cell crowding in cultures of MRC-5 cells and that this constitutes a major difference between the diploid cell and the heteroploid L-132 cell. This reduced ability for nutrient uptake was reflected in a reduced rate of protein synthesis and a requirement for higher extracellular concentrations to achieve the maximum rate of protein synthesis. However, as depression of DNA synthesis is probably the most important result of contact inhibition of growth the data do not explain how continuous medium perfusion (Kruse & Miedema, 1965; Griffiths, in preparation) and frequent medium changes (Griffiths, 1971) overcome the inhibition of DNA synthesis and post-confluent growth.

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REFERENCES


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