The role of HMG CoA reductase and dolichol synthesis in the control of 3T6 cell proliferation: effects of cell crowding, serum depletion and addition of epidermal growth factor

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Summary

The proliferation of 3T6 cells was substantially decreased when the monolayer cultures were allowed to reach confluency. This growth inhibition (so-called density-dependent inhibition) was of the same magnitude as that following serum depletion in non-confluent cultures. Each type of growth inhibition was correlated to a depression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, an enzyme that regulates the biosynthesis of cholesterol and isoprenoid derivatives (e.g. dolichol) by catalysing the reduction of HMG CoA (which is derived from acetyl-CoA) into mevalonate. However, the depression of enzyme activity was more substantial in cells exposed to cell crowding than that in serum-depleted cells (87 and 48 %, respectively). On the other hand, there was a 60–65 % inhibition of the incorporation of mevalonate into dolichol due to serum deprivation, while it remained at normal level in confluent cultures, which implies that the inhibitory effects on dolichol synthesis due to these two experimental conditions were approximately equipotent. Addition of epidermal growth factor (EGF) to the cell cultures, whose proliferation was inhibited due to serum depletion, restored DNA synthesis completely, and these effects were related to a normalization of the activity of HMG CoA reductase and of the incorporation of mevalonate into dolichol. In contrast, in confluent cells addition of EGF only caused a slight increase in DNA synthesis and activity of HMG CoA reductase, and there was no significant increase in the incorporation of mevalonate into dolichol either. Supplementation with mevalonate as well as dolichol to confluent cell cultures induced a significant increase in DNA synthesis, while only dolichol was capable of increasing DNA synthesis in serum-depleted cells.

Taken together, these results propose: (1) the existence of two regulatory sites in the biosynthesis of dolichol, the first one of which is constituted by HMG CoA reductase, and the second one by an unknown enzyme in the distal part of the pathway; (2) that the activity of HMG CoA reductase is stimulated by serum and growth factors, and is inhibited by cell crowding; (3) that the second step is independent of the culture density, but is stimulated by serum and growth factors; (4) that these two regulatory sites are involved in the control of cell proliferation in 3T6 cells.

Key words: HMG CoA reductase, dolichol synthesis, 3T6 cell proliferation.

Introduction

The 3T6 cell line has been found useful in the study of cell cycle control (Larsson & Zetterberg, 1986a; Larsson, 1987). In comparison with 3T3 cells and SV-3T3 cells, 3T6 cells may be considered as being intermediately tumour-transformed (Larsson & Zetterberg, 1986a). Like their ‘normal’ counterparts (3T3 cells), 3T6 cells respond to serum depletion by rapidly decreasing their proliferation in a cell cycle-specific fashion (Larsson & Zetterberg, 1986a). However, unlike 3T3 cells, they do not enter a phase of quiescence (G0). Instead, they continue traversing the cell cycle at a reduced rate (Larsson & Zetterberg, 1986a). Interestingly, the inhibitory effect of serum depletion on the proliferation of 3T3 and 3T6 cells was correlated

to a decrease in the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase in both cell types (Larsson & Zetterberg, 1986a). HMG CoA reductase is an endoplasmic enzyme that controls the biosynthesis of cholesterol and a variety of isoprenoid derivatives (e.g. coenzyme Q, isopentenyladenine, dolichol) by catalysing the conversion of HMG CoA to mevalonate. In a more recent study (Larsson, 1987) the effects of different serum regimens on the metabolism of mevalonate in 3T6 cells were investigated. It was found that the incorporation of mevalonate into dolichol was decreased by 60-75% following a 4-h serum depletion, while that into cholesterol remained high (Larsson, 1987). Considered together with a definite serum dependency in the control of HMG CoA reductase activity, serum factors are likely to play an important role in the de novo synthesis of dolichol from acetyl-CoA. In turn, external addition of dolichol to serum-depleted cells partially counteracted the decrease in cell proliferation, which would otherwise occur (Larsson, 1987). These findings suggest the existence of a dolichol-dependent control of 3T6 cell proliferation.

The present study aims to investigate further the role of HMG CoA reductase and dolichol synthesis in the growth regulation of 3T6 cells. Interest will be especially focused on how cell crowding, in comparison with serum depletion, affects these events. The effects of addition of a purified growth factor, i.e. epidermal growth factor (EGF), will also be studied.

Materials and methods

Chemicals

Mevalonolactone and dolichol (dolichol-20) were purchased from Sigma Company, Stockholm, Sweden, and radiochemicals from Amersham, Stockholm, Sweden. Epidermal growth factor (receptor grade) was purchased from Collaborative Research.

Cell culture

Swiss 3T6 cells (obtained from Flow Laboratories Inc) were maintained in tissue culture bottles. The stock cultures were grown in a humidified 5% CO₂/95% air mixture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 50 units of penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹. For experimental purpose cells were transferred onto plates by treatment with 0.25% trypsin in Tris-buffered saline containing 0.5 mM-EDTA. The experiments were performed on cultures with different cell densities, i.e., 10 000–500 000 cells cm⁻².

Determination of DNA synthesis

DNA synthesis in 3T6 cells growing on glass coverslips was estimated by incorporating [³H]thymidine (1 μCi ml⁻¹, 25 Ci mmol⁻¹) into acid-precipitable material for 30 min before fixation in 95% (v/v) ethanol. Autoradiographs were prepared as described (Zetterberg & Larsson, 1985), and % labelled nuclei was determined by microscopic examination.

Determination of HMG CoA reductase activity

After the indicated experimental procedures, 3T6 cells were washed twice with buffered-phosphate saline, scraped and collected by centrifugation. The cell pellets were frozen in a buffer containing 50 mM-K₂HPO₄, 5 mM-EDTA, 5 mM-dithiothreitol until the time of assay. After thawing the frozen cell pellets, 200 μl of a buffer solution containing 100 mM-K₂HPO₄, 20 mM-dithiothreitol, 40 mM-glucose-6-phosphate, 5 mM-NADPH and 5 units of glucose-6-phosphate dehydrogenase ml⁻¹ was added; 0.9 nmol of [¹⁴C]HMG CoA (57 mCi mmol⁻¹) was then added, and after a 60-min incubation period (37°C) the reaction was ended by addition of 10 μl of 1 M-KOH; 20 min later the basic solution was neutralized with 20 μl 1 M-HCl, and 0.4 pmol [³H]mevalonolactone (1.27 Ci mmol⁻¹) was added as an internal standard. One sample was taken for determination of nucleic acid content (by spectrophotometry) and another for separation of [¹⁴C]mevalonolactone from [¹⁴C]HMG CoA by ion-exchange chromatography (Edwards et al. 1979). The amount of [¹⁴C]mevalonate was then determined by scintillation counting and related to the actual nucleic acid content. Corrections were made for background, quenching and spillover.

Determination of incorporation of [³H]mevalonate into cholesterol and dolichol

Cells on monolayer were labelled with [³H]mevalonolactone (10 μCi ml⁻¹, 1.27 Ci mmol⁻¹) for the indicated intervals, rinsed twice with buffered-phosphate saline, dissolved in 1 ml of 0.1 M-NaOH. From the lysate 50 μl was taken for determination of nucleic acid content. Lipids were then extracted from the dissolved cells by a modification (Faust et al. 1979) of the method of Bligh & Dyer (1959). [¹⁴C]cholesterol was added as an internal standard. Cholesterol and dolichol were separated by HPLC with a radial-PACK CN-cartridge (10 cm × 0.8 cm, 10 jμm) in a radial compression chamber (RCM-8) (Waters Associates) in n-hexane (HPLC-grade): isopropanol (HPLC-grade) (10:000:5) over a 30-min interval. The flow rate was 1 ml min⁻¹ and 1.0-ml fractions were collected for scintillation counting. The detection was made by a variable wavelength detector at 210 nm and a full-scale absorbance of 0.32 was used.

By carrying out normal-phase HPLC on a cyanopropyl(CN) column the total amount of dolichol, irrespective of isoprenoid chain length, could be isolated and measured, as described (Palmer et al. 1984).

Flow cytophotometry

The cells cultivated in bottles were washed with an EDTA solution (0.2 mM) briefly at room temperature. The EDTA was removed and the cells were then exposed to 0.25% trypsin solution at 37°C until the cells detached from the glass surface (normally 2-5 min). The cells were then suspended in medium containing 10% FCS. After a 5-min centrifugation, at 5000 revs min⁻¹, the supernatant was removed. The cells were then washed once with 0.2 M-Tris-buffered saline and fixed by adding 1 ml 95% ethanol at 0°C, drop by drop, under vigorous stirring. The suspension was
kept in a refrigerator (4°C) until further analysis was completed.

The cells were stained in a buffer solution containing 0.14 M NaCl, 0.11 M Tris HCl (pH 7.0), and 50 mg ethidium bromide per 1000 ml buffer solution; 50 μl RNase (100 000 i.u. ml⁻¹ in 0.9% NaCl solution) was added to every 5 ml of stain solution.

Results

Fig. 1 shows how different culture densities of 3T6 cells affect DNA synthesis. Cells were grown up to various culture densities: 10 000 cells cm⁻² ('sparse'), 25 000 cells cm⁻² ('intermediate') and 50 000 cells cm⁻² ('confluent'), after which they were pulse-labelled with [³H]thymidine. As can be seen, the [³H]thymidine labelling index was substantially decreased as the cell cultures reached confluency (from 55–60% to 14%), whereas the labelling index did not differ to any significant extent between the intermediate and sparse cultures. By flow cytophotometry it was confirmed that the decrease in cell proliferation due to cell crowding was attributable to an increased accumulation of cells in the G1 phase (Fig. 2).

By exposing non-confluent (sparse and intermediate) cultures to serum-free medium the percentage of cells synthesizing DNA was substantially depressed (Fig. 3). The labelling index was thereby decreased from 50–60% to 10–12%. As can be seen there was no significant difference between sparse and intermediate cultures in their response to serum depletion.

Since there was no appreciable difference between sparse and intermediate cell cultures in proliferative activity in the presence or absence of serum, sparse cultures are omitted in the following experiments.

In Fig. 4 the effects of different culture densities and serum regimens on the activity of HMG CoA reductase are demonstrated. The HMG CoA reductase activity was decreased by 83% when the cells reached confluency, whereas the enzyme activity in serum-depleted non-confluent cultures was reduced by approximately 50%. Because HMG CoA reductase constitutes the
Fig. 4. Effects of cell crowding and serum depletion on activity of HMG CoA reductase. 3T6 cells were seeded at two different cell concentrations in DMEM + 10% FCS. At 48 h later, when the cultures had reached cell densities of 20 000 (intermediate) and 50 000 cells cm$^{-2}$ (confluent), respectively, the confluent cultures were changed to DMEM + 10% FCS (hatched bar), and the non-confluent cultures to DMEM with (open bar) or without serum (filled bar). At 24 h later the cells were harvested for assay of HMG CoA reductase activity. The data represent the mean values of duplicate determinations from three separate experiments. *, $P < 0.05$; ***, $P < 0.005$.

Fig. 5. Effects of cell crowding and serum depletion on incorporation of $[^{3}H]$mevalonate into dolichol and cholesterol. 3T6 cells were grown up to either non-confluent (20 000 cells cm$^{-2}$) or confluent (50 000 cells cm$^{-2}$) cell densities. The confluent cultures were then exposed to DMEM + 10% FCS + $[^{3}H]$mevalonolactone (10 $\mu$Ci ml$^{-1}$) (A), and the non-confluent cultures to DMEM + $[^{3}H]$mevalonolactone (10 $\mu$Ci ml$^{-1}$), with (C) or without (B) serum, over a 24-h period, after which the cells were harvested for extraction of neutral lipids. Cholesterol and dolichol were separated by normal-phase HPLC with a radial Pak CN-cartridge. Peaks corresponding to reference compounds of cholesterol and dolichol were identified, and 1-ml fractions were collected and counted with a liquid scintillation counter.

Fig. 6 shows the effects of supplementation with mevalonate or dolichol on the cell cultures, at various concentrations (10–1000 $\mu$M, 0.01–1 $\mu$g ml$^{-1}$, respectively), on the proliferation of 3T6 cells exposed to cell crowding or serum depletion. This was measured by determination of the $[^{3}H]$thymidine labelling index after pulse-labelling with $[^{3}H]$thymidine. Dolichol added at concentrations of 0.01 $\mu$g ml$^{-1}$ or 0.1 $\mu$g ml$^{-1}$ was found partially to counteract the decrease in DNA synthesis due to serum depletion, whereas mevalonic acid, irrespective of the concentration added, did not significantly increase the fraction of cells synthesizing DNA. In contrast, supplementation with dolichol as well as mevalonate partially counteracted the decrease in DNA synthesis following cell crowding. A concentration of 100 $\mu$M mevalonate had the best effect on cell proliferation. These results, namely that a metabolite (mevalonate) and/or a product of the pathway (dolichol) exert counteractive effects, even if partial, on the inhibition of cell proliferation, suggest: (1) that the depression of HMG CoA reductase is a cause rather than a result of decrease in cell proliferation; (2) that the product, i.e. dolichol or a dolichol-related metabolite, is involved in the control of cell proliferation.
Table 1. Effects of serum depletion and cell crowding on the incorporation of [3H]mevalonolactone into cholesterol and dolichol

<table>
<thead>
<tr>
<th>Culture density</th>
<th>Incorporation of [3H]mevalonolactone into:</th>
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<tbody>
<tr>
<td></td>
<td>Serum Dolichol Cholesterol</td>
<td></td>
</tr>
<tr>
<td>Non-confluency</td>
<td>(+) 2700 980 3150</td>
<td></td>
</tr>
<tr>
<td>Confluency</td>
<td>(+) 62390 49250 58860</td>
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Values are total radioactivity in peaks corresponding to cholesterol and dolichol (compare with Fig. 5).

Fig. 6. Effects of supplementation with mevalonate or dolichol on DNA synthesis of 3T6 cells exposed to cell crowding and serum depletion. 3T6 cells were seeded at two different cell concentrations in DMEM + 10% FCS. At 48 h later, when the cells had reached 'non-confluent' (approx. 20 000 cells cm⁻²) or confluent (approx. 50 000 cells cm⁻²) culture densities, they were shifted to DMEM + 10% FCS or DMEM without serum (only for non-confluent cells) + indicated concentrations of mevalonolactone (10⁻¹⁻¹⁰⁻⁰ µM) (---), or dolichol-20 (0.01-1 µg ml⁻¹) (-----). At 24 h later the cells were pulse-labelled (30 min) with [³H]thymidine and then fixed. Autoradiographs were processed, and the percentage of labelled cells was determined microscopically. The values obtained represent the mean values and standard deviations of duplicate determinations from three different experiments. *, P<0.05; **, P<0.01.

Fig. 7. Effects of EGF on DNA synthesis of 3T6 cells exposed to cell crowding and serum depletion. 3T6 cells were seeded at two different cell concentrations in DMEM + 10% FCS. At 48 h later, when the cultures had reached cell densities of 20 000 (non-confluent) or 50 000 cells cm⁻² (confluent), respectively, the confluent cultures were exposed to DMEM + 10% FCS, with or without EGF (100 ng ml⁻¹), and the non-confluent cultures to DMEM, with or without EGF (100 ng ml⁻¹). At 24 h later the cells were pulse-labelled (30 min) with [³H]thymidine and then fixed. Autoradiographs were processed, and the percentage of labelled cells was determined microscopically. The indicated values represent the mean values of duplicate determinations from two experiments. n.s., non-significance; ***, P<0.005.

Fig. 7 shows the effects of epidermal growth factor (EGF) on cell proliferation. An EGF dose of 100 ng ml⁻¹ was chosen because this concentration had the best effect on 3T6 cell proliferation (data not shown). As can be seen, EGF efficiently counteracted the inhibitory effect of serum depletion on DNA synthesis, whereas the growth inhibitory effect mediated by cell crowding was hardly affected (Fig. 7). The difference between crowded and serum-arrested cells in their response to stimulation of EGF might be due to medium conditioning by growth-inhibitory factors in crowded cells, which would thereby counteract the stimulatory effect of EGF. However, in a recent study it was demonstrated that conditioned medium from crowded 3T6 cells does exert a stimulatory effect on cell proliferation of serum-depleted cells (Dafgård et al. 1987). Moreover, it has been confirmed that 3T6-conditioned medium does not counteract the stimulatory effect of EGF on DNA synthesis (unpublished results).

The counteractive effect of EGF on growth inhibition due to serum depletion in non-confluent cultures
Fig. 8. Effects of EGF on HMG CoA reductase activity of 3T6 cells exposed to cell crowding and serum depletion. 3T6 cells were seeded at two different cell concentrations in DMEM+10% FCS. At 48 h later, when the cultures had reached cell densities of 20000 (non-confluent) or 50000 cells cm$^{-2}$ (confluent), respectively, the confluent cultures were exposed to DMEM+10% FCS, with (hatched bar) or without (open bar) EGF (100 ng ml$^{-1}$), and the non-confluent cultures to DMEM with (hatched bar) or without (open bar) EGF (100 ng ml$^{-1}$). At 24 h later the cells were harvested for assay of HMG CoA reductase activity. The indicated values represent the mean values of duplicate determination from two to three experiments. n.s., non-significance; ***, $P<0.005$.

was related to a complete counteraction of the decrease in HMG CoA reductase activity (Fig. 8). In addition, supplementation with EGF counteracted the decrease in the incorporation of mevalonate into dolichol (Table 2). In contrast, in cells growth-inhibited due to cell crowding EGF did not prevent the decrease in the activity of HMG CoA reductase (Fig. 8), and only exerted a slight increase in the incorporation of mevalonate into dolichol (Table 2). From this it follows that the synthesis of dolichol was not increased to any appreciable extent in crowded cells stimulated by EGF.

Table 2. Effects of EGF on the incorporation of $[^{3}H]$mevalonolactone into dolichol and cholesterol

<table>
<thead>
<tr>
<th>Culture density</th>
<th>Serum (10%)</th>
<th>EGF (1000 ng ml$^{-1}$)</th>
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<tr>
<td></td>
<td>(+/-)</td>
<td>(+/-)</td>
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<tr>
<td>Non-conflueny</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Conflueny</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
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Incorporation of $[^{3}H]$mevalonolactone into:

<table>
<thead>
<tr>
<th></th>
<th>Dolichol</th>
<th>Cholesterol</th>
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<tbody>
<tr>
<td></td>
<td>(cts min$^{-1}$ mg$^{-1}$ nucleic acid)</td>
<td>(cts min$^{-1}$ mg$^{-1}$ nucleic acid)</td>
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<table>
<thead>
<tr>
<th></th>
<th>980</th>
<th>49250</th>
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<tr>
<td></td>
<td>3060</td>
<td>6540</td>
</tr>
<tr>
<td></td>
<td>3150</td>
<td>58860</td>
</tr>
<tr>
<td></td>
<td>3949</td>
<td>55030</td>
</tr>
</tbody>
</table>

3T6 cells were grown up to either non-conflueny (20 000 cells cm$^{-2}$) or conflueny. Confluent cultures were then shifted to DMEM+10% FCS + $[^{3}H]$mevalonolactone (10 $\mu$Ci ml$^{-1}$), with or without EGF (100 ng ml$^{-1}$), and non-confluent cultures were shifted to DMEM + $[^{3}H]$mevalonolactone (10 $\mu$Ci ml$^{-1}$), with or without EGF (100 ng ml$^{-1}$). Total radioactivity in the peaks corresponding to cholesterol and dolichol was measured.

Discussion

As shown in the present study, the proliferation of 3T6 cells is substantially decreased by cell crowding (so-called density-dependent inhibition). This type of growth inhibition is related to a substantial decrease in HMG CoA reductase activity. A similar inhibition of HMG CoA reductase activity correlated to cell density has been shown to occur in 3T3 cells (Chen, 1983). Thus, as in the case with serum depletion, 3T6 cells seem to exhibit similar growth characteristics to their 'normal' counterparts (3T3 cells). In contrast, there is no detectable decrease in either HMG CoA reductase activity or cell growth of SV40-transformed 3T3 cells (SV-3T3 cells) when they reach confluency (unpublished results). Taken together these data suggest that the regulatory influence of culture density on cell growth may be mediated by mechanisms in which the regulation of HMG CoA reductase is involved. This is supported by the observation that supplementation with mevalonate increases the fraction of cells synthesizing DNA in confluent cells. However, addition of mevalonate to 3T6 cells that were growth-inhibited due to serum depletion did not affect DNA synthesis to any detectable extent. This, considered together with the fact that incorporation of mevalonate into dolichol was decreased in cells growth-inhibited due to serum depletion but not due to cell crowding, and that supplementation with dolichol in fact increased DNA synthesis in both cases, suggests that de novo synthesis of dolichol, or a dolichol-related metabolite, rather than the activity of HMG CoA reductase per se, is involved in growth regulation of 3T6 cells.

Thus, the rate of dolichol synthesis seems to be regulated at two different levels in the pathway: the first level constitutes HMG CoA reductase, and the second level a step in the metabolism of mevalonate, probably a step in the formation of dolichol from farnesyl pyrophosphate. The first step is dependent on both cell density and the presence of serum growth factors (i.e. EGF), while the second step is dependent on serum or growth factors, but not on cell density.
DNA synthesis may play a role in biological membranes by affecting the transmembrane permeability of alkaline cations (Boscoboinik et al. 1985).

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References


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