Effects of Calcium Depletion on Human Cells in Vitro and the Anomalous Behavior of the Human Melanoma Cell Line MM170

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ABSTRACT

Seven strains of normal human cells (fibroblastic, skin epithelial, and amniotic) ceased to proliferate in medium depleted of free calcium ion by titration with ethylenebis(oxyethylenenitri-lo)tetraacetic acid (EGTA), whereas the growth of 9 of 10 human melanoma cell lines was not affected. Fibroblasts showed a rapid drop in thymidine pool size and decreased incorporation of thymidine and uridine when treated with EGTA, followed during the next 48 hr by a decrease in plasma membrane potential and by development of a proliferative block in the G1 phase of the cell cycle. The calcium-independent melanoma line MM96 exhibited an early decrease in thymidine pool size and enhanced incorporation of nucleosides but continued to proliferate with little perturbation of the cell cycle or change in membrane potential. Tumor cell DNA may therefore be selectively labeled in the presence of normal cells. The anomalous, calcium-independent melanoma line (MM170) showed an immediate increase in the thymidine pool size and in nucleoside incorporation and subsequently accumulated in G1 and G2 with diminution of membrane potential and of DNA and RNA synthesis. The proliferative block in MM170 cells could be reversed by addition of calcium ion or by replacement with control medium. Addition to the medium of all 8 nucleosides (50 μM), singly or together, did not prevent EGTA-induced cytostasis in fibroblasts or MM170; transport of thymidine across the cell membrane was enhanced by 24-hr EGTA treatment in fibroblasts, MM96, and MM170. Thus, although calcium affected thymidine utilization rapidly and differently in each of the three cell types, nucleoside starvation per se did not appear to be responsible for either type of proliferative block.

INTRODUCTION

The ability of cells transformed by adenovirus (17) or avian sarcoma virus (1, 2) to proliferate at low calcium levels has since been shown to have a general property of the neoplastic phenotype (4, 5, 22, 24, 30–32). In general, the proliferation of normal human and rodent cells ceases at 25 to 100 μM free calcium of neoplastic cells (human tumor cell lines and chemically transformed rodent cells) at 10 μM, and of virus-transformed cells below 1 μM. Cells are arrested reversibly in the G1 phase of the cell cycle after 1 to 2 divisions (4, 24, 38) and become depleted of calcium (24). The mechanism for the continued growth of neoplastic cells at low calcium levels remains unknown. Calcium affects many cell functions, either directly or indirectly, and transformation is accompanied by various changes in the plasma membrane (3, 20, 24), in mitochondria (7, 20), and in calcium-binding proteins (19) that could affect the role of calcium.

In extending a previous study (22) of human material, particularly with respect to the early effects of calcium depletion on thymidine utilization, we have found that the proliferation of one melanoma cell line is blocked at the same calcium level as that of normal cells, but apparently by a different mechanism. The possible role of mitochondria and changes in cell membrane potential have also been investigated. Apart from opening up new approaches to the comparison of growth control mechanisms in normal and tumor cells, the phenomenon offers an in vitro method for selective growth of human tumor cells, complementary to other techniques such as growth in soft agar, and has been used as such to aid the establishment of tumor cell lines from biopsies (22). In another potential application, the feasibility of which was evident from a study of rat hepatoma (33), in vitro assays of DNA damage for prediction of drug resistance in tumor biopsies would require selective labeling of tumor DNA in the presence of various kinds of normal cells. A secondary aim of this work was therefore to define the conditions needed to achieve such labeling using calcium-depleted medium.

MATERIALS AND METHODS

The derivation and general properties of the human cell lines have been described (6, 23, 26, 37). Cells were cultured in Roswell Park Memorial Institute Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5 mM 4-(2-hydroxyethyl)-1-pipera-zoneethanesulfonic acid. Periodic assays for Mycoplasma (23) were negative. All comparative studies of agents and cell lines were performed simultaneously using the same reagents; cell lines were used 24 hr after passage. Flow cytometric analysis was carried out on a FACS IV instrument (Becton-Dickinson FACSS Systems, Sunnyvale, Calif.) with an argon ion laser (Spectra-Physics Model 164-05; operated at 1 watt and using an excitation wavelength of 514 nm). Cells were treated with RNase and Triton in phosphate buffer (0.15 M NaCl, 10 mM Na2PO4, and 3 mM KH2PO4, pH 7.2) and stained with propidium iodide (50 μg/ml), as described by Taylor (35). Chicken RBC were added to cells with the staining solution to act as an internal standard (34). Cell size was determined using a Particle Data counter (Particle Data, Elmhurst, Ill.).

RESULTS

Cell Proliferation in EGTA Medium. The calcium-selective chelating agent EGTA was used to deplete the medium of free calcium. EGTA may chelate other metal ions, although with lower efficiency. Addition of zinc and cupric ions (1 mM) reversed EGTA effects in lymphocytes (27), but this was attributed to displacement of calcium from the EGTA complex. The calcium-EGTA
complex was not toxic to cells used in this or previous (22, 38) studies. Unless otherwise stated, all EGTA-containing medium was titrated to the level required for blocking fibroblast proliferation, using the cell growth assay, as described previously (22). Different batches of complete medium required different levels of EGTA (0.55 to 1.0 mM) to reach this end point, the free calcium concentration, determined using plasmacorinth B (11), being in the 20 to 30 μM range. Seven cultures of normal cells constituting 3 different cell types (fibroblastic, skin epithelioid, and amniotic) failed to proliferate under these conditions, whereas 9 of 10 melanoma lines were unaffected. Two continuous human lines of supposedly normal origin, Chang liver and AV-2 cells (Commonwealth Serum Laboratories), were found to proliferate in EGTA medium. These cells also grew in agar, indicative of a transformed phenotype, and were therefore excluded from further studies.

To make quantitative comparisons of the growth of cell lines at low calcium levels, sparsely seeded cultures were grown for 7 days at various EGTA concentrations and then pulsed with [3H]thymidine. Of the 9 melanoma lines tested, only 1 (MM170) failed to grow at the EGTA end point (0.6 mM) required to inhibit the 2 fibroblast strains (Chart 1). Inhibition of the EGTA-tolerant melanoma lines occurred above 0.7 mM EGTA, the cells becoming rounded within 24 hr of treatment. The nature of this inhibition was not further studied. The EGTA-induced cytostasis of MM170 cells and PGP fibroblasts was not prevented by addition of 50 μM thymidine, deoxythidine, deoxyadenosine, deoxyguanosine, uridine, cytidine, adenosine, and guanosine, either singly or together. Exogenous ascorbate (50 μM), added to the EGTA medium because of the reported calcium dependence of ascorbate transport (16), also failed to prevent the block. The fastest-growing melanoma lines appeared to be the most tolerant to low calcium, and a subline (MM253c1-11E) derived from one of these by culture in increasing levels of EGTA over 8 weeks (11 cycles of treatment) was made even more resistant. After several passages in control medium, however, MM253c1-11E reverted to normal tolerance. Similar treatment of MM96 cells after exposure to 5-(3-methyl-1-triazeno)imidazole-4-carboxamide, a carcinogenic methylating agent (21), was also unsuccessful in producing stable, enhanced tolerance to calcium depletion.

Cell growth at high densities was assessed by determining the increase in cell number. As reported previously (22), melanoma cells continued to proliferate in EGTA medium, whereas fibroblasts underwent 1 to 2 rounds of replication before growth ceased (Chart 2A). The MM170 line behaved as the latter, the block being reversible by culture in control medium. Since the growth of fibroblasts is anchorage dependent in 10% fetal calf serum (25), it was possible that calcium depletion caused detachment of cells from the plastic surface or prevented reattachment of cells after events such as mitosis, with consequent inhibition of growth. To investigate this possibility, cells prelabeled with [3H]thymidine were cultured in EGTA medium, either as a cell suspension or as preattached monolayers. The results (Chart 2B) showed that melanoma cells and fibroblasts attached to plastic surfaces and remained adhered equally as well in EGTA as in control medium.

Cell Cycle Analysis. Both of the methods used for determination of the growth fraction showed that the majority of cells in control cultures were proliferating (Table 1). Despite the low proportion of fibroblasts in the G2 phase of the cell cycle, it was possible to show that fibroblasts cultured in EGTA medium became blocked in the G1 phase (Chart 3; Table 1). MM253c1 cells were not affected under such conditions. Some G1 accumulation was observed in MM96 and MM127 cells at 48 hr but had almost disappeared by 96 hr. MM170 cells, however, accumulated in the G2 phase (Chart 3). Little significance can be attached to apparent changes in the S phase, because the proportion of cells was quite low (less than 0.25, even for the rapidly proliferating lines) and was difficult to determine accurately because of broadening of the G1 and G2 peaks following EGTA treatment.

The temporal response of these changes (Table 1) showed that the effect in fibroblasts was complete within 1 cell-doubling time.
Human Cells in Calcium-depleted Medium

Table 1

<table>
<thead>
<tr>
<th>Cell</th>
<th>Growth fraction</th>
<th>Fraction in G1</th>
<th>Treatment time (hr)</th>
<th>Control</th>
<th>EGTA</th>
<th>% of change</th>
<th>Control</th>
<th>EGTA</th>
<th>% of change</th>
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<tr>
<td>PGP</td>
<td>Labeling method</td>
<td>Colcemid method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM170</td>
<td>0.94</td>
<td>0.93</td>
<td>48</td>
<td>0.68 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58 ± 0.04 (0.3)</td>
<td>-15</td>
<td>0.22 ± 0.04</td>
<td>0.34 ± 0.02 (2.7)</td>
<td>55</td>
</tr>
<tr>
<td>MM170 + Colcemid (1 μg/ml added at 48 hr)</td>
<td>0.94</td>
<td>0.93</td>
<td>48</td>
<td>0.53</td>
<td>0.50</td>
<td>-6</td>
<td>0.24</td>
<td>0.42</td>
<td>75</td>
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<tr>
<td>MM96</td>
<td>1.0</td>
<td>0.92</td>
<td>96</td>
<td>0.13</td>
<td>0.33</td>
<td>154</td>
<td>0.81</td>
<td>0.63</td>
<td>-22</td>
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<tr>
<td>MM127</td>
<td>0.73</td>
<td>0.94</td>
<td>48</td>
<td>0.79</td>
<td>0.82</td>
<td>3</td>
<td>0.13</td>
<td>0.09</td>
<td>-31</td>
</tr>
<tr>
<td>MM253c1</td>
<td>0.76</td>
<td>0.94</td>
<td>96</td>
<td>0.55</td>
<td>0.59</td>
<td>0</td>
<td>0.32</td>
<td>0.35</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Proportion of labeled nuclei in cells cultured for 2 doubling times in the presence of [³H]thymidine (1 μCi/ml).

<sup>b</sup> Proportion of G2 cells after culturing for 2 doubling times in the presence of Colcemid (1 μg/ml; Grand Island Biological Co. Diagnostics, Grand Island, N. Y.).

<sup>c</sup> Mean ± S.E. of 2 experiments.

<sup>d</sup> Numbers in parentheses, change in modal DNA content per cell as the percentage of modal DNA in controls.

<sup>e</sup> Mean ± S.E. of 4 experiments.

Charts. Effect of EGTA on the cell cycle. After 48 hr of growth in control medium (upper profiles) or EGTA medium (lower profiles), cells were analyzed using flow cytometry, as described in the text. Right-hand peak in each profile, G2 cells. Peak on the extreme left in the PGP profile, a chicken RBC marker.

time (48 hr), whereas 58% of MM170 cells remained in G1 even after 2 doubling times (96 hr). To determine whether these cells were blocked in G1, as well as in G2 or were merely progressing slowly through G1, some cultures were treated with Colcemid. As found using other cell types (12), the proportion of MM170 cells in G1 was decreased 4-fold in normal medium (Table 1), accompanied by a corresponding increase in G2. The cell cycle distribution of MM170 cells cultured in EGTA medium was much less affected by Colcemid treatment.

The DNA content of the fibroblasts decreased by approximately 10% during 48-hr treatment but increased slightly in MM96 and MM170 cells (Table 1). The lysis required for staining of DNA with propidium iodide eliminates mitotic cells from the DNA profile (35). Use of ethanol-fixed cells, however, gave similar results, indicating that few cells were blocked in mitosis. In addition, chromosome analysis revealed a fall in mitotic levels in fibroblasts and MM170 cells at 48 hr (results not shown) consistent with the inhibition of cell growth and DNA synthesis (see below). No chromosome aberrations were found.

Cell Membrane Potentials. The membrane potential of PGP cells in control medium was similar to that reported previously for human fibroblasts (36). As expected for tumor cells (3), the membrane potentials of the melanoma lines were less than those

Chart 4. Effect of EGTA on uptake of [³H]thymidine. Cell monolayers (2 x 10⁶/30-mm plate) cultured in EGTA medium for 4 or 24 hr were treated with [³H] thymidine (10 μCi/ml) and incubated at 37° for the times stated. The cells were rapidly washed with ice-cold phosphate buffer (2 x 2 ml), dislodged in 1 ml of buffer with a rubber scraper, counted (hemacytometer), and transferred to Instagel (10 ml; Packard Instruments, Zurich, Switzerland) for liquid scintillation counting. Uptake of thymidine was calculated after subtraction of the blank (dish without cells; typically, 10,000 to 20,000 dpm) but with no correction for externally bound label. A, MM170 cells; B, MM96 cells; C, PGP fibroblasts. ○, control medium; Δ, EGTA medium for 4 hr; ○, EGTA medium for 24 hr. Points, means of duplicates; all S.D., <10%.
of fibroblasts. Culture in EGTA medium reduced the membrane potential in all cells tested. The maximum change was found after 24 hr and remained constant during the next 3 days. Fibroblasts were affected much more than were MM96 and MM253, the change for MM170 being intermediate in value.

Uptake of Thymidine. The above membrane changes and the intention of measuring DNA synthesis by thymidine incorporation prompted a study of thymidine transport. In control medium, thymidine uptake by melanoma cells was biphasic, with rapid accumulation within the first 2 min, followed by a slower but prolonged increase (Chart 4A and B). Separate experiments using trichloroacetic acid precipitation showed that the latter was due to incorporation into DNA. In fibroblasts, the initial rapid uptake was followed by a plateau during the next 20 min (Chart 4C). As expected for actively proliferating cells, incorporation of thymidine increased linearly during this period but was too low (0.2 dpm/cell at 20 min) to affect the uptake profile. Allowing for differences in cell volumes, the rate and final level of initial accumulation of thymidine by fibroblasts (cell volume, 4.46 x 10^6 cu µm) were approximately 10-fold those of MM96 cells (1.36 x 10^5 cu µm) and MM170 cells (2.24 x 10^5 cu µm). The intracellular thymidine concentration in PGP fibroblasts after 2 min (3 µM) was therefore much higher than the levels in MM96 cells (0.15 µM), in MM170 cells (0.23 µM), and in the extracellular medium (0.2 µM).

EGTA treatment for 4 hr did not have a major effect upon thymidine uptake in any of the 3 cell types studied (Chart 4A). After 24 hr, however, the rate and final level of initial uptake was substantially increased. Thymidine accumulation during the second phase appeared to increase in MM96 (Chart 4B) but, as expected from the growth studies, was strongly inhibited in MM170 (Chart 4A).

Thymidine Pool Sizes. Since preliminary experiments indicated considerable enhancement of [3H]uridine and [14C]thymidine incorporation 1 to 4 hr after EGTA treatment, intracellular thymidine pool sizes were determined at 2 different EGTA levels using the isotopic dilution method of Clarkson (10). The MM170 cell line exhibited an increase in thymidine pool size 2 hr after treatment with end point-level EGTA, but this declined to the control value at a higher EGTA concentration (Chart 5). Thymidine pools decreased in all of the other lines tested, with the higher EGTA level having a lesser effect.

In studying the temporal response of thymidine pool size to EGTA treatment, considerable variation in control values was found in different experiments and during the 6-hr observation period (Chart 6A). The thymidine pool appeared to decrease in MM96 cells and fibroblasts and increase in MM170 cells, but these trends were not statistically significant.

The 0- to 6-hr response to end point-level EGTA revealed a more complex situation than that observed at 2 hr. MM170 and, to a lesser extent, MM96, showed an immediate large increase above controls in the 0- to 1-hr labeling period, followed by a gradual decline to 50% of controls by 6 hr (Chart 6B). This was maintained for two days (results not shown).

Incorporation of Thymidine and Uridine. Fibroblasts underwent immediate inhibition of incorporation of [3H]thymidine but recovered to control levels within 2 to 4 hr of the commencement of EGTA treatment (Chart 7B). The change in DNA synthesis, calculated as the product of the percentage of changes in pool size and thymidine incorporation, therefore corresponds to 30 to 50% inhibition. Thymidine incorporation in MM96 cells and MM170 cells, on the other hand, increased relative to controls during the same period and, combined with the pool size changes, represents little change in DNA synthesis for MM96 and up to a 4-fold increase for MM170. After 12 hr, DNA synthesis responded to EGTA treatment as expected from the respective growth curves, inhibition in fibroblasts and MM170 cells but not in MM96 cells. The EGTA dose response of thymidine incorporation after 24-hr treatment (Chart 8) indicated that EGTA levels above the end point for fibroblast growth inhibited thymidine incorporation in both cell types and therefore did not improve upon the 4-fold enhancement of thymidine incorporation into melanoma cells compared with fibroblasts, obtained at the lower EGTA levels.
The temporal and EGTA dose responses of uridine incorporation were similar to those of thymidine, except that in MM170 cells incorporation reached a maximum 2 hr before thymidine incorporation (Charts 7 and 8).

Mitochondrial Inhibitors. Fibroblasts and melanoma cells were used in clonal assays to determine the effects on cell growth of compounds previously found (7, 20) to evoke different responses from tumor compared with normal cell mitochondria. These substances, each used at a range of concentrations up to the level of overt toxicity, were ruthenium red (0.1 to 10 µg/ml), glucagon (0.01 to 10 µg/ml), ataracyctoside (0.1 to 50 µg/ml), and insulin (0.01 to 5 units/ml). No differential effects on the growth or blocking of melanoma cells and fibroblasts were observed either in control or in EGTA medium.

DISCUSSION

Including the previous study (22), selective growth of human tumor cells in calcium-depleted medium has now been established for 15 of 16 cell lines, the proliferation of all 15 cultures of normal cells being blocked under the same conditions. This applies to primary cultures of tumors (22) and, in this study, of normal cells, indicating that the phenomenon is not merely an artifact of long-term culture in vitro but stems from a unique property of the transformed phenotype. Because calcium is involved in many different aspects of cell metabolism (13, 20, 27, 29, 38), this study focused, where possible, on the early effects of calcium depletion on processes closely related to proliferation. Such analysis of asynchronous cells provides information at a time of minimal perturbation of cell metabolism but, compared with the more usual approach of studying cells released from a prolonged proliferative block (38), could lack sensitivity in detecting changes in small subpopulations.

Alterations in thymidine utilization were the most striking early effects of calcium depletion and differed in the 3 cell types studied. As reported previously for other systems (9, 39), thymidine incorporation per se could not be used to follow changes in the rate of DNA synthesis. However, considering together the incorporation, pool size, and transport data and ignoring uncertainties concerning possible compartmentation effects as found for the thymidine pool in HeLa cells (18), DNA synthesis appeared to be greatly stimulated by EGTA in the first two hr in MM170 cells (200 to 400%), less stimulated in MM96 cells, and strongly inhibited (25 to 50%) in the fibroblasts. Although studied less extensively, the similar response for uridine incorporation points to a common effect on pyrimidine nucleosides.

The dependence of proliferation upon calcium-controlled nucleotide synthesis in late G1 (4, 24, 38), in combination with the above results, suggests that fibroblasts become blocked in G1 because of a calcium-dependent effect related to early inhibition of nucleotide synthesis. According to this view, the other, later effects, such as decreases in membrane potential and complete inhibition of DNA synthesis, would result from rather than cause the G1 block. Inhibition of thymidylate synthetase following calcium depletion has now been established for 15 of 16 cell lines, the proliferation of all 15 cultures of normal cells being blocked under the same conditions. This persists for 15 of 16 cell lines, the proliferation of all 15 cultures of normal cells being blocked under the same conditions. This differs from the previous study (22), where possible, on the early effects of calcium depletion on processes closely related to proliferation. Such analysis of asynchronous cells provides information at a time of minimal perturbation of cell metabolism but, compared with the more usual approach of studying cells released from a prolonged proliferative block (38), could lack sensitivity in detecting changes in small subpopulations.

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for production of the required calcium-binding protein would then account for the temporary, enhanced EGTA resistance observed in the MM253c1-11E line. Alternatively, the enhanced ability of PGP to accumulate thymidine, related perhaps to the high membrane potential (36), may be part of a calcium-dependent proliferation control mechanism which is absent in tumor cells.

The exceptional tumor line MM170 showed an early increase in the thymidine pool and in nucleoside incorporation and accumulation of cells in both the G1 and G2 phases. The decrease in the proportion in G1 at 48 hr suggests that late G1 cells escape the EGTA block. Apart from pleomorphism, the reported properties of MM170, such as high chromosome number and contact-independent growth (26, 37), are typical of tumor cells in general. Passage through G1 and G2 is calcium dependent (4, 13, 24, 28, 38), and cells can become blocked in either phase under a variety of conditions (14, 15, 28). At this stage, therefore, it can only be stated that, although transformation of nonlymphoid cells relaxes the requirement for calcium in G1, the calcium dependence of other phases of the cell cycle may be increased. The nature and stringency of the block are not yet known. One possibility is that the combination of large nucleoside pools and high incorporation of exogenous nucleosides in MM170 cells immediately after calcium deprivation represents an untimely or uncontrolled stimulation of RNA and DNA synthesis which prevents the cell from proceeding past G2.

Study of an even wider range of cell lines may be necessary to distinguish proliferation-dependent effects of EGTA treatment from more distantly related, even artifactual, changes. The apparent drop in thymidine pool sizes in untreated PGP and MM96 cells during the 6-hr observation period, for example, and the opposite trend in MM170 cells may result from small fluctuations in temperature or pH which affect the cells in different ways but which may be unrelated to mechanisms of EGTA cytostasis. The small decrease in the DNA content of fibroblasts during EGTA treatment is also difficult to explain. Conceivably, it results from defective synthesis during S phase and production of DNA-deficient daughter cells unable to proceed beyond the next G1 phase.

Mitochondria play a major role in the regulation of intracellular calcium and exhibit altered calcium transport systems in tumor cells (7, 20), yet none of the inhibitors used in the present study had any differential effect on the growth of various cell types. Since it is possible that some of the compounds were unable to cross the cell membrane, a separate study using permeabilized cells or isolated human mitochondria may be necessary.

Differential labeling of tumor compared to normal cell DNA and RNA by a 24-hr pretreatment with PGP was shown to be feasible. Since EGTA-sensitive tumor lines such as MM170 would appear to be quite rare, this approach should be useful for labeling tumor DNA in primary cultures of biopsies prior to in vitro drug treatment and assay for drug sensitivity markers, such as levels of DNA damage.

ACKNOWLEDGMENTS

The flow microfluoromometric equipment was provided by the Queensland Cancer Fund and operated by T. Mangan.

REFERENCES


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