PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **182,** 315–321 (1986)

Influence of Increased Extracellular Calcium Concentration and Donor Age on Density-Dependent Growth Inhibition of Human Fibroblasts (42345)

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Abstract. Elevated calcium chloride concentration ([$CaCl_2$]) has been shown to increase saturation density for an established mouse fibroblast line and for human fetal lung fibroblasts (WI-38). In order to examine the effect of increased [$CaCl_2$] on human fibroblasts from donors of varying age, fibroblasts were grown in medium (basal level of 1.8 m*M* $CaCl_2$) supplemented with fetal bovine serum (FBS) until confluent. Compared to controls in basal medium, newborn foreskin fibroblasts exposed to additional $CaCl_2$ had a 110–450% increased cell yield that was independent of [$CaCl_2$] within the range of an additional 1.5–5.0 m*M*. The effect was maintained over an eightfold range of FBS concentration. Initial growth rate was unaffected, but a prolongation of exponential phase occurred for cultures exposed to increased [$CaCl_2$]. Confluent cultures refed medium with increased [$CaCl_2$] were stimulated 5- to 10-fold more than cultures refed basal medium. An additional 2 m*M* $CaCl_2$ resulted in a 210% increase for young adult-derived fibroblasts versus a 29% increase for old adult-derived fibroblasts ($P < 0.001$). These data indicate that increased [$CaCl_2$] decreases density-dependent growth inhibition of postnatal human dermal fibroblasts *in vitro* and that this effect is donor age dependent.

Density-dependent growth inhibition is a well-described behavior of cultured fibroblastic cells. This property is of interest because it is not expressed by cultured malignant cells or following malignant transformation *in vitro* (1–3) and because it is a model for the regulation of cellular proliferation that has been shown to be directly affected by *in vitro* aging (4, 5). Saturation density for fibroblasts has been shown to vary directly with concentration of serum in the culture medium (6, 7) and inversely with age of the tissue donor (8) or population-doubling level of fetal fibroblast cultures (5, 9, 10).

Dulbecco and Elkington (11) found that the saturation density of the established mouse embryonic fibroblast line Balb/c 3T3 increased with an increased level of $CaCl_2$ and recently this phenomenon has also been reported for WI-38 human fetal lung-derived fibroblastic cells (12).

The following experiments were undertaken to investigate the possible effect of increased calcium ion concentration on saturation density of postnatal human dermal fibroblasts and the influence of donor age on this phenomenon.

Materials and Methods. *Cell culture.* Fibroblast explant cultures, derived from foreskin specimens of term infants and skin biopsy specimens of the inner upper arm of adult volunteers aged 22–27 or 66–83 years, were established as previously described (13), except that tissue was kept overnight in 0.25% trypsin solution at 4°C to facilitate separation of the epidermis from the dermis. All volunteers were determined by history to be in good general health, without diabetes or skin disease, and using no medication at the time of the biopsy. Explants were refed weekly and harvested at confluence (approximately 4 to 6 weeks), and fibroblasts were serially passaged until needed. Passage 1–5 cultures were used in all experiments.

Fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DME; GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO) at 37°C in a humidified 95% air–5% CO_2 atmosphere. This serum-supplemented medium contained approximately 2 m*M* extracellular calcium (Ca_{ex}) based on the proportional amounts of DME calcium, as specified by the

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0037-9727/86 $1.50

manufacturer (0.9 of 1.8 m*M*, or 1.62 m*M*), and serum calcium (0.1 of 3.5 to 3.9 m*M*, or 0.35 to 0.39 m*M*), as determined by atomic absorption spectroscopy of several serum lots. At each passage cells were detached from cultures vessels with a Ca^{2+}–Mg^{2+}-free 0.25% trypsin solution; during experiments, cells were detached and counted in a solution containing 0.25% trypsin and 0.1% EDTA.

Experiments. Fibroblasts were seeded at 1 $\times$ 10^4 cells/cm^2 (90,000 cells per dish) in 2 ml of medium in 35-mm style plastic dishes (Falcon, Oxnard, Calif.). Beyond the 2 m*M* Ca_{ex} present in the control culture medium, additional Ca_{ex} was added at seeding to experimental cultures from a $CaCl_2$ stock (ACS grade; J. T. Baker, Phillipsburg, N.J.) in deionized distilled water. Cultures were provided with fresh medium only at the time of seeding and in some experiments at 7 days, after attainment of confluence. Earlier studies ((11) and F. C. Praeger, unpublished data) have shown that the medium is not depleted of calcium when cells are cultured under these conditions. Cell number of duplicate cultures was measured with a hemacytometer. Statistical significance was determined by the double-tailed *t* test for two samples, the Dunnett test for comparisons with a control, and the Neumann–Keuls test for pairwise comparisons.

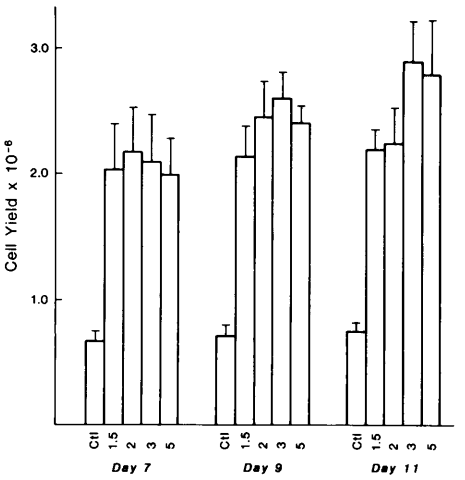

FIG. 1. Effect of increased [$CaCl_2$] on fibroblast yield at confluence. Average cell yields per dish determined on Days 7, 9, and 11 for three experiments using NFF are shown for control cultures (Ctl) and cultures grown in the presence of the following additional amounts of $CaCl_2$: 1.5 m*M* (1.5), 2 m*M* (2), 3 m*M* (3), and 5 m*M* (5). Bar is one SEM. When cell yields determined on Days 7, 9, and 11 were grouped together, Ca^{2+}-treated cultures for each level of additional $CaCl_2$ showed a significant increase in cell yield compared to control cultures ($P < 0.01$). Fibroblasts from a different donor were used in each experiment.

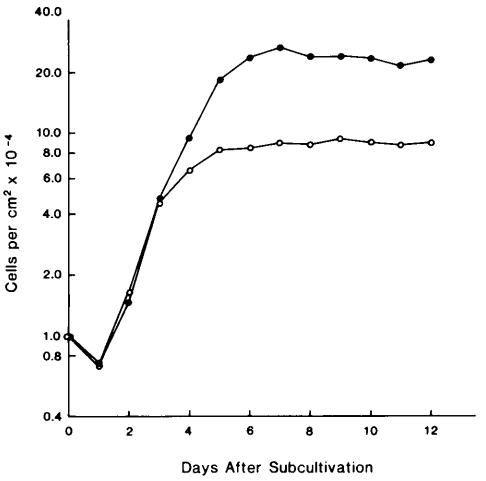

FIG. 2. Growth curves of newborn foreskin fibroblasts. Average cell yields of duplicate dishes of control cultures (open circles) and cultures grown with an additional 2 m*M* $CaCl_2$ (closed circles) were determined on the days indicated.

Results. *Effect of increased Ca_{ex} concentration on confluent density.* Ca^{2+}-treated newborn foreskin fibroblasts (NFF) showed large increases in cell yield at confluence over those of control cultures ranging from an average of 207% at Day 11 with an additional 1.5 m*M* $CaCl_2$ to an average of 308% also at Day 11 with an additional 3 m*M* $CaCl_2$ (Fig. 1). The range between experiments was 110 to 450%. Initial growth rates were identical for Ca^{2+}-treated and control NFF cultures with a prolongation of exponential phase primarily responsible for the increase in saturation density of Ca^{2+}-treated cultures (Fig. 2). Large increases in cell yield (171 to 222%) at confluence of Ca^{2+}-treated NFF cultures relative to controls were maintained over an eightfold range of FBS concentration (Fig. 3) despite the fact that control cultures grown with 20% FBS have nearly twice the average saturation density of control cultures at 2.5% FBS (8.6 $\times$ 10^5 and 4.6 $\times$ 10^5 cells per dish, respectively).

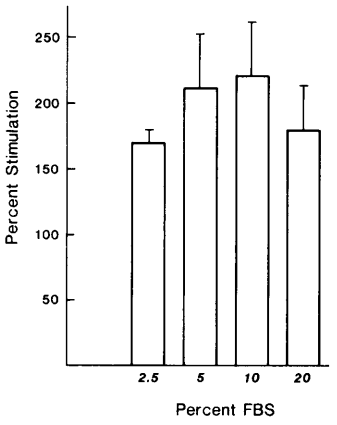

FIG. 3. Effect of serum concentration on cell response to increased [$CaCl_2$]. NFF in the same experiment were grown in the indicated FBS concentrations with and without additional $CaCl_2$. Percentage stimulation of cell yield at confluence of Ca^{2+}-treated cultures relative to control cultures for each FBS concentration was determined as follows: [(Ca^{2+}-treated − control)/control] × 100. Cell yields were determined on Days 7 and 9 and the larger of the two was used as the cell yield at confluence. Percentage stimulations are the averages of three experiments in which 1.5 mM additional $CaCl_2$ was used in one experiment and 2 m*M* additional $CaCl_2$ in two experiments with similar results. Bar is one SEM. Pairwise comparisons were not significant ($P > 0.05$). Fibroblasts from different donors were used in each experiment.

Response of confluent cultures to increased Ca_{ex} concentrations. Confluent NFF cultures refed fresh medium without additional $CaCl_2$ had an average increase of 45% in cell yield over cell counts taken at time of refeeding, while confluent cultures refed fresh medium and an additional 3 or 5 m*M* $CaCl_2$ showed average increases of 287 and 330%, respectively (Fig. 4).

Donor age effect. The average stimulation achieved with 2 m*M* additional $CaCl_2$ for five young adult-derived fibroblasts was 210% versus 29% for five old adult-derived fibroblasts (Fig. 5). This highly significant difference in stimulation ($P < 0.001$) is independent of the confluent density of control cultures since the range of cell yields of the old donor-derived control cultures encompassed the range of the young donor-derived control cultures (2.5×10^5 to 9.4×10^5 cells for old donors and 4.6×10^5 to 5.8×10^5 cells for young donors). Further, the difference could not be ascribed to possible slower growth rate for the calcium-stimulated old adult cultures, since percentage stimulation measured at Days 10 and 14 was no greater than that measured at Day 7. Fibroblast cultures derived from seven newborns, included as a reference standard, showed stimulation similar to the young adult-derived fibroblast cultures.

Microscopic observation of cell morphology and precipitation. Cells exposed to additional $CaCl_2$ (≥ 1.0 m*M*) at seeding appear similar to cells not exposed to additional $CaCl_2$ during initial exponential growth, but as the cultures become increasingly dense (Day 4 or 5) Ca^{2+}-treated cells appear narrower than control cells. This change in morphology persists during confluence (Fig. 6). No difference in precipitation is observable the first few days after

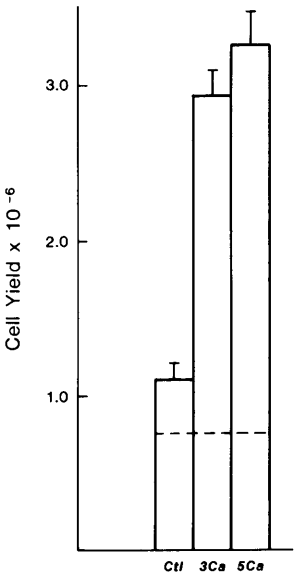

FIG. 4. Effect of increased [$CaCl_2$] on serum-stimulated confluent cultures. Confluent NFF cultures grown in DME supplemented with 10% FBS received a fresh medium change at Day 7 without additional $CaCl_2$ (Ctl) or with an additional 3 m*M* $CaCl_2$ (3Ca) or 5 m*M* $CaCl_2$ (5Ca). Cell yields were determined at refeeding (Day 7) and 5 or 6 days after refeeding. Average cell yields per dish of three experiments are shown. Average cell yield ± SEM at refeeding (indicated by dashed line) was $7.6 \pm 1.0 \times 10^5$ cells per dish. Bar is one SEM. Pairwise comparisons showed significant differences ($P < 0.01$) between controls (Ctl) and either of the Ca^{2+} treatments (3Ca and 5Ca) but not between the two Ca^{2+} treatments ($P > 0.05$). Cells from a different donor were used in each experiment.

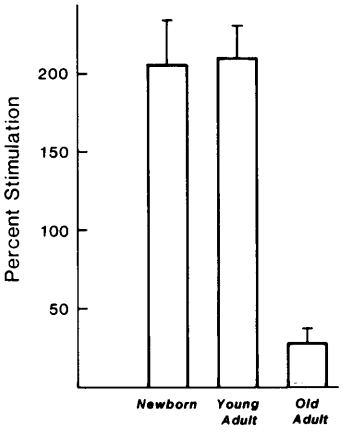

FIG. 5. Effect of donor age on cell response to increased [$CaCl_2$]. Percentage stimulations of cell yield at confluence (Day 7) of Ca^{2+}-treated cultures (2 mM additional $CaCl_2$) relative to control cultures are the averages from independent experiments of seven different NFF lines (newborn), five different young adult (22–27 years)-derived fibroblast lines, and five different old adult (66–83 years)-derived fibroblast lines. Bar is one SEM. See Fig. 3 caption for percentage stimulation formula. Difference in percentage stimulation between young adult- and old adult-derived fibroblasts was significant at $P < 0.001$.

seeding, but usually by Day 7 there is more precipitation on Ca^{2+}-treated cultures ($\geq$2 mM additional $CaCl_2$) versus control cultures. However, the addition of only 1 mM $CaCl_2$ also results in a large stimulation by Day 7 (an average of 161% for two experiments) without any more visible precipitate (100× phase contrast) than that observed on control cultures.

Discussion. Increased Ca_{ex} concentrations result in impressive increases in cell yields at confluence of human neonatal and young adult fibroblast cultures over those of control cultures. These increases are approximately sixfold greater than the stimulation reported for human fetal lung-derived fibroblasts of 20 to 60% (12) and equal or exceed the maximal stimulation reported for embryonic mouse fibroblasts that required 7.2 mM additional $CaCl_2$ (11). These large differences in stimulation between human fetal lung fibroblasts (WI-38) and fibroblasts derived from human neonatal foreskin or young adult inner arm may be related not only to age, but also to body site and/or developmental stage. Similarly, the differences between fibroblasts derived from newborn foreskin vs old adult arm skin cannot unambiguously be ascribed to donor age. The data do indicate that the large effect of additional $CaCl_2$ on the confluent density of cultured human fibroblasts is not limited to a specific body site or stage of development.

A major finding in the present study is the striking age-associated loss of $CaCl_2$-induced confluent density stimulation. The average difference observed between fibroblast cultures derived from five young adult vs five old adult donors (210 vs 29%) is especially striking given the well-recognized large interdonor variability in cultured cell lines. Site differences for the newborn vs young adult fibroblasts (foreskin vs upper arm) make it impossible to determine whether there is truly no loss of confluent density stimulation in the first decades of life or whether the phenomenon is masked by differences in $CaCl_2$ responsiveness between genital vs nongenital fibroblast populations.

The continued exponential growth of Ca^{2+}-treated cultures at a cell density at which control cultures are entering stationary phase indicates a diminished effect of cell density upon proliferation. Compared to control cultures, one to two additional population doublings of Ca^{2+}-treated cultures were required to reach their final cell yield. This indicates that Ca^{2+}-treated cells divide at densities greater than those at which control cultures cease growing. Our present data do not allow us to determine whether the morphological change of Ca^{2+}-treated cells as they approach confluence, also observed for WI-38 cells (12), is cause or effect of their increased cell density relative to control cultures.

Serum stimulation of confluent fibroblasts has been shown to result in a fraction of the total cell population progressing through one cell cycle, at which point the cells again enter quiescence (14, 15). However, in the case of additional $CaCl_2$, even if one assumes that the total population of serum-stimulated confluent human neonatal fibroblasts enters the cell cycle, an average of two rounds of cell division must occur to account for the final cell yield achieved in these experiments. This indicates that increased $CaCl_2$ concentration must act via some mechanism other than a

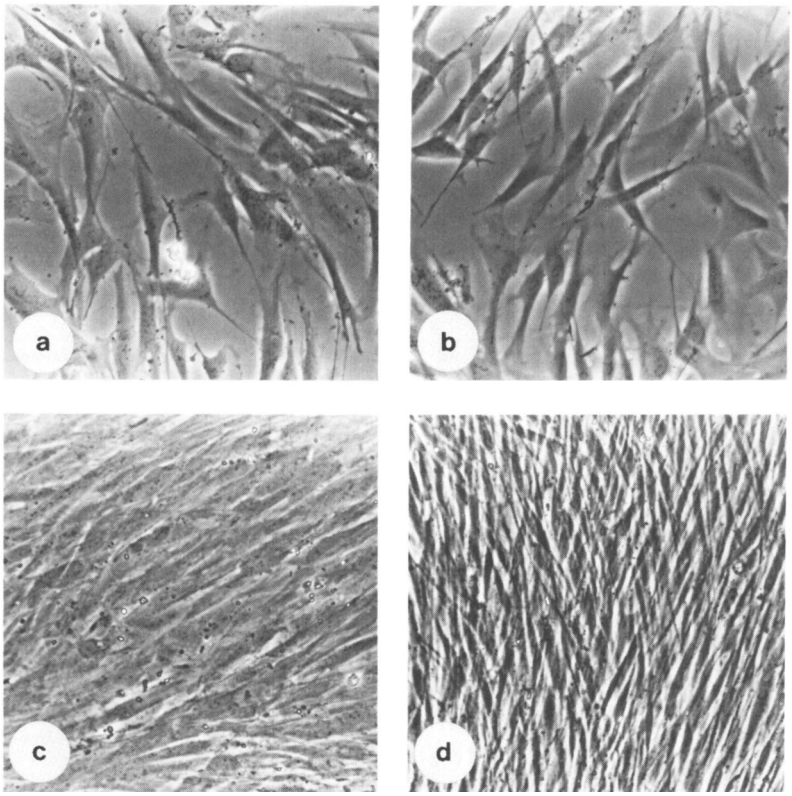

FIG. 6. Morphological effect of increased [$CaCl_2$] on newborn foreskin fibroblasts. Three days after seeding, (a) control cultures and (b) Ca^{2+}-treated cultures (2 m*M* additional $CaCl_2$). Nine days after seeding, (c) control cultures and (d) Ca^{2+}-treated cultures. Magnification = 128×. Phase contrast optics.

simple enhancement of the initial cell response to added serum mitogens.

The stimulatory effect of increased Ca_{ex} concentration on density-arrested 3T3 cells has been suggested to be the result of a corresponding increase in calcium phosphate precipitate (16, 17). Within the model of the competence–progression theory (18), calcium phosphate precipitate is considered to be a competence factor (19). Varying amounts of precipitate are present in our cultures with the addition of $\geq$2 m*M* $CaCl_2$, but the large stimulation observed with the addition of only 1 m*M* $CaCl_2$ was not accompanied by a visible precipitate. Further, Cheung and McCarty (20) have recently presented evidence that increased Ca_{ex} concentration may stimulate confluent NFF by a different mode of action than calcium phosphate precipitation since those authors found that (a) the stimulation was not affected by 0.01 m*M* chloroquine or 10 m*M* ammonium chloride, although these agents did significantly inhibit the stimulatory effects of calcium phosphate, and (b) the onset and peak of [^{3}H]thymidine uptake by cells was earlier in the presence of increased [Ca_{ex}] than in the presence of calcium phosphate crystals. If an increased [Ca_{ex}] resulted in precipitation which then stimulated [^{3}H]thymidine uptake, it would take at least as long to see the onset and peak of [^{3}H]thymidine uptake induced by increasing the level of Ca_{ex} as by the addition of calcium phosphate crystals.

Platelet-derived growth factor (PDGF), another competence factor and a major growth factor in serum (18, 21–24), has been found to have a mitogenic capability inversely related to cell density (25). Consistent with this finding is our observation that an eightfold increase in FBS (2.5 to 20%) resulted in less than a

twofold increase in fibroblast saturation density. In contrast, the same amount of additional $CaCl_2$ resulted in a similar stimulation relative to control cultures over the same eightfold range of FBS, suggesting that cell response to additional $CaCl_2$ is not as affected by cell density as is the response to PDGF. This suggests in turn that even if an increased $CaCl_2$ concentration functions as a competence factor for human fibroblasts, it does so differently from the competence factor PDGF.

The loss of stimulation with increasing donor age observed in this study has potential application as both a marker and a probe for *in vivo* aging research. Previously, large age-associated losses in responsiveness to epidermal growth factor and other mitogens have been demonstrated for fetal lung fibroblasts at early vs late passage (26), newborn vs adult dermal fibroblasts (27), and young vs old adult dermal fibroblasts (28), while only relatively small changes with donor age have been reported in population doubling time, number of replicating cells, and cell yield at confluence in studies of young versus old adult-derived fibroblasts (8). The basis for these differences is not known. Further studies of the mechanism by which Ca_{ex} concentration alters density-dependent growth inhibition should provide insight into both regulation of cell division and age-associated changes in proliferative behavior.

The authors thank Michael Vrabel for his excellent technical assistance. This work was supported by the Agriculture Research Service of the U.S. Department of Agriculture.

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Received November 8, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted March 18, 1986.