Promotion of Human Adipocyte Precursor Replication by 17β-Estradiol in Culture

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ABSTRACT The influence of 17β-estradiol and 17α-estradiol on adult human omental adipocyte precursors grown in a propagating culture system was studied. Cells were grown in subculture in the presence or absence of hormone. 17β-estradiol resulted in significant promotion of adipocyte precursor replication, as determined by cell counting and incorporation of radioactive thymidine into DNA. The hormone stimulated cell multiplication in the concentration range 0.5–500 ng/ml growth medium. The highest level tested was 500 ng/ml. The maximal effects were obtained at 50 ng/ml ($P < 0.001$ by paired t test, 48 h after hormone addition). All 10 cell strains (five were derived from men and five from women) that were tested responded similarly to the hormone. 17β-estradiol did not affect cell size. 17α-estradiol did not promote the replication of adipocyte precursors, nor did it influence cell size. Thus, 17β-estradiol, which is the active isomer in known target tissues, stimulates the multiplication of human adipocyte precursors in culture.

INTRODUCTION

The factors that control adipocyte precursor replication are unknown. Recently, a propagating culture system has been elaborated for these cells (1). It is suitable for studies of regulatory mechanisms operative in adipocyte precursor replication and differentiation.

The total body fat content of girls and boys is the same until puberty; it then increases in girls and decreases in boys (2). The fat content of women is normally higher than that of men (2, 3). The distribution of adipose tissue also becomes different at the time of puberty (4). Subcutaneous adipose tissue is thicker in women than in men (3). The greater thickness is a result of an increased number of adipocytes in several subcutaneous regions; in the gluteal area, however, the difference is mainly accounted for by the larger fat cells present in women (3). These facts suggest that estrogens may have the potential to influence adipose tissue growth.

It is known that estrogens are taken up by adipose tissue and isolated fat cells from mammals including humans (5–7). However, the significance of this uptake has not been established.

We herein report the promotion of cultured adult human adipocyte precursor replication by 17β-estradiol.

METHODS

Source, preparation and culture of adipocyte precursors. Tissue from 10 patients 20- to 60-yr-old (5 males and 5 females) was obtained at the time of abdominal surgery; the patients were not obese according to Metropolitan Life Insurance Company tables. The cells were isolated from omental adipose tissue as reported (1). After centrifugation, the digested stromal fraction was suspended in medium 199 (K. C. Biological, Inc., Lenexa, Kans.) supplemented with 10% (by volume) fetal calf serum (Flow Laboratories Inc., Rockville, Md.), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell culture and preparation for subculture were conducted as described (1). The inocula ($0.1–0.7 \times 10^5$ cells) were plated on 25-cm² Falcon tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and grown in 5 ml of medium. All studies were conducted in first or subsequent subculture.

Cell counting and sizing. Cells were resuspended in 0.15 M NaCl, aliquots were diluted with Isoton (Coulter Electronics, Inc., Hialeah, Fla.) and counted in a Coulter counter model Z_F. The size of cells was determined at the same time in a Coulter Channelyzer II.

Incorporation of [³H]thymidine into DNA. At the beginning of first subculture, 10 μCi of [³H]thymidine (sp act = 45.5 Ci/mmol, New England Nuclear, Boston, Mass.) were added to each flask. 17β-estradiol (Ikapharm, Ramat-Gan, Israel, recrystallized from and dissolved in methanol) or 17α-estradiol (Steraloids, Wilton, N. H., dissolved in methanol) were added to one-half of the flasks. The final concentrations

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were 0.05–500 ng/ml growth medium for 17β-estradiol and 50 ng/ml for 17α-estradiol. The same volume of methanol as that which contained estrogen, was added to the control flasks.

Cells were disrupted by freezing and thawing in water five times. Portions of aqueous broken cell suspensions were then taken for protein determination and for measurement of [^{3}H]thymidine incorporation into DNA. The samples were rendered 5% (vol/vol) with respect to perchloric acid. After centrifugation, the pellets were washed and spun three times with 5% perchloric acid. The pellets were finally dissolved with dilute NaOH (pH of water adjusted to 9.5) and portions were counted with Bray's solution at an efficiency of 25–30%.

Analytical methods. Protein concentrations were determined by a microbiuret procedure (8). 17β-estradiol levels were measured by a radioimmunoassay method (9). The proportion of free 17β-estradiol was determined by equilibrium dialysis.

RESULTS

Characteristics of adipocyte precursors. The human omental adipocyte precursors, which were isolated and cultured as previously described, had the same morphological and enzymological characteristics as reported earlier (1). Under the experimental conditions used in this investigation, these characteristics and the doubling time of all the cell strains were similar in both first and subsequent subculture.

Analysis of 17β-estradiol in growth medium. The mean concentration of 17β-estradiol was 15 pg/ml in assays of four fetal calf serum samples (each performed in triplicate). The same stock of serum was used in all studies. When 17β-estradiol was added to a final concentration of 50 ng/ml (in medium 199 supplemented with 10% by volume fetal calf serum), 40% of the hormone was in its free form.

Effect of 17β-estradiol on cell counts. 17β-estradiol resulted in a significant ($P < 0.001$ by paired t test) increase in the number of adipocyte precursors in culture (Fig. 1). As will be confirmed by the data on [^{3}H]-thymidine incorporation into DNA, these results indicate promotion of cell replication. The maximal effect was observed at a hormone concentration of 50 ng/ml growth medium. Because the lowest level of 17β-estradiol that resulted in an appreciable increase in cell number was 0.5 ng/ml, the contribution of hormone present in fetal calf serum (15 pg/ml) was negligible.

Fig. 2 illustrates the effect of 17β-estradiol on cell counts at different times in first subculture. The data are representative of time-courses using cell strains from 10 patients. At a 17β-estradiol concentration of 50 ng/ml, the greatest promotion of replication occurred 48 h after addition of hormone. The photomicrographs in Fig. 3 show adipocyte precursors in first subculture. After 48 h of growth, the flask that did not contain 17β-estradiol had a smaller number of adipocyte precursors (Fig. 3A) than the one with 17β-estradiol at 50 ng/ml (Fig. 3B).

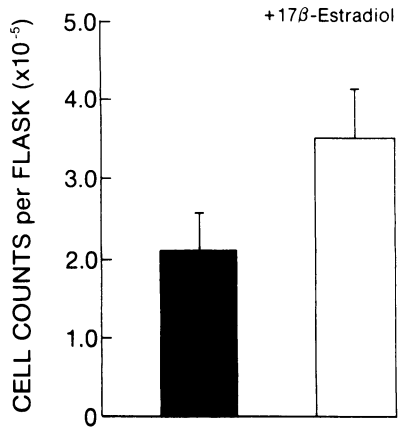


FIGURE 1 Effect of 17β-estradiol on the number of adipocyte precursors. Cell strains were isolated from the omental adipose tissue stromal fraction of five men and five women. At the beginning of first subculture, 17β-estradiol was added to one-half of the flasks at a final concentration of 50 ng/ml growth medium. The means of cell counts and their SE are shown at 48 h in culture. The effect of the hormone was significant by the paired t test at $P < 0.001$.

When the adipocyte precursors were suspended for cell counting and sizing, they became spherical enabling determination of cell diameter. 17β-estradiol did not alter cell size, the diameter being in the range 17–22 μm in each case.

In contrast to its 17β-isomer, 17α-estradiol at a concentration of 50 ng/ml growth medium did not alter the rate of adipocyte precursor replication (Fig. 4).

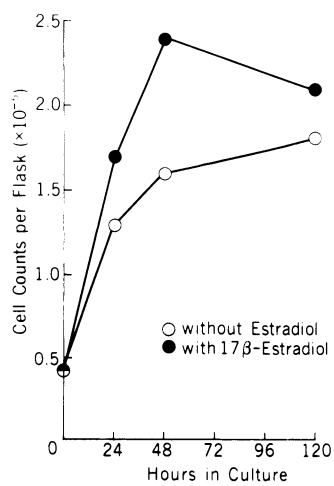


FIGURE 2 Cell counts of an adipocyte precursor strain in the presence and absence of 17β-estradiol. At the beginning of first subculture, the hormone was added to one-half of the flasks at a final concentration of 50 ng/ml growth medium. The data are representative of time-courses using cell strains derived from 10 patients (five men and five women).

The data are representative of time-courses using cell strains from two patients, one a man, the other a woman. For comparison, the same strains were grown within the same week, in the same subculture, in the presence of 17β-estradiol, which promoted replication as described. As was the case for 17β-estradiol, its 17α-isomer did not influence cell size.

Effect of 17β-estradiol on radioactive thymidine incorporation into DNA. 17β-estradiol resulted in a significant ($P < 0.001$) promotion of [^{3}H]thymidine incorporation into DNA (Fig. 5). This finding, which corroborated the evidence obtained by direct cell counting, indicated that the hormone enhanced the replication of adipocyte precursors in culture. The concentration of 17β-estradiol required to obtain the maximal effect and the time when it occurred were the same as described for cell enumeration. As was the case for cell counts, all 10 cell strains responded similarly. 17α-estradiol did not alter the degree of [^{3}H]thymidine incorporation into DNA.

In other experiments, when the same concentration of 17β-estradiol was added again after 48 h in culture, it resulted in renewed promotion of adipocyte precursor replication, as indicated by cell counting and measurement of [^{3}H]thymidine incorporation into DNA. The same degree of promotion was observed under the following two experimental conditions: first, 17β-estradiol was added again to cells grown in culture for 48 h; second, the adipocyte precursors were subcultured at 48 h and, using a similar inoculum size as in the previous passage, were grown again in fresh medium containing 17β-estradiol. Cell replication was promoted to the same extent as observed after the first addition of hormone. Inoculum size, in the range $0.1–0.7 \times 10^5$ cells, did not influence the magnitude of the 17β-estradiol effect. Decreasing availability of free, biologically active hormone is a possible reason for the diminishing effect after 48 h in culture.

In other experiments, 3 μCi of [^{3}H]thymidine were added at two-hourly intervals from 8 to 16 h in culture, and again at 24 and 48 h. The results were consistent with those illustrated in Fig. 2, that is, 17β-estradiol did not result in an appreciably greater rate of [^{3}H]thymidine incorporation into DNA during the earlier periods of time, when compared to the 24–48-h interval. It was not feasible to conduct studies before 8 h of culture because an insufficient number of cells adhered to the substratum during this period of time.

For the sake of consistency and to allow statistical analysis on data obtained with cells in the same passage, most of the studies were done with precursors in first subculture. In addition, each cell strain was tested in at least one subsequent subculture. 17β-estradiol resulted in a similar degree of promotion of adipocyte precursor replication up to the last (fourth) passage that was examined in this regard.

DISCUSSION

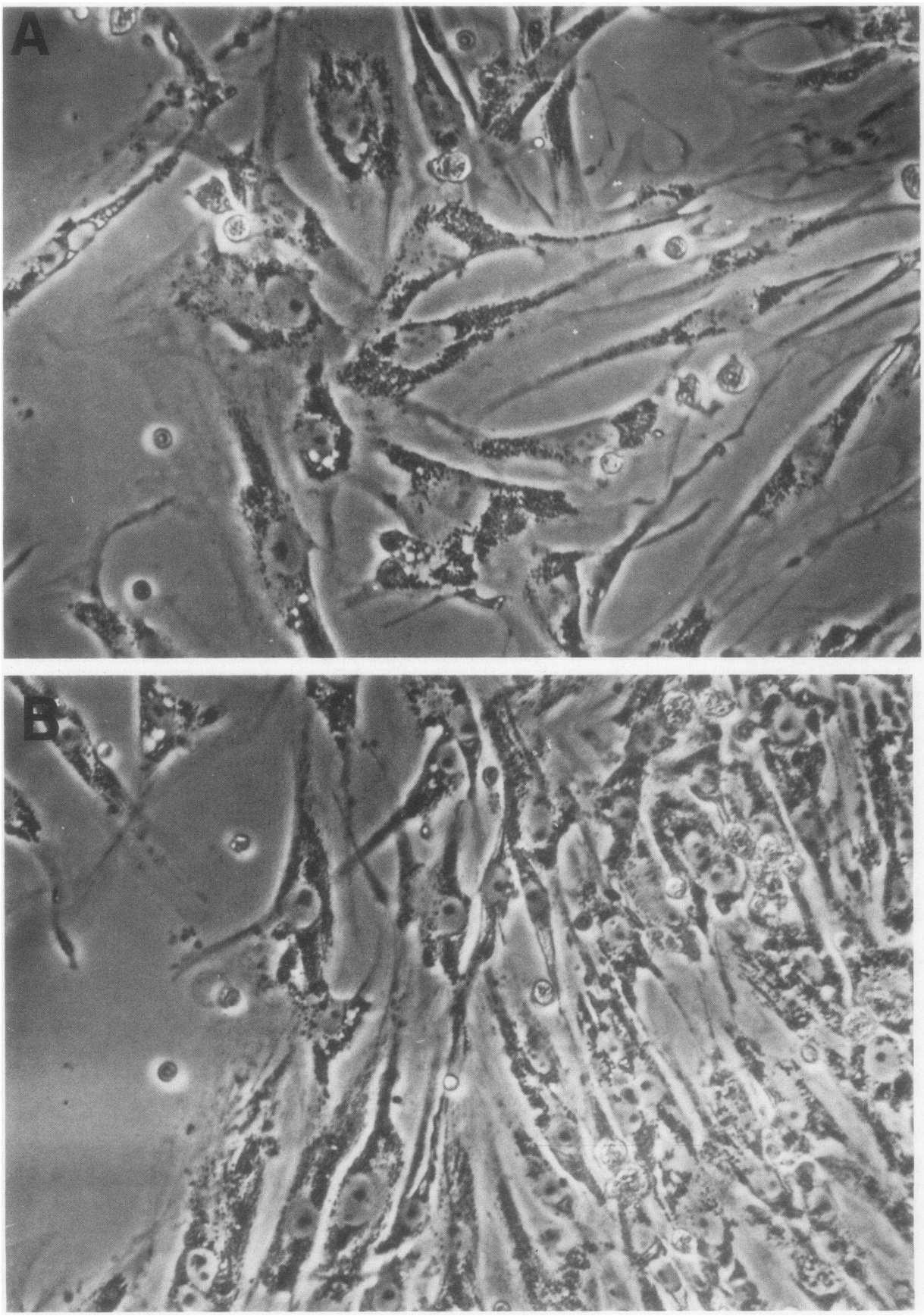
The uterus, vagina, corpus luteum, and the mammary glands have been considered to be the major target organs for estrogens (10, 11). Other sites of female sex steroid action include the hypothalamus and the pituitary (11). In the current study, 17β-estradiol was found to promote the replication of adipocyte precursors in culture. 17α-estradiol, the isomer inactive in known target tissues, was also without effect on fat cell precursors. Information obtained using cell culture systems must be interpreted with caution because it does not always reflect in vivo events. Our findings, however, do suggest that adipocyte precursors may be target cells for estrogens.

17β-estradiol promotes the replication of human endometrial cells in culture at similar concentrations and time periods as observed for adipocyte precursors in our studies (12, 13). As is the case for target tissues in vivo, 17α-estradiol is also biologically inactive on endometrial cells in culture (13).

Appreciable uptake of estrogens by fat cells occurs in vitro and in vivo (5–7). A recently reported study (14) has analyzed the effects of two dosage levels of the long-acting estrogen preparation, polyestradiol phosphate (Estradurin), in rats. After 10 wk, relatively high doses resulted in decreases of both total body weight and mass of subcutaneous and parametrial adipose tissue. The effects were ascribed to the anorexigenic properties of the hormonal preparation. After 4 wk, relatively smaller doses led to an increased weight of perirenal and parametrial fat depots. Body weight was not affected. The alterations in adipose tissue weight appeared to be a result of changes in fat cell size, rather than number. These results are difficult to interpret because of methodological reasons. Adipocyte precursors and more mature fat cells containing relatively small quantities of triglyceride, would not have been detected. Thus, an influence of the estrogen preparation on precursor cell replication may have been missed. In addition, demonstration of such an effect in vivo might require a longer treatment period.

It remains to be proven in vivo whether precursor cells take up estrogens and then replicate at an accelerated rate. If these events occurred under physiological conditions, augmented multiplication could account, at least partly, for the differences with respect to adipose tissue cellularity between men and women (3). These dissimilarities become apparent at puberty, a stage when differences in the synthesis and secretion of sex steroids become manifest.

The effect of 17β-estradiol in culture is reproducible and statistically significant, but not dramatic. Because adipocyte precursors of women are exposed to a different (as compared to men) hormonal environment for several years, a "dramatic" effect might not be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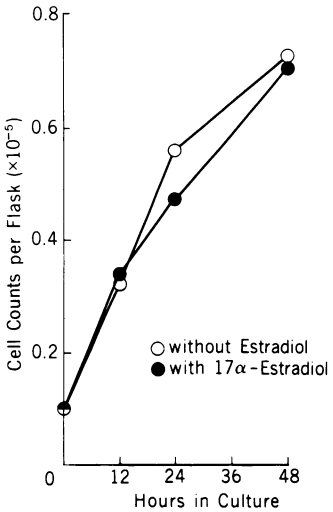

FIGURE 4 Cell counts of an adipocyte precursor strain in the presence and absence of 17α-estradiol. At the beginning of first subculture, 17α-estradiol was added to one-half of the flasks at a final concentration of 50 ng/ml growth medium. The data are representative of time-courses with cell strains derived from two patients (one man and one woman).

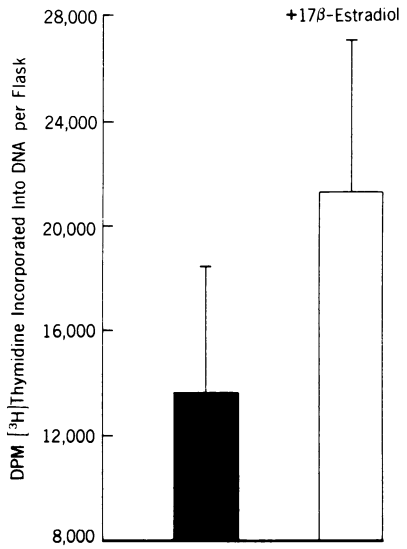

FIGURE 5 Effect of 17β-estradiol on [³H]thymidine incorporation into DNA of adipocyte precursors. Cell strains were isolated from the adipose tissue of the 10 patients referred to in Fig. 1. At the beginning of first subculture, 10 μCi of [³H]thymidine (45.5 Ci/mmol sp act) were added to 5 ml growth medium contained in each flask; at the same time, 17β-estradiol was added to one-half of the flasks at a final concentration of 50 ng/ml growth medium. The means of the disintegrations per minute incorporated and their SE are shown at 48 h in culture. The effect of 17β-estradiol was significant at $P < 0.001$.

expected. A relatively small effect exerted over a rather prolonged period of time could account, at least partly, for the fact that several adipose tissue depots of women contain a greater number of adipocytes than those of men (3). Direct proof of this proposal is of course required before a conclusion can be drawn.

Estrogens not only enhance the growth of such known target tissues as the reproductive organs, but also mediate their differentiation (10, 11). These hormones increase the number of initiation sites for transcription (15). Consequently, specific proteins which participate in target cell differentiation are synthesized (11, 16–20). It is not known whether these biochemical events are related to the stimulation of cell multiplication. While our studies indicate that 17β-estradiol promotes the replication of cultured adipocyte precursors, it is possible that estrogens also bring about specific biochemical changes.

Although the physiological significance of findings in cell culture requires confirmation by in vivo studies, the adipocyte precursor culture system provides a useful approach to the investigations of factors potentially operative in fat tissue growth and adipocyte differentiation.

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FIGURE 3 Photomicrographs of live adipocyte precursors. They were taken after 48 h in first subculture. The control was devoid of added 17β-estradiol (A), whereas the other flask was supplemented with hormone at a final concentration of 50 ng/ml growth medium (B). Original magnification of 160.

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