PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 523–528 (1986)

# Conversion to Adipocytes of a Clonal Bone Marrow Preadipocyte Line (H-1/A) and Fatty Acid Composition of the Resultant Adipocytes[1] (42287)

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*Abstract.* Conversion to adipocytes and fatty acid composition were investigated in a clonal bone marrow preadipocyte line (H-1/A). The growing cells exhibited a fibroblastic appearance. After the cessation of growth, triacylglyceride (TG) synthesis in the cells increased as they incorporated precursor from the growth medium and became adipocytes. Hydrocortisone and insulin accelerated the TG synthesis in H-1/A cells in a dose-dependent manner when they were cultured in the growth medium containing 10% horse serum. The rate of conversion to adipocytes was reduced as the concentration of horse serum was decreased, and this reduction was not influenced by the addition of insulin and/or hydrocortisone. These results suggest that converison to adipocytes of H-1/A cells is primarily dependent on some component(s) of the serum. Conversion to adipocytes of the cells may involve a process of differentiation since the conversion was completely inhibited when the cells were cultured in the presence of bromodeoxyuridine. Fatty acid composition was significantly different between adipose H-1/A cells and adipocytes derived from other marrow preadipocyte line MC3T3-G2/PA6 cells. Unsaturated fatty acids accounted for 76% of the fatty acid composition of adipose H-1/A cells; in contrast, saturated fatty acids constituted 65% of the fatty acid composition of the adipose MC3T3-G2/PA6 cells. These results suggest that there is a heterogeneity of preadipocytes in bone marrow. These two preadipocyte lines thus provide a useful tool for the study of marrow adipocytes and can also be used to analyze the hematopoietic microenvironment through studies of the effect of these cells on hematopoietic cell proliferation.


Preadipose cell lines have been studied as a model for mammalian cell differentiation and as a useful tool for understanding lipid metabolism in adipocytes, although the origin of fat cells is still controversial (1). Several preadipocyte cell lines derived from bone marrow stroma have been established (1–4) and it has been indicated that preadipocytes in the bone marrow differ from those in the other sites of the body in their sensitivity to insulin or hydrocortisone in fat synthesis (5).

A cell line (H-1) derived from the adherent cell layer of 14-week-old Dexter-type bone marrow culture has been established and has been found to have multiple effects on the proliferation and/or differentiation of hematopoietic progenitor cells (2, 6, 7). Morphologically the cells are fibroblastic in nature, produce collagen types I and III, and have none of the specialized structures found in endothelial cells or macrophages. They have neither Fc receptors nor peroxidase activity, which confirms that they are not mononuclear phagocytes (8). These cells develop many fat droplets as they age and become confluent. The nuclei of developing adipocytes are central rather than eccentric, indicating that they are maturing white adipose cells or brown fat cells.

This paper focuses on the conversion to adipocytes and fatty acid composition of this marrow-derived cell line with reference to a preadipocyte line MC3T3-G2/PA6 (3).

**Materials and Methods.** *Cells and culture.* At the 15th passage, H-1 cells were cloned by dilution plating (9). This procedure was repeated twice. One of the cloned lines (H-1/A) was used in this experiment. The H-1/A cells were routinely cultured in Fischer's medium (GIBCO, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% horse serum (Irvine Sci., Santa Ana, Calif.), penicillin, and streptomycin. The cultures were

[1] Research supported by Grants-in-Aid for Science Research from Ministry of Education, Science, and Culture in Japan (57570154, 58480163, 58570178, 59480153, and 59015090).



0037-9727/86 $1.50

incubated at 33°C in an atmosphere of 5% carbon dioxide in air and subcultured weekly at a split ratio of 1:8. Cell monolayers were routinely dissociated by trypsin–EDTA (GIBCO) and $1 \times 10^5$ cells in 5 ml of growth medium were seeded onto screw-capped 25-$cm^2$ plastic culture flasks (Corning Co., Corning, N.Y.) for experimental purposes. The growth medium was changed every third day. All experiments were performed with cells obtained at the least after the 10th passage, corresponding to approximately 30 generations after isolation, although at least 30 passages (90–100 generations) have been performed since then with no detectable change in growth rate or conversion to adipocytes.

Plating efficiency was determined by inoculating each of six petri dishes (5 cm) with 5 ml of growth medium containing 100 cells, the number of cells being determined with a hemocytometer. At 1 week, the number of adherent colonies was counted. All the cultures were fed for 3 additional weeks in order to test each colony for its ability to convert to adipose cells. Tumorigenic activity in the H-1/A cells was checked by subcutaneously injecting $10^6$ cells into 8-week-old male Balb/c-nu/nu mice irradiated with 350 rad. The MC3T3-G2/PA6 cell line was kindly provided by Dr. H. Kodama, Tohoku Dental University, Fukushima, Japan, and has been maintained in α-MEM (GIBCO) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, Utah).

*Incorporation of [$^3H$]thymidine into adipocytes.* H-1/A cells were seeded to 35-mm petri dishes with cover slips, and the growth medium was changed every third day. [$^3$H]Thymidine (0.5 μCi; sp act 48 Ci/mmole) in 10-ml aliquots was added to each dish on Days 6 and 24 and the dishes were incubated for an additional 24 hr. The cells were fixed, stained with oil red O, and then processed for autoradiography using nuclear emulsion NR-M2 (Konishiroku Co., Tokyo) by the dipping method. Cells carrying 10 grains or more over their nuclei were taken as positive for DNA replication. At least 1000 cells were scored for each labeling index value. The adipocytes were taken to be those cells whose nuclei were completely encircled by lipid droplets.

*Factors influencing the synthesis and accumulation of triacylglyceride by H-1/A cells.* To examine the adipogenic activity of the horse serum, confluent 7-day-old H-1/A cultures were washed with plain Fischer's medium two times and replenished every third day with Fischer's medium containing different concentrations of horse serum (10, 5, 2.5, 1, and 0.5%) for another 3 weeks. In some cases, the growth medium was supplemented with one of the following test substances: 5-bromo-2′-deoxyuridine (Sigma Chemical Co., St. Louis, Mo.) in the range $10^{-9}$ to $5 \times 10^{-5}$ *M*, insulin (bovine pancreas, crystalline 25.5 IU/mg, Sigma Chemical Co.) in the range 0.01 to 50 μg/ml, and hydrocortisone sodium succinate (Upjohn Co., Kalamazoo, Mich.) in the range $10^{-9}$ to $10^{-5}$ *M*. The growth medium supplemented with insulin was changed on Days 1, 4, 7, 10, 13, and 16 and the cells were harvested at an appropriate time. As hydrocortisone markedly inhibited the growth of H-1/A cells, this was added on Day 7 when the cultures reached confluence. The triacylglyceride (TG) content of the cells were determined in the ways described below and the rate of conversion to adipocytes was estimated as previously mentioned (10).

*Isolation of TG and determination of fatty acid composition.* Methods for isolating TG and determining fatty acid composition have recently been published in detail (11). After the period of incorporation, the culture was washed and the cells were trypsinized and sedimented. The cell pellets were washed twice in phosphate-buffered saline (pH 7.4) and extracted with methanol:chloroform:water (2:1:0.8). The lipids were separated by chromatography on a Fluorisil column preconditioned with 50% (v/v) methanol in water, and then processed for metanolysis with boron trifluoride–methanol. Finally, gas chromatography of fatty acid methyl esters was carried out on a glass capillary column coated with ethylene glycol succinate. In preconditioned Fluorisil column chromatography, glycerides were eluted by 20% (v/v) methanol in dichloromethane.

*Incorporation of labeled precursors into cellular lipids and removal of radioactivity from growth medium.* The compound used was [2-$^{14}$C]sodium acetate (sp act 3 mCi/mmole) from New England Nuclear (Boston, Mass.). Cellular lipids were labeled for 24 hr with or without [$^{14}$C]acetate (0.5 μCi/5 ml medium) on the 6th and 13th days of culture. Thereafter,

TG was isolated in the aforementioned way. In each group, two flasks were used. To measure the radioactivity incorporated, the TG fraction was dissolved in Liquifluor (New England Nuclear) and counted by liquid scintillation. The removal of radioactivity from the growth medium was also measured before and after the incubation of the medium with radioactive precursor for 24 hr.

**Results.** *Growth behavior of H-1/A cells.* The H-1/A cells in the logarithmic growing phase exhibited a fibroblastic appearance and the sheet of cells at confluence showed a cobblestone-like appearance (Fig. 1). As long as the subculture was performed once in 5 or 6 days, most of the cells were morphologically fibrocytic with a few fat-containing cells. The plating efficiency of the cells was constant at 45% during the course of the experiments. All the adherent colonies gave rise to adipose cells in the center in varying amounts during an additional 3 weeks of culture.

After the 9th day of H-1/A culture, conversion to adipocytes was markedly enhanced and neutral fat had accumulated as lipid droplets which in a small portion of the cells ultimately fused into a few large fat globules. The morphology of the adipocytes was quite similar to those seen in primary bone marrow culture (10). These adipocytes were anabolic for many weeks and continued to accumulate net quantities of lipid the entire time (data not given). H-1/A cells did not show any tumorigenic properties even when examined about 50 generations after isolation.

The incorporation of [$^3$H]thymidine into the nuclei of developing adipocytes was studied autoradiographically. On the 7th day of culture, 11% of nonfatty H-1/A cells incorporated radioactive [$^3$H]thymidine into their nuclei within 24 hr. More than 60% of the cells contained a large amount of stainable lipid on the 24th day of culture, and had not incorporated [$^3$H]thymidine into their nuclei. On the other hand, nonfatty cells were sometimes found between adipocytes even on the 24th day of culture, and about 13% of these nonfatty cells had incorporated large quantities of [$^3$H]thymidine into their nuclei.

*Factors influencing the synthesis and accumulation of TG by H-1/A cells.* The expression of the adipocytes' phenotype can be

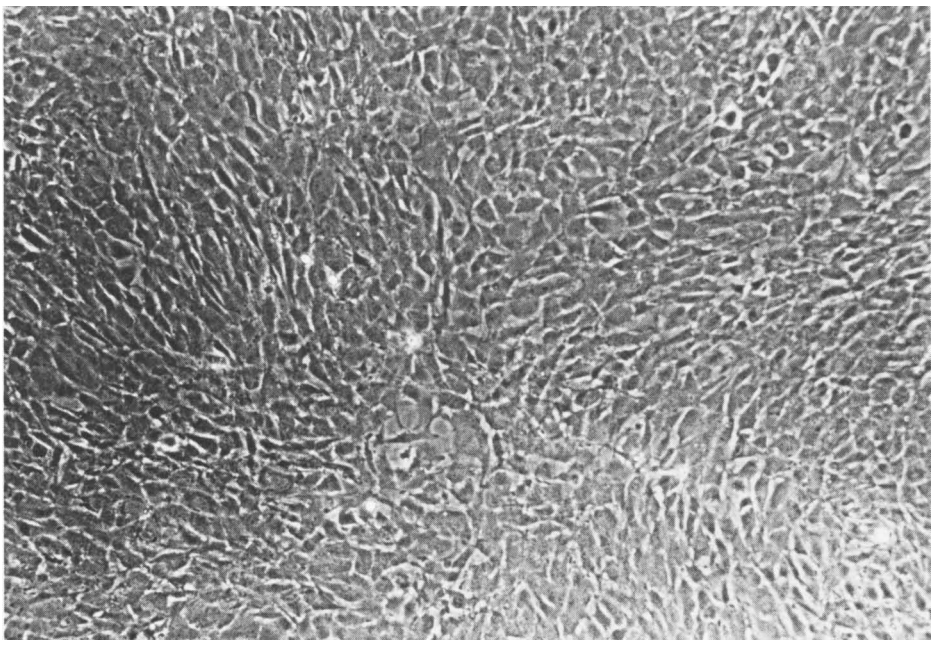

FIG. 1. Morphological appearance of H-1/A cells grown at 33°C and 5% $CO_2$ on the 7th day of culture. A sheet of cells has a cobblestone-like appearance. Some of the cells begin to accumulate small lipid droplets in their cytoplasm.

modified by serum concentration, agents, or hormones. The rate of conversion to adipocytes was reduced as the concentration of horse serum was decreased after 6 days of culture (Table I). There was 74 ± 10% conversion to adipocytes in H-1/A cells cultured in the growth medium supplemented with 10% horse serum for 4 weeks, while 7.2–8.5% adipocytes was found in H-1/A cells when they were maintained in the growth medium containing 1% or lower concentrations of horse serum after confluence of the cells. This reduction of the conversion to adipocytes was not influenced when the cultures were exposed to $10^{-6}$ *M* hydrocortisone and/or 10 μg/ml insulin at the sane time the serum concentration was reduced. Bromodeoxyuridine inhibited the conversion to adipocytes in a dose-dependent manner (Table II). Concentrations of $2 \times 10^{-5}$ *M* or higher completely inhibited conversion to adipocytes, and at lower concentrations the effectiveness of the bromodeoxyuridine diminished. The effect of insulin on fat synthesis was dose-dependent in the concentration range 0.01 μg/ml ($2.4 \times 10^{-4}$ IU/ml) to 10 μg/ml when the TG content of the cultured cells was quantitatively determined, and higher concentrations reduced the fat synthesis (Table III). This effect was almost as obvious in living cultures observed under the microscope, for the round refractile fat cells were larger in cultures maintained in the presence of insulin. Hydrocortisone also accelerated the conversion to adipocytes in a dose-dependent manner in the range $10^{-9}$ to $10^{-5}$ *M* (Table III). Hydrocortisone markedly inhibited the growth of the preadipocytes, but the inhibitory effect of other drugs or hormones on cell growth was minimal at the doses used. Conversion to adipocytes of MC3T3-G2/PA6 cells also decreased as the concentration of FBS was decreased. Few adipocytes were found when they were cultured in α-MEM containing 2.5% FBS after confluence.

*Lipid analysis.* A lipid extract obtained from H-1/A cells on the 13th day of culture contained large amounts of acylglycerides (93% TG and 6.5% monoacylglyceride), but no phosphoglycerides. [$^{14}$C]Acetate (721 cpm/mg

TABLE II. THE FREQUENCY OF ADIPOSE CONVERSION IN H-1/A CELL CULTURES WITH OR WITHOUT VARIOUS CONCENTRATIONS OF BROMODEOXYURIDINE (BrdU)

| Concn. of BrdU (*M*) | Percentage of adipose conversion |
|---|---|
| 0 | 58.0 ± 9.9 |
| $2 \times 10^{-7}$ | 34.6 ± 4.7 |
| $2 \times 10^{-6}$ | 7.8 ± 1.1 |
| $2 \times 10^{-5}$ | 0 |

*Note.* H-1/A cells ($10^5$/flask) were inoculated onto screw-capped 25-cm$^2$ flasks containing Fischer's medium supplemented with 10% horse serum and then the growth medium supplemented with bromodeoxyuridine was changed on Days 1, 4, 7, 10, 13, 16, 19, and 22. Flasks were fixed on the 24th day of culture. Two flasks were used at each point and two photomicrographs were randomly taken in each flask. Three hundred cells were counted in each photograph and the frequency of adipose conversion was determined.

TABLE I. FREQUENCY OF ADIPOSE CONVERSION OF H-1/A CELLS CULTURED IN DIFFERENT CONCENTRATIONS OF HORSE SERUM

| Concn. of horse serum (%) | Percentage of adipocytes |
|---|---|
| 10.0 | 74.0 ± 10.0 |
| 5.0 | 32.0 ± 9.8 |
| 2.5 | 14.8 ± 9.9 |
| 1.0 | 7.2 ± 2.7 |
| 0.5 | 8.5 ± 1.9 |

*Note.* Concn. means concentration. To estimate adipose conversion of H-1/A cultures, 28-day-old cultures were used to calculate the mean percentage of adipocytes ($n$ = 4).

TABLE III. TRIACYLGLYCERIDE (TG) CONTENT (PERCENTAGE CONTROL) OF H-1/A CELLS ON 13th DAY OF CULTURE WITH VARIOUS CONCENTRATIONS OF INSULIN OR HYDROCORTISONE (HDC) ADDED

| Concn. of insulin (μg/ml) | TG content (%) | Concn. of HDC (*M*) | TG content (%) |
|---|---|---|---|
| 0 | 100 | 0 | 100 |
| 0.01 | 160 | $10^{-11}$ | 103 |
| 0.1 | 180 | $10^{-9}$ | 178 |
| 1.0 | 220 | $10^{-7}$ | 249 |
| 10.0 | 249 | $10^{-5}$ | 280 |
| 50.0 | 140 | | |

*Note.* The figures represent percentage of TG content per cell in each group in relation to control (28 pg per cell).

TG) had been incorporated into TG by the 7th day of culture, and 1169456 cpm/mg TG by the 14th day. [$^{14}$C]Acetate incorporation was thus 1550 times higher on the 14th day than on Day 7. H-1/A cells removed 93% of the radioactivity in the supernatants in 24 hr of incubation from Day 13 to Day 14, whereas they removed only 22% of the radioactivity from Day 6 to Day 7. The fatty acid composition of lipids in the adipocyte stage of H-1/A cells and MC3T3-G2/PA6 cells is shown in Table IV. Adipose MC3T3-G2/PA6 cells contained a wider spectrum of fatty acids than H-1/A did. Unsaturated fatty acids (palmitoleate, oleic, and linoleate) were a major component (76%) of adipose H-1/A cells, whereas saturated fatty acids (palmitate and stearate) constituted about 65% of adipose MC3T3-G2/PA6 cells.

**Discussion.** A clonal bone marrow preadipocyte line (H-1/A) was isolated from the H-1 cell line (2) by dilution plating. When the H-1/A cells reached confluence, the incorporation of lipid precursors from the growth medium into the cells became markedly enhanced, the rate of TG synthesis increased, and the cells became adipocytes. DNA synthesis was arrested at the adipocyte stage in H-1/A cells. The plating efficiency of the cells was 45%, and all the adherent colonies gave rise to adipose cells in the center after another 3 weeks of culture. This indicates that all clone H-1/A cells are potentially able to convert to adipose cells.

The conversion of H-1/A cells to the adipocytes in culture containing 10% horse serum was promoted when they were exposed to hydrocortisone or insulin. It has been reported that fat accumulation in bone marrow preadipocytes is not produced by insulin *in vitro* (5), and bone marrow preadipocyte lines established by Lanotte *et al.* (1) and Kodama *et al.* (3) were found not to respond to insulin (0.01–10 μg/ml). In these respects, H-1/A cells differ from other marrow preadipocyte lines.

The rate of conversion to adipocytes was reduced as the concentration of horse serum was decreased after 6 days of culture. On the 7th day of H-1/A culture, 6.0 ± 3.0% adipocytes was found and, therefore, the percentage of adipocytes was regarded as the base line level. Accordingly, conversion to adipocytes did not occur in H-1/A cells when they were cultured in growth medium containing 1% or lower concentrations of horse serum for over 4 weeks after confluence. This reduction of conversion to adipocytes was not reversed by the addition of insulin and/or hydrocortisone. These results suggest that conversion to adipocytes of H-1/A cells is primarily dependent on some component(s) of the serum.

Conversion to adipocytes of H-1/A cells was completely inhibited by previous growth in the presence of bromodeoxyuridine. In all differentiative systems, bromodeoxyuridine inhibits differentiation at some stage of the developmental process (13). Accordingly, conversion to adipocytes in the H-1/A cell line represents a process of differentiation. Green *et al.* (14) reported that TG synthesis and collagen synthesis are unaffected by bromodeoxyuridine in preadipocyte line 3T3-L1 in the logarithmic growth phase, and suggested that incorporated bromodeoxyuridine prevents an increase in the activity of the enzymes responsible for the elevated TG synthesis.

The fatty acid composition of adipose H-1/A cells differed greatly from that of the adipocytes of MC3T3-G2/PA6 cells established from the calvaria of newborn C57bl/6 mice by Kodama *et al.* Furthermore, unsaturated fatty acids accounted for 76% of fatty acid composition of adipose H-1/A cells; in contrast, saturated fatty acids constitute 65% of the fatty acid composition in adipose MC3T3-G2/PA6 cells. It was previously reported that H-1/A cells produce noncytotoxic inhibitor to force cycling stem cells dormant in *in vitro* hematopoiesis (15); on the contrary, MC3T3-G2/PA6 cells promote the replication of stem cells (16). These data strongly suggest that

TABLE IV. FATTY ACID COMPOSITION (PERCENTAGE) OF LIPIDS IN THE ADIPOCYTES DERIVED FROM H-1/A LINE AND MC3T3-G2/PA6 LINE

| Fatty acid | H-1/A | MC3T3-G2/PA6 |
|---|---|---|
| $C_{14-0}$ (myristic) | 0 | 3.4 |
| $C_{14-1}$ (myristoleic) | 0 | 0.4 |
| $C_{16-0}$ (palmitic) | 19.2 | 41.3 |
| $C_{16-1}$ (palmitoleic) | 51.0 | 8.0 |
| $C_{18-0}$ (stearic) | <2.0 | 19.0 |
| $C_{18-1}$ (oleic) | 15.9 | 17.9 |
| $C_{18-2}$ (linoleic) | 9.1 | 0.6 |
| $C_{20-4}$ (arachidonic) | 0 | 9.3 |

there is a heterogeneity of bone marrow preadipocyte.

Bone marrow is a complex structure in all mammals, and the diverse stromal cells play an enigmatic role in hematopoiesis. At present, it is not certain whether cells in culture behave as they do *in vivo*. However, if such is the case, two different types of marrow preadipocyte lines could provide much information of the hematopoietic microenvironment by observing the modulation of hematopoietic cell proliferation and differentiation.


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Received September 5, 1985. P.S.E.B.M. 1986, Vol. 181. Accepted December 9, 1985.