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Modulation of One of Three Murine Bone Marrow Stromal Cell Lines to Adipose Cells by Serum and Insulin

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Adipose cells have been recognized as an integral component of the bone marrow hematopoietic microenvironment in vivo and as an essential cell type required for in vitro maintenance of stem cells. Four stromal cell lines obtained from the adherent cell population of murine bone marrow cultures have been enriched and purified by multiple trypsinizations. We noted that these cell lines exhibited an accumulation of vacuoles of lipid, the extent of which varied between cell lines in response to a change from medium containing 10% fetal calf serum to medium containing 20% horse serum. The lipid was lost when the cell lines were transferred back into the medium supplemented with fetal calf serum. In light of the reported lipogenic and antilipolytic effects of insulin on fibroblasts and adipocytes, we investigated the ability of insulin to induce adipocyte transformation of these bone marrow stromal cell populations. Three cell lines were exposed to bovine insulin at concentrations ranging from 10^{-9} to 10^{-6} M. All three cell lines responded to the insulin by accumulating lipid, but the extent of accumulation and the insulin concentration at which maximum lipid content was attained were population specific. One cell line (MC_1) responded fully at physiological levels of insulin (10^{-9} M), whereas the other two showed lipid accumulation only at pharmacological concentrations. The initial growth of MC_1 was inhibited in the presence of 10^{-9} M insulin which is compatible with the observed differentiation to adipocytes. The growth of MC_3 was unaltered in the presence of physiological concentrations of insulin, whereas that of MC_4 was accelerated. Grafts of organ cultures of the cell lines under the kidney capsule of syngeneic mice developed specific characteristics representative of the different cell lines. In particular, the majority of the grafts of MC_1 consisted primarily of fat cells which were not observed in the grafts of MC_3 and MC_4. These data strongly suggest that these cell lines comprise cells with different potentialities and that the MC_1 line represents a preadipocyte stromal cell of bone marrow.

Key words: bone marrow preadipocyte, bone marrow stroma, cell lines, insulin, insulin-induced marrow stroma

Adipose cells are an integral stromal component of bone marrow, and it has been suggested that an adventitial preadipocyte exists in vivo [1]. Large fat cells

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have also been reported to be essential for the long-term maintenance of hematopoietic stem cells and progenitor cells in vitro [2]. Greenberger et al [3] determined that the addition of hydrocortisone or insulin to long-term liquid cultures of bone marrow increased not only the number of fat cells in the monolayer, but also increased the maintenance and proliferation of hematopoietic stem cells. The existing data imply, therefore, that there exists a stromal cell population in vivo and an adherent cell population in vitro which respond to an appropriate stimulus (eg, insulin) by extensive accumulation of lipid, ie, bone marrow preadipocytes or adventitial cells.

We have enriched and cloned stromal cell populations from in vitro cultures of bone marrow and have described previously some of the morphological characteristics of these cells [4]. In the course of these studies, we noted that these cell lines (designated MC_1 through MC_4) altered their morphology in response to a change from medium containing 10% fetal calf serum to medium containing 20% horse serum. This morphological change was evident as an accumulation of vacuoles of lipid, the extent of which varied between cell lines. This observation suggested that we might be observing either serum-factor or insulin-dependent lipogenesis. This investigation was devised to establish if any of these bone marrow cell lines exhibited the characteristics of serum-factor or insulin-dependent adipocyte differentiation.

This was accomplished by observing the responses of the cell lines after transplanting them back into the in vivo hormonal milieu as organ cultures under the kidney capsule, and by assessing the effects on the doubling times and lipid accumulation following exposure to various molar concentrations of insulin in vitro.

MATERIALS AND METHODS

Female C57B1/6J mice were utilized throughout these studies. Animals were housed in laminar air flow isolation in a limited access animal facility under standard conditions and were allowed food and water ad libitum.

Bone Marrow Stromal Cell Lines

Clusters of cells termed FCFC and PFU-C by Friedenstein et al [5] and Wilson et al [6], respectively, were isolated by partial trypsinization and physical manipulation. Primary monolayers of mouse bone marrow adherent cells were subjected to trypsinization while under observation with an inverted phase-contrast microscope. When the desired cluster of cells was noted to be loosening, a sterile 10-μl pipette was used to remove it from the flask. Colonies isolated in this fashion were placed into wells of a microtiter plate and cultured in Fischer's medium supplemented with 20% horse serum at 33°C in a 5% CO_2 in air atmosphere.

Organ Culture and Transplantation

Confluent cultures of stromal cell lines were trypsinized, centrifuged, and washed, and then resuspended as a thick cell slurry. Aliquots (5 μl) were placed on sterile nucleopore filters floating on 5 ml of media, cultured at 37°C in a 5% CO_2 in air atmosphere for 24 hr and then transplanted under the capsule of the right kidney. The transplanted organ cultures were left in place for 30 days. The ani-

mals were sacrificed, the right kidney was removed, and the grafts were recovered. The grafts and representative organ cultures were fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, processed through ascending alcohols, and embedded in araldite 502. Toluidine blue-stained thick sections (0.5 μm) were evaluated with a Zeiss light microscope.

Insulin Titration

At passage, bovine insulin (Sigma) was added in concentrations from 10^{-9} to 10^{-6} M to groups of cultures of each cell line. Media was changed every 3 days and fresh insulin was added each time. Representative flasks from each group were fixed in buffered Formalin at 3-day intervals, stained with Oil-Red-O [7], counterstained with Ehrlich's hematoxylin [8], and evaluated for stained lipid content using a Zeiss light microscope.

Doubling Time

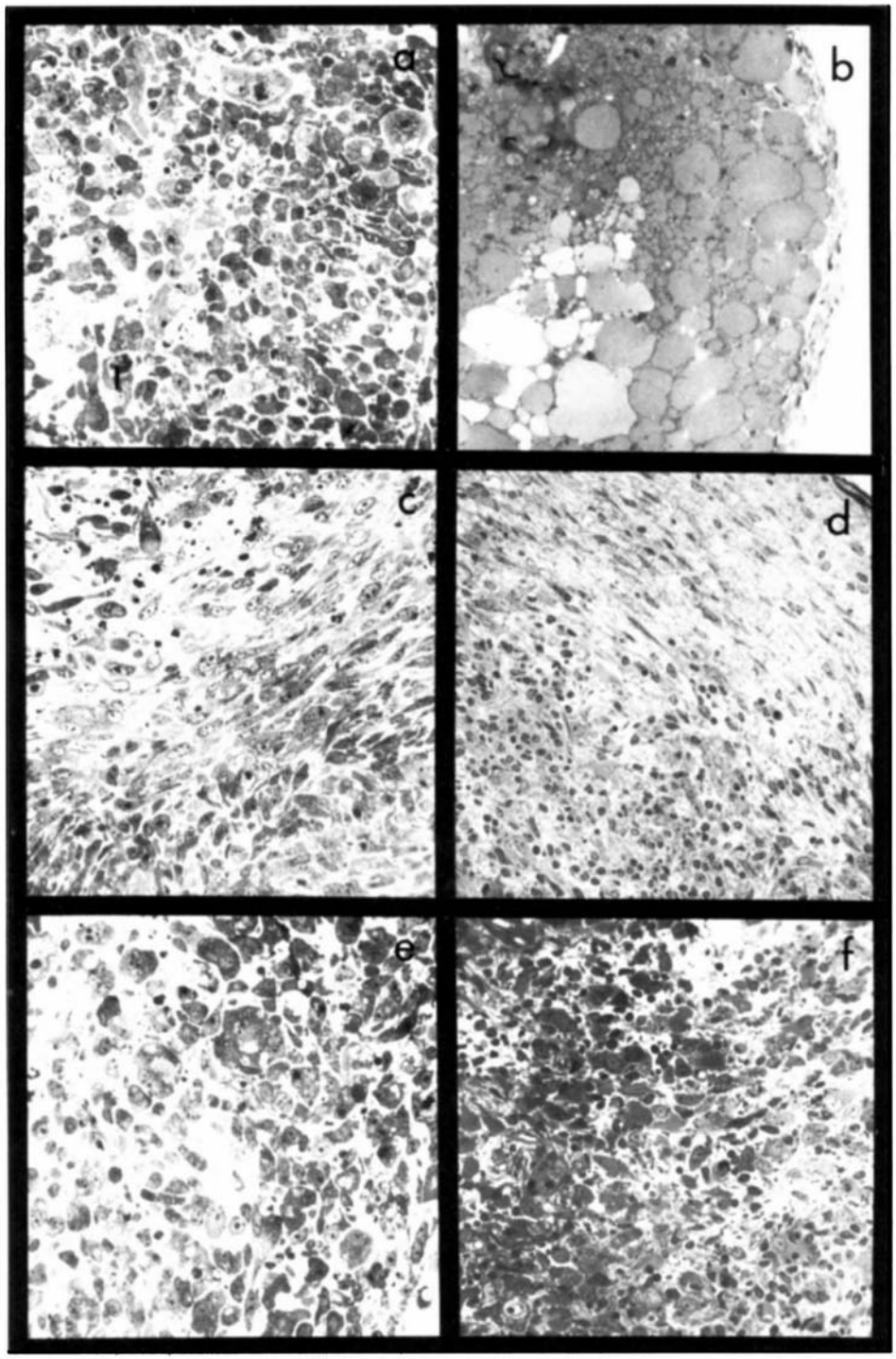
Corning 25-cm^2 flasks were seeded with 4×10^5 cells. Insulin (10^{-9} M) was added to half of the flasks from each cell line. Cells in flasks from each group were harvested and counted using a Coulter Counter (Model ZBI) at 1, 2, and 3 days postinitiation. Linear regression analyses employing the least squares fit were performed using the natural logarithms of cell numbers over the exponential portions of the growth curves [9]. This permitted calculation of the doubling times and provided information on initial growth of the cells with or without the presence of insulin.

RESULTS

Our initial observations of extensive lipid accumulation in the cell lines transferred from media containing 10% fetal calf serum to media supplemented with 20% horse serum were confirmed. Transfer of the cell lines back to media containing 10% fetal calf serum resulted in a loss of previously accumulated lipid vacuoles as evaluated employing phase-contrast microscopy. The cell line denoted as MC_1 appeared to exhibit the greatest response to the change in serum. When 24-hr organ cultures of this cell type were transplanted under the kidney capsule of syngeneic recipients for 30 days, nearly 66% of the grafts were composed almost exclusively of fat cells (Fig. 1b).

Another cell line, MC_3, which has fibroblastoid morphology in monolayer and organ cultures, presented an entirely different type of graft following transplantation. The cells in these grafts were oriented in parallel arrays, which results in a structure with the appearance of a thickened kidney capsule (Fig. 1d). The grafts of a third cell line, MC_4, were unremarkable and were characterized by a pleomorphic cellular array (Fig. 1f).

When exposed to bovine insulin at concentrations ranging from 10^{-9} to 10^{-6} M, all three cell lines responded by accumulating lipid. The extent of lipid accumulation and the dose at which maximum lipid content was achieved were population specific (see Table I). Over the range of insulin doses utilized, MC_1 exhibited the greatest capacity for lipogenesis of the three populations. Furthermore, the greatest response was at the lowest (physiological) dose. MC_3 was sensitive to the same doses of insulin as MC_1, but with a lower level of response. The greatest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degree of lipid accumulation in MC_4 was observed at 10^{-6} M insulin concentration.

Figure 2 shows the growth curves of MC_1, MC_3, and MC_4 with and without the presence of insulin. It appears that the initial growth of MC_1 was inhibited in the presence of insulin. Insulin had little effect on the growth of MC_3, but appeared to accelerate the growth of MC_4. Table II presents the estimated doubling times and the estimate of the initial intercept of these growth curves together with the estimates of the variation for these values. The initial intercept of MC_1 in the presence of insulin was significantly lower than in the absence of insulin, although the doubling time was unchanged. This observation is compatible with the hypothesis that insulin reduces the proportion of proliferating MC_1 cells presumably by inducing their differentiation to adipocytes. The doubling time of MC_4 was decreased significantly in the presence of insulin, although the initial intercept was unaltered. Insulin had no significant effects on the growth of MC_3.

DISCUSSION

In these studies we have shown that three bone marrow stromal cell lines (MC_1, MC_3, and MC_4) exhibit different characteristics of fat cell accumulation when (1) transferred from 10% FCS to 20% HS, (2) when transplanted under the kidney capsule of intact recipients, (3) when grown in the presence of 10^{-9} M insulin, and (4) when exposed to varying molar concentrations of insulin. In particular MC_1, which accumulated lipid on transfer from 10% FCS to 20% HS and on transplantation under the kidney capsule, responded with a delay in initial growth (due to an apparent reduction in the plating of proliferating cells) and maximal lipid accumulation at a physiological dose of 10^{-9} M of added insulin. The other cell lines accumulated lipid under the influence of insulin, but only at much higher (nonphysiological) molar concentrations.

It must be noted that we do not yet know if insulin or other serum factors are responsible for the stimulation of lipid accumulation in MC_1 on transfer from 10% FCS to 20% HS and transplantation under the kidney capsule. We are currently performing RIA to determine insulin levels in these sera in an attempt to resolve these questions. Further, our evaluation of lipid accumulation was based on a morphological assessment similar to that employed by Hiragun et al [7]. We are currently employing radiolabeled precursors to establish that this is, indeed, due to stimulation of lipogenesis and/or the inhibition of lipolysis [10] and not due to nonphysiological pinocytotic processes. In our studies of the effects of 10^{-9} M insulin on the growth of these cell lines, the cells were grown in a basal medium consisting of RPMI 1640 supplemented with 10% FCS. Although we know from our initial studies that this medium does not lead to lipid accumulation in any of the cell lines, it would be preferable to repeat these studies at either a limiting serum concentration or under serum-free conditions. This might also provide information as to whether serum factors, other than insulin, are involved in this process. Finally, we have not yet determined if insulin will stimulate additional

Fig. 1. All preparations are plastic sections stained with toluidine blue. (a) MC_1 cells following 24 hr of organ culture (200×); (b) grafted MC_1 organ culture 30 days after transplantation (200×); (c) MC_3 cells following 24 hr of organ culture (350×); (d) grafted MC_3 organ culture 30 days after transplantation (200×); (e) MC_4 cells following 24 hr of organ culture (350×); (f) grafted MC_4 organ culture 30 days after transplantation (200×).

TABLE I. Estimated Lipid Accumulation in Three Marrow Stromal Cell Lines Grown in Monolayer Culture in the Presence of Selected Doses of Insulin

	Insulin concentration[a]		
Cell line	10^{-9} M	10^{-7} M	10^{-6} M
MC_1	+ + +	+ + +	+
MC_3	+ +	+ +	+
MC_4	+	+	+ +

[a]Scored 9 days postinitiation of culture.

mitoses in these cell lines once they have attained confluence. This experiment technically is difficult to perform, since these bone marrow stromal cells have a tendency to detach from the surface of the flask when they reach confluence. Following the addition of fresh medium, some of the supernatant cells reattach, and regrowth to confluence resumes.

Despite these reservations, we believe that these studies show that the bone marrow stromal cell line MC_1 was much more sensitive to the lipogenic-antilipolytic effects of insulin than were the other two cell lines, MC_3 and MC_4. Based on doubling times, insulin was without effect on MC_1 and MC_3 cells but significantly enhanced the proliferation of MC_4 cells. Straus and Williamson have reported that some cell populations are induced into mitosis by insulin, whereas other populations are not affected in this manner [11]. They demonstrated a lower binding affinity for insulin in unresponsive cell populations and suggested that two types of insulin receptors exist. However, Coppock et al [12] demonstrated similar numbers of insulin receptors and similar insulin affinities in responsive and unrespon-

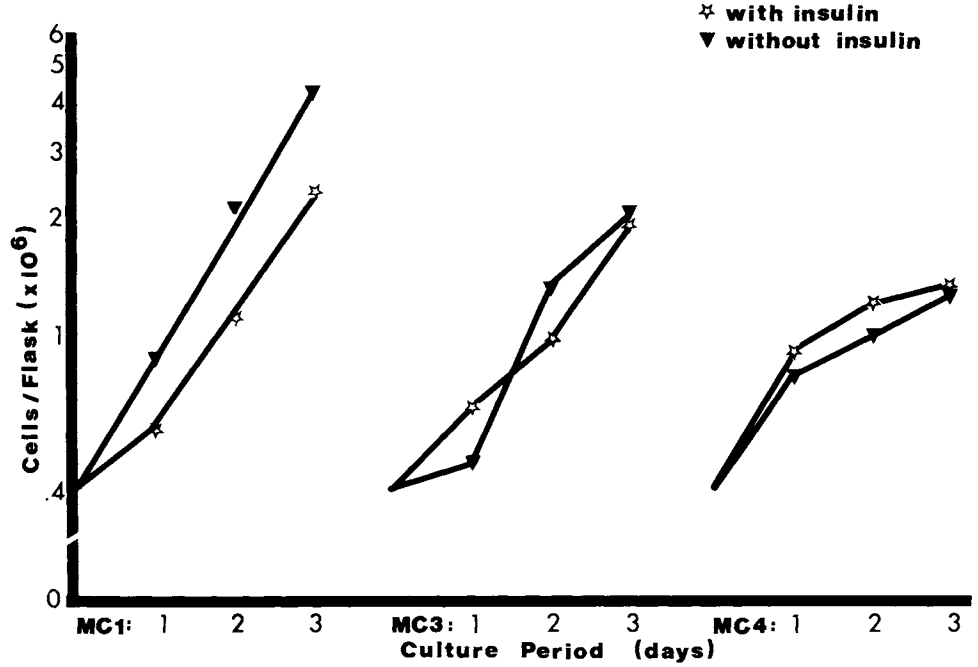

Fig.2. Growth curves of three marrow stromal cell lines in response to presence or absence of 10^{-9} M insulin.

TABLE II. Estimated Doubling Times and Initial Intercepts of Growth Curves for Three Marrow Stromal Cell Lines in Response to Presence or Absence of 10^{-9} M Insulin

Cell population	Estimated doubling time (Td) (hr)	95% Confidence Limits of Td.	Estimated initial intercept (Ii.) (millions of cells)	95% Confidence Limits of Ii.
MC_1	21.5	(18.8–25.2)	0.43	(0.34–0.55)
MC_1 with insulin	23.5	(22.2–24.9)	0.29[a]	(0.26–0.32)
MC_3	22.6	(15.8–39.8)	0.25	(0.12–0.49)
MC_3 with insulin	31.0	(24.5–42.3)	0.37	(0.27–0.49)
MC_4	64.2	(58.6–70.5)	0.60	(0.57–0.63)
MC_4 insulin	49.5[a]	(44.5–55.5)	0.65	(0.60–0.71)

[a]Differs significantly from the value obtained in the absence of insulin ($P < 0.05$).

sive populations. Evidence has been published by Jarett et al suggesting that the insulin receptors are the same in adipocytes and hepatocytes, but that differences in responses to insulin are the result of aggregation of receptor sites which allows for cross-linking [13]. The lipid accumulation in MC_3, and MC_4 cell lines, which occurred in response to pharmacological doses of insulin, is perhaps due to "spill-over" to related hormone receptors [14] and is thus not of physiological significance. When MC_1 was transplanted under the kidney capsule of syngeneic recipients the resultant grafts consisted primarily of adipose tissue (Fig. 1b). This was not seen in any grafts of the other cell lines, thus providing further substantiation of the preadipocyte identity of the MC_1 cell line.

Although there have been many studies of the effects of insulin on cellular metabolism, only in a few instances do these studies concern lymphohematopoietic stromal cells. Hofert and Phillips demonstrated a response of thymocytes to insulin; however, a heterogeneous cell population was used and the precise cell type responding could not be determined [15]. Bar et al [16] have shown that macrophages in the spleen possess insulin receptors. Interactions between macrophages and lymphocytes are very important in the differentiation of the latter group of cells. Recently, it has been shown that a subclass of thymus-processed lymphocytes acquires insulin receptors during the course of differentiation [17,18]. Thus insulin might be important to the maintenance of normal stromal-parenchymal cell interactions in lymphohematopoietic tissues.

This report presents evidence for a bone marrow-derived stromal cell line which responds to insulin by the accumulation of lipid in the cytoplasm. It is interesting to note that in higher mammals, active hematopoietic bone marrow is lost from the long bones about the time of sexual maturation and is replaced by nonhematopoietic yellow (fatty) marrow. Once this process has occurred, it is very difficult to persuade these yellow marrow spaces to revert back to red actively hematopoietic marrow [19], even though there might be a significant advantage to a hematopoietically stressed individual for this to occur, eg, a patient undergoing an intensive chemotherapy regimen. A comparison of the reversible insulin-dependent lipid accumulation observed in this study in cell line MC_1 with the adipocytes of yellow bone marrow might provide some clues as to cellular differences which might exist in these two different situations.

In summary, a stromal cell line derived from bone marrow responded to physiological levels of insulin by accumulating lipid. This accumulation may be due to the lipogenic or antilipolytic influences of insulin. When transplanted back into the in vivo situation, a lipid-filled graft resulted. These features suggest that this cell line represents the preadipocyte of bone marrow, which may correlate with the adventitial cell in vivo described by Weiss [1].

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