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SHORT COMMUNICATION

Initiation of a Process of Differentiation by Stable Transfection of ob17 Preadipocytes with the cDNA of Human A_1 Adenosine Receptor*

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ABSTRACT. A process of differentiation was observed when ob17 preadipocyte cells were stably transfected with a vector containing the cDNA of the human A_1 adenosine receptor of adipose tissue. Growth of the cell lines continued but was slowed relative to untransfected cells and cells transfected with vector alone, never attaining confluence. During this process, cells were observed to differentiate morphologically and to accumulate lipid droplets in their cytoplasm, droplets that stained with Oil red-O. During that same period of time, cells transfected with vector alone multiplied rapidly, attained confluence, and showed no signs of differentiation. We conclude that expression of the A_1 adenosine receptor initiated a differentiation process that resembled aspects of the normal differentiation of preadipocytes. If so, a role for the A_1 receptor in normal preadipocyte differentiation should be considered, perhaps after cell-to-cell contact occurs at confluence. BIOCHEM PHARMACOL **58**;1:167–170, 1999. © 1999 Elsevier Science Inc.

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The process of adipose cell differentiation consists of the following consecutive phenotypic changes: adipoblast to preadipocyte, to immature adipose cell, to mature adipose cell [1]. Preadipocyte lines have been established from mouse embryos (3T3-LI, 3T3-F442A), a hamster embryo (CHEF-18), and adult mice (ob17,BFC-1) [2–4]. When grown in culture, preadipocyte lines undergo changes in morphology only after reaching confluence. The fibroblast-like cells become round, enlarge, start undergoing differentiation, and finally accumulate triglyceride droplets in their cytoplasm. These changes are accompanied by the sequential appearance of enzymes characteristic of mature fat cells [1].

Among the factors shown to influence or be influenced by the differentiation process are the A_1 and A_2 adenosine receptors. Vassaux *et al.* [5], using pharmacological and molecular approaches, demonstrated the presence of the A_2 receptor in preadipocytes from periepididymal fat pads of 4-week-old rats; the A_1 receptor could be detected only in mature adipocytes. They proposed a bimodal action of adenosine and concluded that the adenosine A_2 receptor expressed in preadipocytes is involved in the control of adipose cell differentiation, potentiating differentiation by increasing cyclic AMP synthesis.

Similar findings were reported by Borglum *et al.* [6], who studied ob17 cell differentiation as a function of adenosine receptor expression. By binding studies and northern analysis, their conclusion was that A_2 adenosine receptor agonists stimulate preadipocyte conversion to differentiated mature adipose cells and, moreover, that the A_1 receptor inhibits this conversion.

We recently cloned the cDNAs of human and mouse fat A_1 adenosine receptors [7]. During the course of that study, the human A_1 receptor was stably expressed in the ob17 preadipocytes for the purpose of carrying out binding studies. Expression was confirmed by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) binding and inhibition by various ligands. At that time, we made an unexpected observation that could indicate a role for the A_1 receptor in the normal differentiation of preadipocytes to adipocytes.

MATERIALS AND METHODS

Cell Culture

The ob17 preadipocytes were obtained from W. Blaner of the Institute of Human Nutrition, Columbia University. They were maintained in Dulbecco's Modified Eagle's Medium (low glucose) (Gibco BRL) with 10% fetal bovine serum.

Establishment of Stable Transfectants

Using Lipofectamine reagent (Gibco BRL), ob17 cells were transfected with the pcDNA3 vector alone (Invitrogen) or

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vector into which the human adipose A_1 adenosine receptor cDNA had been inserted [7]. The pcDNA3 vector allows selection for neomycin resistance of cells successfully transfected with receptor.

The ob17 cells were grown in 10-cm dishes to 50% confluence (3.5×10^6 cells) in normal growth medium. Plasmid DNA (8 μg) was diluted with Opti-Mem I Reduced Serum Medium (Gibco BRL No. 31985) to a total volume of 0.8 mL. Lipofectamine (128 μL) was also diluted into Opti-Mem I Reduced Serum Medium to a total volume of 0.8 mL. The two solutions were combined, mixed gently, and incubated at room temperature for 15 min to allow DNA–liposome complexes to form. Meanwhile, cells were rinsed with 8 mL of the Reduced Serum Medium. For each transfection, 6.4 mL of this medium was added to the tube containing the liposome complexes. The suspension was mixed gently and overlaid on the rinsed cells. The cells were incubated with the liposomes for 7 hr, after which the medium was changed to 10 mL of normal growth medium with 10% fetal bovine serum. Forty-eight hours after transfection, the medium was changed to selection medium consisting of normal growth medium with 500, 750, or 1000 μg/mL of Geneticin (Gibco BRL No. 11811), an antibiotic of the neomycin family. Cells were treated with selection medium for 3 weeks in order to select for stably transfected cells displaying neomycin resistance. Between 2 and 3 weeks into the selection process, resistant cells began to appear. They were separated out by serial dilution and allowed to grow from single cells.

The transfected ob17 cells were maintained in Dulbecco's Modified Eagle's Medium (low glucose) (Gibco BRL) with 10% fetal bovine serum. Cells were grown on Lab-Tek Chamber Slides (Nunc, Inc.).

Cell Fixation

Slides with cells were briefly dipped in phosphate-buffered saline to remove any residual medium. Cells were fixed in 10% formalin for 10 min and then immediately stained.

Oil Red-O Staining

Cells were stained with Oil red-O [8]. Briefly, a 0.4% stock solution of Oil red-O was prepared in isopropyl alcohol and warmed to 50° in a water bath. The solution was diluted in distilled water to 0.24%. After mixing well and standing for 7 min at room temperature, the diluted solution was vacuum filtered and poured directly into the staining dish for immediate use.

After fixation, cells were dipped for 1 min in distilled water. This was repeated, followed by a 30-sec 60% isopropanol rinse and an 18-min staining with Oil red-O. Subsequently, cells were again rinsed twice with distilled water for 1-min intervals. Slides were mounted with Gel/Mount (Biomeda Corp. Cat. No. M01) with coverslips, and sealed with clear nail polish.

Cells were photographed using an Olympus BX-40 microscope

RESULTS AND DISCUSSION

Morphology of ob17 Preadipocytes Stably Transfected with the Human A_1 Adenosine Receptor

All experiments were carried out with cells derived from a single cell selected from cells stably transfected with human A_1 receptor in pcDNA3, a vector that allows selection for neomycin resistance. The morphology and growth pattern of the stable line containing receptor differed markedly from untransfected ob17 cells or those stably transfected with the pcDNA3 vector alone. The A_1-transfected cells multiplied more slowly than controls, never reaching confluence during the time we observed them. Most of the cells lost their fibroblastic appearance, becoming larger and more ovoid in shape, mostly polygonal, with some round cells. Finally and most striking, after only 1 week in culture, about 10–20% of the cells contained visible lipid droplets in their cytoplasm. As time went on, the majority of cells contained lipid droplets despite never attaining confluence. At this point, the cells enlarged with time, but did not divide, i.e. they behaved like fully differentiated adipocytes. This was not the case for cells stably transfected with vector only.

Oil Red-O Staining

After growing in culture for 10 days, Oil red-O staining of the A_1 receptor-transfected ob17 cells confirmed the presence of lipid droplets (Fig. 1a). No adipogenic agents such as insulin had been added to the cell culture medium. Cells transfected with vector alone showed negligible staining even at confluence (Fig. 1b).

The same phenomena were observed in three such stable cell lines, each established from a single cell, ruling out the possibility of artifacts introduced during transfection. Cells transfected with vector only were confluent at the time these assays were done, were fibroblastic in appearance, and showed minimal staining for lipid (Fig. 1b).

Our findings would not have been predicted on the basis of current thought about the absence of a role for the A_1 receptor in preadipocyte differentiation [5, 6]. However, there are reports that can be cited as supporting our observation. For example, adenosine, via interaction with the A_1 receptor of adipocytes, enhances the action of insulin, which, like adenosine, inhibits lipolysis and is among the hormones required for differentiation [9]. In studies on cells isolated from epididymal fat pads of Sprague–Dawley rats, adenosine lowered the concentration of insulin necessary to produce half-maximal effects on lipolysis and glucose transport by a mechanism independent of cyclic AMP [10, 11]. The effects of adenosine on insulin action appear to be by promotion of an active conformation of glucose transporters [10–12] and not due to changes in affinity to its receptor, suggesting that adenosine increases

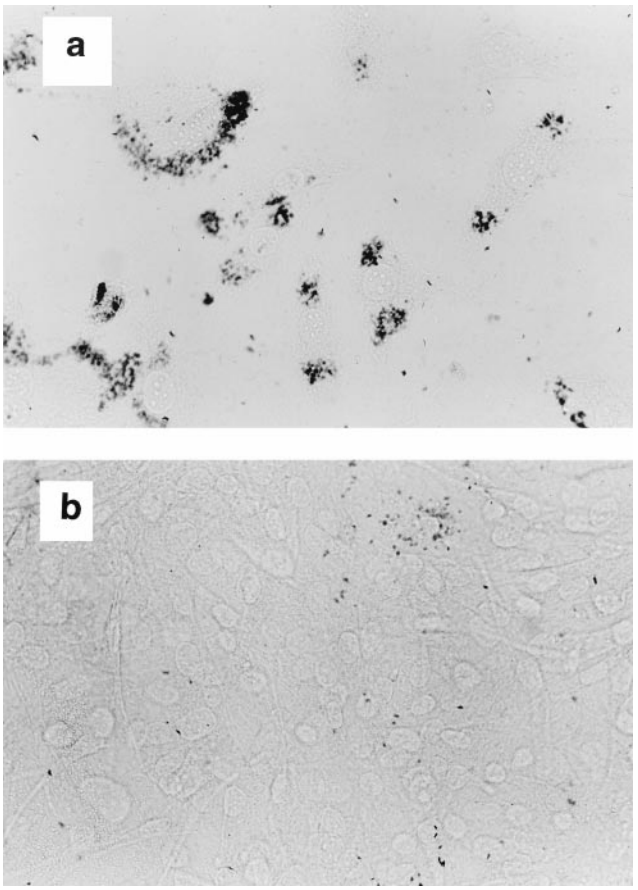

FIG. 1. (a) ob17 cells stably transfected with human A_1 adenosine receptor in pcDNA3 vector. Differentiation and accumulation of Oil red-O positive lipid droplets can be seen. (b) ob17 cells stably transfected with pcDNA3 vector alone. Cells are at confluence with a scatter of lipid droplets stained with Oil red-O.

the efficiency of coupling of insulin receptors to insulin action [11–16]. The ultimate effect of enhanced insulin activity by the action of adenosine on the A_1 receptor is to promote fat storage through inhibition of lipolysis and enhanced import of glucose for triglyceride synthesis, reactions compatible with differentiation of preadipocytes to adipocytes.

To summarize, stable transfection of ob17 preadipocytes with a vector containing the cDNA of the human A_1 adenosine receptor initiated a process of differentiation that included slowing of growth, changes in morphology, and the intracellular accumulation of Oil red O-positive droplets. This would not have been expected if preadipocyte differentiation was influenced solely by A_2 adenosine receptor function [5, 6]. The A_1 receptor might exist at undetectable levels in preadipocytes, gradually increasing in number immediately after confluence is reached, as a result of cell-to-cell contact. At that point it could play its part in the initiation of differentiation and continue to increase in concentration.

The extent of differentiation attained by transfection with the human A_1 adenosine receptor remains to be determined by examining the cells for enzymes characteristic of mature adipocytes [1]. To do this requires harvesting sufficient quantities of cells to allow characterization. As cell multiplication essentially stopped in our system, a different approach will be necessary, e.g. transfection with an inducible expression vector containing the cDNA of the A_1 adenosine receptor, which can be turned on after a sufficient number of preadipocytes have accumulated [17, 18].

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