Cyclic AMP Effects on Cell-to-Cell Junctional Membrane Permeability during Adipocyte Differentiation of 3T3-L1 Fibroblasts

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ABSTRACT Mouse 3T3-L1 fibroblast cells, also known as preadipocytes, differentiate in vitro into adipocytes when treated with promoting agents and acquire numerous properties characteristic of mature fat cells. We studied junctional cell-to-cell communication by measuring the incidence of electrical coupling and transfer of carboxy-fluorescein among these cells. When 3T3-L1 cells were induced to differentiate into adipocytes, they lost virtually all cell-cell communication. Preadipocytes that remained nondifferentiated after the treatment maintained normal communication. Loss of communication in the adipocytes invariably coincided with appearance of lipid droplets and not with other phenotypic changes. In the differentiating cells, loss of cell-to-cell communication and lipid accumulation was prevented if dibutyryl cyclic AMP and caffeine were present in the culture medium. Addition of dibutyryl cyclic AMP and caffeine to already differentiated adipocytes resulted in loss of lipid and simultaneously improved junctional permeability. The results demonstrate that in the in vitro 3T3-L1 cell system, (a) cell-to-cell communication and lipid synthesis are intimately related during the adipose conversion and (b) cAMP affects the expression of the two phenotypes.

The in vitro conversion of 3T3-L1 fibroblasts into adipocytes has been recognized as a process of cellular differentiation that resembles the development of the mammalian adipose tissue in many respects (10, 11). Under appropriate culture conditions, the cells continue to accumulate lipid droplets that subsequently coalesce and ultimately occupy nearly the entire cell volume, except for a thin cytoplasmic rim and the flattened eccentric nucleus, characteristic of mature fat cells (12). Adenosine 3',5'-monophosphate (cAMP) was shown to mediate the hormonal regulation of lipid metabolism in fat cells from rat epididymal fat pads (3). Moreover, dibutyryl cyclic AMP (dbcAMP) and epinephrine greatly reduce lipid accumulation in 3T3-L1 adipocytes (10, 11). Underlying these effects is cAMP regulation of the expression of lipogenic enzymes (24).

cAMP also seems to play a role in the regulation of the permeability of cell-cell junctional membrane (15). It has been shown that elevation of intracellular cAMP concentration, by treatment with dbcAMP or agents known to increase the adenylate cyclase activity, leads to an increase in junctional membrane permeability and in the number of gap junction particles (1, 5, 7). Among such agents, catecholamines, in particular, were found to increase the junctional permeability (18). The gap junction is the membrane structure that presumably contains specialized cell-cell channels (4, 19, 26). These channels are direct hydrophilic pathways that interconnect cell interiors (14) and permit relatively unrestricted exchange of small molecules between cells (8, 9, 22).

In this paper we examined the junctional permeability during the 3T3-L1 preadipocyte conversion to adipocytes and the possible relationship between this permeability and cAMP. We found that the junctional permeability decreases during the conversion and that cAMP affects both the junctional permeability and lipid accumulation in a predictable and reversible manner in the differentiated adipocytes.

MATERIALS AND METHODS

Cell Culture and Growth Conditions

The mouse 3T3-L1 (Swiss Albino) fibroblast cell line was obtained from American Type Culture Collection (Rockville, MD). Stock cultures of cells...
were maintained in exponential growth by subculturing every 7 days. Experimental cultures were inoculated with 10^6 cells per square centimeter and grown to confluence (6–7 days) in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. Cells were fed three times weekly with 5 ml of medium per 60-mm culture dish and the cultures were maintained in a humidified 95% air and 5% CO₂ atmosphere at 37°C. Cultures were examined routinely and lipid synthesis was verified by staining cells with Oil-Red-O.

**Induction of Adipocyte Conversion**

Confluent cultures of 3T3-L1 cells were maintained on Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. We promoted differentiation by incubating confluent cultures with fresh medium containing 10% fetal bovine serum (FCS) supplemented with 0.5 mM methyl-isobutylxanthine (MIX), 0.25 μM dexamethasone (DEX), and 1 μg/ml insulin (INS) for 3 days. In some experiments the cultures were treated with dbcAMP (1 mM) and caffeine (1 mM) in an attempt to elevate the intracellular level of cAMP. Only cultures with fewer than 12 passages were used. Cultures maintained beyond the 12th passage were less efficient in adipocyte conversion.

**Determination of Cell-to-Cell Junctional Permeability**

**Electrical Coupling Measurements**: Contiguous cells, namely cells in contact as seen by phase-contrast microscopy, were randomly impaled with glass microelectrodes filled with 1 M KCl and with tip resistances of 100–120 MΩ. Each microelectrode was connected to a preamplifier (WP-I instruments, Inc., New Haven, CT., model M44A), which served the dual purpose of passing electric current and recording voltages. Square pulses of negative (hyperpolarizing) DC current of 50-ms duration and ~2.5 nA were microinjected into cell 1 (see Fig. 1) and, with a 100-ms delay, cell 2 (see Fig. 1) and, with a 100-ms delay, cell 2 once every 1.5 s. Traces of the resulting voltage displacements were displayed on a storage oscilloscope (Fig. 1, C and F). Measurements on a single culture plate were limited to 15 min and were performed in room air warmed to 30–33°C. Incidence of electrical coupling was determined as the frequency of coupled cell pairs among the pairs tested. The limit of resolution was a coupling coefficient of 0.02 (16). Cell membrane potentials were routinely recorded and the percentage of cells that were fibroblast-like cells that underwent no morphological changes and acquired no lipid droplets.

**Junctional Permeability Changes**.

There was no significant change in the incidence of the electrical coupling and fluorescence transfer of the preadipocytes during the treatment with the promoting agents (stage II) (Fig. 1, D–F), as evident from maximum incidence of electrical coupling present (100%) and relatively high incidence of carboxy-fluorescein transferring junctions (59–67%; Table I). In sharp contrast, the junctional membrane permeability changed dramatically in the third stage upon the removal of MIX and DEX from the culture medium. Within 24 h after removal of these agents, the incidence of communication fell: the junctional transfer of carboxy-fluorescein was zero in all the preadipocytes tested and the incidence of the electrical coupling had fallen to 50% (Table I, stage III and Fig. 1, D–F). Over the next 48 h the coupling incidence leveled off at 6% with no further reduction in the incidence beyond the third day of stage three. Measurements performed 3 days later yielded very similar results indicating a stabilization at a very low level, which persisted even in the presence of normal medium (Table I). By contrast, the incidence of permeable junctions did not diminish amongst the preadipocytes still present that had not undergone adipose conversion and were easily identified by their fibroblast-like features. Junctional communication in these cells was not different from the preadipocytes in normal medium (Table I). Thus, treatment with the promoting agents per se was not sufficient cause to diminish the junctional membrane permeability of the cells.

**RESULTS**

**Adipocyte Differentiation**

As previously reported (17, 20), the 3T3-L1 cells underwent rapid morphological changes when treated with agents promoting adipocyte differentiation. These changes were associated with lipid synthesis and storage as seen in Fig. 1D. We distinguish three stages in the in vivo adipose conversion. During stage one (preadipocyte population maintained on normal medium), the cell cultures were densely packed, stationary monolayers of flat cells. Stage two was promoted with the aid of MIX-DEX-FCS-INS. Within 4 h after the addition of the promoters, the cells became round and refractile and by 24 h numerous mitotic cells appeared. This cellular proliferation persisted for 3 days, approximately doubling the cell densities of the starting cultures (Fig. 1A)(21).

Removal of MIX and DEX from the culture medium was the starting point for stage three of the adipocyte differentiation and marked the beginning of dramatic changes in the junctional membrane permeability described below. Interestingly, although during stage three FCS and INS were still present in the medium, the cellular proliferation ceased—even though both FCS and INS are known to be potent mitogenic factors. The most striking feature of stage three was the rapid appearance of cytoplasmic lipid droplets, in some instances as early as 6 h after the removal of MIX and DEX. By 24 h, 40–60% of the cells contained some lipid droplets. In the next 2 days the fraction of cells containing lipid reached 80–90% and it remained constant thereafter (Fig. 1D). Accumulation of lipid by these cells continued unabated in the presence of calf serum alone. By day 8 they had become engorged with very large lipid droplets. The remaining 10–20% of the population were fibroblast-like cells that underwent no morphological changes and acquired no lipid droplets.
When dbcAMP and caffeine were added to already differentiated adipocyte cultures, within 24 h the cells lost virtually all lipid droplets and the incidence of electrical coupling increased to the 46\% level. During the next 2 d the communication incidence continued to increase, reaching a maximum level (100\%) on the third day (Table II, experiment b).

**DISCUSSION**

The major finding in this report is that during the in vitro differentiation of 3T3-L1 preadipocytes into adipocytes, the
cells lose cell-to-cell communication. More than 90% of the adipocyte population have reduced junctional permeability during the first 3 of the adipose conversion (Table I). Since the change in the junctional membrane permeability is intimately associated with the conversion of the preadipocytes into adipocytes, it may be an integral part of its differentiation program. Moreover, the observed loss of junctional permeability seems to be a relatively stable phenotypic change in the adipocyte, as is its capacity to synthesize lipids (10). The junctional permeability changes during the differentiation of the adipocyte did not correlate with changes in cell shape, nor was it a consequence of the changes observed in cell morphology. In fact, if anything, as seen in the phase-contrast microscope the cells appeared in better contact with each other during the critical period when the cells had lost communication (Fig. 1 and Table I). However, the reduction in
the junctional permeability invariably coincided with the onset of lipid synthesis (Table I). This suggests that the latter two phenotypes are intimately related developmentally in the 3T3-L1 adipocytes.

The junctional membrane permeability and the synthesis of lipids in the 3T3-L1 adipocyte are likely to be regulated by changes in the concentration of intracellular cAMP. Elevation of intracellular cAMP concentration, by the addition of exogenous dbcAMP, improved the junctional permeability and at the same time brought about a decrease in visible lipid droplets. Conversely, removal of the exogenous source of cAMP had the exact opposite effects: junctional permeability returned to the previous low levels and lipid synthesis was resumed (Table II). The ability of cAMP to regulate junctional permeability and to regulate junction formation has been demonstrated in various normal and tumor cell lines (1, 5), and the same mechanism for regulating junctional permeability may also exist in the adipocytes. However, the reduction in adipocyte junctional permeability suggests that the differentiation somehow may involve an abatement of the channel formation mechanism. This in turn could follow from a depression of cytoplasmic levels of cAMP. The latter suggestion is consistent with the finding that the cAMP content of untreated adipocytes is lower than that of the preadipocytes (20).

We do not know if the in vitro findings in the 3T3-L1 adipocytes represent the events in vivo. There is some evidence that fat cells from adipose tissue may have a low level of cell-to-cell communication. These studies, which were performed on neon fat body and mouse brown fat, have shown that although electrical coupling was present in these tissues, it was found with considerable difficulty, i.e., coupling could not be detected with every impalement (23). These findings were ascribed to possible injury during the preparation of the tissues. However, in the light of the findings reported here, we can not rule out the possibility that the findings were correct and therefore that the in vitro findings represent their in vivo counterpart.

The interesting possibility emerging from these observations is that the reduction of cell-to-cell communication may be a necessary event for the development of adipocyte function, which is the metabolism of lipids. Under basal conditions the adipocyte in situ accumulates lipids, whereas during conditions of stress it releases free fatty acids. The latter is in the main, mediated by direct action of hormones, for example, epinephrine (11) and adrenocorticotropin (20), on the adipocytes. Furthermore, adipocyte differentiation results in increased sensitivity to β-adrenergic agonists and adrenocorticotropin (20). These hormones are potent lipolytic agents that effect breakdown of lipids in mature fat cells by increasing the intracellular cAMP levels (3). Finally, cAMP was shown to prevent lipid synthesis by reducing specifically the accumulation of mRNA for lipogenic enzymes (24). There is already convincing evidence that the junctional permeability of cells is enhanced by the action of hormones capable of interacting with mammalian cells in culture and elevating the intracellular cAMP concentration (18). All of this taken together suggests that the channels may be instrumental in adipose tissue responsiveness to the action of hormones. For example, increased cell-to-cell channel formation in response to lipolytic hormones may result in a more efficient tissue response, by permitting a more efficient spread of cAMP itself. Previous studies suggest that cAMP possibly may flow from one cell to its neighbors (2, 13, 25), and in addition, this molecule is certainly of a size one would expect to be admitted by the cell-to-cell channel (22).

We thank our colleagues Werner R. Loewenstein, Sidney J. Socolar, and Ren-ye Ho for discussion and critical reading of this manuscript. This work was supported by National Institutes of Health grants CA 14464 and AM 21575.

Received for publication 15 September 1983, and in revised form 4 September 1984.

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