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Adipose conversion of 3T3 cells depends on a serum factor

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ABSTRACT **The adipose conversion of 3T3-F442A cells depends on an adipogenic factor in serum. In the presence of this factor, cells grown to confluence become spherical, greatly increase the activity of their lipogenic enzymes, and accumulate triglyceride. In the absence of the adipogenic factor, the cells grow normally, but when they reach confluence they do not become spherical, do not accumulate triglyceride, and do not undergo any increase in activity of lipogenic enzymes. In cattle there is a great deal more of the adipogenic factor in the serum before birth than in the serum of grown animals. The nature of the adipogenic factor suggests that it may play an important role in the development of adipose tissue.**

3T3 cells may undergo adipose conversion when their growth is reduced or arrested. The frequency of this conversion varies in different cloned populations, and it has been possible to obtain clones with high or low frequency of conversion (1, 2). The adipose conversion is accompanied by an increasing rate of triglyceride synthesis (3) due to an increasing activity of lipogenic enzymes (4–10).

The rapidity of the adipose conversion is affected by hormones (1, 3). In some cases the cells become more sensitive to hormones when the adipose conversion takes place (11, 12). The adipose conversion is also affected by prostaglandin F2α and methylisobutylxanthine, agents known to affect cyclic nucleotide levels (13).

Because of its role in enzymatic carboxylation, biotin is important in the adipose conversion. In the absence of biotin, the increase in fatty acid synthesis that usually accompanies the adipose conversion cannot occur, but enzymatic differentiation does occur, though it is modified in some respects (10). Biotin is therefore not a regulating factor in the overall process of differentiation.

The adipose conversion also depends on a non-lipid factor in the serum. This factor is essential for all aspects of the conversion so far examined and may have a regulating function in the overall process of differentiation.

MATERIALS AND METHODS

Cell Cultures. 3T3-F442A cells (2) were grown and maintained in fortified Eagle's medium. Cells were inoculated into medium supplemented with 10% calf serum. Usually 2 or 3 days later, the cultures were refed with definitive medium, containing different serum supplements, insulin (10 μg/ml), and biotin (0.1 μM). Cultures were refed three times weekly until they were fixed and stained. Sera of different species were purchased from Colorado Serum Company. The calf serum came from animals 6 months–1 year old.

The methods for preparation of cell extracts and determination of enzyme activity have been described (5). Delipidation of serum was performed by the method of Rothblat *et al.* (14). For further details see ref. 9.

Oil Red O Stain. A stock was prepared as follows (15): 0.5 g of Oil Red O was dissolved in 100 ml of isopropanol. Before use, the solution was diluted 6:4 with distilled water, allowed to stand for 10 min, and then filtered through Whatman no. 1 paper. Cultures were fixed with 10% formalin in isotonic phosphate buffer, stained for 1 hr, and washed with water.

RESULTS

Increased adipose conversion in presence of fetal serum

Our attention was first directed to the existence of an adipogenic factor by study of the adipose conversion in the presence of fetal serum. 3T3-F442A cells were grown to confluence in medium supplemented with 10% calf serum. Thereafter some cultures were refed with the same medium and others with medium supplemented with fetal calf serum, either untreated or previously delipidized. Insulin was present at 10 μg/ml. Eight days after reaching confluence, the cultures were fixed and stained with Oil Red O. Fig. 1 shows that fetal calf serum was much more effective in supporting the adipose conversion than calf serum. The fat cell clusters produced in the presence of fetal calf serum were more numerous, and microscopic examination showed that the proportion of fatty cells in the clusters was greater.

Though the total lipid content of fetal calf serum is generally below that of calf serum, it might be thought that the greater lipogenic activity of fetal calf serum was due to preferential uptake of some of its lipids. For this reason it is important to note that after delipidation with solvents, fetal calf serum retained its greater ability to support the adipose conversion of 3T3-F442A cells (Fig. 1). The factor in fetal serum that promotes the conversion is therefore not itself a lipid.

The greater activity of fetal serum could also be demonstrated by allowing the adipose conversion to take place in mixtures of fetal calf and calf serum. Cultures were grown to confluence in medium containing 10% calf serum. The serum supplement was then changed to one consisting of mixtures of calf serum and fetal calf serum in different proportions, to a total concentration of 10%. When cultures were fixed and stained (Fig. 2), it was clear that the presence of fetal serum at a concentration of 2% or higher resulted in a more marked adipose conversion. Some factor important to the adipose conversion is therefore present in fetal serum at a concentration many times higher than in calf serum. Because this factor seems necessary for all aspects of the adipose conversion, we will refer to it as an adipogenic factor. Since its activity was preserved after exhaustive dialysis, the factor is probably a macromolecule.

Effect of sera of different species and the particular importance of cat serum

Sera of different species were compared for their ability to support the adipose conversion. Adult human serum and pig

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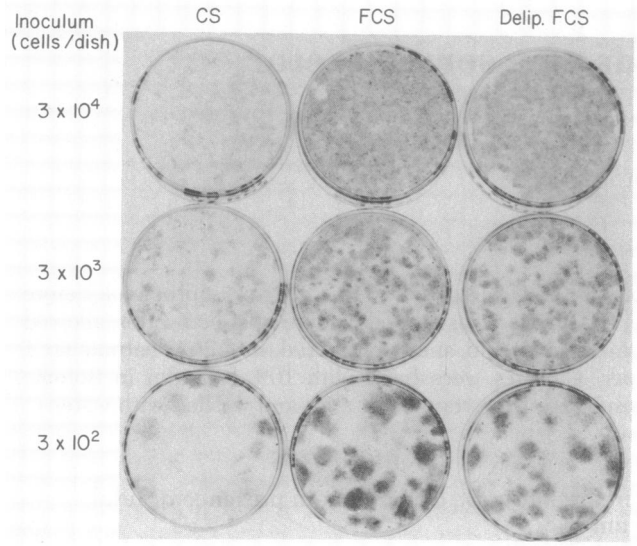

FIG. 1. Comparison of calf serum with fetal calf serum for ability to promote the adipose conversion of 3T3-F442A cells. Cultures were grown nearly to confluence in 50-mm dishes in medium containing 10% calf serum. The serum supplement of some cultures was then changed to fetal calf serum, either untreated or delipidized with solvents. Cultures were fixed and stained with Oil Red O 8 days after reaching confluence. Red color of stained triglycerides shows as black on the photograph. Note the greater adipose conversion in the presence of fetal serum, whether delipidized or not. Fat cell clusters are larger when the inoculum size is smaller (2). CS, calf serum; FCS, fetal calf serum; Delip. FCS, delipidized fetal calf serum.

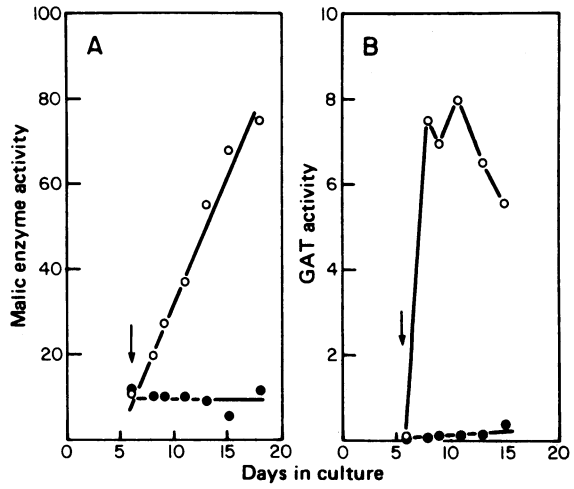

FIG. 3. Failure of lipogenic enzymes to develop in cultures supplemented with cat serum. Cultures were inoculated in the usual way and grown to confluence (arrows) in medium supplemented with either 10% fetal calf serum or with 10% cat serum; insulin and biotin were added to all cultures. At intervals, cultures were harvested and extracted for measurement of enzyme activity. In the presence of fetal calf serum, the activity of the two enzymes increased 10-fold and 50-fold. No increase in activity developed in the cultures receiving cat serum. ○, Medium supplemented with 10% fetal calf serum; ●, medium supplemented with 10% cat serum. (*A*) Malic enzyme activity in nmol of NADPH per min per mg of protein. (*B*) Glycerophosphate acyltransferase activity in nmol of [^{14}C]glycerophosphate incorporated into lipid per min per mg of protein.

serum supported the adipose conversion about as well as calf serum. Horse serum was less effective. Fetal calf serum was by far the most effective. No fetal serum other than calf could be obtained.

The serum of the domestic cat differed from that of all the other species tested in that it was virtually unable to support the adipose conversion. 3T3-F442A cells grew normally in medium supplemented with 10% cat serum, but even after 2½ weeks in the confluent state, no fat cell clusters developed.

The adipose conversion can be interrupted at different stages; for example, in the absence of biotin, the earliest stages of the adipose conversion take place, though lipid accumulation may be prevented (10). The inability of cat serum to support adipose conversion was due to failure of the earliest stages that could be identified. For example, a characteristic part of the conversion is the change in shape of the cell to the spherical (2, 10). This change did not occur in medium supplemented with cat serum; the cells remained flattened and highly extended.

The next stage of the conversion is increasing activity of lipogenic enzymes. The effect of cat serum was tested on two enzymes, malic enzyme and glycerophosphate acyltransferase. Cultures were inoculated in the usual way in medium containing 10% calf serum. Two days later, the supplement was changed to 10% fetal calf serum or 10% cat serum. At intervals, beginning when the cells were nearly confluent, cultures were harvested and assayed for the two enzymes. In the presence of fetal calf serum, the activity of malic enzyme and of glycerophosphate acyltransferase rose by 10-fold and 50-fold, respectively (Fig. 3). In the presence of cat serum, no increase was observed.

In order to study the nature of the adipogenic factor of fetal calf serum, conditions are required in which growth occurs but adipose conversion does not. Calf serum is much less adipogenic than fetal calf serum, but would produce a rather high background for assay of adipogenic factor. Because 3T3 cells do not survive in serum-free medium, some serum proteins are required. For this purpose cat serum was found to be a very useful supplement. The sensitivity of such an assay system is shown in Fig. 4. Cultures were inoculated in medium supplemented with 10% calf serum. After 2 days, the serum supplement was changed to 10% cat serum together with different concentrations of fetal calf serum. Cultures were fixed and stained 1 week after the cells reached confluence. A supplement of 10% cat serum alone supported virtually no adipose conversion, but the

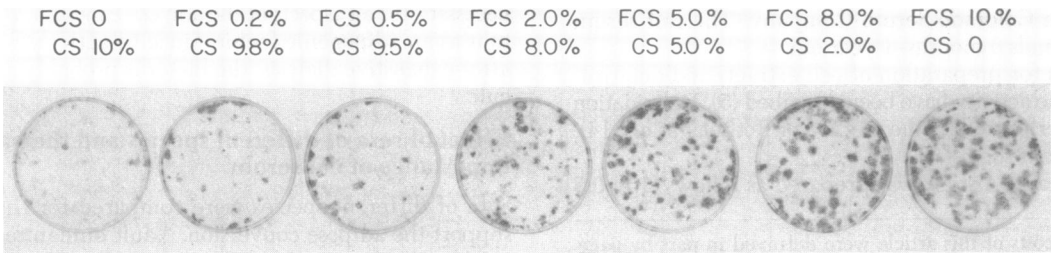

FIG. 2. Effect of concentration of fetal calf serum on adipose conversion. F442A cells (3×10^3) were inoculated into 32-mm dishes and grown to confluence in medium supplemented with 10% calf serum. The serum supplement was then changed to mixtures of calf serum (CS) and fetal calf serum (FCS) as shown. Cultures were fixed and stained 14 days after reaching confluence. The greater ability of fetal calf serum to promote the adipose conversion is evident at a concentration of less than 2%, against a background of nearly 10% calf serum.

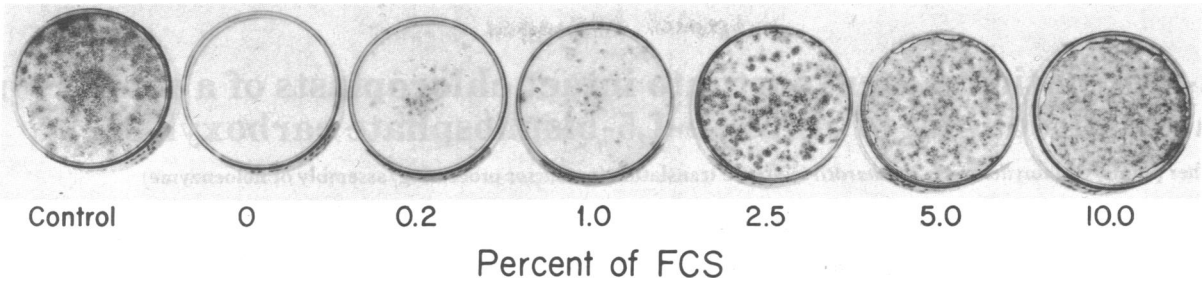

FIG. 4. Assay of adipogenic factor of fetal calf serum on a cat serum background. Dishes (35 mm) were inoculated with 3×10^3 cells in medium supplemented with 10% calf serum. Two days later the medium was changed to one supplemented with 10% cat serum and different concentrations of fetal calf serum (FCS). A control received 10% fetal serum as sole supplement. Insulin and biotin were added to all cultures. Two weeks after inoculation (1 week after reaching confluence), the cultures were fixed and stained. Photograph shows that the amount of adipose conversion, as estimated from stainable lipid content, was proportional to the concentration of fetal serum. Cat serum alone did not support appreciable conversion. The sensitivity of the assay was less than 0.2% of fetal calf serum.

addition of increasing amounts of fetal calf serum produced increasing amounts of adipose conversion. The adipogenic effect of 0.2% of fetal calf serum was readily detected.

DISCUSSION

These experiments have disclosed the presence in serum of a factor necessary for the adipose conversion. This factor acts quite differently from biotin, which is required for only certain parts of the conversion. A deficiency of biotin does not prevent change in cell shape to the spherical, and enzymatic differentiation is not prevented, though it is modified (10). In contrast, in the absence of the adipogenic factor described here we can detect no change characteristic of the adipose conversion.

We have called this factor adipogenic to distinguish it from other agents acting more directly on lipid synthesis. For example, insulin has a lipogenic action, for in its presence triglyceride accumulates more rapidly and the activity of some of the lipogenic enzymes increases (9); but the adipose conversion takes place in the absence of insulin with apparently unaltered frequency, judging by the number of fat cell clusters in surface cultures. In the virtual absence of the adipogenic factor (medium supplemented with cat serum), insulin, even at 10 μg/ml, produces no adipose conversion. The adipogenic factor probably acts earlier than insulin and seems to be required for the entire program of differentiation.

The virtual absence of the adipogenic factor from cat serum will be very useful for assay of the factor during its purification, but the significance of this unusual property is not clear. It is possible that the cat serum contains an adipogenic factor that does not interact with mouse cells, though the corresponding factor of all other species tested was able to do so. The age factor was not controlled, and it may be that in cats the adipogenic factor declines more strikingly with age. Calf serum is obtained from relatively young animals.

The only fetal serum tested was bovine; it contained more of the adipogenic factor than the serum of grown animals. The 3T3 cells did not grow better in fetal serum, so that the greater adipogenic activity is not associated with any other superior properties that might improve the adipose conversion; the greater content of adipogenic factor becomes evident only when the cells become confluent and enter a predominantly resting state. Since fat cells develop predominantly in early life, we suggest that the adipogenic serum factor may be a determinant of this form of differentiation.

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