Changes in Angiotensinogen Messenger RNA in Differentiating 3T3-F442A Adipocytes

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Angiotensinogen messenger RNA (mRNA) has been identified in both brown and white adipose tissue. Recently we have shown that when 3T3-L1 cells were treated with isobutylmethylxanthine (IBMX) to accelerate differentiation, angiotensinogen mRNA increased markedly in adipocytes as compared with preadipocytes. To determine if a correlation existed between the regulatory events associated with the differentiation process, we compared the change in angiotensinogen mRNA in spontaneously differentiating 3T3-F442A cells with two established parameters of differentiation in adipocyte cell lines. Differentiation was assessed by visual examination of cells for lipid droplets, fluorescent staining of the F-actin fibers, and increases in glycerol phosphate dehydrogenase mRNA. F-actin fibers were highly structured in preadipocytes, becoming disassembled and very disorganized as cells differentiated into adipocytes. The quantity of angiotensinogen mRNA increased as the number of lipid-containing cells increased within a culture. Glycerol phosphate dehydrogenase mRNA accumulated in differentiated adipocytes to about the same extent as angiotensinogen mRNA. Thus, increases in angiotensinogen mRNA were associated with the morphological and biochemical changes that occur during the phenotypic modulation of 3T3-F442A cells. (Hypertension 1990;15:867–871)

Methods

3T3-F442A cells (provided by H. Green, Harvard Medical School, Cambridge, Massachusetts) and control 3T3-C2 cells, a clonal line with a low frequency of differentiation into adipocytes, were cultured as previously described. 3T3-F442A cells were grown to confluence and allowed to differentiate spontaneously without the addition of drugs. Angiotensinogen mRNA accumulation was compared with accumulation of glycerol phosphate dehydrogenase (GPD) mRNA, an established marker of differentiation in adipocytes, and with the change in the intermediate actin fibers that change with differentiation.

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and GPD with a cDNA provided by B. Spiegelman (Harvard Medical School). Blots were probed with angiotensinogen cDNA, exposed to film, then washed extensively with 0.1 SSPE, and probed with GPD cDNA.

Cells were grown in Falcon (Fisher Scientific, Pittsburgh, Pennsylvania) 35-mm culture plates and stained for F-actin (intermediate actin) with rhodamine-phalloidin, a fluorescent probe, by the method of Borak et al. Results

F442A cells were allowed to differentiate spontaneously in the absence of exogenous drugs; C2 cells were maintained in the same medium and matched for time in culture with the F442A cells. Figure 1
FIGURE 1. Photomicrographs showing changes in F-actin fibers during spontaneous differentiation of F442A cells. Left panels are phase-contrast photomicrographs of cells, whereas right panels show the F-actin fibers from the same field visualized with rhodamine-phalloidin and a fluorescent filter. All photographs were taken at ×400. Panels A and B: Preconfluent cells with the characteristic fibroblastic morphology with highly developed and well-organized F-actin fibers. Panels C and D: Confluent, contact-inhibited cells that show no morphological changes associated with differentiation and have well organized F-actin fibers. Panels E and F: Heterogenous cell population from a culture that had approximately 60% lipid-containing cells; most of the cells have become spherical, and many have accumulated lipid droplets. Stress fibers lack the parallel linear arrangement seen in panel D.

illustrates the transformation of the F-actin fibers in the F442A cells from preconfluency to confluency to spontaneously differentiated adipocytes. Preconfluent cells stained for F-actin showed a well-developed stress-fiber pattern with numerous parallel fibers. This pattern is also seen in the confluent but undifferentiated cells (Figure 1, panels C and D). As the F442A cells became confluent and contact inhibited, cells began to retract their processes and round up, an indication that differentiation had begun. This was paralleled by a disruption of the highly organized pattern of the F-actin fibers. As cells continued to undergo phenotypic modulation and began to accumulate lipid droplets, the F-actin fibers were disassembled and became very disorganized. This correlates with the decrease in actin in differentiated adipocytes. This is illustrated in Figure 1 (panels E and F) where many cells have become rounded and have accumulated lipid droplets; concomitantly, the F-actin fibers (Figure 1, panels E and F) appear to be undergoing disassembly, and many of the remaining fibers no longer exhibit the parallel linear arrangement found in undifferentiated cells.

When cells were allowed to differentiate spontaneously during long-term culture, morphological changes were usually observed first. Random cells then began to accumulate lipid droplets. As the percentage of F442A cells containing lipid droplets increased, a flask of F442A cells and a flask of control C2 cells were removed for extraction of RNA. Angiotensinogen mRNA accumulation increased as the percentage of cells containing lipid droplets increased (Figure 2). When approximately 20% of the cells contained lipid droplets (as assessed by microscopic examination), angiotensinogen mRNA was detected at low levels. In Figure 2, lane 7 represents approximately 60% differentiation and correlated with the changes in F-actin fibers illustrated in Figure 1 (panels E and F). As seen in Figure 2, the expression of angiotensinogen mRNA was increased dramatically with complete differentiation of F442A cells and did not change over the same time course in C2 cells.

GPD is an established marker of differentiation in many adipocyte model systems. To establish if the angiotensinogen gene was regulated by the same phenotypic modulatory events that regulate GPD, we examined RNA extracted from two sets of spontaneously differentiated F442A cells (Figure 3). Densitometric analysis of dot blots probed for angiotensinogen and GPD revealed that the message levels for
both proteins increased approximately the same amount in the spontaneously differentiating F442A cells. When dot blots from time-matched C2 cells were probed for angiotensinogen and GPD, there were no detectable signals from either cDNA probe (data not shown).

**Discussion**

Treatment of control 3T3-C2 cells and 3T3-L1 cells with dexamethasone and IBMX to induce differentiation was shown to increase angiotensinogen mRNA in adipocytes and undifferentiated C2 cells. Part of the increase in angiotensinogen mRNA in adipocytes was because of dexamethasone stimulation of the angiotensinogen gene and was reflected by the increase in mRNA seen in the C2 cells. The additional increase in angiotensinogen mRNA in L1 cells over C2 cells, however, was postulated to reflect regulation by the events that occur during the differentiation process. To address this issue, we compared the changes in angiotensinogen mRNA with those of GPD mRNA and cytoskeletal elements as F442A cells differentiated spontaneously. GPD represents one of several cDNA clones isolated that increases as preadipocytes differentiate into adipocytes, whereas F-actin mRNA decreases as the number of fat cells increases within a culture. Changes in the F-actin fibers and GPD mRNA correlated with changes in angiotensinogen mRNA, indicating that the differentiation process might play a major role in the increased accumulation of angiotensinogen mRNA.

Ringold et al have isolated cDNAs from TA1 adipocytes that represent mRNAs up-regulated by the differentiation process. These mRNAs were divided into two distinct classes as follows: Class I represents RNAs expressed solely in the differentiated adipocyte, whereas class II clones represent RNAs expressed at low levels in preadipocytes that increase several times on differentiation to adipocytes. Angiotensinogen appears to belong to this second class of genes. Addi-
tionally, Ringold et al. have identified a class II clone, clone 5, that, like angiotensinogen, can be regulated by steroids in the preadipocyte and the adipocyte. Clone 5, however, does not encode angiotensinogen because it hybridizes to a 1.1 kilobase mRNA, whereas angiotensinogen mRNA is 1.8 kb. We have identified another mRNA (angiotensinogen) in adipocytes that is increased by the differentiation process and can be regulated by steroids in both the undifferentiated and differentiated state.

The role of angiotensinogen in preadipocytes and adipocytes is unknown. Developmental regulation of angiotensinogen gene expression suggests that angiotensinogen or the angiotensin peptides might have a functional role in the differentiated cell. Although it has not been demonstrated that renin is synthesized by adipocytes, Schelling et al. have reported renin-like activity in lysates of wild-type 3T3 cells. We have detected angiotensin I-like immunoreactivity in the media from 3T3-L1 and 3T3-F442A cells in the absence of exogenous renin (unpublished observations from our laboratory). Thus, it appears that adipocytes are capable of processing angiotensinogen to angiotensin peptides. Because angiotensin II stimulates the production of prostaglandins in isolated epididymal adipocytes, these observations suggest that a paracrine angiotensin system might exist to regulate some aspect or aspects of white adipose tissue function.

Lee et al. have suggested a possible role for angiotensinogen in the early development of the rat. This is based on their findings that angiotensinogen mRNA was detected at low levels in embryo bodies and yolk sac on day 11 of embryogenesis and increased until a plateau was reached at day 17. This angiotensinogen mRNA was about 200 nucleotides larger than that expressed in adult liver. The authors hypothesized that most of the angiotensinogen mRNA expressed in the body of the embryo was of hepatic origin. A study by Gomez et al. identified the perirenal and interscapular brown fat and kidney as the sources of embryonic angiotensinogen mRNA. Fetal liver angiotensinogen mRNA was detected at day 20 of gestation only with poly A+-enriched liver RNA. Both studies detected low levels of angiotensinogen mRNA in fetal brain on day 15, which increased significantly by day 20. Lee et al. postulated that the increase in angiotensinogen mRNA in fetal brain might be associated with the delayed differentiation of nervous tissue. Thus, expression of the angiotensinogen gene is tissue specific during ontogeny and probably is regulated by the processes associated with differentiation of at least some of the tissues.

Therefore, in spontaneously differentiating 3T3-F442A cells, F-actin fibers are disassembled as the phenotypic expression of the cells is modified. Concomitantly, angiotensinogen mRNA levels increase as the percentage of fat cells increases within a culture, whereas no change occurs in the time-matched C2 cells. The changes in angiotensinogen mRNA levels in spontaneously differentiated F442A cells are similar to the changes in GPD mRNA, an established marker of adipocyte differentiation. Thus, the increases in angiotensinogen mRNA in spontaneously differentiating cells parallel both the structural changes associated with adipocyte differentiation and the increases in GPD mRNA. We conclude that the expression of angiotensinogen mRNA in spontaneously differentiating 3T3-F442A cells appears to be controlled by a developmental regulatory mechanism.

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