

Tumor Necrosis Factor- α Stimulates Cell Proliferation in Adipose Tissue-Derived Stromal-Vascular Cell Culture: Promotion of Adipose Tissue Expansion by Paracrine Growth Factors

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Abstract

KRAS, KRYSZYNA M., DOROTHY B. HAUSMAN, AND ROY J. MARTIN. Tumor necrosis factor- α stimulates cell proliferation in adipose tissue derived stromal-vascular cell culture: promotion of adipose tissue expansion by paracrine growth factors. *Obes Res.* 2000;8:186–193.

Objective: Elevated levels of tumor necrosis factor- α (TNF- α) protein and mRNA have been reported in adipose tissue from obese humans and rodents. However, TNF- α has catabolic and antiadipogenic effects on adipocytes. Addressing this paradox, we tested the hypothesis that paracrine levels of TNF- α , alone or together with insulin-like growth factor-I (IGF-I), support preadipocyte development.

Research Methods and Procedures: Cultured stromal-vascular cells from rat inguinal fat depots were exposed to serum-free media containing insulin and 0.2 nM TNF- α , 2.0 nM TNF- α , or 0.2 nM TNF- α + 1.0 nM IGF-I at different times during 7 days of culture.

Results: TNF- α inhibited adipocyte differentiation as indicated by a reduction in both immunocytochemical reactivity for the preadipocyte-specific antigen (AD3; early differentiation marker) and glycerol-3-phosphate dehydrogenase activity (late differentiation marker). Early exposure (Days 1 through 3 of culture) to 0.2 nM TNF- α did not have a long term effect on inhibiting differentiation. Continuous exposure to 0.2 nM TNF- α from Days 1 through 7 of culture resulted in a 75% increase in cell number from control. There was a synergistic

effect of 0.2 nM TNF- α + 1 nM IGF-I on increasing cell number by Day 7 of culture to levels greater than those observed with either treatment applied alone.

Discussion: These data suggest that paracrine levels (0.2 nM) of TNF- α alone or in combination with IGF-I may support adipose tissue development by increasing the total number of stromal-vascular and/or uncommitted cells within the tissue. These cells may then be recruited to become preadipocytes or may alternatively serve as infrastructure to support adipose tissue growth.

Key words: development, insulin-like growth factor-I, cytokines, preadipocytes, differentiation

Introduction

Research has shown that adipocyte-derived paracrine factors can affect adipose tissue development (1–4). Identification and elucidation of the mechanisms by which these paracrine factors affect adiposity may lead to potential targets for therapeutic intervention in the development of obesity. Tumor necrosis factor- α (TNF- α), a cytokine, is synthesized by and secreted from adipose tissue (5) and its expression and production increases with increasing adipocyte size (6). An association between TNF- α and fat cell size has been described in several models of obesity. In humans, fat tissue from obese subjects secreted 2-fold more TNF- α than did that of lean controls (7). In diet-induced obese rats, adipose tissue TNF- α activity was positively correlated with adiposity (8). Furthermore, adipose tissue-derived TNF- α activity is elevated in older rats in relation to adipose cell size (6). Elevated levels of TNF- α may be associated with the development of secondary metabolic complications in obese individuals. There is a large body of

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evidence supporting a role of TNF- α in promoting insulin resistance during the development of obesity (5,7,9–14).

However, TNF- α has also been well characterized for its catabolic and antiadipogenic effects on adipocytes (9,15–17). It is thus a paradox that TNF- α , which is a strong antiadipogenic cytokine, would be high in the obese state. Previous research examining the effect of TNF- α on adipose tissue development focused primarily on very high levels of the cytokine (5 to 50 nM TNF- α). Those levels better represent septic or bacterial infection situations not physiological levels in adipose tissues of healthy animals (18,19). It is possible that the *in vivo* role of TNF- α on adipose tissue development may have been masked in these studies through the use of supraphysiological levels of the cytokine. Furthermore, *in vitro* studies typically examine specific factors in isolation; whereas *in vivo* multiple paracrine factors would interact to produce the observed cellular response. Insulin-like growth factor-I (IGF-I) is another cytokine that is produced in adipocytes (20–21) and may act as a paracrine factor for adipose tissue growth. IGF-I receptors have been detected in both preadipocytes and stromal-vascular (S-V) cells (22). The addition of IGF-I to preadipocyte cell lines and primary cultures induced both mitogenic and differentiation responses (22–25). Whether IGF-I stimulates differentiation or proliferation may be dependent upon the presence or absence of other growth factors such as TNF- α . Likewise, the specific response(s) of developing preadipocytes to TNF- α may be influenced by the presence of other paracrine factors such as IGF-I.

The present study examined the effects of low levels of TNF- α on early and late differentiation of preadipocytes and total cell proliferation in a primary S-V culture system. The interaction of IGF-I and TNF- α on adipocyte development was also examined. We report that low levels of TNF- α stimulate proliferation of adipose tissue-derived S-V cells and acts synergistically with IGF-I to further stimulate proliferation.

Materials and Methods

Cell Culture Techniques

Inguinal fat pads were excised aseptically from pentobarbital-anesthetized, male Sprague-Dawley rats (80 to 100 g body weight). Adipose tissues from two rats were pooled, and S-V cells and preadipocytes were isolated as described (26). Briefly, tissues were minced and incubated with 5 mL/g tissue of digestion buffer (0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] with 3.2 mg/mL collagenase). Undigested tissue was removed by filtering through 240- and 20- μ m nylon mesh. Filtered cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 Ham's medium (containing 50 mg/L gentamicin sulfate, 50 mg/L cephalothin, and 2.5 mg/L amphotericin B) and centrifuged at 600g for 10 min to separate the

fat cells from the pelleted S-V cells. Aliquots of S-V cells were stained with Rappaport's stain and counted on a hemocytometer. Cells were seeded on 35-mm dishes with 2 mL of plating medium (DMEM/F12 Ham's medium, antibiotics, 10% fetal bovine serum) at a density of 1×10^4 cells/cm². Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere. After 24 hours of plating, medium was removed and DMEM/F-12 Ham's with antibiotics was applied for 2 hours to rinse residual plating medium. Upon rinsing, cells were exposed to treatment media, which contained DMEM/F-12, antibiotics as listed previously, 2 nM thyroxine (Sigma Chemical Co., St. Louis, MO) and varying levels of TNF- α and/or IGF-I. In Experiments 1 and 2, control and all treatments also contained 100 nM insulin, 60 ng/mL transferrin, and 60 pg/mL sodium selenite (ITS, Sigma). In Experiment 3, control and all treatments also contained 1 nM insulin, 0.6 ng/mL transferrin, and 0.6 pg/mL sodium selenite (ITS, Sigma). Recombinant rat TNF- α was purchased from Genzyme (Cambridge, MA). Recombinant human IGF-I was purchased from Bachem (Torrance, CA). Fresh treatment media were applied every 2 days.

Early Differentiation Assay

Preadipocytes were identified by immunocytochemistry with an antiadipocyte monoclonal antibody, AD3, which recognizes a cell surface antigen that is expressed before overt preadipocyte differentiation in both porcine (27) and rat (28) primary cultures. At Days 3 and 7 of the experiment, dishes were fixed in 4% paraformaldehyde then exposed to 1% hydrogen peroxide. Cells were rinsed with 0.1 M phosphate-buffered saline after all steps. Cells were incubated in a humidity chamber with a 1:100 dilution of the AD-3 monoclonal antibody (27), then incubated with biotinylated goat anti-rabbit/mouse IgG followed by ExtrAvidin-Peroxidase, and finally exposed to AEC (3-amino-9-ethyl-carbazole) peroxidase substrate (Sigma). AD-3-positive cells were quantified by image analysis (see below).

Late Differentiation Assay

Briefly, cultures were homogenized by sonic dismemberment in 1.0 mL of ice-cold sucrose buffer. Measurements of glycerol-3-phosphate dehydrogenase activity (GPDH; EC 1.1.1.8) were made using the methods of Wise and Green (29), as modified by Ramsay et al. (26). One unit of activity corresponds to oxidation of 1 nmol NADH/min/mg of protein. Soluble protein concentrations of homogenates were determined using protein assay (Bio-Rad, Melville, NY). Oil Red-O was used to stain additional culture dishes for the presence of lipid-filled cells (30) and dishes were counterstained with Harris hematoxylin.

Quantitative Analysis of Stained Culture Dishes

Cell counting was done using Image Pro Plus software (Media Cybernetics, Silver Springs, MD) and a camera (CCD-72; Dage-MT1, Michigan City, IN). Cells were quan-

tified under 10 \times magnification, with the pixel size on the video screen set at 6.6 \times 5.3 μ m and the gray values set between 0 (black) and 256 (white). AD3-stained dishes were analyzed at a threshold that delineated the reaction product. Total object count (AD3-reactive cells) in three fields (one field = 1 mm²) per dish were determined and averaged. The dishes were subsequently counterstained with hematoxylin and quantified for total cell number at a size threshold that delineated nuclei. Total object count (nuclei) in three fields (one field = 1 mm²) per dish were determined and averaged. Data were expressed as number of preadipocytes or total cells/unit area (field).

Cell Death

Media were collected from cell plates at several time points following exposure to treatment and sonicated. Lactate dehydrogenase activity (EC 1.1.1.2.7) of the media was measured spectrophotometrically (Sigma kit DG1340-K).

Statistical Analysis

All data were expressed as least-square means \pm SEM. All experiments were replicated at least three times. One-way ANOVA was used to determine significant effects of treatment (SuperANOVA program; Abacus Concepts, Inc., Berkeley, CA). Differences were accepted as significant at the level of $p < 0.05$.

Results

Experiment 1

The effect of physiological levels of TNF- α on preadipocyte development were examined. First, the effect of exposure to either 0.2 nM TNF- α (low TNF- α) or 2.0 nM TNF- α (high TNF- α) on early differentiation was examined in serum-free cultures containing 100 nM insulin, conditions optimal for inducing cell differentiation. Exposure to either level of TNF- α for 2 days immediately after plating (Days 1 through 3 of culture) suppressed early differentiation as indicated by a significant reduction in preadipocyte number (AD3-positive cells; Figure 1). Coincidentally, exposure to low TNF- α treatment for 2 days also resulted in a consistent and significant increase in overall cell number. This occurred despite the fact that culture conditions had been optimized for differentiation rather than proliferation. In contrast, high TNF- α treatment for 2 days resulted in either a significantly lower total cell number than control (Figure 1) or no difference from control (replicate experiments, data not shown). There were no treatment differences in lactate dehydrogenase activity of the culture media (data not shown). Thus, the lower total cell number of the high TNF- α -treated cultures was not likely due to necrosis or apoptosis.

Experiment 2

To determine the effect of exposure time, cell cultures were exposed to low or high TNF- α either continuously

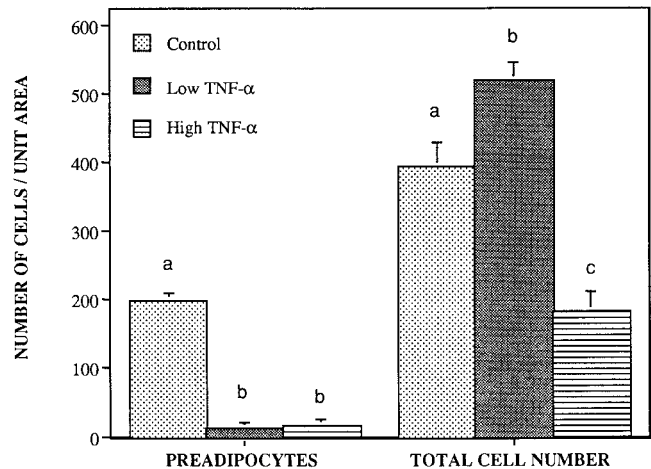


Figure 1. The effect of TNF- α on preadipocyte and total cell number at Day 3 of culture. After 24 hours of plating, S-V cells were exposed to serum-free media containing 100 nM insulin (ITS, see Materials and Methods) supplemented with 0.2 nM TNF- α (Low TNF- α), 2.0 nM TNF (High TNF- α), or without TNF- α (Control) for 2 days (Days 1 through 3 of culture). Preadipocytes were identified by immunocytochemistry for the presence of AD3. Total cell number was quantified using image analysis. Data are expressed as the number of preadipocytes or total cells per unit area (1 mm²). Values represent least-squares mean \pm SEM and are representative of at least three experiments. Means within a cell type with different superscripts represent significant effects ($p < 0.05$) of TNF- α treatment.

(Days 1 through 7), early (Days 1 through 3), or late (Days 3 through 5) in culture. When not exposed to TNF- α , cultures were maintained on serum-free medium containing 100 nM insulin (see Materials and Methods). Control cultures were maintained throughout the experiment on serum-free medium containing 100 nM insulin. Continuous exposure of cell cultures to TNF- α suppressed preadipocyte development to less than 30% of control by Day 7, regardless of the level of TNF- α used ($p < 0.05$; Figure 2). Exposure to low TNF- α early in culture suppressed preadipocyte development to 75% of control; however, high TNF- α exposure early in culture had a greater, lasting inhibitory effect on early differentiation (15% of control). Preadipocyte development was not affected by either low or high TNF- α when exposed between Days 3 and 5 of culture only.

GPDH activity was used as an indicator of adipocyte late differentiation. Continuous treatment with TNF- α , regardless of level, inhibited late differentiation to 5% of the activity of control cells at Day 7 (Figure 3). A significant difference in GPDH activity between low and high TNF- α treatment was only seen with early exposure ($p < 0.05$). Cells exposed early in culture to low or high TNF- α had 75% or 30% of GPDH activity, respectively, as did control cells. This parallels a greater rebound in preadipocyte num-

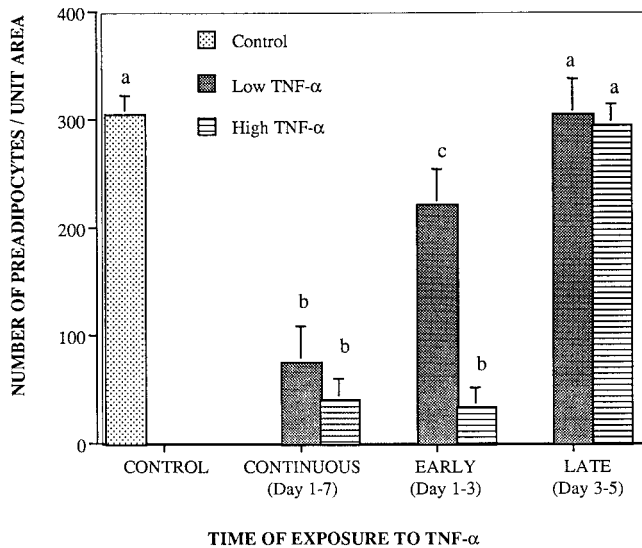


Figure 2. Early differentiation of S-V cells to preadipocytes as affected by TNF- α exposure at 7 days of culture. After 24 hours of plating, S-V cells were exposed to serum-free media containing 100 nM insulin (ITS, see Materials and Methods) supplemented with 0.2 nM TNF- α (Low TNF- α), 2.0 nM TNF- α (High TNF- α), or without TNF- α (Control) for up to 6 days. Cultures were treated with either low or high TNF- α continuously (Days 1 through 7), early (Days 1 through 3), or late (Days 3 through 5) in culture. When not exposed to TNF- α treatment, cultures were maintained on control medium. Preadipocytes were identified by immunocytochemistry for the presence of AD3. Data are expressed as number of preadipocytes/unit area (1 mm²). Values represent least-squares mean \pm SEM and are representative of at least three experiments. Means with different superscripts represent significant effects ($p < 0.05$) of TNF- α treatment.

ber upon removal of low TNF- α . A 50% decrease in GPDH activity from control was seen when cells were exposed to either low or high TNF- α between Days 3 and 5 of culture. Oil Red-O staining also indicated decreased number and size of lipid droplets in all TNF- α -treated cells compared to control cells (data not shown).

Total cell number was significantly increased from control with continuous, low TNF- α treatment but unchanged with continuous exposure to high TNF- α treatment (Figure 4). There was a 75% increase above control when cultures were treated with low TNF- α continuously for 6 days (Days 1 through 7 of culture). There were no significant differences in total cell number from control with exposure to either low or high TNF- α for 2 days only early or late in culture. The effect of various TNF- α doses on cellular proliferation was also determined through concomitant exposure of the cells to TNF- α and [³H]thymidine during the exponential growth phase of primary culture. Either 2 or 4 days of exposure to 0.02 or 0.2 nM TNF- α resulted in a significant increase ($p < 0.02$) in [³H]thymidine incorporation into the total cell pellet (data not shown). In contrast,

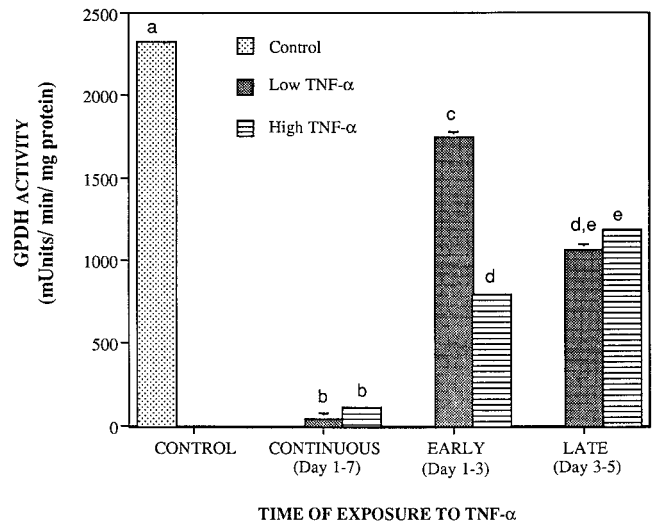


Figure 3. GPDH activity of adipocytes as affected by TNF- α exposure. After 24 hours of plating, S-V cells were exposed to serum-free media containing 100 nM insulin (ITS, see Materials and Methods) supplemented with 0.2 nM TNF- α (Low TNF- α), 2.0 nM TNF- α (High TNF- α), or without TNF- α (Control) for up to 6 days. Cultures were treated with either low or high TNF- α continuously (Days 1 through 7), early (Days 1 through 3), or late (Days 3 through 5) in culture. When not exposed to TNF- α treatment, cultures were maintained on control medium. Values represent least-squares mean \pm SEM and are representative of at least three experiments. Means with different superscripts represent significant effects ($p < 0.05$) of TNF- α treatment.

lower (0.002 nM) and higher (2.0 nM) doses of TNF- α had no effect on [³H]thymidine incorporation.

Experiment 3

IGF-I stimulates proliferation and differentiation in several cell types. Experiments were therefore done to test the possibility of a synergistic relationship between TNF- α and IGF-I in primary preadipocyte cell culture. In these studies, all treatments contained only 1 nM insulin (see Materials and Methods) as opposed to 100 nM insulin used in the previous experiment. Because insulin is a stimulator of adipocyte development, a low level of insulin was used to allow for the examination of IGF-I effects on early differentiation and total cell proliferation, without the overriding influence of insulin. Quantification of differentiation and cell number was made after 6 days of treatment. At 1 nM, IGF-I did not stimulate preadipocyte development above control levels (Figure 5A) nor did this level of IGF-I overcome the inhibition of early differentiation induced by 0.2 nM TNF- α (IGF-I + TNF- α). However, when applied together, TNF- α and IGF-I were synergistic in promoting total cell proliferation whereby a 2-fold increase in cell number above control values was observed in cultures treated with IGF-I + TNF- α (Figure 5B) ($p < 0.05$).

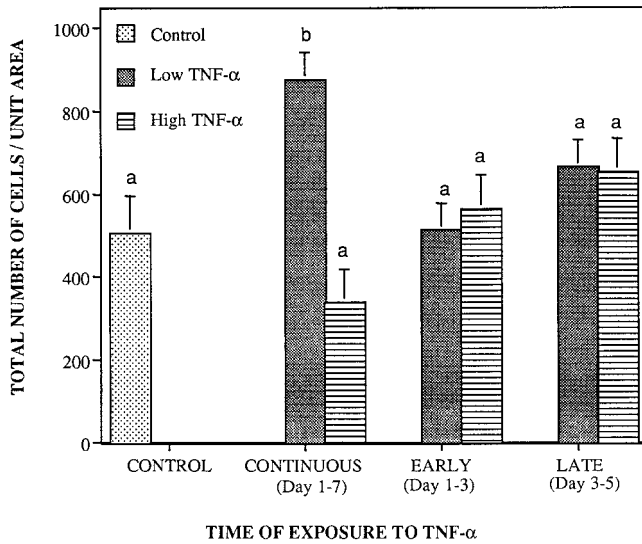


Figure 4. Total cell number in cultures as affected by TNF- α exposure. After 24 hours of plating, S-V cells were exposed to serum-free media containing 100 nM insulin (ITS, see Materials and Methods) supplemented with 0.2 nM TNF- α (Low TNF- α), 2.0 nM TNF- α (High TNF- α), or without TNF- α (Control) for up to 6 days. Cultures were treated with either low or high TNF- α continuously (Days 1 through 7), early (Days 1 through 3), or late (Days 3 through 5) in culture. When not exposed to TNF- α treatment, cultures were maintained on control medium. Total cell number was quantified using image analysis. Data are expressed as number of total cells/unit area (1 mm²). Values represent least-squares mean \pm SEM and are representative of at least three experiments. Means with different superscripts represent significant effects of treatment ($p < 0.05$).

Discussion

These results are the first to demonstrate a growth-promoting effect of TNF- α in adipose tissue-derived S-V cell culture. This paper also reports the effect of physiological levels of TNF- α on early differentiation of preadipocytes. TNF- α was shown to be a potent stimulator of proliferation in adipose tissue-derived primary S-V culture. Treatment of S-V cells with 0.2 nM TNF- α and 100 nM insulin resulted in an approximate 75% increase in total cell number from control. Furthermore, the addition of IGF-I with TNF- α had a synergistic effect on enhancing cell proliferation, approximately doubling cell number from control by Day 7 of culture. These results also show that TNF- α at the levels tested here is a potent inhibitor of early preadipocyte differentiation. Cultures exposed early (Days 1 through 3) to physiological levels of TNF- α (0.2 nM) were able to partially rebound in terms of preadipocyte number and differentiation activity upon TNF- α removal, whereas exposure to supraphysiological levels of TNF- α (2.0 nM) early (Days 1 through 3) in culture resulted in long-term suppression of preadipocyte development.

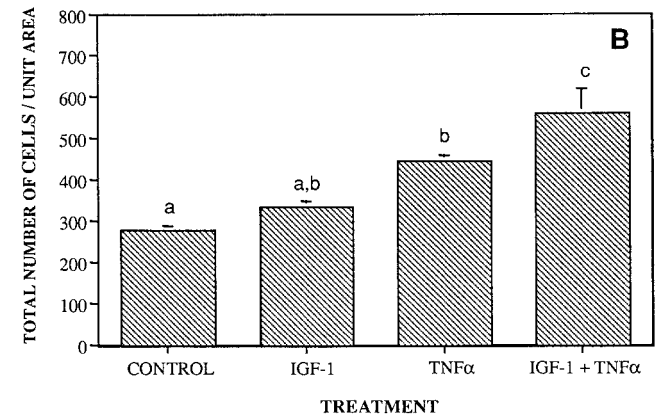
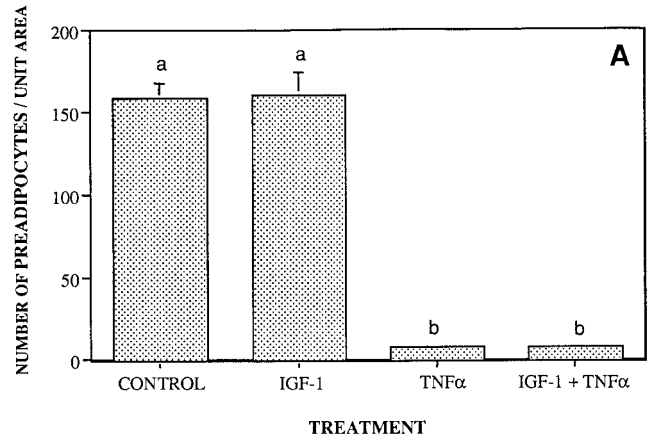


Figure 5. (A) Early differentiation of S-V cells to preadipocytes as affected by TNF- α and IGF-I exposure. (B) Total cell number in cultures as affected by TNF- α and IGF-I exposure. After 24 hours of plating, S-V cells were exposed to serum-free media containing 1 nM insulin (ITS, see Materials and Methods) supplemented with 0.2 nM TNF- α (TNF), with 1.0 nM IGF-I (IGF), or with both 1.0 nM IGF-I and 0.2 nM TNF- α (IGF + TNF) for 6 days. Control treatment received only 1 nM insulin. Preadipocytes were identified by immunocytochemistry for the presence of AD3. Total cell number was quantified using image analysis. Data are expressed as number of preadipocytes or total cells/unit area (1 mm²). Values represent least-squares mean \pm SEM and are representative of at least three experiments. Means with different superscripts represent significant effects of treatment ($p < 0.05$).

The studies reported here show that TNF- α is an inhibitor of early differentiation of preadipocytes. Use of the AD3 monoclonal antibody enabled the identification and quantification of preadipocytes before overt differentiation to adipocytes occurred. Inhibition of early differentiation was dependent upon level and time of exposure to TNF- α . Continuous exposure to either 0.2 or 2.0 nM TNF- α prevented early differentiation. However, transient exposure of cells to low levels (0.2 nM) of TNF- α for 2 days early in culture did not prevent preadipocyte differentiation once

TNF- α treatment was removed. This rebound of early differentiation was not seen with high TNF- α treatment. All cultures were maintained on serum-free media containing 100 nM insulin with or without TNF- α . TNF- α has been implicated in promoting insulin resistance (5,9–14). Insulin has adipogenic activity by stimulating differentiation and accelerating lipid accumulation (31). The inhibition of early and late differentiation by TNF- α suggests that there might have been insulin insensitivity and that the duration of insensitivity was dependent upon the concentration and time of exposure to TNF- α . Treatment of cells with TNF- α early in culture had a greater effect on preadipocyte development than did treatment applied late in culture. This is supported by previous observations that early differentiation of preadipocytes primarily occurs within 2 days after plating (32).

TNF- α is an inhibitor of late differentiation of adipocytes (33). Suppression of late differentiation has been reported using 10- to 20-fold higher levels of TNF- α (17,34) than those used in the experiments reported here. The low levels of TNF- α (0.2 nM) used in the present study also prevented late differentiation of adipocytes. Late exposure of cells to TNF- α resulted in decreased GPDH activity without a change in preadipocyte number from control cells. Others have also reported an antiadipogenic effect of TNF- α on fat cells without the complete reversion to preadipocytes (35) by means of decreasing expression of lipogenic enzymes and increasing lipolysis (15, 36, 37)

Low levels of TNF- α stimulated an increase in total cell number suggesting an effect on increasing cell proliferation. Several fibroblast cell lines, including BALB/c 3T3 (38) and 3T3-L1 (39) have been shown to be responsive to the proliferative action of TNF- α . IGF-I is a known stimulator of proliferation in a variety of cell types (22–25) as is TNF- α (40). The addition of IGF-I to 0.2 nM TNF- α treatment significantly increased proliferation above that observed with the control or with either cytokine treatment alone. This is not surprising as synergy between TNF- α and other growth factors has been previously reported (38). For example, TNF- α was synergistic with epidermal growth factor and platelet-derived growth factor in stimulating DNA synthesis in fibroblasts (38).

The specific cell type undergoing proliferation as a result of the TNF- α /IGF-I treatment is unknown. The preadipocyte/stromal cell primary culture used in this study contained a heterogeneous population of cells. The cell pellet from which these cultures were plated contained a variety of cell types, including endothelial cells, fibroblasts, macrophages, and pericytes in addition to preadipocytes (41). An increase in one or more of these cell types could be responsible for the increase in total cell number that was observed with TNF- α or TNF- α + IGF-I treatment. Microvascular endothelial cells represent a major component of the S-V cell population. However, as propagation of endothelial cells requires the continued presence of serum (42,43), it is

unlikely that the conditions in our serum-free culture system were favorable for the proliferation of these cells. An increase in fibroblasts could also account for the increase in total cell number with TNF- α or TNF- α + IGF-I treatment. Preliminary evidence indicates robust fibronectin and troponin staining in cultures treated for 2 days with 0.2 nM TNF- α (D. Hausman, unpublished observations). Further studies are required to verify the effects of low dose TNF- α treatment on the proliferation of specific cell types within this culture system.

Adipose depot expansion may occur by adipocyte hyperplasia, hypertrophy, or both (44). Faust (45) suggested that adipose cells reaching a critical size will trigger the events that result in the proliferation of adipocytes. Kanarek and Orthen-Gambeill (46) reported an increase in mean fat cell size before the onset of adipocyte hyperplasia in diet-induced obese rats. TNF- α expression and production increases with increasing fat cell size (6). IGF-I production may also correspond to fat cell size. Marques et al. (47) have shown increased total content of IGF-I in adipose tissue from high-fat diet-induced obese rats. Adipose tissue levels of TNF- α and IGF-I are higher with obesity, but these paracrine factors have seemingly opposing effects on adipose tissue development. IGF-I induced both mitogenic and differentiation responses in preadipocyte cultures (22–25), whereas TNF- α inhibits the expression of adipogenic enzymes (6, 16, 35). Based on the results presented here, it is hypothesized that TNF- α may also be permissive for adipose tissue expansion, whereby its production increases when existing fat cells reach a critical size. Because it is a very potent inhibitor of differentiation, TNF- α may act to drive cells into a mitogenic program directly or by other mechanisms such as increasing growth factor responsiveness. IGF-I, produced in response to enlarging fat cells, may act synergistically with TNF- α to further enhance proliferation. The net result would be an expansion of the cell pool whereby these cells may then be recruited to become adipocytes or may alternatively serve as infrastructure to support adipose tissue growth.

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References

1. Lau DCW, Shillabeer G, Li Z-H, Wong K-L, Varzaneh FE, Tough SC. Paracrine interactions in adipose tissue development and growth. *Int J Obes*. 1996;20:S16–S25.
2. Lau DCW, Shillabeer G, Wong KL, Tough SD, Russell JC. Influence of paracrine factors on preadipocyte replication and differentiation. *Int J Obes*. 1990;14:193–201.
3. Serrero G, Lepak N. Endocrine and paracrine negative regulators of adipose differentiation. *Int J Obesity*. 1996;20:S58–S64.

4. **Hausman GJ, Wright JT.** Genetic, endocrine and paracrine/autocrine aspects of porcine adipocyte differentiation. *Proc Recip Meats Conf.* 1990;43-73.
5. **Hotamisligil GS, Shargill NS, Spiegelman BM.** Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science.* 1993;259:87-91.
6. **Morin C, Pagliassoni M, Windmiller D, Eckel R.** Adipose tissue-derived tumor necrosis factor- α activity is elevated in older rats. *J Gerontol.* 1997;52A:B190-B5.
7. **Hotamisligil GJ, Arner P, Caro JF, Atkinson RL, Spiegelman BM.** Increased adipose tissue expression of tumor necrosis factor- α in human obesity and insulin resistance. *J Clin Invest.* 1995;95:2409-15.
8. **Morin C, Eckel R, Pagliassoni M.** High fat diets elevate adipose tissue-derived tumor necrosis factor- α activity. *Endocrinology.* 1997;138:4665-71.
9. **Hotamisligil GS, Murray D, Choy LN, Spiegelman BM.** TNF- α inhibits signaling from insulin receptor. *Proc Natl Acad Sci USA.* 1994;91:4854-8.
10. **Hauner H, Petruschke T, Russ M, Rohrig K, Eckel J.** Effects of tumour necrosis factor alpha (TNF α) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. *Diabetologia.* 1995;38:764-71.
11. **Hotamisligil GS, Budavari A, Murray D, Spiegelman BM.** Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes: central role of tumor necrosis factor- α . *J Clin Invest.* 1994;94:1543-9.
12. **Hotamisligil GS, Spiegelman BM.** Tumor necrosis factor: a key component of the obesity-diabetes link. *Diabetes.* 1994;43:1271-8.
13. **Liu LS, Spelleken M, Rohrig K, Hauner H, Eckel J.** Tumor necrosis factor- α acutely inhibits insulin signaling in human adipocytes: implication of the p80 tumor necrosis factor receptor. *Diabetes.* 1998;47:515-22.
14. **Saghizadeh M, Ong JM, Garvey WT, Henry RR, Kern PA.** The expression of TNF α by human muscle: relationship to insulin resistance. *J Clin Invest.* 1996;97:1111-8.
15. **Torti FM, Dieckmann B, Beutler B, Cerami A, Ringold GM.** A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science.* 1985;229:867-9.
16. **Zhang B, Berger J, Erding H, et al.** Negative regulation of peroxisome proliferator-activated receptor- γ gene expression contributes to the antiadipogenic effects of tumor necrosis factor- α . *Mol Endocrinol.* 1996;10:1457-66.
17. **Pape ME, Kim K-H.** Effect of tumor necrosis factor on acetyl-coenzyme A carboxylase gene expression and preadipocyte differentiation. *Mol Endocrinol.* 1988;2:395-403.
18. **Argiles JM, Lopez-Soriano J, Busquets S, Lopez-Soriano FJ.** Journey from cachexia to obesity by TNF. *FASEB J.* 1997;11:743-51.
19. **Nakane A, Numata A, Minagawa T.** Endogenous tumor necrosis factor, interleukin-6, and gamma interferon levels during listeria monocytogenes infection in mice. *Infect Immun.* 1992;60:523-8.
20. **Nougues J, Reyne Y, Barenton B, Chery T, Garandel V, Soriano J.** Differentiation of adipocytes precursors in a serum-free medium is influenced by glucocorticoids and endogenously produced insulin-like growth factor-1. *Int J Obes.* 1993;17:159-67.
21. **Chen NX, Hausman GJ, Wright JT.** Influence of thyroxine in vivo on preadipocyte development and insulin-like growth factor-I and IGF binding protein secretion in fetal stromal vascular cell cultures. *Obes Res.* 1996;4:357-66.
22. **Ramsay TG.** Insulin-like growth factors and adipose tissue. In: Bray GA, Ryan DH, eds. *Molecular and Genetic Aspects of Obesity.* Baton Rouge, LA: Louisiana State University Press; 1996, pp. 373-403.
23. **Wabitsch M, Hauner H, Heinze E, Teller WM.** The role of growth hormone/insulin-like growth factors in adipocyte differentiation. *Metabolism.* 1995;44:45-9.
24. **Wright JT, Hausman GJ.** Insulin-like growth factor-1 (IGF-1)-induced stimulation of porcine preadipocyte replication. *In Vitro Cell Dev Biol.* 1995;31:404-8.
25. **Schmidt W, Poll-Jordan G, Loffler G.** Adipose conversion of 3T3-L1 cells in serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP. *J Biol Chem.* 1990;265:15489-95.
26. **Ramsay TG, Hausman GJ, Martin RJ.** Preadipocyte proliferation and differentiation in response to hormone supplementation of decapitated fetal pig sera. *J Anim Sci.* 1987;63:735-44.
27. **Wright JT, Hausman GJ.** Monoclonal antibodies against cell surface antigens expressed during porcine adipocyte differentiation. *Int J Obes.* 1990;14:284-91.
28. **Kras KM, Hausman DB, Hausman GJ, Martin RJ.** Adipocyte development is dependent upon stem cell recruitment and proliferation of preadipocytes. *Obes Res.* 1999;7:491-7.
29. **Wise LS, Green H.** Participation of one isoenzyme of cytosolic glycerophosphate dehydrogenase in adipose conversion of 3T3 cells. *J Biol Chem.* 1979;254:273-81.
30. **Hausman GJ.** Techniques for studying adipocytes. *Stain Technol.* 1981;56:149-54.
31. **Wiederer O, Loffler G.** Hormonal regulation of the differentiation of rat adipocyte precursor cells in primary culture. *J Lipid Res.* 1987;28:649-58.
32. **Kras KM, Hausman DB, Hausman GJ, Martin RJ.** Development of a system for studying early differentiation of adipose cells in culture. *FASEB J.* 1995;10:1252.
33. **Smas CM, Sul HS.** Control of adipocyte differentiation. *Biochem J.* 1995;309:697-710.
34. **Ron D, Brasier AR, McGehee RE Jr, Habener JF.** Tumor necrosis factor-induced reversal of adipocytic phenotype of 3T3-L1 cells is preceded by a loss of nuclear CCAAT/enhancer binding protein (C/EBP). *J Clin Invest.* 1992;89:223-33.
35. **Xing H, Northrop JP, Grove JR, Kilpatrick KE, Su JL, Ringold GM.** TNF α -mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPAR γ without effects on Pref-1 expression. *Endocrinology.* 1997;138:2776-83.
36. **Morin CLS, Schlaepfer IR, Eckel RH.** Tumor necrosis factor- α eliminates binding of NF- κ B and octamer-binding protein to the lipoprotein lipase promoter in 3T3-L1 adipocytes. *J Clin Invest.* 1995;95:1684-9.

37. **Holden RJ, Pakula IS.** The role of tumor necrosis factor- α in the pathogenesis of anorexia and bulimia nervosa, cancer cachexia and obesity. *Med Hypotheses*. 1996;47:423–38.
38. **Palombella VJ, Vilcek J.** Mitogenic and cytotoxic action of tumor necrosis factor in BALB/c 3T3 cells: role of phospholipase activation. *J Biol Chem*. 1989;264:18128–36.
39. **Cornelius P, Marlow M, Lee MD, Pekala PH.** The growth factor-like effects of tumor necrosis factor- α : stimulation of glucose transport activity and induction of glucose transporter and immediate early gene expression in 3T3-L1 preadipocytes. *J Biol Chem*. 1990;265:20506–16.
40. **Bemelmans MHA, van Tits LJH, Buurman WA.** Tumor necrosis factor: function, release and clearance. *Crit Rev Immunol*. 1996;16:1–11.
41. **Ramsay TG, Rao SV, Wolvertson CK.** In vitro systems for the analysis of the development of adipose tissue in domestic animals. *J Nutr*. 1992;122:806–17.
42. **Novakofski JE.** Primary cell culture of adipose tissue. In: Hausman GJ, Martin RJ, eds. *Biology of the Adipocyte: Research Approaches*. New York: Van Nostrand Reinhold Company; 1987, pp. 160–97.
43. **Lau DCW, Wong KL, Tough SC.** Regional differences in the replication rate of cultured rat microvascular endothelium from retroperitoneal and epididymal fat pads. *Metabolism*. 1987;36:631–6.
44. **Bouchard C, Després JP, Mauriege P.** Genetic and nongenetic determinants of regional fat distribution. *Endocr Rev*. 1993;14:72–93.
45. **Faust IM.** Role of the fat cell in energy balance physiology. In: Stunkard AJ, Stellar E, eds. *Eating and Its Disorders*. New York: Raven Press; 1984, pp. 97–107.
46. **Kanarek RB, Orthen-Gambeill N.** Dietary induced obesity in experimental animals. In: Beynen AC, West CE, eds. *Comparative Animal Nutrition, Vol 6. Use of Animal Models in Human Nutrition Research*. New York: Karger; 1988, pp. 83–110.
47. **Marques BG, Hausman DB, Latimer AM, Kras KM, Mullen Grossman B, Martin RJ.** Insulin-like growth factor I mediates high fat diet-induced adipogenesis in Osborne-Mendel rats. *Am J Physiol Regul Integr Comp Physiol*. 2000. (in press).