Connective tissue growth factor inhibits adipocyte differentiation

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Tan JT, McLennan SV, Song WW, Lo LW, Bonner JG, Williams PF, Twigg SM. Connective tissue growth factor inhibits adipocyte differentiation. Am J Physiol Cell Physiol 295: C740–C751, 2008. First published July 2, 2008; doi:10.1152/ajpcell.00333.2007.—Adipocyte differentiation is a key process implicated in the pathogenesis of obesity and insulin resistance. Its regulation is triggered by a cascade of transcription factors, including the CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor-γ (PPARγ). Growth factors such as transforming growth factor-β1 (TGF-β1) are known to inhibit adipocyte differentiation in vitro, via the C/EBP pathway, and in vivo, but whether a downstream mediator of TGF-β1, connective tissue growth factor (CTGF), also known as CCN2, has a similar role is unknown. Mouse 3T3-L1 cells were differentiated into adipocytes by using standard methods, and effects and regulation of CTGF were studied. Intervention with recombinant human CTGF during differing stages of differentiation caused an inhibition in the development of the adipocyte phenotype, according to the gene expression of the differentiation markers adiponectin and PPARγ, as well as suppression of lipid accumulation and expression of the lipogenic enzyme glycerol-3-phosphate dehydrogenase. Whereas CTGF gene expression promptly fell by 90% as 3T3-L1 preadipocytes differentiated into mature adipocytes, CTGF mRNA expression was induced by added TGF-β1. CTGF applied to cells early in the course of differentiation inhibited total cell protein levels and nuclear localization of the β1-isofom of C/EBP (C/EBP-β) and, subsequently, total cell C/EBP-α levels. CTGF also inhibited the adipocyte differentiation program in primary cultures of mouse preadipocytes. Expression of CTGF mRNA was twofold higher in the central fat depots of mice compared with subcutaneous fat, suggesting a potential role for CTGF in vivo. In summary, these data show that CTGF inhibits the adipocyte differentiation program.

NIH/3T3; CCN2

ADIPOGENESIS, OR FORMATION OF NEW ADIPOSE TISSUE, OCCURS IN ADULT MAMMALS IN RESPONSE TO EXCESS ENERGY SUPPLY TO THE ORGANISM (43). A KEY ADIPOGENIC PROCESS IS ADIPOCYTE DIFFERENTIATION. RECENT STUDIES HAVE REPORTED THAT IN VISCERAL SITES, THE FAT CELLS ARE LESS INSULIN SENSITIVE THAN THOSE FOUND IN SUBCUTANEOUS SITES, AND THEY APPEAR LESS ABLE TO DIFFERENTIATE INTO MATURE ADIPOCYTES (55), INDICATING THAT IMPAIRED ADIPOCYTE DIFFERENTIATION OCCURS IN A SITE-SPECIFIC MANNER.

ADIPOCYTES ARE DERIVED FROM A MESENCHYMAL STEM CELL POPULATION THAT COMMIT TO PREADIPOCYTES AND THEN DIFFERENTIATE TO ADIPOCYTES VIA A SERIES OF WELL-CHARACTERIZED MORPHOLOGICAL AND BIOLOGICAL CHANGES, EVENTUALLY ACCUMULATING LIPID DROPLETS (13, 57). THE DIFFERENTIATION PHASE INVOLVES A TRANSITION FROM THE UNDIFFERENTIATED FIBROBLAST-LIKE PREADIPOCYTES INTO MATURE, ROUND LIPID-LADEN ADIPOCYTES AND IS CHARACTERIZED BY A CHANGE IN CELL MORPHOLOGY FROM FIBROBLASTIC TO THE UNILATERAL APPEARANCE OF MATURE ADIPOCYTES (20). THESE MORPHOLOGICAL CHANGES RESULT FROM ALTERATIONS OF EXPRESSION AND ORGANIZATION OF THE EXTRACELLULAR MATRIX AND CYTOSKELETON COMPONENTS (11).

STUDIES HAVE ESTABLISHED THAT TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) IS A REGULATOR OF ADIPOCYTE DIFFERENTIATION. INCREASED TGF-β1 GENE EXPRESSION HAS BEEN OBSERVED IN ADIPOSE TISSUE OF BOTH ANIMAL AND HUMAN MODELS OF OBESITY (3, 39). IN VITRO, TGF-β1 BINDS TO HIGH-AFFINITY RECEPTORS ON THE PLASMA MEMBRANE OF 3T3-L1 FIBROBLASTS AND POTENTLY INHIBITS THEIR ADIPOGENIC CONVERSION (10, 24, 53). TGF-β1 TREATMENT DURING DIFFERENTIATION INHIBITS THE DEVELOPMENT OF THE COMPLETE ADIPOCYTE PHENOTYPE (24).

THE PROTEIN CONNECTIVE TISSUE GROWTH FACTOR (CTGF), ALSO KNOWN AS CCN2, IS A HEPARIN BINDING 36- TO 38-KDA CYSTEINE-RICH PROTEIN AND A MEMBER OF THE HIGHLY CONSERVED CCN EARLY RESPONSE GENE FAMILY OF PEPTIDES (1, 2, 14, 25, 34). IT IS MULTIFUNCTIONAL AND IS PRODUCED BY ENDOTHelial CELLS AND MENSECHYMAL STEM CELLS AND EPITHELium-DERIVED CELLS. CTGF ACTS VIA AUTOCRINE AND PARACRINE MECHANISMS TO REGULATE METABOLIC FUNCTION INCLUDING CELL PROLIFERATION (5, 15, 23, 27, 28, 32). CTGF IS A DOWNSTREAM MEDIATOR OF THE ACTIVITIES OF TGF-β1 (31, 38). ALTHOUGH TGF-β1 HAS BEEN SHOWN TO INHIBIT ADIPOCYTE DIFFERENTIATION, WHETHER CTGF HAS A SIMILAR AUTOCRINE ROLE HAS NOT BEEN REPORTED, AND CTGF HAS NOT BEEN DESCRIBED IN FAT IN VIVO.

THE CCN2 FAMILY OF TRANSCRIPTION FACTORS INVOLVED IN DIRECtORIZATION AND DNA BINDING, WITH SIX MEMBERS (α TO γ) BEING CHARACTERIZED TO DATE (13, 16, 48). C/EBP-β AND C/EBP-α EXPRESSION ARE TANGENTLY INCREASED AT THE EARLY PHASE OF ADIPOCYTE DIFFERENTIATION AND CAN ACTIVATE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ (PPARγ) DIRECTLY, WHICH IN TURN ACTIVATES C/EBP-α (57). AS FOR PPARγ, C/EBP-α IS REQUIRED FOR THE DIFFERENTIATION OF PREADIPOCES TO MATURE ADIPOCYTES IN MOST WHITE ADIPOSE TISSUE DEPOTS (16). TGF-β1 HAS BEEN ShOWN TO IMPACT NEGATIVELY ON C/EBP-α, C/EBP-β, AND PPARγ BIOACTIVITY (9, 16, 24).

WE HYPOTHEZIZED THAT CTGF INHIBITS ADIPOCYTE DIFFERENTIATION AND THAT IT DOES SO IN AN AUTOCRINE FASHION SIMILAR TO THAT OF TGF-β1. WE EXAMINED THE EFFECTS OF CTGF ON ADIPOCYTE DIFFERENTIATION AND POTENTIAL MECHANISTIC PATHWAY(S) BY WHICH IT ACTS.

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MATERIALS AND METHODS

Cell culture and adipocyte differentiation. NIH/3T3-L1 fibroblast cells (obtained from American Type Culture Collection, Manassas, VA) were maintained in DMEM containing 4.5 g/l d-glucose and 4 mM l-glutamine and supplemented with 10% (vol/vol) fetal calf serum (FCS) at 37°C in 5% CO₂-95% air with cells passedage before reaching confluence. The cells used in this study were between passages 7 and 15. Each experiment was performed three times independently in triplicate.

Cells were induced to differentiate with the use of standard methods (8). At 80% confluence, they were treated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 2 μM dexamethasone, and 20 μM insulin in DMEM supplemented with 10% FCS (day 0). Three days later (day 3), the differentiation medium was replaced with DMEM supplemented with 10% FCS and containing 20 μM insulin. This medium was refreshed every second day for a further 7 days (day 10).

In some parallel studies, cells were differentiated in the presence of adiponectin and peroxisome proliferator-activated receptor-γ agonist ciglitazone (20 μM) instead of the IBMX, dexamethasone, and insulin mix. The degree of differentiation was assessed by measurement of the induction of adipocyte differentiation markers PPARγ and adiponectin using quantitative real-time RT-PCR (described below) and also by lipid accumulation determined by Oil Red O staining (47) in combination with the induction of the lipogenic enzyme glycerol-3-phosphate dehydrogenase (G3PDH) (33, 35). The maintenance of gene expression of plasminogen activator inhibitor type 1 (PAI-1) was also taken as an indicator of inhibition of adipocyte differentiation (52).

Real-time RT-PCR conditions. Cells used for experiments were washed with PBS, and the RNA was extracted with TRI reagent (Sigma Aldrich, St. Louis, MO). The amount of RNA was quantitated spectrophotometrically using the SmartSpec Plus spectrophotometer (Sigma Aldrich, St. Louis, MO). The amount of RNA was quantitated spectrophotometrically using the SmartSpec Plus spectrophotometer (Sigma Aldrich, St. Louis, MO). The amount of RNA was quantitated spectrophotometrically using the SmartSpec Plus spectrophotometer (Sigma Aldrich, St. Louis, MO). The amount of RNA was quantitated spectrophotometrically using the SmartSpec Plus spectrophotometer (Sigma Aldrich, St. Louis, MO). The amount of RNA was quantitated spectrophotometrically using the SmartSpec Plus spectrophotometer (Sigma Aldrich, St. Louis, MO).

Table 1. Primer sequences used for measurement of mouse CTGF, adiponectin, PPARγ, and PAI-1 by real-time RT-PCR

<table>
<thead>
<tr>
<th>Differentiation Markers</th>
<th>Primers Sequences</th>
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<tbody>
<tr>
<td>CTGF (in-house)</td>
<td>Forward 5'-GCATCTGACCAGGATTCT-3'</td>
</tr>
<tr>
<td>ADiponecin (in-house)</td>
<td>Reverse 5'-TTGAGACGGTGCGGT-3'</td>
</tr>
<tr>
<td>PPARγ (40)</td>
<td>Forward 5'-CTGGCTGTTTGGAAGGTCCT-3'</td>
</tr>
<tr>
<td>PAI-1 (40)</td>
<td>Reverse 5'-CCCACTGCTGACCTGATGCC-3'</td>
</tr>
<tr>
<td>Resistin (in-house)</td>
<td>Forward 5'-GGCTGGCGCTTGAGAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ATCCGCCTGGCAATC-3'</td>
</tr>
</tbody>
</table>

CTGF, connective tissue growth factor; PPARγ, peroxisome proliferator-activated receptor-γ; PAI-1, plasminogen activator inhibitor type 1.

CTGF inhibits adipocyte differentiation. Recombinant human CTGF (rhCTGF) was produced and purified in our laboratory using a recombinant adenoviral expression system, AdEasy, as detailed previously (41, 58). Cultured 3T3-L1 fibroblastic cells were differentiated as described earlier. Several treatment regimens were conducted to observe the effects of CTGF on adipocyte differentiation. Effects of either a single treatment of CTGF at day 0 or a continuous exposure for the duration of the differentiation process on the adipocyte phenotype were first examined. For these experiments, cells were cultured in the presence of either rhCTGF (50 ng/ml) or active rhTGF-β1 (0.2 ng/ml), as a positive control, from the induction of differentiation and were replenished each time the medium was replaced until day 10, when the degree of differentiation was determined based on the three end points as described earlier.
The second set of experiments observed effects of addition of CTGF at the time at which the cells had committed to the differentiation process or had already fully differentiated into mature adipocytes. For these experiments, where one treatment only was given, a single dose of rhCTGF (250–500 ng/ml as indicated) or active rhTGF-β1 (2 ng/ml) was applied midway during the differentiation process (day 5) or at near-terminal differentiation (day 6). After 48 h, the RNA was extracted. In another protocol, 3T3-L1 preadipocytes were allowed to differentiate into fully mature adipocytes at day 10. The mature adipocytes were cultured in serum-free conditions for 24 h and then treated once with rhCTGF (250–500 ng/ml as indicated) or active rhTGF-β1 (2 ng/ml) for 48 h, after which the degree of differentiation was measured.

Effects of TGF-β1 on expression of CTGF. Cells were differentiated as described earlier, and two treatment regimens were conducted. The first involved differentiation of 3T3-L1 preadipocytes to mature adipocytes at day 10 and in parallel wells, with observation of the effects of either a single treatment of TGF-β1 (2 ng/ml) at day 0 or a repeated exposure of TGF-β1 at a lower concentration (0.2 ng/ml), which was added at each medium change. The second treatment regimen examined intervention with a single TGF-β1 treatment of 2 ng/ml at the early stage of differentiation with treatment added to cells at either day 0 or day 3 and analyzed at day 3 or day 5, respectively.

Potential mechanistic pathways by which CTGF acts to inhibit differentiation. For the in vitro mechanistic studies, protein expression of C/EBP-α and C/EBP-β were measured by Western immunoblot analysis. C/EBP-β protein expression was measured in whole cell lysate from cells treated with CTGF or TGF-β1 for 24 and 48 h following the addition of the differentiation media, whereas C/EBP-α protein expression was measured in whole cell lysates at both the early and later phases of differentiation. Cells were washed with PBS and lysed with lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 0.2% Triton X-100, pH 7.4). Samples were then applied to SDS-PAGE, and each lane had the same amount of total protein loaded (40 μg) as determined by the Bio-Rad DC Protein Assay (Bio-Rad, Richmond, CA). Separation of the proteins was resolved on SDS-PAGE using 12.5% separation gels and 4% stacking gels in running buffer (3 g of Tris, 14.4 g of glycine, and 10 g of SDS). For detection of C/EBP by Western immunoblot analysis, the primary antibodies were the rabbit polyclonal IgG anti-C/EBP-α (2 μg/ml; sc-61) and anti-C/EBP-β (2 μg/ml; sc-150; both Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed as previously described for the immunoblot method for CTGF detection. Bands were then visualized using the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG at a 1:10,000 dilution (Vector Laboratories) and chemiluminescence (Amersham). The protein α-tubulin was used as a loading control, with its detection by the Abcam α-tubulin HRP antibody (ab 40742) at 1:5,000 dilution followed by the goat-anti-rabbit HRP antibody at 1:10,000 dilution. The α-tubulin 57-kDa band was detected after stripping of nitrocellulose membranes with stripping buffer.

For nuclear localization studies, the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL) was used. Briefly, ~500 μl of ice-cold PBS was added to wells, and scraped cells were collected. Cells were then pelleted by centrifugation at 500 g for 3 min at 4C, and supernatant was removed, collecting the packed cell volume. Reagents CERI and CERII were added sequentially to the cells according to the manufacturer’s instructions, allowing the nonnuclear (supernatant) fraction to be carefully removed and stored. In addition to the NER reagent followed by vortexing, the supernatant containing the nuclear fraction was then transferred into a separate, fresh, prechilled tube and stored at −80°C until further use. The protein concentration in each fraction was determined using the Bio-Rad DC Protein Assay, as described earlier.
terminated by exsanguination, and epididymal fat was excised. The method of isolation of primary cultures of preadipocytes was adapted from Fasshauer et al. (13). In brief, after collagenase I digestion, tissue was seized and cells were maintained and grown under the same conditions as described for the NIH/3T3-L1 fibroblast cells. For primary cultures of P2–P3 cells, a standard protocol of adipocyte differentiation was undertaken, except that triiodothyronine was not added to the differentiation mix. Cells were maintained (P0–P1) in DMEM supplemented with 10% FCS and 20 mM HEPES. Differentiation cocktail mix (0.5 mM IBMX, 2 μM dexamethasone, and 20 μM insulin in DMEM supplemented with 10% FCS and 20 mM HEPES) was used on days 0–3 to differentiate the preadipocytes into adipocytes over a subsequent total 10-day time course. In some parallel wells at day 0, cells were treated with a single application of rhCTGF (500 ng/ml) or rhTGF-

H2O2 in methanol) for 20 min, followed by rinsing in TBS. Nonimmune block against false positives was achieved by incubation in 10% normal goat serum-TBS for 30 min. Sections were then incubated with the in-house-generated primary anti-CTGF antibody (1:100 dilution) for 1 h at room temperature. This antibody was generated by immunizing rabbits using a CTGF COOH-terminal peptide sequence that has complete homology with mouse CTGF (41, 56, 58). After washings, secondary antibody was then added as biotin-conjugated goat anti-rabbit IgG (DAKO, Copenhagen, Denmark) at 1:200 dilution for 1 h at room temperature. Images shown are representative of 3 independent experiments conducted in triplicate. Animal data were generated from groups of six animals as indicated in the text. All results are means ± SD. All data were compared using either unpaired t-test or one-way ANOVA, followed by post hoc comparisons using Bonferroni’s multiple comparison test. Statistical significance was accepted when P < 0.05.

Gene expression of CTGF in vivo. For the 30-wk-duration animal studies, RNA was extracted from 100 mg of mouse fat tissue using Qiagen RNeasy Lipid Tissue mini kit (Valencia, CA). The amount of RNA was quantitated spectrophotometrically as described earlier, and 2 μg of RNA were reverse-transcribed to cDNA. The expression of CTGF was determined by quantitative real-time PCR using SYBR green fluorophore as described earlier.

Immunohistochemistry detection of CTGF protein in vivo. Slides were deparaffinized with xylene and rehydrated through graded ethanol, and washed slides then underwent antigen retrieval by heat treatment with a microwave for 2 min at 100°C in TBS (100 mM Tris, and 300 mM NaCl) at pH 7.5. Slides were then cooled to room temperature before blocking of endogenous peroxidase activity with hydrogen peroxidase [1% (vol/vol) H2O2 in methanol] for 20 min, followed by rinsing in TBS. Nonimmune block against false positives was achieved by incubation in 10% normal goat serum-TBS for 30 min. Sections were then incubated with the in-house-generated primary anti-CTGF antibody (1:100 dilution) for 1 h at room temperature. This antibody was generated by immunizing rabbits using a CTGF COOH-terminal peptide sequence that has complete homology with mouse CTGF (41, 56, 58). After washings, secondary antibody was then added as biotin-conjugated goat anti-rabbit IgG (DAKO, Copenhagen, Denmark) at 1:200 dilution for 1 h at 21°C. Immuno-reactive signal was detected using the Vectastain ABC kit (Vector Laboratories) with visualization of the immunoperoxidase reaction using 3,3'-diaminobenzidine (Sigma-Aldrich) as the chromogen, and the slides were lightly counterstained with hematoxylin and coverslipped in xylene.

Statistical analysis. Cell culture experiments were performed at least three times independently in triplicate. Animal data were generated from groups of six animals as indicated in the text. All results are means ± SD. All data were compared using either unpaired t-test or one-way ANOVA, followed by post hoc comparisons using Bonferroni’s multiple comparison test. Statistical significance was accepted when P < 0.05.
RESULTS

Confirmation of adipocyte differentiation. The 3T3-L1 cells were induced to differentiate from day 0, and the differentiation process was monitored at various time points. To confirm and measure adipocyte differentiation, we studied the mRNA of adiponectin and PPARγ as positive markers of differentiation and PAI-1 maintenance as a negative marker, expressed as the percent change from day 0, taken as 100%. The expression of adiponectin (Fig. 1A) was significantly higher as the cells differentiated throughout the differentiation process, with a 10-fold increase detected as early as day 3 (1,887 ± 3,659%) and a 300-fold increase at day 5 (34,100 ± 30,640%), reaching a 3,000-fold increase by day 10 (381,800 ± 615,800%). Expression of PPARγ (Fig. 1B) also increased during differentiation, although with less change compared with adiponectin, with a twofold increase at day 3 (227.6 ± 92.4%) and, as reported by others (36), peaking at day 5 (390.8 ± 222.5%). Also as expected, the mRNA expression of PAI-1 (Fig. 1C) followed a different pattern compared with both adiponectin and PPARγ, and its expression decreased during differentiation (43.1 ± 52.9% at day 5 and 29.1 ± 22.2% at day 10).

Figure 2 shows the cell morphology of differentiating cells at various time points (days 0, 5, and 10). Cells changed from a fibroblast-like shape at day 0 (Fig. 2A) to a combination of fibroblast-like and round, lipid-laden cells at day 5 (Fig. 2B), and then by day 10, nearly all cultured cells exhibited a round, unilocular lipid-laden-like shape (Fig. 2C). Figure 2, D–F, shows that at day 0, no cells detectably stained with Oil Red O (Fig. 2D), but as differentiation progressed to day 5 (Fig. 2E), a small proportion of the cells began to accumulate lipid. By day 10, the majority of the cells had accumulated lipid (Fig. 2F). The amount of Oil Red O dye detected at day 5 was 2.4-fold higher (238.8 ± 110.4) compared with that at day 0, with an ever higher amount extracted at day 10, nearly a 4-fold increase (392.6 ± 173.5%) (Fig. 2G). The enzyme activity of the lipogenic enzyme G3PDH was measured at days 0 and 10.

Fig. 3. Expression of differentiation markers adiponectin (A) and PAI-1 mRNA (B), lipid accumulation as quantitated from Oil Red O staining (C), and G3PDH enzyme activity from cells harvested at day 10 during either a single (S) treatment of either recombinant human connective tissue growth factor (rhCTGF; 500 ng/ml) or positive control, recombinant human transforming growth factor-β1 (rhTGF-β1; 2 ng/ml), at day 0 or a continuous (C) treatment every 48 h with either rhCTGF (50 ng/ml) or rhTGF-β1 (0.2 ng/ml). Results are means ± SD expressed as percent change from same-day untreated cells (Ctrl; 100%). *P < 0.05; **P < 0.01; ***P < 0.001, significantly different from untreated cells. ###P < 0.001, significantly different from single treatment of TGF-β1 (2 ng/ml). Images shown in E–J are representative of cells that were stained with Oil Red O and photographed at ×400 magnification. E: day 0. F: day 10, untreated control. G: single treatment of CTGF (500 ng/ml) at day 0. H: continuous treatment of CTGF (50 ng/ml) every 48 h. I: single treatment of TGF-β1 (2 ng/ml). J: continuous treatment of TGF-β1 (2 ng/ml) every 48 h.

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The G3PDH enzyme activity present in differentiated cells at day 10 (Fig. 2H) was significantly higher compared with undifferentiated cells (day 10, 0.61 ± 0.96 units/ml vs. day 0, 0.02 ± 0.50 units/ml; P < 0.05). Collectively, the gene expression, Oil Red O, and lipogenic enzyme data confirm the differentiation of 3T3-L1 preadipocytes into mature adipocytes.

Effects of CTGF treatment before commitment to adipocyte differentiation. Potential effects of either a single treatment before commitment to differentiation or a continuous exposure of lower dose CTGF on the differentiation phenotype were then examined. Cells were cultured in the presence of either rhCTGF (50 ng/ml) or active rhTGF-β1 (0.2 ng/ml) throughout the induction of differentiation and replenished every 48 h until day 10. In addition, in a parallel study, a single exposure of either rhCTGF (500 ng/ml) or active rhTGF-β1 (2 ng/ml) was added to the cells at the induction of differentiation. The degree of differentiation was again measured by gene expression of differentiation markers as well as lipid accumulation by Oil Red O staining and the lipogenic enzyme G3PDH.

Figure 3 shows the mRNA expression of the differentiation markers adiponectin (A) and PAI-1 (B). Effects of both a single treatment of rhCTGF at induction of differentiation and continuous exposure inhibited the mRNA expression of adiponectin compared with its corresponding same-day untreated control (Ctrl, 100.0 ± 34.1%); a single rhCTGF treatment inhibited adiponectin expression (31.1 ± 13.4%), whereas continual exposure had a similar effect (44.0 ± 28.3%). The positive control of rhTGF-β1 also showed a similar pattern. However, no significant difference was observed in the PAI-1 mRNA expression.

The degree of differentiation as determined by quantitation of lipid accumulation by Oil Red O staining showed that treatment of cells with either CTGF or TGF-β1 significantly inhibited Oil Red O accumulation, irrespective of whether treatment was a single dose at initiation of differentiation or a continual exposure (Fig. 3C). The rhCTGF, as either a single or continuous treatment, inhibited lipid accumulation by a modest but significant ~23% compared with control untreated cells. A similar pattern was seen for the positive control of rhTGF-β1.

A single treatment of rhCTGF or a continuous exposure decreased lipogenic enzyme G3PDH activity, as did rhTGF-β1 compared with untreated controls (Fig. 3D). Figure 3, E–J, shows representative images of the mature adipocytes seen at day 10 following the various treatment regimens after Oil Red O stain. There was a larger population of rounded lipid-laden adipocytes at day 10 in the differentiated controls (Fig. 3F, Ctrl) compared with day 0 (Fig. 3E). In the wells that were treated with either a single or a continuous exposure of CTGF (Fig. 3, G and H, respectively), there were fewer cells that had accumulated lipid. These observations were similar to those seen in the cells treated with TGF-β1 (Fig. 3, I and J).

Regulation of CTGF gene expression during adipocyte differentiation and by TGF-β1. The mRNA expression of CTGF was then measured during differentiation. As the cells began to differentiate and change in morphology, the mRNA expression of CTGF significantly fell compared with that at day 0, as early as day 3 (9.4 ± 16.4%), with the mRNA expression remaining low at day 5 (18.1 ± 15.9%), day 8 (24.9 ± 13.5%), and day 10 (8.0 ± 7.6%) (Fig. 4A). A similar reduction in CTGF mRNA levels was seen when ciglitazone was used to differentiate cells, rather than the cocktail of insulin, dexamethasone, and IBMX (data not shown).

In contrast to the fall in CTGF mRNA occurring during differentiation, a continual exposure of cells to added TGF-β1 throughout the differentiation process increased CTGF mRNA twofold by day 10 (Fig. 4B). In contrast, a single treatment of TGF-β1 at day 0 had no significant persistent effect on CTGF gene expression by day 10 (Fig. 4B). At days 3 or 5, a single TGF-β1 treatment added to the cells at day 0 caused a detectable increase in CTGF mRNA (Fig. 4C). These data suggest...
that CTGF may act downstream of TGF-β1 in this model system.

**Potential mechanistic pathways by which CTGF inhibits adipocyte differentiation.** Because we had shown that CTGF inhibited the differentiation of preadipocytes into fat cells, potential cellular pathways of this effect were then examined. Some cells were treated at the initial stage of differentiation with rhCTGF, and the β isoform of C/EBP was measured up to 48 h after addition of differentiation reagents. Figure 5A shows a representative Western immunoblot of C/EBP-β protein in whole cell lysates at the early stages of differentiation with the immunoreactive bands quantitated using Phoretix and expressed as percent change compared with expression at the induction of differentiation (0 h, 100%). Addition of the induction differentiation media at 0 h resulted in a significant transitory twofold increase in C/EBP-β protein expression at 24 h postaddition (225.2 ± 65.6%, P < 0.05), and expression decreased to 155.7 ± 46.1% at the 48 h time point. Within the same time frame, cells treated with either rhCTGF (500 ng/ml) or active rhTGF-β1 (2 ng/ml) showed a significantly reduced amount of C/EBP-β protein compared with the corresponding same-day control.

Whole cell lysates were separated into nuclear and non-nuclear fractions, and C/EBP-β protein was again measured by Western immunoblot. Whereas the differentiation mix at 24 h after addition increased the relative fraction of C/EBP-β in the nucleus (Fig. 5B), CTGF and TGF-β1 treatment both inhibited the amount of nuclear C/EBP-β.

Since C/EBP-α increases following the induction of C/EBP-β, detection of the α isoform of C/EBP was measured during the latter stages of differentiation. Figure 5C shows a representative Western immunoblot of C/EBP-α in cells treated with...
either CTGF or TGF-β1 before protein extraction at day 8 or 10. Compared with the untreated controls, there was a significant fivefold increase in C/EBP-α at day 8 (525.7 ± 48.0%, \(P < 0.001\)) and at day 10 (597.3 ± 80.0%, \(P < 0.001\)). At day 8 of differentiation, cells that were treated with either rhCTGF or rhTGF-β1 showed a significantly lower C/EBP-α expression compared with the respective same-day control (Ctrl, 525.7 ± 48.0% vs. CTGF, 334.6 ± 56.3%, \(P < 0.05\); and TGF-β1, 254.1 ± 85.4%, \(P < 0.01\)). This pattern was again observed at day 10 (Ctrl, 597.3 ± 80.0% vs. CTGF, 380.0 ± 68.0%, \(P < 0.05\); and TGF-β1, 194.5 ± 56.9%, \(P < 0.001\)).

**Effects of CTGF during later adipocyte differentiation.** Cells treated with one dose of CTGF before being committed to the differentiation process thus showed a less well-defined adipocyte phenotype, consistent with the possibility that CTGF could inhibit the adipocyte differentiation process by preventing an increase of C/EBP-β at the early stages of induction. We then observed whether CTGF addition was able to delay differentiation when cells were treated at the later stages of differentiation. Cultured 3T3-L1 fibroblastic cells were differentiated into adipocytes as described. As indicated in Table 2, cells were treated with rhCTGF (250 ng/ml) or active rhTGF-β1 (2 ng/ml) midway during the differentiation process (day 5), closer to terminal differentiation (day 8), or when cells had more fully differentiated (day 11, following 24-h serum-free conditions). Gene expression markers were then measured after 48 h of growth factor treatment. Addition of rhCTGF midway through the differentiation process partially but significantly prevented the increase in mRNA expression of both adiponectin and PPARγ but had no effect on PAI-1 expression. These results were similar to those seen when rhTGF-β1 was added, where treatment significantly prevented an increase in adiponectin and PPARγ while maintaining a higher expression of PAI-1.

**Table 2. Expression of differentiation markers after indicated treatment with either rhCTGF or rhTGF-β1 as control at various time points during differentiation**

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<thead>
<tr>
<th>mRNA Species</th>
<th>Adiponectin</th>
<th>PPARγ</th>
<th>PAI-1</th>
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<tr>
<td><strong>Days 5–7</strong></td>
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<td></td>
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<tr>
<td>Diff. alone</td>
<td>100.0 ± 27.2</td>
<td>100.0 ± 17.5</td>
<td>100 ± 20.0</td>
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<tr>
<td>Diff. + CTGF</td>
<td>62.0 ± 18.4†</td>
<td>71.2 ± 6.1*</td>
<td>84.5 ± 25.3</td>
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<tr>
<td>Diff. + TGF-β1</td>
<td>18.4 ± 8.0‡</td>
<td>60.5 ± 15.0‡</td>
<td>246 ± 91.8‡</td>
</tr>
<tr>
<td><strong>Days 8–10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diff. alone</td>
<td>100.0 ± 31.8</td>
<td>100.0 ± 29.3</td>
<td>100 ± 57.4</td>
</tr>
<tr>
<td>Diff. + CTGF</td>
<td>50.5 ± 21.6*</td>
<td>58.5 ± 14.9†</td>
<td>136.8 ± 19.2</td>
</tr>
<tr>
<td>Diff. + TGF-β1</td>
<td>34.0 ± 28.3†</td>
<td>62.5 ± 18.2†</td>
<td>154 ± 35.2*</td>
</tr>
<tr>
<td><strong>Days 11–13</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diff. alone</td>
<td>100.0 ± 23.2</td>
<td>100.0 ± 21.0</td>
<td>100 ± 22.8</td>
</tr>
<tr>
<td>Diff. + CTGF</td>
<td>66.6 ± 14.4*</td>
<td>68.9 ± 16.2‡</td>
<td>144 ± 53.5</td>
</tr>
<tr>
<td>Diff. + TGF-β1</td>
<td>54.3 ± 24.3‡</td>
<td>70.0 ± 23.0‡</td>
<td>135 ± 40.5</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as percentage change from untreated cells (100%). Expression of differentiation markers after treatment for 2 days as indicated with either recombinant human (rh)CTGF (250 ng/ml) or rhTGF-β1 (2 ng/ml) as control, with detection either midway during differentiation (day 7), at the latter phase of differentiation (day 10), or when cells are more fully differentiated (day 13). In each case, gene expression of adiponectin, PPARγ, and PAI-1 was measured from cells harvested 48 h after treatments. *\(P < 0.05\); †\(P < 0.01\); ‡\(P < 0.001\); significantly different from same-day differentiated alone cells (by 1-way ANOVA; Bonferroni’s multiple comparison test). Diff., differentiation.

With treatment at the latter stages of differentiation (Table 2, days 8–10 and 11–13), the pattern of change was similar to that observed for the midway treatment (day 5). When mature day 11 adipocytes were treated with CTGF and TGF-β1, there was, however, no observable change in lipid accumulation (data not shown).

**Effects of CTGF on primary cultures of preadipocytes.** To determine whether the main finding in NIH/3T3-L1 cells, that CTGF inhibits adipocyte differentiation, also occurs in primary cell cultures, preadipocytes were isolated from epididymal fat stores and grown in vitro, as described in MATERIALS AND METHODS. Cells were differentiated into adipocytes, as demonstrated at day 10 by lipid accumulation (Fig. 6, A–C) and induction of resistin and adiponectin mRNA (Fig. 6, E and F). Addition of rhCTGF clearly inhibited subsequent fat cell differentiation as indicated by all three measures: lipid accumulation (Fig. 6D) and the two gene expression markers (Fig. 6, E and F). TGF-β1 as a control showed a similar effect to CTGF (Fig. 6, E and F).

**Expression of CTGF gene and protein levels in vivo.** To detect whether CTGF is present in vivo in fat and determine its relative distribution, we measured the regulation of CTGF gene and protein expression after 30 wk of standard laboratory chow feeding at two fat depots: central (epididymal) fat and subcutaneous fat. The gene expression of CTGF in the epididymal fat was approximately twofold higher than in the subcutaneous fat depot (Fig. 7A, \(P < 0.05\)). Figure 7, B and C, depicts the immunohistochemical localization of CTGF protein in the adipocytes of the epididymal fat depot. CTGF localized to the region of the plasma membrane and surrounding interstitium of the adipocytes, with more intense staining at the site of the smaller adipocytes, which have not accumulated substantial lipid. Staining with the corresponding negative isotype control (Fig. 7, D and E) showed no immunoperoxidase signal.

**DISCUSSION**

This study demonstrates the novel data indicating that CTGF is downregulated during adipocyte differentiation and that addition of CTGF can inhibit adipocyte differentiation in 3T3-L1 cells and in primary cultures of murine preadipocytes. The 3T3-L1 cells are a well-published model for the study of adipocyte differentiation, with the ability to convert from preadipocyte fibroblast-like morphology into mature, well-rounded, lipid-laden adipocytes (11, 17, 44, 62). The adipocyte differentiation process in these cells was monitored using three complementary end points: the gene expression of differentiation markers (adiponectin, PPARγ, and PAI-1), lipid accumulation as detected by Oil Red O staining, and lipogenic enzyme activity of G3PDH.

Significant reduction in endogenous CTGF expression at various times during differentiation from the preadipocyte stage was detected. This change parallels that previously described for TGF-β1 (10, 24, 53). A recent report also described a fall in CTGF mRNA levels during adipogenesis, in that case by 50%, as cells progressed from the mesenchymal stem cell stage through to terminally differentiated adipocytes (50). That study did not examine CTGF mRNA changes from the specific stage of committed preadipocytes into differentiated adipocytes, as did the current work, possibly explaining the lesser regulation of CTGF observed. It would be of interest to...
determine whether CTGF mRNA levels fall progressively from the earliest stages of multipotent mesenchymal cells, as they undergo determination into the adipocyte lineage, and whether the recognized stage of mitotic clonal expansion also affects CTGF mRNA. In contrast to our current findings in adipogenesis, CTGF is upregulated during differentiation of chondrocytes and osteoblasts, and it stimulates these processes (42). Such findings suggest that CTGF may be one of the factors that directs mesenchymal cellular differentiation along one path at the cost of another.

Although dexamethasone has been shown to increase CTGF mRNA in some cell systems (12), in contrast, during adipocyte differentiation at an early time point, CTGF mRNA levels fall and protein levels in whole cell lysate subsequently plateau. Presumably, the program of adipocyte differentiation takes priority over effects of individual reagents. The fact that the amount of steady-state CTGF mRNA falls during adipocyte differentiation induced by ciglitazone also indicates that the finding of a reduction in CTGF is generalizable to adipocyte differentiation and is not dependent on any one of the prodifferentiating agents added.

The current work showed that CTGF is positively regulated by TGF-β1 in 3T3-L1 cells. This is consistent with findings reported in other cell types (5, 15, 23, 27, 28, 32). Indeed, CTGF has been shown to mediate some of the actions of TGF-β in fibrosis (29, 38), and we have shown, albeit in other cell types, that CTGF bioactivity is dependent on cellular TGF-β signaling mechanisms (45). Being a downstream mediator of TGF-β1 in some cells, it is plausible that CTGF would have an effect similar to TGF-β1 in inhibition of the adipocyte differentiation process. Studies of CTGF regulation, including modification of endogenous CTGF bioactivity, are now required to address whether CTGF mediates effects of exogenously added or endogenous TGF-β1.

CTGF was shown in the current work to inhibit adipocyte differentiation both when it was added during the early stage of commitment as a single dose and also during the adipocyte differentiation process. CTGF had a potent effect to inhibit the early increase in C/EBP-β in whole cell lysates, potentially explaining how it negatively affects at least the early differentiation process. TGF-β1 exerts its effects via the downregulation of C/EBP-α, C/EBP-β, and PPAR-γ (9, 10, 16). This action of TGF-β1 has been reported to occur through the repression of C/EBP activity (49). Inhibition of adipogenesis by TGF-β1 is mainly mediated via Smad3, as Smad3 physically associates with adipocyte transcription factors C/EBP-β and C/EBP-δ to repress their transactivating capacity (37, 59). TGF-β1 also has been shown to downregulate PPAR-γ expression (49). Our
studies implicate CTGF in inhibition of nuclear localization of C/EBP-β. Further studies are required to determine whether CTGF affects C/EBP-β mRNA and/or the activity of C/EBP-β and whether this is the main way in which CTGF exerts its effect to prevent initial adipocyte differentiation. Previous reports have indicated that CTGF has the ability to have an impact on many transcription factors, including those affecting cell differentiation (7), and the finding that CTGF affects C/EBP-β in this context is consistent with those publications.

The concentration of rhCTGF used, which showed a biological effect in this study, is not dissimilar to other published CTGF bioactivities (7). RhCTGF has been reported to vary in its action from a dose of 10 ng/ml to 5 μg/ml (15, 21), depending on the rhCTGF preparation and the cell type and end point studied. We showed that 250 and 500 ng/ml had persisting effects, when applied as a single treatment, to inhibit adipocyte differentiation. A lower dose of 50 ng/ml reapplied every second day also inhibited the differentiation process.

The cellular mechanism for the effect of CTGF during the adipocyte differentiation process is not clear. A precedent exists where TGF-β inhibits both initial and later adipocyte differentiation, and yet only the early effects of TGF-β are through C/EBP-β (10, 24, 53). The MAP kinase second messenger systems, specifically proadipogenic ERK-1 (6) and antiadipogenic p38 MAP kinase (4), have been implicated in the regulation of preprogrammed adipocyte differentiation, and especially since CTGF has been shown to impact on MAP kinase pathways in other cell types (61), MAP kinases are a candidate pathway for further study in determining the mechanism of CTGF effect during committed adipogenesis. The fact that CTGF does not have one single defined cell surface receptor but can in differing systems function by binding specific integrins (22), cell surface chaperone proteins (51), other growth factors (26), and the TrkA receptor (60) requires that a broad and systematic study of CTGF cellular action be defined in the 3T3-L1 cells.

When mature adipocytes were treated with CTGF, the expression of the differentiation markers adiponectin and PPARγ were significantly reduced, although there was no change in lipid accumulation in the 3T3-L1 cells. Previous studies by
others have shown that TGF-β1 can reverse the adipocyte phenotype or reduce expression of differentiation markers in mature adipocytes (10, 24, 53). As for TGF-

phenotype or reduce expression of differentiation markers in

REFERENCES

the National Health and Medical Research Council of Australia for providing

C750 CTGF INHIBITS ADIPOCYTE DIFFERENTIATION

In summary, this study shows that CTGF is downregulated during adipocyte differentiation. Intervention with exogenous CTGF protein before commitment or during differentiation was effective in the inhibition of adipocyte differentiation. CTGF also inhibited the expression of early C/EBP-β and later C/EBP-α, both of which are crucial transcription factors involved in the adipogenesis program. A higher CTGF expression seen in the central fat depots also suggests that CTGF may influence the degree of adipocyte differentiation in vivo and thus the formation of insulin-resistant adipocytes, and these studies form a precedent for exploring functions of CTGF in fat tissue. Future studies expanding on the mechanistic pathways by which CTGF affects adipocyte differentiation, including the regulation of C/EBPs, as well as in vivo models of obesity and insulin resistance, would complement the novel findings reported in this work.

GRANTS

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REFERENCES


