Transmembrane Tumor Necrosis Factor (TNF)-α Inhibits Adipocyte Differentiation by Selectively Activating TNF Receptor 1*

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Tumor necrosis factor α (TNFα) is a potent cytokine with multiple biological activities and exists in two forms as follows: a 17-kDa soluble form that is a cleaved product of the 26-kDa transmembrane form (mTNFα). It has been suggested that the transmembrane form of TNFα is mainly responsible for localized responses via cell-cell contact. Here, we have examined the activities of transmembrane TNFα in cultured adipocytes. A noncleavable transmembrane form of TNFα (mTNFΔ1–9K11E) was expressed in several preadipocyte cell lines using retroviral gene transfer. In wild type preadipocytes carrying both TNF receptors, expression of mTNFΔ1–9K11E resulted in inhibition of the differentiation program. The extent of this varied depending on the nature and strength of the adipogenic stimuli. The TNF receptor responsible for this function was determined by expressing mTNFΔ1–9K11E in preadipocyte cell lines lacking either TNF receptor 1 (TNFR1), 2 (TNFR2), or both. In order to confirm the results in the same cellular background, TNF receptors were also reconstituted in the cell lines lacking corresponding receptors. These experiments demonstrated that TNFR1 was necessary and sufficient for mediating mTNFΔ1–9K11E-induced inhibition of adipogenesis and that this action was similar to that of soluble TNFα. In conclusion, our results indicate that mTNFΔ1–9K11E is biologically active in cultured adipocytes and can alter the adipogenic program of these cells by selectively activating TNFR1. This may have physiological implications where local TNFα actions are thought to be generated at sites such as adipose tissue.

Originally identified as a mediator of necrosis of certain tumor cells, tumor necrosis factor α (TNFα)† has now been shown to have a wide array of biological activities (1, 2). It is also implicated in the pathogenesis of several diseases such as septic shock, rheumatoid arthritis, autoimmune disorders, and insulin resistance (1, 3).

TNFα is primarily produced by activated macrophages and lymphocytes but is also expressed in endothelial cells and other cell types including adipocytes (1, 2, 4). It exists in two forms as follows: a 17-kDa soluble form (sTNFα) that is cleaved from the 26-kDa transmembrane protein (mTNFα) at the cell surface by TNFα-converting enzyme (5, 6). Although the majority of TNFα-induced responses has been attributed to sTNFα, a few studies have shown that mTNFα is also biologically active and capable of mediating similar responses including apoptosis, proliferation, B cell activation, and some inflammatory responses (7, 8). Furthermore, mTNFα has been implicated in some disease states such as experimental hepatitis where serum sTNFα levels were found to be within the normal range (9), indicating the relevance of localized TNFα responses. The existence of two different forms of TNFα makes its physiology more complicated. Furthermore, the fact that mTNFα relies on cell contact-dependent signaling may render the actions of mTNFα cell type-specific in vivo. In support of this, mTNFα has been reported to trigger inflammatory responses in astrocytes but not in neurons, whereas sTNFα can induce similar effects in both cell types (10).

The biological functions of both mTNFα and sTNFα can be signaled by two distinct TNF receptors: TNFR1 (55 kDa) and TNFR2 (75 kDa). The lack of homology in intracellular domains of two TNF receptors indicates that they can mediate distinct biological activities. Indeed, whereas a broad array of cellular responses has been attributed to TNFR1, many other effects are mediated by TNFR2 (11, 12). These two receptors can also act in concert under many circumstances (9, 13). The role of TNFR1 and TNFR2 in mediating the actions of sTNFα and the downstream signaling mechanisms has been studied extensively. In contrast, little information is available regarding the pathways and mechanisms utilized by mTNFα. Some early studies have demonstrated that transmembrane TNFα is superior to sTNFα in activating TNFR2 (7, 12). However, subsequent reports have indicated that transmembrane TNFα can signal through both receptors depending on the cellular context (8). Other studies have used TNFR-deficient mice to demonstrate that both receptors were required as in the case of experimental hepatitis (9) and arthritis (14), whereas TNFR2 alone is sufficient to mediate the effects of transmembrane TNF in experimental cerebral malaria (15).

Soluble TNFα plays an important role in regulation of energy metabolism. It has profound effects on adipocytes, including mobilization of triglycerides and inhibition of insulin action (2, 3). In adipocytes, it can regulate the expression of several genes (4) and modulate the secretion of free fatty acids and leptin which play active roles in systemic energy balance (16, 17). Recent studies demonstrated that TNFα is a candidate mediator of insulin resistance in obesity. The expression level of TNFα in adipose tissue is elevated in a variety of rodent obesity models (4) and also in obese humans (18, 19). Soluble TNFα has...
been shown to inhibit insulin action in cultured adipocytes (20) and other cell types (21, 22) as well as in whole animals (23–25). Several studies on various models of rodent obesity demonstrated increased insulin sensitivity upon genetic loss of TNFa function (26–28), although one recent report could not demonstrate this in TNFR1−/− R2−/− mice with dietary obesity (29). Similar to genetic studies, pharmacological blocking of TNF activity also results in significant reversal of insulin resistance in obesity (4, 30).

Despite strong evidence of a role for TNFa in obesity-related insulin resistance, circulating levels of sTNFa appear to be very low or undetectable (3). It is therefore possible that obesity might be one example where TNFa action is localized to the site(s) of production, such as adipose tissue. Thus, mTNFa may be a potential candidate mediator of such local events. However, the effects of mTNFa on adipocyte biology and energy metabolism remain unknown.

In this study, we have examined the effects of transmembrane TNFa on cultured 3T3-F442A adipocytes and determined the TNF receptor responsible for its functions by using TNFR1−/−, TNFR2−/−, and TNFR1−/− R2−/− preadipocyte cell lines developed in our laboratory. These studies demonstrate that transmembrane TNFa is indeed biologically active in cultured adipocytes and that it alters the differentiation program of adipocytes by selectively activating TNFR1.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—** TNFR1−/−, TNFR2−/−, and TNFR1−/− R2−/− fibroblast cell lines were established from day 16–17 mouse embryos with targeted mutations in the corresponding TNFR(s) using the classic 3T3 protocol. Multiple fibroblast cell lines were established for each genotype and tested for their capacity to differentiate into adipocytes. For each genotype, one cell line with the highest rate of differentiation was selected and used for the experiments. 3T3-F442A, TNFR1−/−, TNFR2−/−, and TNFR1−/− R2−/− preadipocytes were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 10% bovine calf serum (HyClone). Infected cells were maintained in the same medium in the presence of appropriate selection drugs. For differentiation, cells were seeded at 1.5 × 105 cells per well on 6-well plates in DMEM supplemented with 10% cosmic calf serum (HyClone). Cells were grown to confluence and exposed to adipogenic reagents for 4–6 days, followed by culturing for 4 more days in medium containing insulin only. Recombinant murine soluble TNFa (Genzyme, MA) treatments were started at confluence and continued throughout the experiments with a new dose applied every 2 days at the indicated concentrations. Cells were then either stained with oil red O for microscopy or processed for RNA collection. Unless otherwise indicated, insulin was used at a concentration of 5 μg/ml, dexamethasone at 1 μg/ml, isobutylmethylxanthine at 0.5 mM, and BRL49653 at 1 μM. The polyclonal rabbit anti-murine TNF antibody (Genzyme, MA) was purchased from Jackson ImmunoResearch (West Grove, PA). The polyclonal rabbit anti-rat Na,K-ATPase antibody was provided by Dr. Lorraine Santy (Harvard University). The anti-human insulin receptor and aP2, mouse ACRP30, and rat Na,K-ATPase, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, and 200 μg/ml Pefabloc) and centrifuged at 14,000 rpm in a microcentrifuge. The supernatants, which contain solubilized membrane proteins, were collected, and equal amounts of protein from each fraction were used for immunoblot analysis. To analyze secretion of sTNFa, 48-h conditioned medium (CM) was collected from each cell line and concentrated to a final volume of 1 ml. The polyclonal rabbit anti-murine TNF antibody was used for immunoprecipitation, as described previously (26). Immunoblots were performed using polyclonal rabbit antibodies against human insulin receptor and aP2, mouse ACRP30, and rat Na,K-ATPase, respectively.

**Quantitation of Differentiation—** Cells were stained with oil red O (Sigma) for visualizing the lipid droplets and hematoxylin (Sigma) for nuclei according to conventional methods. The percentage of differentiation was calculated as number of cells containing visible lipid droplets divided by the total number of nuclei per microscopic field, under 400-fold magnification. Three representative fields were counted for each sample, and the mean ± S.D. was used for comparisons.

**Vector Construction—** The cDNA of the nonleucine murine membrane TNFa (mTNFa1–9K11E) was provided by Dr. Els Decoster and Dr. Walter Fiers (Gent University, K. L. Ledegangkstraat, Belgium) in vector pSV235 (7). The coding region was amplified by polymerase chain reaction (5′ primer, CTGAGTCTCCCTCCAGAAAGAC, and 3′ primer, GAGATCAGATTAAAGGTCAGATG) and cloned into the EcoRI (Invitrogen). The integrity of the PCR product was confirmed by sequencing. The coding region was then ligated with Xhol/BamHI digestion followed by Klenow fill-in. The 0.76-kilobase pair mTNFa1–9K11E cDNA fragment was cloned in sense orientation into the SnaBI site of the retroviral vector, pBabe-hygro, which contains the hygromycin B resistance gene (32). The cDNA of TNFR1 was obtained by performing reverse transcription-PCR (5′ primer, TGCGAGGCTCTGGAGAC, and 3′ primer, AAGGGTCTGGGTGGCTTATAT) using mouse spleen cDNA (strain C57BL6). The final 1.37-kilobase pair PCR product was cloned into PCRII vector. One point mutation was detected by sequencing and corrected by site-directed mutagenesis based on the published sequence (33). The coding region of TNFR1 was excised with NaeI/EcoRI digestion and cloned into the SnaBI/EcoRI site of the retroviral vector, pBabe-puro, which contains the puromycin resistance gene (32). The cDNA of murine TNFR2 was obtained from Immunix (34). The coding sequence was excised with BamHI (followed by Klenow fill-in/EcoRI digestion and cloned into the SalI site (followed by Klenow fill-in/EcoRI site of the retroviral vector, pBabe-hygro, which contains the bleomycin resistance gene (32). All the expression constructs were sequenced to confirm the integrity and correct orientation of the cloned cDNA sequences.

**Transfection and Infection—** Packaging of the viral particles was achieved by transfecting the expression plasmids into Bosc 23 cells, a human kidney cell line (35), with the Cell Phect calcium phosphate coprecipitation kit (Amersham Pharmacia Biotech). Forty-eight hours post-transfection, supernatant from packaging cells was collected and filtered through sterile 0.45-μm syringe filters. Twenty-four hours before infection, recipient cells were seeded at a density of 75,000 cells/cm2. For infection, recipient cells were incubated with viral supernatant plus fresh DMEM (3:1) containing a final concentration of 4 μg/ml Polybrene (Sigma). After a 24-h incubation, cells were fed with fresh DMEM and allowed to grow to 80% confluency in 2–3 days. Cells were re-seeded at a density of 6 × 104 per 75 cm2 for selection, and corresponding antibiotics were added the following day. Puromycin (Sigma) selection was completed in 3–4 days, while hygromycin B (Sigma) and zeocin (a derivative of bleomycin, Invitrogen) lasted 1 week and 1 month, respectively. Unless otherwise indicated, cells were maintained in appropriate antibiotics throughout experiments to maintain stable expression levels of mTNFa1–9K11E, TNFR1, and TNFR2.

**Total RNA Preparation and Northern Blotting—** RNA samples were extracted according to the guanidinium thiocyanate method (4). Following denaturation, RNAs were loaded on a 1% agarose gel containing 3% formaldehyde (4). After electrophoresis, RNAs were transferred to a biotran membrane (ICN), UV cross-linked, and baked at 80 °C for 1 h. Hybridization with [α-32P]dCTP (NEN Life Science Products)-labeled cDNA probes and subsequent washings were done as described previously (4). Northern blots were quantitated by using NIH image program image analysis software and 18 S RNA was used for loading normalization.

**Immunofluorescence—** Cells were grown on coverslips in 6-well plates. After being rinsed 5 times with modified phosphate-buffered saline (PBS containing 1 mM MgCl2 and 0.1 mM CaCl2), cells were fixed in 3% paraformaldehyde. Following 5 min incubation in PBS containing 50 mM NH4Cl, cells were rinsed 3 times with PBS and 2 times with PBS containing 0.5% bovine serum albumin. This was followed by a 45-min

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Expression of a Non-cleavable Transmembrane Form of TNFα in Preadipocyte Cell Lines—To study the potential effects of transmembrane TNFα in cultured adipocytes, we have ectopically expressed a non-cleavable transmembrane form of murine TNFα in several preadipocyte cell lines. These include the 3T3-F442A cells and the newly developed preadipocyte cell lines deficient in TNFR1, TNFR2, or both. The non-cleavable form of TNFα (mTNFΔ1–9K11E) has been generated by delet- ing amino acids 1–9 and mutating residue 11 from Lys to Glu in the N-terminal part of mature TNFα and previously char- acterized in L929, CT6, PC60-R55/R75, and U937 cells (8). In this study, we chose a retroviral expression system to express constitutively the non-cleavable mutant (Fig. 1A) because of its high integration efficiency. This system also allowed expres- sion in a large number of cells and prevented the common problem of clonal variability. The exogenous TNFα message was readily identified in infected cells since it is larger than the endogenous one produced by LPS-stimulated Raw264.7 macro- phages (Fig. 1B). Comparable levels of mTNFΔ1–9K11E ex- pression were demonstrated by Northern blot analysis in all stably infected preadipocyte cell lines (Fig. 1B). Messenger RNA levels of both TNF receptors were also determined in all cell types to document the presence and potential regulation of the relevant TNF receptors. The TNFR2 mRNA level in 3T3-F442A cells expressing mTNFΔ1–9K11E was substan- tially higher than that expressed by vector-alone (Fig. 1B, lanes 3 and 4). Expression of mTNFΔ1–9K11E in TNFR1−/− cells did not affect TNFR2 mRNA level (Fig. 1B, lanes 5 and 6). This indicates that either the regulation of TNFR2 mRNA expres- sion by mTNFΔ1–9K11E requires the presence of TNFR1, or base-line TNFR2 expression in these cells is already at maxi- mum levels. Endogenous TNFR1 mRNA levels were unaffected by the presence of mTNFΔ1–9K11E. Proper expression and localization of mTNFΔ1–9K11E as a membrane protein was determined by both indirect immuno- fluorescence in intact cells and immunoblot analysis of cellular fractions. As shown in Fig. 1C, expression of mTNFΔ1–9K11E protein on TNFR1−/− R2−/− cells could be visualized by the use of a polyclonal rabbit anti-murine TNFα antibody (left panel) followed by a fluorescein-conjugated anti-rabbit IgG. The peripheral distribution of fluorescence is consistent with plasma membrane-associated localization. To confirm this fur- ther, we performed immunoblot analysis of cellular fractions of adipocytes (Fig. 1D, top panel) which showed that mTNFΔ1– 9K11E was predominantly detected in a high density mem- brane-containing fraction. This fraction is enriched with plasma membranes as confirmed by the detection of Na,K- ATPase (Fig. 1D, middle panel) which is a commonly used plasma membrane marker (37). Insulin receptor was also de- tected in this fraction (data not shown). The mTNFΔ1–9K11E protein was expressed as a 25-kDa protein compared with the wild type 26-kDa mTNFα. In both mTNFΔ1–9K11E and wtTNFα-expressing cells, two additional smaller molecular weight bands have been consistently detected in the membrane fractions. These are likely to be products of alternative initiation sites as previously reported (38). Transmembrane TNFΔ1– 9K11E protein level in total cell extracts from different cell lines was also similar as determined by immunoblotting (data not shown). The protein expression level of the non-cleavable mutant in our system is estimated to be 5% of the endogenous counterpart produced by LPS-stimulated macrophages quanti- tated by densitometry scanning of immunoblots. No mTNFΔ1– 9K11E protein was detected in cytosol. This fraction is indeed enriched with cytotoxic proteins as confirmed by immunoblotting for cytotoxic adipocyte fatty acid-binding protein, aP2 (Fig. 1D, bottom panel). Finally, we also examined whether in our experimental system, the mTNFΔ1–9K11E could be aberrantly cleaved at other sites to yield sTNFα products. Immunoblot analysis of concentrated conditioned media did not reveal any detectable TNFα immunoreactivity from vector and mTNFΔ1– 9K11E-expressing cells but did show the presence of sTNFα from wt-TNFα-expressing cells (Fig. 1E, top panel). ACRP30/ Adipok (adipocyte complement-related protein of 30 kDa) (39, 41), a secreted protein exclusively made in adipocytes, was used as a control for proper protein secretion to the conditioned media from these cells (Fig. 1E, right panel). These data demon- strated that mTNFΔ1–9K11E was expressed on the cell surface and did not produce detectable sTNFα. These results are essentially identical to those observed in lymphocytes using the same construct (8). Effects of mTNFΔ1–9K11E on the Differentiation of Adipocytes—The 3T3-F442A preadipocyte cell line is commonly used as an experimental model for adipocyte differentiation in vitro (40, 42). These cells express both TNF receptors but do not produce detectable levels of endogenous TNFα (Fig. 1B). In these cells, we first examined whether ectopic expression of mTNFΔ1–9K11E could lead to alterations in the differentiation process. Four different permissive conditions for adipocyte dif- ferentiation were used to compare control 3T3-F442A cells to those expressing mTNFΔ1–9K11E. The induction conditions used were as follows: (a) insulin alone (5 μg/ml); (b) insulin, dexamethasone (1 μM), and isobutylmethylxanthine (0.5 mM); (c) insulin and BRL49653 (1 μM), an activator for the adipogenic transcription factor peroxisome proliferator-activated recep- tor γ (PPARγ); and (d) a mixture containing all four of the above reagents. After the induction of differentiation with these reagents for 3 days, cells were maintained in 5 μg/ml insulin for 4 more days. Experiments were then stopped for morphological and molecular comparison of vector-infected control cells with those expressing mTNFΔ1–9K11E. In the absence of any adipogenic stimuli, 6 ± 0.6% of control 3T3- F442A cells spontaneously differentiated into adipocytes, whereas no morphological sign of differentiation was detecta- ble in mTNFΔ1–9K11E-expressing cells (Fig. 2A). The use of insulin as the only inducer resulted in the differentiation of 37 ± 2.9% control cells, but this effect was completely blocked by the presence of mTNFΔ1–9K11E (Fig. 2A). When a mixture of insulin, dexamethasone, and isobutylmethylxanthine was used, all control F442A cells differentiated. However, only 36 ± 2.1% of mTNFΔ1–9K11E-expressing cells differentiated into fat cells under this condition. The addition of BRL49653, a thiazolidinedione compound which acts as a high affinity li- gand for PPARγ, significantly reduced the effect of mTNFΔ1– 9K11E on adipocyte differentiation. The effect of BRL49653 was incomplete (18 ± 3.7% differentiation) when used with insulin alone but complete when used in combination with insulin, dexamethasone, and isobutylmethylxanthine.
FIG. 1. Expression of a non-cleavable murine TNFΔ1–9K11E in preadipocyte cell lines. A, map of the mTNFΔ1–9K11E retroviral expression vector. LTR, long terminal repeat. B, expression of endogenous TNFα message in LPS-induced macrophages (Raw 264.7) and mTNFΔ1–9K11E in 3T3-F442A, TNFR1Δ−/−, TNFR2Δ−/−, and TNFR1Δ−/−R2Δ−/− proliferating preadipocyte cell lines. V, vector-infected; T, mTNFΔ1–9K11E-infected. Lanes 1 and 2, endogenous TNF message of Raw 264.7 macrophages before and after treatment with 100 ng/ml lipopolysaccharide (LPS) for 3 h. C, expression of mTNFΔ1–9K11E on the cell surface. Left, mTNFΔ1–9K11E-expressing TNFR1Δ−/−R2Δ−/− cells stained with a polyclonal rabbit anti-murine TNFα antibody followed by staining of a fluorescein-conjugated anti-rabbit IgG. Right, vector-infected cells with the same treatment. D, ectopic TNFα expression in subcellular fractions of adipocytes. Mφ, macrophages (Raw264.7). Top panel shows a TNFα immunoblot of membrane and cytosolic fractions (100 μg of protein each lane) from differentiated vector, mTNFΔ1–9K11E and wild type (wt/TNFα-expressing TNFR1Δ−/−R2Δ−/− cells. Fraction 1 (Frac 1, low speed spin fraction) contains plasma membrane; fraction 2 (Frac 2, high speed...
To compare these effects of mTNFΔ1–9K11E to sTNFα, 3T3-F442A cells were also treated with recombinant murine soluble TNFα. At a concentration of 1 ng/ml, sTNFα generated inhibitory effects similar to those observed with the mTNFΔ1–9K11E. The percentages of differentiated cells under the four different induction conditions were 0% (insulin), 26 ± 6.5% (insulin and BRL49653), 41 ± 6.3% (insulin, dexamethasone, and isobutylmethylxanthine), and 99 ± 3.5% (combination of all reagents). At a concentration of 10 ng/ml, sTNFα completely blocked differentiation under all conditions (data not shown). During differentiation, experiments with both mTNFΔ1–9K11E and soluble TNFα, cells were closely monitored every day under microscope, and no obvious cytotoxic effect was observed. At the end of differentiation, trypan blue uptake was performed to examine cell viability, and no difference was observed between vector- and mTNFΔ1–9K11E-infected cells.

**FIG. 2. Effects of mTNFΔ1–9K11E on differentiation of 3T3-F442A cells.** A, effects of mTNFΔ1–9K11E on the morphology of 3T3-F442A cells during differentiation. Upper panel shows vector-infected 3T3-F442A cells treated with (from left to right) medium (control), insulin (Ins), insulin + BRL49653 (Ins+BRL), insulin + dexamethasone + isobutylmethylxanthine (Mix), and all four reagents (Mix+BRL); lower panel shows mTNFΔ1–9K11E-infected cells with the same treatments. Cells were fixed with glutaraldehyde and stained with oil red O. B, effects of mTNFΔ1–9K11E on gene expression in 3T3-F442A cells during differentiation. U, uninfected; V, vector-infected; T, mTNFΔ1–9K11E-infected. For the first group of controls (lanes 1–3), RNA was collected at confluency. For the second group of controls (lanes 4–6), cells were maintained at confluency, and RNA was collected simultaneously with the samples from induced cells. In lanes 7–9, cells were induced by 5 μg/ml insulin; lanes 10–12, induced by insulin + 1 μM BRL49653 (lanes 10–12). C, gene expression in vector (V) or mTNFΔ1–9K11E-infected (T) 3T3-F442A cells induced by Mix+BRL in the presence (+) or absence (−) of hygromycin B (HygB). Results showed here are representative of three independent experiments.

Spin fraction) contains intracellular membranes; soluble (Sol) fraction contains cytosolic materials. Lanes 1, 4, and 7, vector-infected cells. Lanes 2, 5 and 8, mTNFΔ1–9K11E-expressing cells. Lanes 3, 6, and 9, wtTNFα-expressing cells. Lane 10, 500 pg of recombinant soluble murine TNFα. Lanes 11 and 12, 20 μg of protein from plasma membrane-containing fraction of control and lipopolysaccharide (LPS)-stimulated macrophages, respectively. Middle and bottom panels, same samples were used for blotting with a polyclonal rabbit anti-rat Na⁺K-ATPase antibody (1:5000 dilution) and a polyclonal rabbit anti-murine αP2 antibody (1:1000 dilution) as controls for membrane and cytosolic fractions, respectively. E, immunoreactive TNFα in conditioned media from vector, mTNFΔ1–9K11E, and wtTNFα-expressing TNFR1−/−R2−/− cells. Lanes 1 and 2, conditioned media from control and LPS-stimulated macrophages. Lanes 3–5, conditioned media from differentiated vector, mTNFΔ1–9K11E, and wtTNFα-expressing TNFR1−/−R2−/− cells. Upper panel, immunoprecipitates were immunoblotted with an anti-TNFα antibody. Bottom panel, direct immunoblot analysis of the same samples with an anti-ACRP30 antibody (1:5000 dilution) as a control for proper protein secretion.
The morphological changes that occur during adipocyte differentiation are accompanied by changes in expression patterns of fat-specific genes, most of which are involved in creating and maintaining the adipocyte phenotype (42). Therefore, expression of a panel of adipose-specific genes can be used to serve as molecular indicators of the state of differentiation. In order to evaluate the effects of mTNFα–9K11E on adipocyte differentiation at the molecular level, we next examined the mRNA levels of four genes that are expressed in a differentiation-dependent manner in adipocytes. These were the adipogenic transcription factor PPARγ, the adipocyte fatty acid-binding protein aP2, the serine protease adipsin, and the insulin-dependent glucose transporter 4 (Glut4). Northern blot analyses were consistent with the morphological changes described above. The expression levels of all of these genes were also significantly decreased in mTNFα–9K11E-expressing cells compared with the controls. Fig. 2B (left panel) shows the comparison of the expression levels of PPARγ, Glut4, adipsin, and aP2 in uninfected, vector-infected, and mTNFα–9K11E-expressing cells. The presence of mTNFα–9K11E completely inhibited expression of these genes in cells induced to differentiate with insulin alone (lanes 7–9), whereas no obvious difference was observed between uninfected and vector-infected F442A cells. Addition of BRL49653 or dexamethasone and isobutylmethylxanthine dramatically increased the expression of all of these fat-specific genes in uninfected and vector-infected cells. This also partially antagonized the effect of mTNFα–9K11E on adipocyte differentiation (Fig. 2B, lanes 10–14). The combination of all adipogenic reagents completely prevented the effect of mTNFα–9K11E, and no difference in gene expression could be detected between vector-infected and mTNFα–9K11E-expressing cells under this condition (Fig. 2B, lanes 15 and 16). However, when cells were induced to differentiate in the presence of continuous selection pressure, the inhibitory effect of mTNFα–9K11E could still be observed even when the strongest induction mixture was applied (Fig. 2C). This was consistent with a higher expression level of mTNFα–9K11E in the presence of continuous selection (Fig. 2C, bottom panel). For these reasons, continuous antibiotic selection was used throughout the remaining experiments described below.

TNFR1 Is Required for Mediating the Anti-adipogenic Effect of mTNFα–9K11E—The TNF receptor responsible for mediating the inhibitory action of mTNFα–9K11E on adipocyte differentiation was determined by expressing this molecule in TNFR1−/−, TNFR2−/−, and TNFR1−/−R2−/− preadipocyte cell lines. In TNFR1−/−R2−/− preadipocytes, which lack both TNF receptors, the expression of mTNFα–9K11E had no effect on differentiation as judged by both morphology and adipocyte-specific gene expression (Fig. 3A, top panel; B, lanes 15 and 16). Identical results were obtained in TNFR1−/− preadipocytes which only express wild type TNFR2 but do not carry a functional TNFR1 gene. No obvious difference was observed between vector-infected and mTNFα–9K11E-expressing TNFR1−/− cells based on morphology of the cells or fat-specific gene expression (Fig. 3A, middle panel; B, lanes 7 and 8). This demonstrates that TNFR2 alone cannot mediate this action. In TNFR2−/− preadipocytes carrying wild type TNFR1 but no functional TNFR2, the expression of mTNFα–9K11E resulted in inhibition of differentiation (Fig. 3A, bottom panel; B, lanes 11 and 12). Only 18.2 ± 3.3% of mTNFα–9K11E-expressing TNFR2−/− cells differentiated under this condition. Further quantitation of fat-specific genes indicated that the expression levels of PPARγ, Glut4, aP2, and adipsin were 31 ± 8, 18 ± 12, 40 ± 5, and 9 ± 12%, respectively, of those detected in vector-infected control cells. These results demonstrate that the presence of TNFR1 alone is sufficient for signaling the inhibitory effect of mTNFα–9K11E on adipocyte differentiation. To rule out the possibility that TNFR2 function might be generally deficient in TNFR1−/− cells, mTNFα–9K11E-stimulated NF-κB activation was determined in these cells. As shown in Fig. 3C, mTNFα–9K11E could effectively activate NF-κB in both TNFR1−/− and TNFR2−/− cells but not in TNFR1−/−R2−/− preadipocytes, confirming that the signaling capacity of TNFR2 was intact in TNFR1−/− cells. These results demonstrate that TNFR1 is necessary for the inhibition of differentiation induced by mTNFα–9K11E.

We also examined the effect of soluble TNFα on adipocyte differentiation in these cellular systems. Recombinant murine TNFα was applied every 2 days at two doses of 1 and 10 ng/ml, respectively. Treatment of TNFR1−/−R2−/− or TNFR1−/− cells with sTNFα did not affect differentiation at both concentrations used in experiments (data not shown). In contrast, in the presence of 1 ng/ml sTNFα, differentiation was evident in only 53 ± 6.4% of the TNFR2−/− cells. At the concentration of 10 ng/ml, sTNFα almost completely inhibited adipocyte differentiation (<1% differentiation). These data indicate that both sTNFα and mTNFα can inhibit adipocyte differentiation through TNFR1, and the extent of inhibition is dose- or expression level-dependent, respectively.

mTNFα–9K11E Inhibits Adipocyte Differentiation through TNFR1 Alone—To confirm the role of TNFR1 in mediating the anti-adipogenic effect of mTNFα–9K11E within the same cellular background, we next introduced intact TNFR1 back into the TNFR1−/− preadipocytes expressing mTNFα–9K11E. Since these cells are already resistant to neomycin and hygromycin B, the retroviral vector containing puromycin resistance gene was used for exogenous TNFα expression. Four stably-infected TNFR1−/− cell lines were established, expressing 1) vectors with hygromycin B and puromycin resistance genes, 2) vector with hygromycin B resistance gene and TNFR1, 3) mTNFα–9K11E and vector with puromycin resistance gene, and 4) mTNFα–9K11E and TNFR1. In cells expressing both mTNFα–9K11E and TNFR1, exogenous TNFα expression levels were significantly lower than those expressing only TNFR1 (Fig. 4). Since direct regulation of the exogenous gene is not expected, it is likely that overexpression of high levels of both mTNFα–9K11E and TNFR1 simultaneously is cytotoxic, so only cells with low TNFα expression levels survived the selection protocol. After the initial selection period, no cytotoxicity was observed when cells were kept growing in appropriate antibiotics. All cell lines were also tested for differentiation. As shown in Fig. 4, mTNFα–9K11E-induced inhibition of adipogenesis was only observed when both mTNFα–9K11E and TNFR1 were expressed simultaneously in TNFR1−/− cells. To determine whether TNFR2 cooperates with TNFR1 in inhibiting adipocyte differentiation, TNFR2 expression was also reconstituted in TNFR2−/− preadipocytes expressing mTNFα–9K11E. The retroviral vector containing bleomycin resistance gene was used to express TNFR2. The stably infected (TNFR2−/−) cell lines were established expressing the following: 1) vectors with hygromycin B and bleomycin resistance genes, 2) vector with hygromycin B resistance gene and TNFR2, 3) mTNFα–9K11E and vector with bleomycin resistance gene, and 4) mTNFα–9K11E and TNFR2. Exogenous TNFR2 expression levels were significantly higher in (TNFR2−/−) cells expressing both mTNFα–9K11E and TNFR2 than in cells expressing only TNFR2. The ability of mTNFα–9K11E to inhibit adipocyte differentiation was not enhanced in (TNFR2−/−) preadipocytes overexpressing TNFR2 when compared with the parental (TNFR2−/−) cells (Fig. 5). Hence, TNFR2 did not potentiate the inhibitory func-
tions of mTNF$\Delta$–9K11E on adipocyte differentiation mediated by TNFR1. These results demonstrate that the transmembrane TNF$\alpha$ inhibits adipocyte differentiation by engaging TNFR1 alone.

**DISCUSSION**

The wide array of biological actions of TNF$\alpha$ is regulated at many levels. The presence of both transmembrane and secreted forms of functional TNF$\alpha$ ligands adds a spatial mode of control to TNF$\alpha$ actions. As the local actions of this molecule are recognized in both physiological and pathological states, understanding the biology of its cell surface-associated form becomes more critical. However, in contrast to the well characterized function and signaling of sTNF$\alpha$, information regarding mTNF$\alpha$ is still limited.

The actions of sTNF$\alpha$ on adipocytes have also been extensively investigated. Numerous reports have shown that sTNF$\alpha$ has strong negative effects on adipocyte differentiation (43–46). Furthermore, the use of human TNF$\alpha$ in cultured murine adipocytes has indicated that TNFR1 can mediate this effect (43, 44). On the other hand, the biological activities and signaling mechanisms of mTNF$\alpha$ have not yet been determined in this respect. In this study, we have generated several preadipocyte cell lines stably expressing a non-cleavable form of TNF$\alpha$ (mTNF$\Delta$–9K11E) to examine its actions in adipocytes. These studies demonstrate that mTNF$\Delta$–9K11E is biologically active in several independent preadipocyte cell lines and can induce marked alterations in the adipocyte differentiation program. In wild type cells carrying both functional TNF receptors, expression of mTNF$\Delta$–9K11E led to significant inhibition of terminal differentiation into adipocytes. The biological activities generated by mTNF$\Delta$–9K11E in our experimental system are unlikely to be supra-physiological since the protein expression level of the non-cleavable mutant is about 5% of the endogenous counterpart produced by LPS-stimulated macrophages.

Our parallel experiments with sTNF$\alpha$ generated results consistent with previous reports and demonstrated that adipocyte differentiation can be altered similarly by both mTNF$\Delta$–9K11E and sTNF$\alpha$. These findings further support the notion that the transmembrane TNF$\alpha$ can functionally engage receptors to modulate adipocyte differentiation.
Transmembrane TNFα Selectively Activates TNFR1

Fig. 4. Effects of reconstituted TNFR1 expression on mTNFα1–9K11E-expressing TNFR1−/− preadipocytes. The expression levels of PPARγ, Glut4, aP2, and adipin mRNAs were compared in TNFR1−/− adipocytes expressing mTNFα1–9K11E with or without exogenous TNFR1. Lanes 1, 5, and 9, TNFR1−/− cells expressing hygromycin B (V)– and puromycin-resistant vectors (used to express TNFR1); lanes 2, 6, and 10, TNFR1−/− cells expressing mTNFα1–9K11E (T) and puromycin-resistant vector; lanes 3, 7, and 11, TNFR1−/− cells expressing hygromycin B-resistant vector (V); and TNFR1, and lanes 4, 8, and 12, TNFR1−/− expressing both mTNFα1–9K11E (T) and TNFR1. Lanes 1–4, control; lanes 5–8, induced by Mix in the presence of 200 μg/ml hygromycin B; lanes 9–12, induced by Mix+BRL in the presence of 200 μg/ml hygromycin B. Results shown are representative of at least two independent experiments.

9K11E and sTNFα under the experimental conditions used in this study. In addition, the observation that the use of BRL49653 can reverse the anti-adipogenic effects of mTNFα1–9K11E is also consistent with previous reports, which demonstrated that similar compounds could block the inhibitory effects of sTNFα on adipocyte differentiation (47, 48). The extent of inhibition of differentiation by both mTNFα1–9K11E and sTNFα was dependent on the amount of ligand and on the strength of the induction conditions. Under the experimental conditions where mTNFα1–9K11E expression is stable, it is possible that stronger induction conditions could deliver stronger or multiple adipogenic signals, which cannot be completely blocked by a constant level of mTNFα1–9K11E. Consistent with this, we observed that high levels of both sTNFα (10 ng/ml) and mTNFα1–9K11E (obtained by continuous selection, Fig. 2C) can still block adipocyte differentiation even under the most permissive condition. Therefore, it is reasonable to assume that there is a balancing point between adipogenic and anti-adipogenic signals, the outcome of differentiation will be determined by the relative strength of signals from either side.

In this study, we have also determined the TNF receptor responsible for mediating this action of mTNFα1–9K11E by utilizing newly developed preadipocyte cell lines deficient in TNFR1, TNFR2, or both receptors. These cells can differentiate into adipocytes with high efficiency and thus provided a valuable experimental system to study signaling through each TNF receptor. Expression of mTNFα1–9K11E in TNFR1−/−,
membrane ligand.

Finally, earlier studies have shown that TNFR1 can mediate other sTNFα functions in adipocytes, in addition to signaling the anti-adipogenic activity. These include modulation of leptin production (51, 52) and inhibition of insulin signaling in murine adipocytes (31). Further studies will be necessary to elucidate whether mTNFα exerts similar actions, and selective usage of TNFR1 is a general mechanism in adipocytes.

The fact that TNFα is biologically active in adipocytes when retained on the cell surface makes it a candidate mediator of other local TNFα-induced responses. It is tempting to speculate that this might potentially be relevant in disease states involving adipose mass and/or altered local levels of tissue TNFα, such as obesity, or lipodystrophies. It is also possible that mTNFα produced in the stroma-vascular component of adipose tissue, from either preadipocytes or macrophages, could also affect adipocytes through cell-cell contact. Thus, if mTNFα is active in mediating other effects that influence the metabolism and/or number of adipocytes, it might also have a strong impact on systemic energy metabolism. However, the limitations of the applicability of the studies in cultured cells to whole animals are obvious. Further in vivo studies, including gain of function transgenic mice, are needed to address the role of this form of TNFα in regulating adipocyte biology locally under physiological or pathological conditions.

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