TNF-related apoptosis-inducing ligand promotes human preadipocyte proliferation via ERK1/2 activation

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ABSTRACT Upon obesity, adipose tissue is excessively expanded and characterized by pathologic processes like hypoxia, fibrosis, and inflammation. Death ligands belonging to the TNF superfamily such as TNF-α are important contributors to these derangements and exert a pronounced influence on the metabolic and cellular homeostasis of adipose tissue. Here, we sought to identify the effect of the death ligand TNF-related apoptosis-inducing ligand (TRAIL) on the adipose tissue precursor cell pool and therefore investigated its influence on preadipocyte proliferation. Treatment of human preadipocytes with TRAIL resulted in a time- and dose-dependent increase in proliferation (EC50 3.4 ng/ml) comparable to IGF-1. Although no apoptosis was observed, TRAIL triggered a rapid cleavage of caspase-8 and -3. Neither inhibition of caspase activity by zVAD.fmk (20 μM) nor ablation of caspase-8 expression by lentivirus-delivered small hairpin RNA (shRNA) abolished the proliferative response. TRAIL triggered a delayed and sustained activation of ERK1/2, leaving Akt, p38, JNK, and NF-κB unaffected. Importantly, inhibition of ERK1/2 activation by PD0325901 (300 nM) or AZD6244 (5 or 10 μM) completely abolished the proliferative response. We thus reveal a hitherto unknown function of TRAIL in regulating adipose tissue homeostasis by promoting the proliferation of tissue-resident precursor cells.—Funcke, J.-B., Zoller, V., Abd El Hay, M., Debatin, K.-M., Wabitsch, M., Fischer-Posovszky, P. TNF-related apoptosis-inducing ligand promotes human preadipocyte proliferation via ERK1/2 activation. *FASEB* J. 29, 000–000 (2015). www.fasebj.org

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With the growing epidemic of obesity, white adipose tissue has come to the center of metabolic research. It has long been regarded a passive organ for energy storage, but is nowadays well recognized as an active endocrine organ (1). Adipose tissue releases more than 100 different factors to the circulation (1). These hormones, growth factors, inflammatory cytokines, and metabolites exert their functions either locally in an auto-/paracrine manner or systemically and thereby regulate whole-body energy balance and metabolism (1).

Adipose tissue displays an exceptionally high plasticity (2, 3). It can rapidly expand or shrink in response to changes in the energy state of the body (2, 3). In obesity, it is excessively enlarged and characterized by pathologic alterations such as hypoxia, fibrosis, and inflammation involving the infiltration and accumulation of macrophages (2, 3). These local alterations play a causal role in the development of obesity-associated comorbidities, especially insulin resistance and type 2 diabetes mellitus, but also hepatic steatosis and cardiovascular diseases (4).

TRAIL belongs to the TNF superfamily (5). In the canonical pathway, TRAIL binding to its cognate receptors at the cell surface (TRAIL-R1 and TRAIL-R2) leads to formation of a primary complex also called death-inducing signaling complex, consisting of receptor oligomers, the adaptor molecule Fas-associated death domain (FADD), the initiator caspases 8 or 10, and the regulatory protein cellular FLICE-like inhibitory protein (cFLIP) (5). The oligomerization of initiator caspases in the complex triggers their activation by self-cleavage, allowing further downstream events and induction of apoptosis to occur (5–7). In a noncanonical pathway, FADD, cFLIP, as well as caspase-8 and -10 can be released from the primary complex and contribute to the formation of a cytosolic complex, which additionally encompasses the adaptor protein TNF receptor type 1-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIPK1), and TNF receptor-associated factor 2 (TRAF2) (8–10). This secondary complex can activate diverse cellular signaling cascades including the Akt, mitogen-activated protein kinase (MAPK, i.e., p38, JNK, ERK1/2), and NF-κB pathways (10). However, the biological significance of this kinase cascade activation is still elusive.

TRAIL has gained fame as an anticancer agent. It is a promising candidate for the treatment of malignancies because it selectively induces apoptosis in cancer cells, but
MATERIALS AND METHODS

Materials

Recombinant human TRAIL was obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). Fully human agonistic monoclonal TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab) antibodies were kind gifts of Human Genome Sciences (11). Recombinant human FasL was obtained from Enzo Life Sciences (Lorrach, Germany) and recombinant human TNF-α from Bioschim (Berlin, Germany). zVAD.fmk was obtained from Bachem (Bubendorf, Switzerland), P0325901 and AZD6244 from Selleckchem (Munich, Germany).

Experimental subjects and human primary stromal-vascular cell isolation

Subcutaneous adipose tissue samples were obtained from 4 female patients undergoing plastic surgery. The mean age of the patients was 31.8 ± 5.7 years, the mean BMI was 30.0 ± 8.3 kg/m². All procedures were performed in accordance with the Declaration of Helsinki guidelines and approved by the ethics committee of Ulm University. Written informed consent was obtained from all patients in advance. Stromal-vascular cells were isolated from adipose tissue samples by established protocols (17).

Cell culture

Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes (18) and human primary stromal-vascular cells from adipose tissue were maintained in their undifferentiated state in growth medium consisting of DMEM/F-12 (1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Darmstadt, Germany), 17 μM pantothenic acid, and 33 μM biotin (Sigma-Aldrich, Munich, Germany).

Microscopic cell counting

SGBS preadipocytes were seeded into 12 well plates (7000 cells/well). For each treatment, triplicate wells were prepared. On the next day, the cells were given fresh growth medium with or without TRAIL (1, 10, or 100 ng/ml). At 0, 24, 48, and 72 hours after stimulation the number of adherent cells was determined by directly counting 3 random microscopic fields (1 mm² area each) per well using a net micrometer.

[3H]-thymidine incorporation

SGBS preadipocytes (5000 cells/well) and human primary stromal-vascular cells (10,000–20,000 cells/well) were seeded into 24 well plates. For each treatment, at least triplicate wells were prepared. On the next day, the cells were given fresh growth medium supplied with the indicated substances. After 48 hours, 2.5 μCi of methyl-[3H]-thymidine (Hartmann Analytic, Braunschweig, Germany) were added. After 16 hours, the cells were washed with Dulbecco’s phosphate-buffered saline (DPBS), trypsinized, and harvested using a Filtermat Harvester (Inotech Biosystems, Brandon, FL, USA). Solid scintillation counting of β decay events was performed on a MicroBeta TriLux 1450 (Perkin Elmer, Rodgau, Germany).

Carboxyfluorescein diacetate succinimydyl ester fluorescence staining

SGBS cells were trypsinized, centrifuged, and resuspended in basal medium consisting of DMEM/F-12 (1:1) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 17 μM pantothenic acid, and 33 μM biotin (Sigma-Aldrich). 5/6-carboxyfluorescein diacetate N-succinimidyl ester (Affymetrix eBioscience, Frankfurt am Main, Germany) was added to a final concentration of 4.5 μM, and the cells were incubated in the dark for 15 minutes at 37°C. The cells were washed 3 times with basal medium and seeded into 6 well plates (30,000 cell/well) in growth medium. For each treatment, at least triplicate wells were prepared. On the next day, the cells were given fresh basal medium or growth medium supplied with the indicated substances. After 72 hours, the cells were trypsinized and analyzed on a FACScalibur flow cytometer (BD, Heidelberg, Germany).

Hoechst 33342 fluorescence staining

SGBS cells were seeded into 4 well chamber slides (BD; 10,000 cells/well). On the next day, the cells were given fresh growth medium supplied with the indicated substances. After 72 hours, the cells were washed twice with DPBS, fixed with formaldehyde, washed with DPBS and incubated with 500 ng/ml Hoechst 33342 (Sigma-Aldrich) in the dark for 15 minutes at room temperature. The cells were washed with DPBS, mounted with fluorescence mounting medium (DAKO, Hamburg, Germany) and analyzed on an AX-70 fluorescence microscope (Olympus, Hamburg, Germany).

Nicoletti assay

SGBS preadipocytes were seeded into 12 well plates (7000 cells/well). For each treatment, at least triplicate wells were prepared. Cell death by apoptosis was quantified as described by Nicoletti et al. (19, 20). Specific apoptosis was calculated by the following formula: 100% × [experimental apoptosis (%) − spontaneous apoptosis in growth medium (%)] / [100% − spontaneous apoptosis in growth medium (%)].
Whole protein extraction and Western blot analysis

The cells were washed once with ice cold DPBS, detached using a cell scraper, and lysed for 20 minutes at 4°C in whole protein lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol) freshly supplemented with 1 mM DTT, 1X Complete protease inhibitors, and 1X phosphatase inhibitors (Roche, Mannheim, Germany). Lysates were cleared by high-speed centrifugation at 4°C.

Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, Munich, Germany). SDS-PAGE and Western blotting were carried out using NuPAGE and iBlot products obtained from Life Technologies. Briefly, 20–30 μg of sample protein were separated on NuPAGE 4–12% Bis-Tris gradient gels in 1X NuPAGE MES SDS running buffer and transferred onto iBlot nitrocellulose membranes. The following primary antibodies were used: rabbit anti-phospho-Akt (S473), rabbit anti-Akt, mouse anti-phospho-ERK1/2 (T202/Y204), rabbit anti-phospho-JNK2 (T183/Y185), rabbit anti-phospho-JNK1 (T183/Y185), rabbit anti-phospho-p38 (T180/Y182), mouse anti-p38, mouse anti-phospho-ikBα (S32/36), rabbit anti-IkBa, and rabbit anti-caspase-3 (Cell Signaling Technology), mouse anti-β-actin, and rabbit anti-ERK1/2 (Sigma-Aldrich), mouse anti-caspase-8 (Enzo Life Sciences), mouse anti-α-tubulin (Calbiochem/EMD Millipore, Darmstadt, Germany), mouse anti-JNK (BD). Horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Enhanced chemiluminescence was carried out using Amersham ECL Western blotting detection reagent (GE Healthcare, Munich, Germany).

Lentiviral-mediated knockdown of caspase-8

Lentiviral-mediated knockdown of caspase-8 was carried out using the BLOCK-iT inducible H1 Lentiviral RNAi kit, the HEK293FT cell line, Lipofectamine 2000, and ViraPower lentiviral packaging mix according to the manufacturer’s recommendations. SGBS preadipocytes were transduced with lentiviral supernatants supplemented with 8 μg/ml Sequabrene (Sigma-Aldrich) at a multiplicity of infection of 1–1.5. Stably transduced cells were selected with Zeocin (Life Technologies). Caspase-8 knockdown was controlled by Western blot.

NFκB p65 immunofluorescence staining

SGBS cells were seeded into 4 well chamber slides (BD; 10,000 cells/well). After 72 hours, the cells were given fresh growth medium supplemented with the indicated substances. At 30 minutes and 2 hours after stimulation the cells were washed twice with DPBS, fixed with formaldehyde, washed twice with DPBS and permeabilized with Triton X-100. The cells were washed twice with DPBS, incubated in blocking buffer (DPBS supplemented with 10% fetal bovine serum) for 1 hour at room temperature. The cells were washed three times with blocking buffer and then transferred into pEntr4/BLOCK-iT-DEST by targeted recombination. Lentiviral particles were produced in HEK293FT cells using the ViraPower lentiviral packaging mix according to the manufacturer’s recommendations. SGBS preadipocytes were transduced with lentiviral supernatants supplemented with 8 μg/ml Sequabrene (Sigma-Aldrich) at a multiplicity of infection of 1–1.5. Stably transduced cells were selected with Zeocin (Life Technologies). Caspase-8 knockdown was controlled by Western blot.

Nuclear protein extraction and NFκB electrophoretic mobility shift assay

Nuclear protein extraction was performed as described by Andrews and Faller (21) with modifications by Kasperczyk et al. (22). In brief, the cells were washed once with ice cold DPBS, detached using a cell scraper, and incubated for 15 minutes at 4°C in low salt buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-potassium hydroxide (HEPES-KOH) pH 7.9, 1.5 mM MgCl2, 10 mM KCl) freshly supplemented with 500 μM DTT, 200 μM PMSE, 1 mM Na3VO4, and 1.33X protease inhibitor (Sigma-Aldrich). Nonidet P-40 substitute (0.6%; Sigma-Aldrich) was added, the cells were vortexed, and the cytosolic extract was separated by centrifugation for 5 minutes at 2500 rpm, 4°C. The cell pellet was incubated for 20 minutes shaking at 4°C in high salt buffer (20 mM HEPES-KOH pH 17.9, 1.5 mM MgCl2, 420 mM NaCl, 200 μM EDTA, 25% glycerol) freshly supplemented with 500 μM DTT, 200 μM PMSE, 1 mM Na3VO4, and 1.33X protease inhibitor (Sigma-Aldrich). Remaining cellular debris were removed by centrifugation for 10 minutes at 13,000 rpm, 4°C. The nuclear extract was collected, snap-frozen in liquid nitrogen, and stored at −80°C until use. Before use, the protein concentration of the nuclear extracts was determined using the Pierce BCA protein assay kit obtained from Thermo Scientific.

NFκB sense probe (5’-AGT TAG GGG GAC TTT CCC AGG C-3’) was labeled with γ-[32P]-ATP (Perkin Elmer) using T4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany). After labeling, a 2-fold molar excess of unlabeled NFκB antisense probe (5’-GGT CAT GCT GTA CTA GAT TT-3’) was added and annealed by short heating to 90°C and slow cooling overnight. The resulting double-stranded labeled NFκB probe was purified on Micro Bio-Spin P30 columns (BioRad, Munich, Germany).

Binding reactions were prepared on ice in binding buffer (10 mM Tris-HCl pH 7.5, 1 mM MgCl2, 50 mM NaCl, 500 μM EDTA, 4% glycerol, 500 μM DTT) with 5 μg nuclear extract protein, 1 μg poly(dIdC) (Sigma-Aldrich), and 10,000-12,000 cpm of labeled NFκB probe and incubated for 30 minutes at room temperature. The complexes formed were separated on a non-denaturing 6% polyacrylamide gel with 0.3X TBE as the running buffer. The polyacrylamide gel was then dried onto Whatman paper (GE Healthcare), and the radioactive signal was detected using Amersham Hyperfilm ECL films (GE Healthcare).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software version 6.01 (GraphPad Software, La Jolla, CA, USA). A 1-way ANOVA was used to analyze the data displayed in Figs. 1B, 1E, 2B, 3A, 3B, 4C, 4E, 6C, and 6D. A 2-way ANOVA was used to analyze the data displayed in Fig. 1A. Where either a 1-way or 2-way ANOVA were employed, a Bonferroni post-test comparing the indicated conditions was performed. A 2-tailed, paired Student’s t-test was used to analyze the data displayed in Fig. 1D. In all cases, the significance level (α) was set to be 0.05.

RESULTS

To study the impact of TRAIL on preadipocyte proliferation, we took advantage of the human SGBS cell
strain. These cells are neither transformed nor immortalized and behave functionally like human primary stromal-vascular cells isolated from adipose tissue (18, 23). They are characterized by a high capacity for adipogenic differentiation and yield adipocytes that closely mimic ex vivo differentiated human primary adipocytes (18, 23). Therefore, the SGBS cell strain represents a useful tool to study both preadipocyte and adipocyte biology.

**TRAIL induces human preadipocyte proliferation**

SGBS preadipocytes were treated with increasing doses of human recombinant TRAIL, and their proliferative behavior was studied. Utilizing microscopic cell counting, a time- and dose-dependent increase in the number of cells adherent to the cell culture dish was observed upon treatment with TRAIL (Fig. 1A). As estimated by [3H]-thymidine incorporation, already at concentrations as low as 1 or 3 ng/ml, TRAIL induced a slight increase in proliferation (Fig. 1B). A maximal increase in proliferation by ~57% was detected at 30 ng/ml TRAIL. The mitogenic effect of this death ligand was further confirmed by a nuclear staining, which clearly indicated higher cell numbers in TRAIL-treated cultures (Fig. 1C), and by flow cytometry of CFSE-labeled and TRAIL-treated cells (Fig. 1D). TRAIL-induced proliferation was dependent on TRAIL binding to its cognate receptors as addition of a human recombinant TRAIL-R2/Fc construct completely abolished its proliferative effect (Supplemental Fig. 1A). Importantly, TRAIL exerted a comparable or even stronger mitogenic function in human primary stromal-vascular cells isolated from white adipose tissue samples of subjects undergoing plastic surgery (Fig. 1E). Moreover, treatment of SGBS preadipocytes with agonistic antibodies specific for either TRAIL-R1 (mapatumumab, mapa) or TRAIL-R2 (lexatumumab, lexa) after 72 hours, [3H]-thymidine incorporation was measured. Data are expressed as means ± SEM of 3 independent experiments performed.

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**Figure 1. TRAIL stimulates human preadipocyte proliferation.** A–C) SGBS preadipocytes were stimulated with growth medium alone or with increasing doses of TRAIL. A) At the indicated time points, the number of adherent cells was counted using a net micrometer. Data are expressed as means ± SEM of 3 independent experiments performed. *P ≤ 0.05 for TRAIL 100 ng/ml compared with growth medium alone at 72 hours. B) After 72 hours, [3H]-thymidine incorporation was measured. Data are expressed as means ± SEM of at least 3 independent experiments performed. **P ≤ 0.01; ***P ≤ 0.001 compared with growth medium alone. C) After 72 hours, Hoechst 33342 fluorescence staining was performed. One representative of 3 independent experiments performed is shown. Scale bars, 200 µm. D) SGBS preadipocytes were stained with CFSE and stimulated with basal medium, growth medium alone or with TRAIL. After 72 hours, the remaining CFSE fluorescence was measured. A representative histogram of 1 of 3 independent experiments performed is shown. The loss of CFSE mean fluorescence intensity in comparison to cells stimulated with basal medium was calculated. *P ≤ 0.05 for TRAIL 30 ng/ml compared with growth medium alone. E) Human primary stromal-vascular cells were isolated from adipose tissue samples of 4 patients and stimulated with growth medium alone or with increasing doses of TRAIL. After 72 hours, [3H]-thymidine incorporation was measured. Data are expressed as means ± SEM. *P ≤ 0.05; **P ≤ 0.01 compared with growth medium alone. F) SGBS preadipocytes were stimulated with growth medium alone or human agonistic antibodies specific for either TRAIL-R1 (mapatumumab, mapa) or TRAIL-R2 (lexatumumab, lexa). After 72 hours, [3H]-thymidine incorporation was measured. Data are expressed as means ± SEM of 3 independent experiments performed.
induces apoptosis favorably in cancer cells, sparing non-malignant cells (11).

Death ligands (TRAIL, FasL, and TNF-α) induce human preadipocyte proliferation in an extent comparable to IGF-1

We then wanted to know whether the proliferative effect of TRAIL also extends to other death ligands of the TNF-α family. Interestingly, both Fas ligand (FasL) and TNF-α stimulated proliferation in SGBS preadipocytes with FasL being comparable to TRAIL, but TNFα even being more efficient (maximal increases by ~57% for TRAIL, ~53% for FasL, and ~107% for TNF-α at 30 ng/ml, respectively) (Fig. 3A). These data suggest that death ligands in general are important and potent mitogenic factors in preadipocytes. This is further supported by the finding that IGF-1, a well-known mitogen in many cell types (25, 26), exerted a proliferative response in a comparable range to that of the death ligands used in this study (maximal increase by ~51% at 300 ng/ml IGF-1) (Fig. 3B).

TRAIL triggers caspase activation, though caspase activity and presence are not required for its proliferative effect

In canonical signaling, the TRAIL-induced cleavage and activation of caspases constitutes a central event (5). From earlier studies of our group, we knew that TRAIL stimulation triggers caspase cleavage in mature adipocytes without inducing apoptosis (14). Therefore, we studied the occurrence of caspase activation in SGBS preadipocytes under the experimental conditions used in this study. Strikingly, in response to 30 ng/ml TRAIL, a rapid cleavage of caspase-8 was observed with the intermediate p43/41 fragment detectable after only 5–10 minutes and the active

![Figure 2. TRAIL does not induce human preadipocyte apoptosis. A, B) SGBS preadipocytes were stimulated with growth medium alone or with increasing doses of TRAIL in the absence or presence of cycloheximide (CHX). After 48 hours, apoptotic cell death was measured by flow cytometry. A) Representative histograms of 1 of 3 independent experiments performed are shown. B) Specific apoptosis was calculated and analyzed statistically. Data are expressed as means ± SEM of 3 independent experiments performed. ***P ≤ 0.001 compared with growth medium alone.

![Figure 3. Death ligands stimulate human preadipocyte proliferation. A) SGBS preadipocytes were stimulated with growth medium alone or with increasing doses of either FasL or TNF-α. After 72 hours, [3H]-thymidine incorporation was measured. Data are expressed as means ± SEM of 3 independent experiments performed. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 compared with growth medium alone. B) SGBS preadipocytes were stimulated with growth medium alone or with either TRAIL or increasing doses of IGF-1. After 72 hours, [3H]-thymidine incorporation was measured. Data are expressed as means ± SEM of at least 3 independent experiments performed. ***P ≤ 0.001 compared with growth medium alone.](image-url)
p18 fragment detectable after 30 minutes (Fig. 4A). Furthermore, cleavage of caspase-3 to the active p17 fragment was evident after 30 minutes and peaked after 2 hours (Fig. 4A). No difference was observed between control and TRAIL-treated cells concerning the activation state of the caspases 10, 6, 2, and 1 (data not shown). To probe the role of caspase activity in the proliferative effect of TRAIL, the pan-caspase inhibitor zVAD.fmk was used. A concentration of 20 μM was sufficient to completely block the activation of caspase-8 and -3 after 2 hours (Fig. 4B). However, the inhibition of caspase activity had no significant impact on the proliferative effect of TRAIL (increase by ~67% for TRAIL alone compared with ~45% for TRAIL with zVAD.fmk) (Fig. 4C). To corroborate this finding, caspase-8-deficient SGBS preadipocytes were generated using a lentiviral-mediated shRNA approach. Two different constructs targeting caspase-8 (shC8.1 and shC8.2) were designed, both of them causing a knockdown of caspase-8 protein expression by ~80% compared with cells expressing a hyper-random sequence (shHRS) without any known target, which was used as negative control (Fig. 4D). Both the control cells and the caspase-8 knockdown cells displayed a robust proliferative response upon TRAIL treatment, and no significant difference was detectable between the 3 used cell types (Fig. 4E). This set of data demonstrates that caspases are not involved in mediating the mitogenic effect of TRAIL on human preadipocytes.

### TRAIL does not alter the activation state of the Akt and NF-κB cascades

By noncanonical signaling, TRAIL can activate diverse cellular cascades including the Akt, NF-κB, and mitogen-activated protein kinase (MAPK, *i.e.*, p38, JNK, and ERK1/2) pathways (10). To study the activation of these kinases, SGBS preadipocytes were stimulated with 30 ng/ml TRAIL for several time points and Western blot analysis was carried out (Fig. 5A). In control as well as TRAIL-treated cells, a phosphorylation of Akt was detectable from 5 minutes to 2 hours with no difference between treatments. As a surrogate of NF-κB activation, the abundance of phosphorylated and nonphosphorylated IκBα was analyzed. Although a positive control (macrophage-conditioned medium) induced a phosphorylation and subsequent degradation of IκBα, we detected only weak and inconclusive phosphorylation signals upon treatment with TRAIL (Fig. 5A). We therefore decided to apply additional,
more specific methods to study NF-κB activation. Immuno-
fluorescence of the NF-κB subunit p65 revealed a clear
translocation of p65 to the nucleus upon treatment with
30 ng/ml TNF-α, whereas upon treatment with 30 ng/ml
TRAIL p65 remained sequestered in the cytoplasm (Fig.
5B). Likewise, in EMSA, a treatment with 30 ng/ml TNF-
α robustly triggered NF-κB transcriptional activity in contrast
to a treatment with 30 ng/ml TRAIL, which did not evoke
any signal (Fig. 5C). This set of data demonstrates that
neither Akt nor NF-κB play a causal role in mediating the
effect of TRAIL on human preadipocyte proliferation.

**TRAIL does not lead to phosphorylation of p38 and
JNK, but activates the ERK1/2 cascade, which proves
indispensable for the induction of proliferation**

We then continued to investigate the activation of MAPKs
in response to TRAIL treatment. Western blot analysis
revealed hardly any activation of p38 and a weak phos-
phorylation of JNK after 5 to 15 minutes, which was
comparable between control and TRAIL-treated cells (Fig.
6A). Also for ERK1/2, a similar initial wave of comparable
activation was observed under both conditions, which is
most likely caused by the media change at the beginning of
the experiment. Importantly though, we detected a strong,
delayed, and sustained phosphorylation of ERK1/2 after
6 hours, which persisted up to 24 hours. The activation of
the ERK1/2 cascade upon TRAIL treatment was com-
pletely blocked using a TRAIL-R2/Fc construct (Supple-
mental Fig. 1B). To define the role of the observed ERK1/2
activation for the mitogenic effect of TRAIL, we decided to
block the ERK1/2 pathway. To do so, 2 small molecule
inhibitors of MEK1/2, the speci-
cific upstream kinases of
ERK1/2, PD0325901, and AZD6244 (selumetinib), were
chosen. For both these inhibitors, clinical studies exploring
their anticancer potential are currently in progress
(clinicaltrials.gov). In SGBS preadipocytes, not only the basal
but also the TRAIL-induced activation of ERK1/2 was
completely abolished by both inhibitors at concentrations of
300 nM for PD0325901 as well as 5 and 10 μM for AZD6244
(Fig. 6B). Moreover, both PD0325901 and AZD6244

![Figure 5.](image-url)
robustly inhibited the basal proliferation and also the TRAIL-induced proliferation of SGBS preadipocytes (Fig. 6C). Of note, a significant increase in \([3H]\)-thymidine incorporation was observed at 100 nM PD0325901, a concentration where the TRAIL-induced phosphorylation of ERK1/2 was not fully blocked by the inhibitor. Importantly, no significant increase in apoptosis was observed upon treatment with TRAIL in presence of 100 nM and 300 nM PD0325901 as well as 5 μM AZD6244 (Fig. 6D). This set of experiments underlines the general importance of the ERK1/2 signaling pathway for human preadipocyte proliferation and suggests that ERK1/2 mediates the proliferative effect of TRAIL.

**DISCUSSION**

We identified TRAIL as a potent mitogen in human preadipocytes. We initially started to explore the effect of TRAIL on proliferation when we observed an increase in the viability of SGBS preadipocytes upon treatment with TRAIL in a study, which primarily focused on the apoptotic function of the death ligand in human preadipocytes and adipocytes (24). In the present study, TRAIL was found to evoke a pronounced increase in proliferation in both the SGBS cell strain as well as in human primary stromal-vascular cells isolated from white adipose tissue. A proliferative effect was also observed for FasL and TNF-α, suggesting that death ligands in general are important and potent mitogenic factors in human preadipocytes. FasL and TNF-α were identified as proliferative factors in human mesenchymal stem cells and adipocyte precursor cells, respectively, earlier (27–29). In contrast, this to the best of our knowledge is the first study to demonstrate that TRAIL can increase preadipocyte numbers by stimulating their proliferation. The relevance of this finding is underlined by the observation that the proliferative effect of TRAIL was comparable to IGF-1. The latter is a well-known mitogen in many types of malignant and nonmalignant cells (25, 26) and according to Hausman _et al._ constitutes...
the most important trigger of proliferation in human preadipocytes (30).

After the discovery of TRAIL, as an apoptosis-inducing member of the TNF superfamily in 1995/96 (31, 32), the TRAIL/TRAIL-R system rapidly became an attractive target for anticancer treatment strategies, because it seemed that TRAIL selectively only induced cell death in transformed cells (11). However, almost 20 years of research succeeding its initial discovery revealed that the initial view of TRAIL as a mere cell death-inducing agent was far too simplistic (10).

In the clinical trials conducted so far, the vast majority of cancers were found to be resistant to cell death induction by targeting the TRAIL/TRAIL receptor system (11). Quite on the contrary, cancer cells not succumbing to cell death frequently show a proliferative, prosurvival, proinflammatory, promigratory, and proinvasive outcome upon TRAIL treatment due to activation of noncanonical signaling pathways (10). Besides that, the occurrence of both apoptotic and nonapoptotic effects of TRAIL has been discovered in benign cell types (e.g., in the immune, hematopoietic, and vascular systems) (33–35). In the vascular system, TRAIL was identified as a mitogenic factor in human primary vascular smooth muscle cells and vascular endothelial cells (36–39).

Our group recently added human adipocytes to the list of nonmalignant cell types regulated by TRAIL in a manner not involving cell death induction (14). In adipocytes, TRAIL impacts key metabolic functions by a rapid activation of caspases resulting in the cleavage and inactivation of PPARγ (14). In the present study, caspase-8 and caspase-3 cleavage was also observed in preadipocytes. However, neither the inhibition of caspase activity with a pan-caspase inhibitor nor the lentiviral-mediated knockdown of caspase-8 expression had any significant impact on TRAIL-induced proliferation. This clearly demonstrates that the mitogenic effect of TRAIL is independent of caspase activity and presence.

TRAIL did not significantly alter the activation pattern of Akt, NF-κB, and the MAPKs p38 and JNK. However, we detected a delayed and sustained activation of ERK1/2 upon treatment with TRAIL. TRAIL-stimulated proliferation via ERK1/2 activation was reported in malignant small cell lung cancer (40) and glioma cells (41), pseudomalignant rheumatoid arthritis synovial fibroblasts (42, 43), as well as nonmalignant vascular smooth muscle and vascular endothelial cells (36, 37). In these studies, ERK1/2 activation typically ensued within 5–15 minutes and lasted for ~30 minutes (36, 37, 40–43). We also observed an early wave of ERK1/2 phosphorylation within 5 minutes to 2 hours, which was seen upon both control and TRAIL treatment, and is most likely attributable to the addition of fresh serum-supplemented medium at the beginning of the experiment. The fact that a strong ERK1/2 phosphorylation upon TRAIL treatment was detected after 6 hours that lasted for up to 24 hours raised the question whether this effect is secondary and caused by TRAIL-induced changes in protein production and/or secretion resulting in auto-/paracrine stimulation. However, experiments performed with brefeldin A to block protein secretion did not reveal any alterations in the phosphorylation of ERK1/2 by TRAIL (data not shown) ruling out this possibility.

The ERK1/2 pathway is a central regulator of growth and proliferation in many cell types. Likewise, blocking ERK1/2 phosphorylation by inhibition of the specific upstream kinases MEK1/2 using the small molecule inhibitors PD0325901 and AZD6244 (selumetinib) strongly reduced the basal, serum-induced proliferation of human preadipocytes. Importantly, blocking the ERK1/2 pathway did not sensitize the cells for TRAIL-induced apoptosis. This highlights that ERK1/2 activity is of importance for the proliferation but not the survival of human preadipocytes and is supported by previous findings from our group, demonstrating the Akt pathway to constitute the main antiapoptotic signaling route in human preadipocytes (20). Most importantly, though, blocking ERK1/2 phosphorylation completely abrogated TRAIL-induced proliferation demonstrating that it is the activation of ERK1/2 that mediates the proliferative effect of TRAIL.

We also compared the TRAIL-induced intracellular signaling cascades to FasL and TNFα-stimulated signaling. The 3 ligands displayed distinct activation patterns of signaling cascades in human preadipocytes (Supplemental Fig. 2). This is interesting since the TRAIL/TRAIL-R, FasL/Fas, and TNFα/TNF-R1 systems do not only share structural features but also possess comparable signaling capacities (5, 10).

Our study has several important implications. We propose that TRAIL represents an important regulator of adipose tissue homeostasis. The most important determinants of adipose tissue mass are the volume and number of present adipocytes. We have shown earlier that TRAIL inhibits the insulin-stimulated glucose uptake and de novo lipogenesis in human adipocytes thereby regulating adipocyte volume (14). Moreover, we have found that TRAIL interferes with the adipogenic differentiation of human preadipocytes thus also regulating adipocyte numbers (44). The current study elucidates that TRAIL stimulates preadipocyte proliferation and might thereby increase the number of adipose tissue resident precursor cells. Adipose tissue is a very dynamic organ. Its capability to expand contributes considerably to metabolic flexibility and thereby systemically to metabolic health (2, 3). Healthy adipose tissue expansion is defined by the controlled recruitment of precursor cells for adipogenic differentiation along with the establishment of appropriate vascularization (2, 3).

We suggest that TRAIL exerts differential effects in adipose tissue in the lean and the obese state. It is rather probable that TRAIL expression is spatially restricted within adipose tissue in the lean state. The stem and progenitor cell compartment of adipose tissue mostly resides in close association with resident vascular, especially microvascular structures (45). The cells potentially contributing to the local expression of TRAIL in these structures are vascular smooth muscle cells and vascular endothelial cells. Although the expression of TRAIL by vascular smooth muscle cells is well documented (35), its expression by vascular endothelial cells is still a subject of debate (46, 47). Strikingly, SGBS preadipocytes as well as human primary stromal-vascular cells express TRAIL (data not shown), thus also implying that the stem and progenitor cells in adipose tissue add to the local presence of TRAIL at sites of their accumulation. By regulating proliferation and differentiation, the local expression of TRAIL in vascular...
and microvascular structures might contribute decisively to the maintenance of the adipose stem and progenitor cell compartment. Moreover, TRAIL might sustain the structure of the adipose tissue stem cell niche by regulating proliferation, survival, and migration of the involved vascular smooth muscle and endothelial cells (36–39). Thus, in the lean state, TRAIL likely contributes to the preservation of adipose tissue function primarily by affecting the adipose tissue stem cell niche. Detailed studies focusing on the expression of TRAIL by the different types of cells found in adipose tissue will be necessary to explore this theory.

By contrast, it is well conceivable that TRAIL expression is spatially unrestricted within adipose tissue in the obese state. Upon excessive pathologic expansion, adipose tissue gets infiltrated by different types of immune cells, especially classically activated proinflammatory M1 macrophages, which are known to express high levels of TRAIL (48). In agreement with this, TRAIL mRNA levels are increased in adipose tissue of obese mice (14). Under these conditions, high levels of TRAIL within adipose tissue might not only impact the pool of resident precursor cells, but also exert additional functions, such as influencing the metabolic properties of adipocytes (14).

Interestingly, the serum levels of TRAIL correlate positively with numerous obesity-related parameters such as the BMI, waist circumference, total body fat, serum triglyceride, and cholesterol levels (13, 49, 50). Based on our in vitro data, which showed that TRAIL inhibited insulin-stimulated metabolic processes in human adipocytes (14), one might conclude that elevated TRAIL levels lead not only to adipose tissue but also to systemic insulin resistance. However, recent data from animal experiments gave contrary results (51, 52). The intraperitoneal application of recombinant TRAIL to mice decreased the severity of adiposity, glucose intolerance, and systemic inflammation upon high-fat feeding (52). Similarly, a systemic lack of TRAIL increased the severity of the glucose intolerance and systemic inflammation in TRAIL knockout mice on a high-fat diet (51). In both cases, the observed beneficial effects of circulating TRAIL might primarily be explained by its immunosuppressive and immunoregulatory functions that counteract inflammation (33). Supporting the latter notion, the immunosuppressive and immunoregulatory functions of TRAIL, especially its effects on T cells but likely also on macrophages, also protect from the development of type 1 diabetes mellitus in several mouse models of this disease (53–55). The distinct impact of a local versus a systemic elevation of TRAIL levels, however, will have to be elucidated in cell type-specific knockout mice.

Taken together, the present study identified TRAIL as a potent mitogen in human preadipocytes. TRAIL stimulated a delayed and sustained phosphorylation of ERK1/2, which was responsible for mediating the proliferative response. An increase in preadipocyte proliferation might contribute to the maintenance of the adipose tissue-resident precursor pool, thereby supporting the formation of new adipocytes and healthy expansion of adipose tissue. In conclusion, manipulating the activity of the TRAIL/TRAIL-R system either in an adipose tissue-selective or systemic manner might thus provide a novel approach to restore adipose tissue function in pathophysiological states.

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