Adipogenic function of tetranectin mediated by enhancing mitotic clonal expansion via ERK signaling

Seulgi Go1,*, Jihyun Park1,#, Safikur Rahman2, Juno Jin1, Inho Choi1 & Jihoe Kim1,*

INTRODUCTION

Tetranectin (TN), an adipogenic serum protein, enhances adipocyte differentiation, however, its functional mechanism has yet to be elucidated. In the present study, we investigated the adipogenic function of TN by using medium containing TN-depleted fetal bovine serum (TN-del-FBS) and recombinant mouse TN (mTN). The adipocyte differentiation of 3T3-L1 cells was significantly enhanced by mTN supplementation essentially at differentiation induction, which indicated a potential role of the protein in the early differentiation phase. The adipogenic effect of mTN was more significant with insulin in the differentiation induction cocktail, implicating their close functional relationship. mTN enhanced not only the proliferation of growing cells, but also mitotic clonal expansion (MCE) that is a prerequisite for adipocyte differentiation in the early phase. Consistently, mTN increased the phosphorylation of ERK in the early phase of adipocyte differentiation. Results of this study demonstrate that the adipogenic function of mTN is mediated by enhancing MCE via ERK signaling. [BMB Reports 2021; 54(7): 374-379]

*Corresponding author. Tel: +82-53-810-3032; Fax: +82-53-810-4769; E-mail: kinjihoe@ynu.ac.kr
#These authors contributed equally to this work.

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the early phase of adipocyte differentiation. Results in this study demonstrate that the adipogenic function of mTN is mediated by enhancing MCE via ERK signaling.

RESULTS

Adipogenic effect of mTN in the early phase of adipocyte differentiation

The adipogenic effect of mTN was examined by inducing adipocyte differentiation of post-confluent and growth-arrested 3T3-L1 cells as described in the scheme (Fig. 1A). Adipocyte differentiation in TN-del-FBS medium was significantly decreased to < 50% of the control level obtained in the FBS medium following the standard differentiation process (Fig. 1B). Supplementation of TN-del-FBS medium with mTN markedly enhanced adipocyte differentiation, consistently indicating the adipogenic effect of the protein (Fig. 1B) (19). mTN supplementation on the day 0 with the induction cocktail MDI (E1) resulted in 180% adipocyte differentiation compared to the control level obtained in TN-del-FBS medium without protein supplementation. mTN supplementation from the day 0 to the day 5 (E2) and the day 0 to the day 8 (E3) resulted in similar adipogenic effect (170-180% differentiation). After two days post-induction, however, mTN did not show any significant adipogenic effect (Fig. 1B, E4-E6). In FBS medium, mTN supplementation did not change the adipocyte differentiation, likely due to the presence of sufficient amount of TN derived from FBS (see the discussion).

Enhancement of adipocyte differentiation by insulin and mTN

The effects of MIX, DEX, and insulin as components of the induction cocktail were examined on adipocyte differentiation in TN-del-FBS medium. Any significant adipocyte differentiation was not observed by induction with MIX, DEX, or insulin alone or with mTN supplementation (data not shown). Both MIX and DEX (MD) were essential for inducing adipocyte differentiation at a detectable level (Fig. 2A). Insulin with MD

mTN enhances cell proliferation and MCE

The effect of mTN on cell proliferation was examined by growing 3T3-L1 cells in TN-del-FBS medium. mTN dose-dependently enhanced the proliferation of 3T3-L1 cells, which showed ~50% increase in cell numbers at 7 μM protein concentration (Fig. 3A). Next, the effect of mTN on MCE was examined by determining the cell numbers after inducing adipocyte differentiation of growth-arrested 3T3-L1 cells in TN-del-FBS medium. The cell numbers were increased 1.3- and 1.5-fold at 24 h and 48 h, respectively, by induction with MDI, indicating MCE (Fig. 3B and C). In addition, mTN further increased the cell number by 1.7-fold at 48 h, but the change was insignificant at 24 h. Differentiation induction with MD caused insignificant changes in cell numbers with slight decreases, however, mTN supplementation significantly increased the cell number at 48 h (Fig. 3C).

mTN increases the phosphorylation of ERK

ERK phosphorylation was analyzed for 48 h after the induction of adipocyte differentiation of 3T2-L1 cells in TN-del-FBS medium. The analysis showed a rapid increase in ERK expression and phosphorylation upon inducing differentiation with MDI (Fig. 4A). Phosphorylation of ERK increased, reaching a maximum level at 30 min and subsequently decreasing to a basal level at 6 h, while the change in ERK expression was insignificant (Fig. 4A and B). The second maximum level of ERK phosphoryla-

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Fig. 1. Adipogenic effect of mTN in the early phase of adipocyte differentiation. (A) Scheme for the adipocyte differentiation of 3T3-L1 cells following the standard process (upper). TN-del-FBS media were supplemented with 5 μM mTN on different days as indicated for different days (E1-E6). (B) Oil red O staining of differentiated cells (upper) and determination of lipid contents by isopropanol extraction (lower). E1-E6 are the same as in the scheme A. Data with P values < 0.05 (*) are indicated.
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Fig. 2. Enhancement of adipocyte differentiation by insulin and mTN. (A) Adipocyte differentiation of 3T3-L1 cells induced with 5 μM mTN and the indicated induction cocktails: MD, 3-isobutyl-1-methylxanthine + dexamethasone; MDI, MD + 5 μg/ml insulin. Adipocyte differentiation was estimated by oil red O staining (upper) and microscopy (lower). (B) Induction of differentiation with MD and the indicated concentrations of insulin ± 5 μM mTN. (C and D) Determination of adipogenic marker gene expression by quantitative real-time PCR. No ind., no induction. Data with P values < 0.05 (*) and < 0.01 (**) are indicated.

Fig. 3. Enhancement of cell proliferation and MCE by mTN. (A) Proliferation of growing 3T3-L1 cells in TN-del-FBS medium supplemented with mTN at the indicated concentrations. (B and C) Adipocyte differentiation of growth-arrested 3T3-L1 cells was induced in TN-del-FBS medium with the indicated induction cocktails and ± 5 μM mTN supplementation. No Ind., no induction. Cell numbers were determined at 24 h (B) and 48 h (C) post-induction using a cell counter. Data (n ≥ 4) with P values < 0.05 (*) and < 0.01 (**) are indicated.

Enhancement was detected at 24 h, which decreased to a basal level at 48 h. Differentiation induction with MD showed similar changes in the phosphorylation of ERK, the levels of which peaked at 30 min and 24 h, although the relative phosphorylation levels were different from those obtained by induction with MDI. The effect of mTN on the phosphorylation of ERK was examined at 30 min and 24 h post-induction (Fig. 4C–E). mTN supplementation did not alter the expression and phosphorylation of ERK at 30 min post-induction (Fig. 4C). However, at 24 h post-induction with MDI, mTN significantly increased ERK phosphorylation by 1.8-fold of the p-ERK/ERK ratio (Fig. 4D and E). A similar effect of mTN was obtained at 24 h post-induction with MD, showing a 1.7-fold (p-ERK/ERK ratio) increase in the phosphorylation of ERK (Fig. 4D and E).
**DISCUSSION**

For adipocyte differentiation, 3T3-L1 preadipocytes were grown to arrest growth in medium containing CS instead of FBS, which contains adipogenic factors that cause spontaneous basal-level differentiation without induction of differentiation. Insulin is an adipogenic serum protein identified by early studies, and it has been included in differentiation induction cocktails (20, 21). Previously, we analyzed the bovine serum proteome and identified TN as an adipogenic protein in FBS (18). Another study revealed that mTN requires the conserved kringle-4-binding domain for its adipogenic effect (19). However, there was no evidence that the adipogenic function of mTN is mediated by the activation of Plg involved in remodeling ECM.

In the present study, we demonstrated that the adipogenic function of mTN is mediated by enhancing MCE via ERK signaling, which is a prerequisite for adipocyte differentiation. Since FBS contains ~10-15 mg/L TN depending on batch-to-batch variations, TN in FBS was removed, and TN-del-FBS medium was used for the adipocyte differentiation of 3T3-L1 cells. Differentiation was markedly reduced by removing TN, and the supplementation of the TN-del-FBS medium with mTN significantly increased the differentiation level. This adipogenic effect was obtained by mTN supplementation at the induction of differentiation, but not after 2 days post-induction, clearly indicating that the protein plays a role in an early phase of adipocyte differentiation (Fig. 1). Moreover, the adipogenic effect of mTN was more significant when inducing differentiation with MDI than it was with MD, and it appeared to be dependent on the insulin dose (Fig. 2A and B). These results suggested that the adipogenic function of mTN could be closely related with the function of insulin in the early phase of adipocyte differentiation.

TN has been suggested as a cancer marker in serum, and is considered to be involved in cancer development and metastasis via the remodeling of ECM and/or the enhancement of cell proliferation (7-9). The effect of TN on cell proliferation is not yet clearly understood, as the results are inconsistent. One study showed that TN enhanced the cell proliferation and progression of colorectal cancer (9), whereas another study indicated that TN inhibited the proliferation of renal carcinoma cells (22). In this study, we found that mTN enhanced the proliferation of growing 3T3-L1 cells (Fig. 2A). In addition, mTN enhanced the proliferation of some cancer cells, but not all tested cells (data not shown). It can be speculated that the effect of mTN (TN) on cell proliferation might be specific to cells with different sensitivities.

More importantly, we found that mTN enhanced MCE, which is the proliferation of growth-arrested 3T3-L1 cells in the early phase of adipocyte differentiation upon induction (Fig. 3C). Moreover, mTN increased ERK phosphorylation, which was consistent with the enhancement of MCE (Fig. 4D and E). Phosphorylation of ERK is a key step in activating MCE via ERK signaling, which has been shown to be enhanced by insulin (15, 23). Induction of differentiation in TN-del-FBS medium revealed changes in the phosphorylation of ERK, which rapidly increased to reach the first peak at 30 min and the second peak at 24 h (Fig. 4A and B). These results are similar to the changes in ERK phosphorylation upon the induction of differentiation in FBS medium (23). However, in TN-del-FBS medium, phosphorylation of ERK at 24 h was highly significant, while the corresponding phosphorylation in FBS medium was close to a basal level. Interestingly, in the TN-del-FBS medium, insulin significantly enhanced ERK phosphorylation at 24 h post-induction, but insignificant at 30 min (Fig. 4C-E). This delayed effect of insulin on the phosphorylation of ERK could be due to unknown serum factors that were removed in the preparation of TN-del-FBS. mTN increased ERK phosphorylation significantly.
at 24 h, but insignificant at 30 min, which agreed well with the significant enhancement of MCE at 48 h. These results indicate that the enhancement of MCE by mTN is mediated via ERK signaling.

In summary, we discovered that the adipogenic protein mTN plays its role in the early phase of adipocyte differentiation. mTN enhanced MCE that is prerequisite for adipocyte differentiation in the early phase. Consistently, mTN increased the phosphorylation of ERK in the early phase of adipocyte differentiation. Results in this study demonstrate that the adipogenic function of mTN is mediated by enhancing MCE via ERK signaling, although the detailed signaling pathway and regulatory mechanism have yet to be elucidated.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise indicated. FBS and calf serum (CS) were from Welgene (Gyeongsan, Korea). TN-del-FBS was prepared by partial protein precipitation and fractionation with the addition of polyethylene glycol 8000 (10%, v/v) to fetal bovine serum (FBS), as previously described (24, 25). Recombinant mTN was prepared in the full-length form fused with 6 × His-tag at the N-terminus, as described in the previous report (19). Primary antibodies (anti-ERK1/2, anti-pErk1/2, anti-C/EBP-β, and anti-β-actin) and secondary antibodies for western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Mouse-derived 3T3-L1 preadipocytes were maintained in CS medium (DMEM/10% calf serum) by incubation at 37°C in a humidified atmosphere of 5% CO₂. Adipocyte differentiation was induced as described in (26) with modifications. Preadipocytes 3T3-L1 were grown to 100% confluency (Fig. 1A D-2) and then incubated for another 2 days to reach growth arrest (Fig. 1A D0). Adipocyte differentiation was induced in FBS medium (DMEM/10% FBS) or TN-del-FBS medium (DMEM/10% TN-del-FBS) by the addition of 0.5 mM MIX, 1 μM DEX, and 5 μg/ml of insulin. After inducing differentiation for 2 days, cells were incubated in FBS medium or TN-del-FBS medium supplemented with 5 μg/ml of insulin for another 6 days. Media were changed every 2 or 3 days. Differentiated cells were stained with oil red O, and lipid contents were estimated by measuring the absorbance of isopropanol-extracted dye at 519 nm (18).

Quantitative real-time PCR

To determine the expression of adipogenic marker genes, cells were harvested on day 5 post-induction. Total RNA was isolated using TRIzol reagent, and cDNA was synthesized using a cDNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Quantitative PCR amplification was carried out with the following primers: 5’-CCTTTTTCACCTCCACCTA-3’ and 5’-TCTGGCATGACATCAGATG-3’ for C/EBP-α, and 5’-CCATTCTGCCCACCAAC-3’ and 5’-AATTGCC AGTGGCTTCCATCA-3’ for PPAR-γ, using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR green Supermix (Bio-Rad, Hercules, CA, USA).

Determination of cell proliferation and MCE

Cell proliferation was determined with 3T3-L1 by seeding 1-3 × 10⁵ cells/well in a 96-well culture plate containing TN-del-FBS medium and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 h. Then, the culture media were supplemented with the indicated concentrations of mTN. After another 24 h of incubation, the number of viable cells was determined using a D-Plus CCK kit (Donginbiotech, Seoul, South Korea) according to the manufacturer’s protocol. For the determination of MCE in TN-del-FBS medium, adipocyte differentiation of post-confluent and growth-arrested 3T3-L1 cells was induced with MIX + DEX (MD) or MIX + DEX + insulin (MDB), with or without 5 μM (100 μg/ml) mTN. Cell numbers were determined at 24 and 48 h post-induction using a LUNA II automated cell counter (Logos Biosystems, Anyang, South Korea).

Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline and harvested at the indicated incubation times. Harvested cells were lysed in buffer containing 50 mM Tris·HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.2% sodium dodecyl sulfate, 1 × protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and 1 × phosphatase inhibitor cocktail (Roche Diagnostics). Soluble proteins were separated by centrifugation at 14,000 rpm and 4°C for 30 min, and protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific, Waltham, MA, USA). Proteins were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane, which was blocked in TBS-T buffer (20 mM Tris pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 5% skim milk. The membrane was incubated with the indicated primary antibodies and, after washing, with a horseradish peroxidase-conjugated secondary antibody. Immunoactive proteins were visualized using a chemiluminescence detection kit, D-Plus ECL Pico (Donginbiotech, Seoul, Korea), according to the manufacturer’s instructions. Western blot signals were quantified using Image X software and normalized using β-actin to determine relative signal intensities.

Statistical analysis

Data are expressed as means ± standard errors (SEs). The significance of differences between intracellulr lipid contents and gene expression levels was analyzed by an unpaired Student’s t-test. A P value < 0.05 was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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