Thrombin Peptide (TP508) Promotes Adipose Tissue-Derived Stem Cell Proliferation via PI3 Kinase/Akt Pathway

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Key Words

Proliferating capacity • Stem cells • Wound healing

Abstract

A synthetic peptide representing the receptor-binding domain of human thrombin (TP508) promotes angiogenesis and accelerates wound healing in animal models. However, the mechanisms underlying the therapeutic effects of TP508 have not been clearly defined. In this study, we set out to determine whether TP508 could stimulate stem cell proliferation. Adipose tissue-derived stem cells (ASCs) were incubated with TP508 (5 µg/ml) and cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation. Our data showed that TP508 treatment significantly stimulated BrdU incorporation in ASCs ($p < 0.01$). The increased BrdU incorporation induced by TP508 was abolished by the PI3 kinase (PI3K) inhibitor LY294002 at 50 µM. Western blot analysis of ASCs revealed increased phosphorylation of Akt in response to TP508 when compared to unstimulated controls. These results indicate that TP508 exerts proliferative effects on ASCs via the PI3K/Akt pathway.

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Introduction

Thrombin is a serine protease that is activated upon tissue damage during the ongoing coagulation cascade [1–3]. Thrombin exerts cellular effects via G-protein-

coupled protease-activated receptors (PARs), in addition to its role in the formation of a blood clot. PARs are activated by proteolytic cleavage of a specific site in the extracellular domain, which, in turn, acts as a ligand. Among the four known PAR receptors, thrombin activates PAR-1, PAR-3 and PAR-4 but not PAR-2. However, growing evidence suggests that PAR-2 may mediate thrombin signaling by complexing with PAR-1 and PAR-4 [4–6]. TP508 is a peptide which represents residues 508–530 of human prothrombin and was originally identified for its ability to compete for high-affinity α -thrombin binding to fibroblasts and to enhance proliferation of fibroblasts co-stimulated with proteolytically active α -thrombin or molecules that activate downstream signaling events, normally elicited by PAR-1 activation [7–11]. TP508 binds to high-affinity thrombin receptors and mimics cellular effects of thrombin at sites of tissue injury [11]. Unlike thrombin, TP508 has no enzymatic activity and does not promote or interfere with blood coagulation [9].

Previous studies have shown that TP508 promotes wound healing in various animal models. In full-thickness incisional wounds in rats, a single topical application of TP508 increased the breaking strength of wounds by approximately 82% over saline controls when measured at day 7 postincision [12]. Significant effects of TP508

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1018–1172/09/0462–0098\$26.00/0

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were also seen on incisional wounds in rats with radiation-induced healing impairment [13].

Treatment of full-thickness excisional wounds in normal rats with a single topical application of TP508 reproducibly accelerates wound closure (40% greater in TP508-treated than vehicle-treated wounds) [11]. TP508 treatment of ischemic flap wounds restored the normal rapid resolution of the inflammatory phase [14]. TP508 also accelerated repair of rat fresh fractures [15] and promoted bone formation in rabbit critical-size segmental bone defects [16] and in a rabbit model of distraction osteogenesis [17]. TP508 stimulates healing of diabetic foot ulcers in a placebo-controlled phase I/II study [18].

The mechanisms underlying the beneficial effect of TP508 remain largely unknown. Some studies have indicated that angiogenesis may be involved [14]. We have shown previously that adipose tissue-derived stem cells (ASCs) produce a significant amount of VEGF which is required for angiogenesis [19]. To define the mechanisms of TP508-mediated wound healing, we set out to determine whether TP508 could stimulate the proliferation of ASCs which in turn promote wound healing.

Materials and Methods

Isolation and Culture of Human ASCs

Subcutaneous adipose tissue was obtained from 3 patients undergoing elective operations (not liposuctions) following the University Institutional Guidelines. Cells were isolated from the fat tissue as described previously [20] with modifications. Fat tissue was minced and incubated for 90 min at 37°C on a shaker with Liberase Blendzyme 3 (Roche) at a concentration of 4 units/g of fat tissue in PBS. The digested tissue was sequentially filtered through 100- and 40- μ m filters (Fisher Scientific) and centrifuged at 450 g for 10 min. The supernatant-containing adipocytes and debris was discarded and the pelleted cells were washed twice with Hanks' balanced salt solution (Cellgro) and finally resuspended in growth media. Growth media contained alpha modification of Eagle's medium (α MEM, Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine (Cellgro), 100 U/ml penicillin with 100 μ g/ml streptomycin (Cellgro). Plastic adherent cells were designated human ASCs (hASCs) and grown in Nunclon culture vials (Nunc) at 37°C in a humidified atmosphere containing 5% CO₂ followed by daily washes to remove red blood cells and non-attached cells.

Cell Proliferation

Cell proliferation was studied using a colorimetric bromodeoxyuridine (BrdU) kit (Roche Diagnostics) according to the manufacturer's instructions. LY294002 was purchased from Cell Signaling (Danvers, Mass., USA). 2×10^4 hASCs at passage 2 were seeded in 96-well plates for 24 h in serum-free medium (α MEM). After 24 h incubation, either human thrombin (Sigma) or TP508 (MD Anderson Cancer Center peptide core lab) was

added to cells and the incubation continued for another 24 h. BrdU solution was then added into the medium. The medium was discarded after 2 h, and cells were fixed with FixDenat solution for 30 min at room temperature. After removing FixDenat, cells were incubated with 100 μ l freshly diluted 1:100 anti-BrdU peroxidase solution for 30 min. The cells were washed 3 times, 100 μ l of substrate solution was added to incubate for 30 min at room temperature, 25 μ l 1 M H₂SO₄ was added to terminate the reaction, and then absorbance at 450 nm was measured in an ELISA plate reader.

Analysis of mRNA Expression of Thrombin Receptor Using Reverse Transcription-Polymerase Chain Reaction Technique

Total RNA was extracted from hASCs using Trizol reagents (Life Technologies). cDNA was synthesized from 1 μ g total RNA using SuperScript II reverse transcriptase (Life Technologies) in 20 μ l according to the manufacturer's instructions. cDNA samples were subjected to polymerase chain reaction (PCR) amplification using AccuPrime SuperMix I (Invitrogen) with specific primers for human thrombin receptor (F: 5'-TATTTGACCA-GCTCCTGGCTGACA-3'; R: 5'-AACAGCAGCTGACAGGAA-CAAAGC-3'). Control reactions consisted of the above-mentioned PCR amplification mix with primers but no cDNA template. PCR was performed with an Eppendorf mastercycler gradient (Eppendorf). Cycles were programmed as follows: 94°C for 10 min, 35 cycles of 30 s denaturation at 94°C, 30 s at annealing temperature (55°C), 60 s extension at 72°C, with a final extension at 72°C for 5 min. The product size was confirmed by running 10 μ l of sample on 1.5% agarose gel electrophoresis.

Western Blot

hASCs were lysed in RIPA lysis buffer (Upstate) including protease inhibitor cocktail (Roche Diagnostics) according to the manufacturer's instructions. The proteins were separated on sodium dodecyl sulfate/polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk, the membranes were incubated for 1 h at room temperature with rabbit anti-p-Akt (Cell Signaling). Following incubation with goat anti-rabbit horseradish peroxidase secondary antibody (Pierce), labeled proteins were detected using the SuperSignal chemiluminescence detection system (Pierce). For PAR-1, goat anti-PAR-1 and donkey anti-goat IgG-horseradish peroxidase were purchased from R&D Systems.

Data Analysis

All values are presented as means $\pm$ standard deviation. Statistical analysis was performed using Student's t tests. A level of $p < 0.05$ was considered as statistically significant.

Results

ASCs Express Thrombin Receptor

The ASCs are plastic-adherent, spindle-shaped cells (fig. 1). These cells are positive for CD44, CD90, CD105 and negative for CD11b, CD14, CD34, CD45 and HLA-DR as we have described previously [21, 22]. We also

Fig. 1. Phase contrast photomicrographs of hASCs at passage two. hASCs are spindle shaped and plastic adherent.

showed previously that the ASCs are multipotent [21, 22].

Reverse transcription-PCR was performed to detect thrombin receptor mRNA. The PCR product showed the expected size (fig. 2a) and its identity was confirmed by sequencing. A blast search of the GeneBank identified the PCR product as *Homo sapiens* coagulation factor II (thrombin) receptor (F2R) (NM_001992). Western blot analysis using anti-PAR-1 antibody detected a clear band of 47 kDa with the expected size for human PAR-1 (fig. 2b).

Effects of TP508 on BrdU Incorporation

Incubation of 2×10^4 ASC with thrombin resulted in an increase of BrdU incorporation compared to the control (fig. 3). Similarly, incubation of ASC with TP508 also resulted in an increase of BrdU incorporation (fig. 4). The increased BrdU incorporation induced by TP508 was abolished by PI3 kinase (PI3K) inhibitor LY294002 at 50 μ M. LY294002 alone has no effect on BrdU incorporation.

Effects of TP508 on Akt Phosphorylation

ASCs were grown for 16 h in serum-free medium, and then treated with TP508 at a concentration of 5 μ g/ml for 1 h. Western blot analysis of ASCs revealed increased phosphorylation of Akt in response to TP508 when compared to unstimulated controls (fig. 4). The protein level of total Akt was not altered by TP508 treatment (fig. 5).

Fig. 2. Human ASCs express thrombin receptor. **a** mRNA expression of thrombin receptor from hASCs by RT-PCR. M = DNA marker (100-bp ladder). **b** PAR-1 expression by Western blot.

Fig. 3. Thrombin induces BrdU incorporation in ASCs. Proliferative effects were assessed by BrdU incorporation. Thrombin: 1 μ g/ml. * $p < 0.05$.

Fig. 4. TP508 induces BrdU incorporation in ASCs. Proliferative effects were assessed by BrdU incorporation. TP = TP508 at 5 μ g/ml; LY294002 = PI3K inhibitor at 50 μ M. * $p < 0.05$.

Fig. 5. Western blot analysis of Akt. C = Control; TP = TP508 at 5 µg/ml incubated for 1 h with ASC.

Discussion

The present study demonstrates that ASC express the thrombin receptor. To our knowledge, the presence of thrombin receptor on ASCs has never been reported before. However, PAR-1 is widely distributed and has already been described on CD34+ hematopoietic stem cells and on endothelial progenitor cells. Smadja et al. [23] showed that human late endothelial progenitor cells express PAR-1, and that PAR-1 activation induces proliferation, migration and increased capillary-like structure formation in Matrigel. Tarzami et al. [24] showed that thrombin or PAR-1-activating peptide produced a 2- to 3-fold increase in the total number of endothelial progenitor cells.

It has been shown that thrombin induces increased expression and secretion of VEGF from human FS4 fibroblasts, DU145 prostate cells and CHRF megakaryocytes via the PI3K pathway [25]. However, in endothelial cells, the stimulation by thrombin as well as thrombin peptide led to DNA synthesis through activation of the ERK pathway [26]. Kataoka et al. [27] showed that PAR1 is required for ERK activation in response to low concentrations of thrombin. Samdja et al. [28] showed that PAR-1-induced proliferation of endothelial progenitor cells involves angiopoietin 2, which in turn activates Akt pathway [29]. It has also been shown that immobilized TP508

peptide stimulated phosphorylation of mitogen-activated protein kinases and focal adhesion kinase and supported $\alpha_v\beta_3$ integrin-dependent endothelial cell attachment and haptotactic migration [30].

In this in vitro model, TP508 promote the proliferation of ASC and the proliferative effect of TP508 was reduced by PI3K inhibitor. These data suggest that TP508 exerts its proliferative effects involving PI3K/Akt signaling.

To further investigate the mechanism of TP508-induced, PI3K/Akt-dependent cell proliferation, a Western blot analysis of the phosphorylated protein was performed. Phosphorylation of Akt was increased, whereas the protein levels of total Akt were not altered. Our findings are in line with a previous report that TP508 promotes cell growth and wound healing via PI3K/Akt pathway [31].

Consistent with in vivo observations that TP508 promoted neovascularization of the wound tissues [10, 14, 32, 33], our data showed that TP508 promotes ASC proliferation. We have shown previously that ASCs differentiated into vascular cells when injected into infarcted hearts [34]. In light of previous reports that TP508 promotes wound healing [12–18], our findings suggest that the beneficial effect of TP508 may be mediated by stem cells. To this end, it has been shown that autologous mesenchymal stem cells increase vascularity and reduce wound size of nonhealing diabetic ulcer [35]. Importantly, a recent report showed that TP508 also stimulates healing of diabetic foot ulcers in a placebo-controlled phase I/II study [18]. It would be interesting to find out whether the beneficial effects of TP508 on diabetic ulcer are mediated by endogenous stem cells.

Acknowledgment

This work has been supported in part by grant 543102 from the Alliance of Cardiovascular Researchers (to E.A.).

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