Substance P and Thiorphan Synergically Enhance Angiogenesis in Wound Healing

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Impaired angiogenesis is a common pathological characteristic of chronic wounds. Therefore, the regulation of angiogenesis is important for proper tissue repair. It was reported that substance P (SP) accelerates wound healing in a skin injury model. SP is degraded by neutral endopeptidase (NEP). Our study shows that systemic co-treatment of SP and thiorphan, an inhibitor of NEP synergically increased the number of α -smooth muscle actin positive-blood vessels in skin wounds. However, there was no synergic improvement in wound contraction and extracellular matrix deposition. Therefore, inhibition of endogenous NEP activity by thiorphan treatment might modulate the effects of SP treatment specifically on accelerating angiogenesis during wound healing. However, the molecular mechanism(s) of the synergic increase in angiogenesis by SP and thiorphan treatment is still unknown. **Tissue Eng Regen Med 2016;13(2):149-154**

Key Words: Substance P; Thiorphan; Neutral endopeptidase; Angiogenesis; Wound

INTRODUCTION

Substance P (SP) is an 11-amino acid peptide that plays essential roles in pain perception. In addition, studies show that it modulates inflammation and mediates the mobilization of bone marrow-derived stem cells into peripheral blood [1,2]. SP is released by unmyelinated fibers in epidermis and papillary dermis in response to injury in skin where it accelerates the wound healing process both in normal and diabetic conditions [3,4].

Neutral endopeptidase (NEP), also known as common acute lymphoblastic antigen or CD10, is a membrane-bound metalloproteinase that degrades neuropeptides including SP in the extracellular fluid [5]. NEP is expressed on various types of cells such as fibroblasts, keratinocytes, and endothelial cells in skin [6,7]; bone marrow-derived stromal cells including osteoblastic cells [8]; *ex vivo* expanded bone marrow-derived mesenchymal stem cells [9] and lymphoid lineage progenitors [10]. NEP is also expressed on tumor cells, tumor stromal cells, and infiltrating inflammatory cells and is involved in the regulation of invasion of tumor cells [11]. NEP also degrades physiologically active peptides including endothelin-1 and basic fibroblast growth

factor (FGF-2) in order to regulate angiogenesis [12,13]. NEP competes with SP receptor to regulate the effects of SP on various biological processes including inflammation, vasodilation, vascular permeability, and wound healing. Therefore, the regulation of NEP activity is important in SP- and/or other peptides-mediated biological phenomena. Several synthetic NEP inhibitors such as phosphoramidon and thiorphan have been developed in order to modulate the important biological phenomena following the inhibition of NEP activity [14]. For example, the inhibition of NEP activity through the treatment of phosphoramidon or thiorphan increased SP-mediated augmented inflammation in pancreas and lung tissues in an acute pancreatitis model [15].

Importantly, the activity and/or the expression level of NEP change in pathological conditions. For instance, it was shown that NEP activity increased in chronic ulcers and in skin of diabetic subjects, compared to acute ulcers and normal skin, respectively [16]. In addition, another study reported that the inhibition of NEP activity in wounds enhanced wound closure through the increase in re-epithelialization in a diabetic mice model. However, it is unknown whether the inhibition of NEP activity could affect angiogenesis, a critical regulator of wound healing. Therefore, we decided to examine whether the inhibition of NEP activity by thiorphan would accelerate angiogenesis and whether co-treatment of SP and thiorphan could have additive or synergic effects.

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MATERIALS AND METHODS

Mice

C5BL/6 male mice were purchased from Central Lab. Animal Inc. (Seoul, Korea). Mice were maintained under controlled temperature (21–22°C) and light (12 hours light/12 hours dark cycles) with free access to drinking water and food. Eight to ten-weeks old mice were used for experiments. All animal experiments were performed in compliance with protocols approved by the Kyung Hee University Animal Institutional Review Board.

Wound healing model

Eight-week-old mice were anesthetized with ketamine (8 mg/kg, Yuhan Corporation, Seoul, Korea) and rompun (Xylazine, 5.6 mg/kg, Bayer Korea, Seoul, Korea). The dorsal skin was shaved and wounded using a 4 mm biopsy punch. Hydrogel (Poloxamer F127, Sigma, St. Louis, MO, USA) was applied to the wounds. Then, the wounds were dressed with Mepitel (Mölnlycke Health Care, Gothenburg, Sweden) and Tegaderm (3M, Maplewood, MN, USA). SP (10 nmoles/kg, Calbiochem, San Diego, CA, USA) was subcutaneously injected for two consecutive days after the wound was inflicted. When necessary, thiorphan (5 mg/kg, Sigma), an inhibitor of NEP, was intraperitoneally injected for five consecutive days after the wound was inflicted. Digital images of the wounds were obtained at 5 days post-wound. Wound size was analyzed using Adobe Photoshop CS6. The wounds were harvested for further analyses at 5 days post-wounding.

Tissue processing and histological analysis

The harvested wound tissues were fixed with 4% paraformaldehyde (3M, Maplewood, MN, USA) at 4°C overnight and were

then bisected down the center. The fixed tissues were dehydrated and embedded in paraffin and 4 µm sections were prepared. For histological analysis, the sections were stained with hematoxylin/eosin or Masson's trichrome (Sigma). Images were collected using an Olympus BX41 light microscope. Granulation tissue area was analyzed using Image J software (NIH).

Immunohistochemistry

The sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.5% H₂O₂ in methanol. Antigen retrieval was performed by heating sections in 10 mM sodium citrate solution. The primary antibody against α -smooth muscle actin (α -SMA) (1:200, Dako, Carpinteria, CA, USA), ABC kit (Vector labs, Burlingame, CA, USA), and DAB kit (Vector labs, Burlingame, CA, USA) were used for the staining, according to the manufacturer's instructions. Images were collected using an Olympus BX41 light microscope.

Statistical analysis

Quantitative data were presented as mean $\pm$ SD. Unpaired Student t-test was applied to evaluate differences between two groups. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad version 5.01 (GraphPad Software, La Jolla, CA, USA; <http://www.graphpad.com>).

RESULTS

Subcutaneous injection of SP accelerates wound contraction independently of endogenous neutral endopeptidase activity

Previously, we observed that SP accelerates wound healing

Figure 1. Effects of substance P (SP) and thiorphan on wound contraction. (A) Representative images of wounds from control (CON), SP-treated group, thiorphan-treated group (Thior), and SP and thiorphan co-treated group (SP/Thior) 5 days after wounding. Red arrows indicate the wounds. (B) Quantitative results of wound contraction in control (CON, $n=4$ mice), SP-treated group (SP, $n=4$ mice), thiorphan-treated group (Thior, $n=4$ mice), and SP and thiorphan co-treated group (SP/Thior, $n=4$ mice). Data are expressed as mean $\pm$ SD.

(our unpublished data) and that SP is degraded by NEP, a cell surface enzyme, in skin [5,17]. Therefore, we examined whether simultaneous treatment of SP and a NEP inhibitor would additively or synergically accelerate wound contraction. Immediately after wounding, SP (10 nmoles/kg, s.c.) and/or thiorphan (NEP inhibitor; 5 mg/kg, i.p.) [18], were injected into mice. We observed that SP treated mice showed accelerated wound contraction compared to control mice ($76.09 \pm 6.62\%$, $n=4$ mice vs. $29.64 \pm 9.42\%$, $n=5$ mice) (Fig. 1). NEP inhibitor also accelerated wound contraction similarly to SP treatment ($64.90 \pm 5.94\%$, $n=4$ mice vs. $76.09 \pm 6.62\%$, $n=4$ mice; $p < 0.25$) (Fig. 1). Therefore, it is likely that the inhibition of endogenous NEP activity mediated by thiorphan is effective in promoting wound contraction. Importantly, the co-injection of SP and thiorphan did not show an additive or synergic effect on wound contraction, but it was similar to that of SP or thiorphan only treated groups (Fig. 1). These gross observations were confirmed with histological analysis of the wounds (Fig. 2). SP enhanced re-epithelialization, compared with the control, but thiorphan did not (Fig. 2A). Importantly, there were no additive or synergic effects of SP/thiorphan co-treatment on re-epithelialization (Fig. 2A). We also observed the effects of SP on histological changes in the granulation tissues. As wound contraction, the treatment of SP or thiorphan increased granulation tissue area, compared to the control (Fig. 2B). However, there were neither additive nor synergic effects of SP/thiorphan co-treatment on changes in granulation tissue area (Fig. 2B). In summary, these results suggest that subcutaneous injection of SP would accelerate wound contraction independently on endogenous NEP activity, although blocking endogenous NEP activity itself by thiorphan treatment accelerated wound contraction.

SP and thiorphan increase ECM deposition in wounds independently of endogenous neutral endopeptidase activity

Restoration of tissue integrity following extracellular matrix (ECM) deposition is essential for wound healing. ECM deposition is also important in providing cells with an adhesion surface and in restoring the availability of cytokines and growth factors [19]. Type I and type III collagens are the major components among the ECM proteins of the dermis [20]. Importantly, previously we found that SP increased the ECM deposition in wounds (our unpublished data). Therefore, we examined additive or synergic effects of SP treatment and the inhibition of NEP on ECM deposition in wounded tissues, even though we were not able to observe any additive or synergic effect in wound contraction. We analyzed ECM deposition using Masson's trichrome staining. Masson's trichrome stained pre-existing collagens of normal dermis in deep blue and newly synthesized

Figure 2. H/E staining of wound tissues. (A) Representative images of H/E stained wounds from control (CON), substance P-treated group (SP), thiorphan-treated group (Thior), and SP and thiorphan co-treated group (SP/Thior). Arrows indicate wound edges. (B) Measurement of granulation tissue area. Data are expressed as mean $\pm$ SD. * $p < 0.04$, ** $p < 0.02$. Scale bar, 100 μ m.

collagens in margins and centers of the wounds in pale blue (Fig. 3). Our data show that SP increased ECM deposition at wound margins, compared to the control group (Fig. 3A and B). However, thiorphan treatment did not affect ECM deposition (Fig. 3C). Moreover, there were no additive effects of SP and thiorphan co-treatment on ECM deposition compared to SP treatment alone (Fig. 3B and D). Overall, these results suggest that subcutaneous treatment of SP would increase ECM deposition independently on endogenous NEP activity.

SP and thiorphan synergically enhance angiogenesis in wounds

Angiogenesis is one of most essentially required factors for accelerating wound healing [19,21]. Therefore, we examined the effects of SP and NEP inhibitor, thiorphan, on angiogenesis in wounds. SP increased by 3.5-fold the number of α -SMA positive blood vessels in wound margins, compared to the control group ($8.19 \pm 1.01\%$, $n=6$ sections vs. $2.32 \pm 0.57\%$, $n=8$ sections) (Fig. 4). Thiorphan also slightly increased angiogenesis in the wounds, compared to the control group ($3.84 \pm 1.09\%$, $n=7$ sec-

tions). However, the effect of thiorphan on angiogenesis was much lower than SP effect. To our surprise, the co-treatment of SP and thiorphan synergically increased the number of α -SMA

positive blood vessels in wound margins, compared to only SP treated group ($30.45 \pm 6.39\%$, $n=6$ sections vs. $8.19 \pm 1.01\%$, $n=6$ sections; $p<0.0063$) (Fig. 4). Therefore, these results suggest that

Figure 3. Masson's Trichrome staining of wound tissues. (A) Control group (CON). (B) Substance P-treated group (SP). (C) Thiorphan-treated group (Thior). (D) SP and thiorphan co-treated group (SP/Thior). Arrows indicate wound edges. The black boxed regions in a, b, c, and d are magnified in a', b', c', and d', respectively. Black broken lines indicate wound edges and arrow heads indicate Masson's Trichrome stained collagens in wound margins. Scale bar, 100 μ m.

Figure 4. Immunostaining for α -smooth muscle actin (α -SMA) of wound margins. (A) α -SMA was stained in brown and nuclei were stained with Fast-Red in pink. Control (CON), substance P-treated group (SP), thiorphan-treated group (Thior), and SP and thiorphan co-treated group (SP/Thior). Black arrows indicate α -SMA positive blood vessels. Scale bar, 100 μ m. Quantitative results are shown in (B). Data are expressed as mean $\pm$ SD.

the inhibition of endogenous NEP would affect synergically the effects of SP on angiogenesis.

DISCUSSION

This study shows that the co-treatment of SP and thiorphan, an inhibitor of NEP, synergically increased angiogenesis in acute skin wounds 5 days after induction of the wounds. However, the co-treatment did not additively or synergically affect wound contraction or ECM deposition.

What is the mechanistic basis(s) of the synergic increase of angiogenesis by SP and thiorphan? The expression level of NEP is regulated by various factors such as oxygen level and aging. The regulation of NEP expression on HUVECs is mediated by HIF1- α under hypoxic condition [22] and aging affects NEP expression levels on dermal fibroblasts [7]. Therefore, it could be expected that the expression of NEP would be different on various cell types such as fibroblasts and endothelial cells in wounds and that they would have different sensitivity to NEP inhibitors. This would lead to the different effects of thiorphan on various biological processes carried out by the different cell types present in skin. Because fibroblasts might have lower expression level of NEP compared to endothelial cells, we were able to observe the synergic effect of SP and thiorphan only on angiogenesis, but not on wound contraction that follows ECM deposition in wounds. It would be interesting to further compare the expression level or the activity of NEP on fibroblasts which synthesize ECM with that on endothelial cells which form blood vessels. However, it would be also possible that endogenous level of SP stabilized by thiorphan would be a threshold level to induce angiogenesis, regardless of a sensitivity of endothelial cells to thiorphan (or an expression level of NEP on endothelial cells). Therefore, SP injection was able to induce angiogenesis and the co-treatment of SP and thiorphan, a NEP inhibitor, was able to synergically facilitate angiogenesis.

NEP is known to degrade various peptides such as basic FGF-2 and endothelin-1 [12,13], both essential in angiogenesis and vascular homeostasis. SP stimulates the expression and secretion of endothelin-1 in melanocytes [23] and also modulates the expression of FGF-2 in fibroblasts. Therefore, NEP inhibitor thiorphan would indirectly regulate SP effects on angiogenesis through the stabilization of angiogenic factors induced by SP.

The synergic increase in angiogenesis by SP and thiorphan co-treatment is very useful not only in wound healing but also in regeneration of various tissues and organs. More importantly, the co-treatment did not show any synergic or additive deposition of ECM. Therefore, the co-treatment of SP and thiorphan would become a therapeutic strategy for accelerating wound healing and regeneration of blood vessel rich tissues/organs through the

enhancement of angiogenesis without any risk of fibrosis.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Kyung Hee University Animal Institutional Review Board.

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