Mini Review

Nicotine at a low concentration promotes wound healing

Naoki Morimoto*, Satoru Takemoto, Takeshi Kawazoe, Motoko Naito and Shigehiko Suzuki

Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Kyoto University

The adverse effects of smoking on wound healing of the skin are known clinically. Recently, an endogenous cholinergic pathway for angiogenesis mediated by endothelial nicotinic acetylcholine receptors was discovered. The objective of this study was to investigate the appropriate concentration of nicotine that accelerated angiogenesis and wound healing. Experiments on tube formation were conducted using an Angiogenesis Kit. Basic fibroblast growth factor (10ng/ml) and nicotine (10^{-10}M, 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-4}M, 10^{-2}M) were added to the conditioned medium. The conditioned medium was used as a control. The area and length of each tube were calculated using an Angiogenesis Image Analyzer. Full-thickness skin defects (8mm) were created on the dorsum of C57BL mice and a silicone sheet (8mm) was sutured. PBS (10 μl), bFGF (1 μg), and nicotine (10^{-1}M, 10^{-4}M, 10^{-7}M) were topically injected for seven days (n=5). Significant differences in area and length of newly formed tubes were seen between the control group and bFGF and the 10^{-10}M nicotine-added groups. The wound area was significantly decreased in the wound treated with bFGF and 10^{-4}M of nicotine. The epithelium length was significantly longer in the wounds treated with bFGF and 10^{-4}M of nicotine. In this study, nicotine accelerated angiogenesis and promoted wound healing.

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* Correspondence should be addressed to:
N.Morimoto, Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Phone: +81-75-751-3613, Fax: +81-75-751-4340, e-mail: mnaoki22@kuhp.kyoto-u.ac.jp

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Introduction

It is generally accepted that cigarette-smoking delays wound healing. Nicotine is one of the toxic constituents of cigarette smoke. Nicotine increases vasoconstriction and reduces nutritional blood flow to the skin[1]. Nicotine also increases platelet adhesiveness and damages the endothelial layers of the vessels, resulting in microvascular occlusion and tissue ischemia[2]. In addition, proliferation of red blood cells, fibroblasts, and macrophages is reduced by nicotine[3,4]. Recently, it was reported that nicotine stimulated angiogenesis via stimulation of nicotinic acetylcholine receptors[5,6]. The cytotoxicity of nicotine has been observed at higher blood concentrations (>10^{-6}M), although nicotine stimulated DNA synthesis and proliferation of endothelial cells at lower concentrations (<10^{-6}M)[7,8]. In this study,

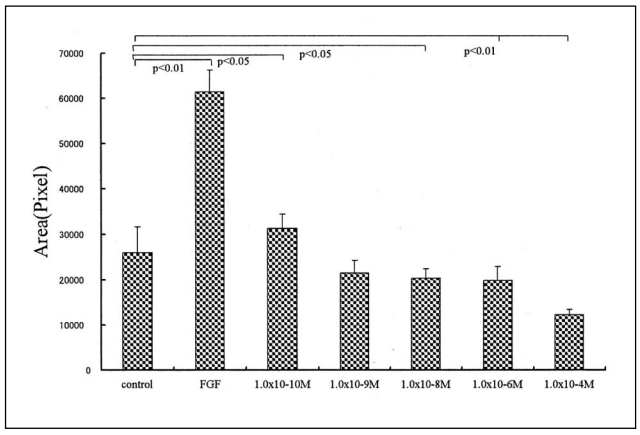

Fig.1 Quantitative analyses of the tube area

Tube area is expressed as pixel mean ±SD. The area in FGF was larger compared with the control ($p < 0.01$). The areas of nicotine of $1.0x10^{-10}$M and $1.0x10^{-8}$M were larger compared with control ($p < 0.05$). The areas of nicotine of $1.0x10^{-6}$M and $1.0x10^{-4}$M were smaller compared with the control ($p < 0.01$).

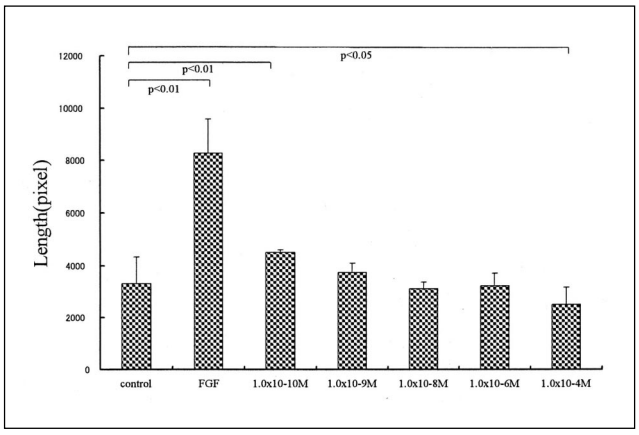

Fig.2 Quantitative analyses of the tube length

Tube length is expressed as pixel mean ± SD . The length in FGF was longer compared with the control ($p < 0.01$). The length of nicotine of $1.0x10^{-10}$M was longer compared with the control ($p < 0.01$). The length of nicotine of $1.0x10^{-4}$M was shorter compared with the control ($p < 0.01$).

we investigated the appropriate concentration of nicotine that accelerated angiogenesis and wound healing in comparison with basic fibroblast growth factor *in vitro* and *in vivo*. Basic fibroblast growth factor (bFGF) is a cytokine known to stimulate the proliferation of fibroblasts and capillary endothelial cells and promote angiogenesis. Recombinant human bFGF has already been used clinically to treat skin ulcers in Japan. We observed tube formation of cultured human umbilical vein endothelial cells (HUVEC) in a HUVEC/fibroblasts co-culture system in the presence or absence of various concentrations of nicotine and bFGF. Then, we examined the wound healing using a full-thickness skin defect created on the backs of mice.

Nicotine promotes tube formation of HUVECs *in vitro*

An angiogenesis assay kit (Kurabo, Okayama, Japan) was used according to the manufacturer's instructions. Briefly, HUVECs co-cultured with human fibroblasts were cultured in conditioned medium and a medium containing 10ng/ml bFGF (Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) and nicotine (10^{-10}M, 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-4}M, 10^{-2}M). The medium was changed every three days. After 12days, cells were fixed with ethanol, and newly formed tubes were visualized with a tubule staining kit (Kurabo, Okayama, Japan). Mouse anti-human CD31 (Kurabo, Okayama, Japan) was used as a primary antibody and 3,3-diamino-benzidine-tetrahydrochloride (DAB) was the substrate. Micrographs of six different fields per well were taken at a magnification of 40x. The area and length of each tube were quantified using angiogenesis imaging software (Kurabo, Okayama, Japan). Quantitative analyses of tube area and tube length revealed that bFGF and nicotine of $1.0x10^{-10}$M promoted tube formation significantly (**Fig.1, 2**). The tube formation decreased as the density of nicotine increased, and significant differences both in the area and length were found between the control group and nicotine of $1.0x10^{-4}$M group. In the presence of nicotine at $1.0x10^{-2}$M, HUVECs and fibroblasts did not proliferate and no tube formation was observed.

In this assay, bFGF promoted the greatest tube formation of HUVECs *in vitro*. Nicotine of $1.0x10^{-10}$M also promoted tube formation *in vitro*, but higher concentrations of nicotine damaged cells. It was reported that the nicotine concentration in the blood during smoking was around $1.0x10^{-7}$M[9, 10]. In our experimental result, during smoking nicotine did not promote angiogenesis.

Nicotine accelerates wound healing *in vivo*

Animal experiments were carried out according to a protocol approved by the Animal Experimental Committee of Kyoto University Graduate School of Medicine. Seven-week-old C57BL/6JJcl mice (n=25, Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) were used. Mice were anesthetized by intraperitoneal injection of a mixture (1:1) of pentobarbital (Abbott Laboratories, North Chicago, USA) and atropine sulfate (Tanabe Seiyaku Co., Ltd., Tokyo, Japan) (0.3 mg/mouse). After shaving and depilation, 8mm full-thickness skin defect including the panniculus carnosus was created on the back of the animal. A

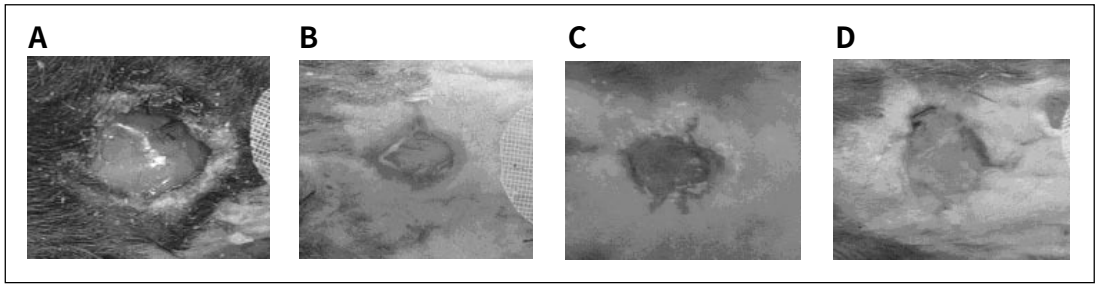

Fig.3 Appearances of wounds on day 8
Wounds were treated with PBS (A), with bFGF solution (B), with nicotine solution of 1.0 × 10^{-4}M (C), and with nicotine solution of 1.0 × 10^{-7}M (D).

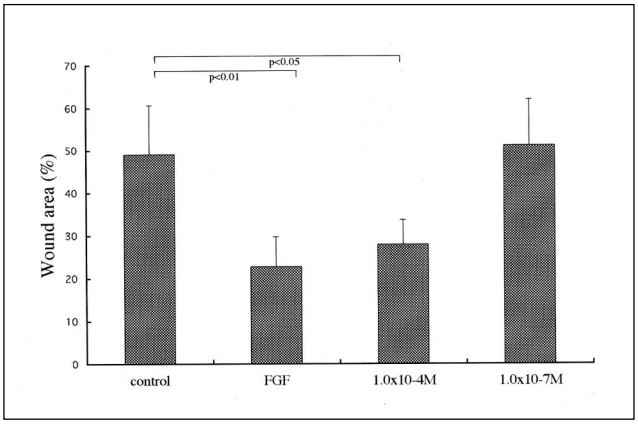

Fig.4 Comparison of wound area on day 8
Wound area is expressed as mean ±SD . The wound area in FGF was smaller compared with the control ($p < 0.01$). The wound area of nicotine of 1.0x10^{-4}M was smaller compared with the control ($p < 0.05$).

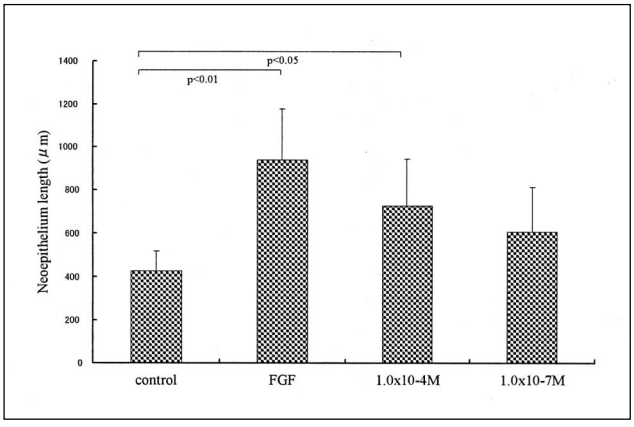

Fig.5 Comparison of neoepithelium length
Neoepithelium length is expressed as mean ± SD. The neoepithelium length in FGF was longer compared with the control ($p < 0.05$). The neoepithelium length in nicotine of 1.0x10^{-4}M was longer compared with the control ($p < 0.05$).

silicone sheet 8mm in diameter (Gunze Ltd, Ayabe, Japan) was applied to the wound and it was sutured using 5-0 nylon. PBS of 10μl, bFGF solution (1μg/10 μl) 10μl, and nicotine solution (10^{-1}M, 10^{-4}M, 10^{-7}M) of 10 μl were administered under the silicon sheet using a micropipette once daily for seven days. Five mice were used in each group. Solutions of nicotine and bFGF were prepared in PBS. The applied dose of bFGF (2 μg/cm^2) was set higher than the clinical dose (1 μg/cm^2), because some amount of bFGF was lost for a movement of the mouse. Nicotine solution of the 10^{-4}M density disturbed tube formation, but no cellular toxicity was observed *in vitro* assay (**Fig.1, 2**).

On day 8, mice were sacrificed by intraperitoneal injection of pentobarbital. After the silicone sheet was removed, we photographed the wounds and measured the epithelized area using NIH imaging software (version 1.62, National Institutes of Health, U.S.A.). The epithelized area was expressed as a percentage of the original wound area. Tissue specimens were harvested and sectioned axially. Samples were fixed with formalin and paraffin-embedded, then stained with hematoxylin and eosin. Using a light microscope, the neoepithelium length of each specimen was measured twice, from one edge and from the other edge, and the average of these two lengths was used for statistical analysis.

The appearances of wounds on day 8 are shown in **Fig.3**. The wound areas administered with bFGF solution and nicotine solution of 1.0x10^{-4}M were significantly smaller compared with the control (**Fig.4**). The neoepithelium lengths administered with bFGF solution and nicotine solution of 1.0x10^{-4}M were significantly larger compared with the control (**Fig.5**). In the granulation tissue beneath the neoepithelium, newly formed vessels were observed in bFGF and nicotine of 1.0x10^{-4}M administrated groups. Mice administered with nicotine solution of 1.0x10^{-1}M died within a few hours.

It was difficult to use nicotine systemically because of its side effects. Nicotine at 1.0x10^{-4}M promoted wound healing and

neoepithelization the same as bFGF. This density was higher than the experiment *in vitro*. This was because nicotine was diluted after it had been administered. At lower concentrations, nicotine produced no effect, and at higher concentrations mice died due to nicotine toxicity.

BFGF has already been used clinically and significant side effects were not reported. In this study, bFGF promoted angiogenesis and accelerated the wound healing most. Nicotine also promoted angiogenesis and wound healing, but it was inferior to bFGF. Nicotine stimulated angiogenesis via stimulation of nicotinic acetylcholine receptors and bFGF stimulated via FGF receptors. Though there might be a multiplier effect of bFGF and nicotine, it was difficult to use nicotine clinically for various kinds of side effects.

All data were analyzed by Fisher's protected least significant difference (Fisher's PLSD) and expressed as mean ± SD (standard deviation). A value of $p < 0.05$ was accepted as statically significant.

Conclusion

Nicotine at an appropriate concentration accelerated angiogenesis *in vitro* and promoted wound healing *in vivo*. Although, nicotine has cellular toxicity and it is difficult to use systemically.

Acknowledgement

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