Topical Application of Plasma Fibronectin in Full-Thickness Skin Wound Healing in Rats

A-Hon Kwon,*,[1] Zeyu Qiu,* and Yutaka Hirao†

*Department of Surgery, Kansai Medical University, Osaka, Japan; and †Research & Development Division, Hirakata Laboratory, Benesis Corporation, Osaka, Japan

Fibronectin (Fn) has been shown to play an important role in wound healing because it appears to be the stimulus for migration of fibroblasts and epidermal cells. The purpose of this study was to investigate whether topical application of plasma Fn (pFn) improves healing of full-thickness skin wounds in rats. A round section of full-thickness skin (diameter of approximately 15 mm) was resected in rats. Animals were then divided into two groups, and wounds were treated topically with a single application of human plasma albumin (control group) or human pFn (FN group). Wound closure rate, hydroxyproline concentration, and histologic features (immunohistochemical staining) were evaluated. The FN group had a significantly higher wound closure rate and hydroxyproline level in the skin than the control group. Histologic analysis of macrophage and fibroblast migration, collagen regeneration, and epithelialization were significantly increased in the FN group compared with the control group. A single topical application of pFn increased the migration of macrophages, myofibroblasts, and fibroblasts. Moreover, further release of transforming growth factor-β1 from activated fibroblasts, keratinocytes, and epithelial cells may also contribute to the beneficial effect of pFn on wound healing. Exp Biol Med 232:935–941, 2007

Key words: fibronectin; wound healing; transforming growth factor-β1; fibroblast; hydroxyproline

Introduction

It has been documented that cytokines such as transforming growth factor-β1 (TGF-β1), platelet-derived growth factors, and migratory and proliferative fibroblasts are key factors for achieving optimal wound healing. TGF-β1 is released immediately after a wound is incurred at the inflammatory stage; it initiates the granulation of tissue (1, 2), promotes subsequent re-epithelialization and proliferation of fibroblasts and keratinocytes, and increases deposition of collagen (3). Infiltrating fibroblasts migrate to and proliferate in the wound bed; they are responsible for the synthesis of new extracellular matrix (ECM) proteins and collagen, which are the initial components of granulated tissue and lead to the formation of scar tissue (4). Impaired wound healing, including excessive scaring, delayed wound healing, and dehiscence of the operative wound, is a significant clinical problem, and wound care is an important factor in clinical situations. Many techniques, including wound bed preparation, biosurgery, wound dressings, topical negative pressure therapy, and topical adjuvants such as growth factor and bioengineered tissue have been used in attempts to accelerate wound healing (5), and more effective approaches to the problem of wound healing are needed.

Fibronectin (Fn) is a dimeric glycoprotein that interacts strongly with other components of the ECM and is involved in a number of biologic processes, such as cellular adhesion, motility, differentiation, hemostasis, and wound healing (6–8). Wide immunologic cross-reactivities among human and other mammalian species of Fn have been reported, and several monoclonal antibodies to human Fn have been used to identify Fns in a wide range of mammals (9). Two forms of Fn have been identified: plasma fibronectin (pFn), which is expressed by hepatocytes and secreted in a soluble form into the plasma, and cellular Fn, an insoluble form expressed locally by fibroblasts and other types of cells and deposited into and assembled in the ECM (10). Fn has been shown to play an important role in wound healing because it appears to be the stimulus for migration of fibroblasts and epidermal cells (11, 12). The skin functions primarily as a protective barrier against the environment. Injury, illness, or surgery results in the loss of integrity of large portions of the skin and leads to major disturbances of this barrier function. The purpose of this study was to investigate whether topical application of pFn improves full-thickness skin wound healing in rats.

This study was supported in part by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (16591372).

[1] To whom correspondence should be addressed at Department of Surgery, Kansai Medical University, 10-15 Fumizono, Moriguchi, Osaka, 570-8507, Japan. E-mail: kon@takii.kmu.ac.jp

Received May 18, 2006.
Accepted March 8, 2007.

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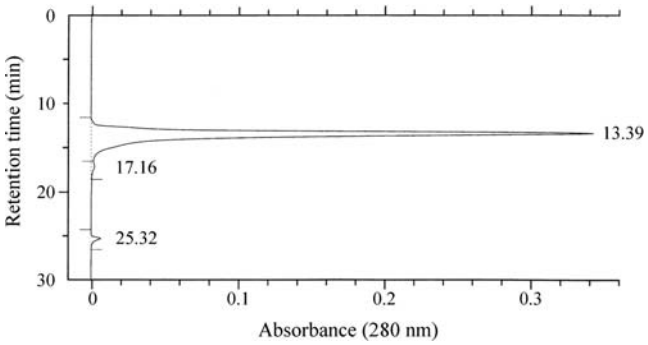

Figure 1. Purity of pFn was analyzed by high-performance liquid chromatography. The main peak is the pFn fraction (approximately 440 kDa); retention time, 13.39 mins; area percent: 97.77%.

Materials and Methods

Purified pFn. Human pFn was purified from plasma cryoprecipitate (Benesis Co., Osaka, Japan). The purification process included heat-defibrinogenation for 30 mins at 50°C in EDTA, citrate, and aprotinin, fractionation with 25% ammonium sulfate, and ion-exchange chromatography using diethyaminoethyl-Sephadex A50 (Pharmacia Biotech Inc., Uppsala, Sweden). Heat treatment of purified pFn was performed for 10 hrs at 60°C in a solution containing sucrose and citrate.

Analysis of the purity of pFn (approximately 440 kDa) was performed by high-performance liquid chromatography. A 7.5-cm SWP column was used as a precolumn and a 30-cm G3000SWXL column was used as the fractionation column (Toyo Soda Manufacturing, Tokyo, Japan). Both columns were equilibrated with 0.05 *M* acetate buffer containing 0.3 *M* NaCl (pH 6.8). Thirty microliters of a sample solution was passed through the columns at a flow rate of 0.5 ml/min. Protein concentration was monitored by absorbance at 280 nm. The purity of the pFn used in this study was approximately 98% (Fig. 1). The purified pFn was stored at a concentration of 44.4 mg/ml in 0.15 *M* NaCl at –40°C. pFn concentrations were determined using an Fn enzyme immunoassay kit (Takara Shuzo Co., Shiga, Japan).

Animals. Male Wistar rats (Simizu Co., Ltd., Kyoto, Japan) weighing 180–200 g were housed individually in rooms maintained at a constant temperature and humidity. The animals had free access to food and water and were subjected to a 12:12-hr light:dark cycle. Food was withdrawn overnight prior to experiments. All experimental animals used in this study were treated according to guidelines set by the Animal Care and Use Committee of Kansai Medical University Animal Center.

Surgical Procedure. Rats were anesthetized with an intraperitoneal (ip) injection of sodium pentobarbital (45 mg/kg body wt). The dorsal regions were shaved with an electric clipper, and the surgical area was disinfected with 70% alcohol. A round section of full-thickness skin (diameter of approximately 15 mm) was resected with scissors, and hemostasis was obtained by direct pressure using sterile gauze. Animals were then divided into two groups, and wounds were treated topically with several single-dose (0.1, 1.0, and 2.5 mg) applications of human pFn (FN group) or rats were given the 1.0-mg dose of human plasma albumin (Benesis Corp.) and served as the control group.

Wounds and surrounding areas were then covered with an adhesive-permeable dressing (Bioclusive; Johnson and Johnson Medical, Skipton, UK).

Estimation of Wound Healing (Wound Closure). Curative effect on the wound (wound closure) was evaluated by tracing the outer margins of the wound on each rat. Wound tracings were scanned using an image scanner (EPSON GT-8000; Seiko Epson Corp., Nagano, Japan), and images were exported to an image processing program (NIH Image, version 1.52; public domain software). Wound areas were traced manually and calculated in mm^2. The wound closure rate was expressed as the percentage of wound area compared with that on postoperative day (POD) 0 (100%). Rats were sacrificed on POD 1, 3, 5, 7, or 14 using an overdose of sodium pentobarbital (300 mg/kg ip) for hydroxyproline analysis and histologic evaluation.

Hydroxyproline Analysis. Granulation tissue from the skin wounds and adjoining normal skin was harvested and stored at –20°C until analysis. Tissues were dried to a constant weight and were hydrolyzed in 6 *M* HCl for 18 hrs at 110°C. Samples were dried on a hot plate and then washed three times with distilled water. The acid-free samples were reconstituted in 2.0 ml of acetate-citrate buffer (1.2% sodium acetate trihydrate, 5% citric acid, 12% sodium acetate, 3.4% sodium hydroxide [pH 6.0]). Five hundred microliters of 0.05 *M* chloramine-T was added to 1 ml of sample, after which samples were incubated for 20 mins at room temperature, followed by the addition of 0.5 ml of 15% perchloric acid and 15% 4-dimethyl aminobenzaldehyde in 1-propanol. After incubation for 15 mins at 60°C, each sample was transferred to a microtiter plate and absorbance read at 550 nm. Hydroxyproline concentrations of the unknowns were calculated from a linear standard curve and are presented as μg/mg dry tissue weight (13).

Histologic Analysis. The wound and surrounding tissues were fixed with 10% formalin, embedded in paraffin, and sectioned. Sections of 5-μm thickness were stained using the naphthol AS-D chloroacetate esterase technique (14) for neutrophil infiltration. Macrophages, fibroblasts, myofibroblasts, vascularization, and epithelialization were examined by immunohistochemical stains.

For macrophage staining, anti-macrophage marker mouse monoclonal antibody (NCL-MAC387; Novo Castra Lab, Newcastle, UK) was diluted 1:1000 in 1% bovine serum albumin in 0.05 *M* phosphate-buffered saline (pH 7.5) as the primary antibody, and biotinylated goat anti-mouse IgG (Nichirei Co., Tokyo, Japan) was used as the secondary antibody. Fibroblasts were identified by TGF-β1 staining: anti-human TGF-β1 rabbit polyclonal antibody (Yanaihara Inc., Shizuoka, Japan) was diluted 1:500 as the

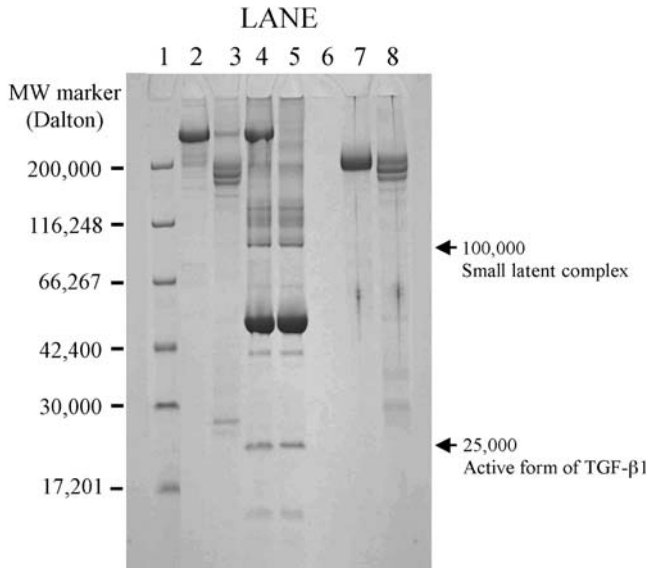

Figure 2. Separation of the purified pFn specimens by SDS-PAGE. Lane 1, Molecular weight marker. Lane 2, Purified pFn (nonreducing conditions) gave a main band at approximately 440 kDa, which dissolved into smaller molecules when heated. The faint band at approximately 220 kDa could be distinguished. Lane 3, Commercial pFn (nonreducing conditions). Lane 4, Purified pFn+ TGF-β1 (from human platelets). Lane 5, TGF-β1. Lane 6, empty. Lane 7, Purified pFn (reducing conditions) gave a main band at approximately 220 kDa. Lane 8, Commercial pFn (reducing conditions).

primary antibody, and biotinylated goat anti-rabbit IgG (Nichirei Co.) was used as the secondary antibody. The myofibroblasts also were evaluated by measuring α-smooth muscle actin (α-SMA) immunoreactivity. The sections were stained with monoclonal mouse anti–α-SMA antibody (1:500; DAKO, Carpinteria, CA) and biotinylated goat anti-rabbit IgG (Nichirei Co.) as the secondary antibody. Vascularization was estimated from vessel staining using anti–von Willebrand factor rabbit polyclonal antibody (1:1200; DAKO) as the primary antibody and biotinylated goat anti-rabbit IgG (Nichirei Co.) as the secondary antibody. Cytokeratin was used as the marker of epithelialization: anti-cytokeratin wide-spectrum screening rabbit polyclonal antibody (1:2000; DAKO) and biotinylated goat anti-rabbit IgG (Nichirei Co.) were used as the primary and secondary antibodies, respectively. Collagen type I and type III were stained using anti–collagen type I and anti–collagen type III goat polyclonal antibodies (1:400; Southern Biotechnology Associates Inc., Birmingham, AL), respectively, as the primary antibodies and biotinylated rabbit anti-goat IgG (DAKO) as the secondary antibody. The chromogenic reaction was performed with 3,3′-diaminobenzidine-4HCl. Positive staining was indicated by a brown color.

Three separated sections of each wound were examined by light microscopy. The number of neutrophils, macrophages, fibroblasts, and vessels were counted in five high-power fields (×100) over three separated sections. The stained section of collagen type I and type III and cytokeratin were displayed at ×40 magnification on a monitor connected to a computer system. The areas stained in brown were measured using an image processing program (analySIS; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Statistical Analyses. All data are expressed as means ± SEM. Comparisons among groups were performed using one- or two-way ANOVA, followed by Bonferroni's post hoc test when variances across groups were equal or by Dunnett's T3 post hoc test when variances were not equal. Variance equality was tested by Levene statistical analysis. $P < 0.05$ was considered to be statistically significant.

Results

Separation of Purified pFn Specimens by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). To confirm that no small amounts of TGF-β1 were mixed in with the purified pFn specimens, we further separated the purified pFn specimens and commercial human pFn (Chemicon International, Temecula, CA) by SDS-PAGE under both reducing and nonreducing conditions (Fig. 2). In Figure 2, the positive control (Lane 5, TGF-β1 from human platelets; Calbiochem Co., San Diego, CA) illustrates the abundant active form of TGF-β1 band at 25 kDa and latent TGF-β1 band at 100 kDa (arrows in Fig. 2). These bands of TGF-β1 were not found in the purified and commercial pFn specimens (Lanes 2, 3, 7, and 8). When TGF-β1 was added to purified pFn specimens (Lane 4), bands corresponding to the active form of TGF-β1 and latent TGF-β1 were again visible. These results suggest that there were no traceable amounts of TGF-β1 in our purified pFn specimens; therefore, the possibility that the improved effect on diabetic wound healing is due to TGF-β1 mixed in with the purified pFn could be excluded.

In addition, purified pFn (nonreducing conditions) gave a major band at approximately 440 kDa, which was degraded to molecules of lower molecular mass upon heating (Lane 2); however, only a faint band at approximately 440 kDa could be distinguished in the commercial pFn (Lane 3). Both preparations of pFn showed the same behavior under reducing conditions (Lanes 7 and 8). These results suggest that commercial pFn contained several fragments of pFn.

Effect of Human pFn on Wound Healing. Several single doses (0.1, 1.0, and 2.5 mg) of pFn were topically administered, and serial changes in the relative wound area (wound closure) were measured on POD 0, 3, 5, 7, 10, and 14. A single administration of topical pFn resulted in rapid wound healing by POD 10. Significant differences were observed at doses of 1.0 and 2.5 mg of pFn compared with the control group ($P = 0.003$ for 1.0-mg dose; $P = 0.018$ for 2.5-mg dose) (Fig. 3). However, because the wound closure rate did not increase in a dose-dependent manner, we used a dose of 1.0 mg of pFn on POD 0 in subsequent experiments.

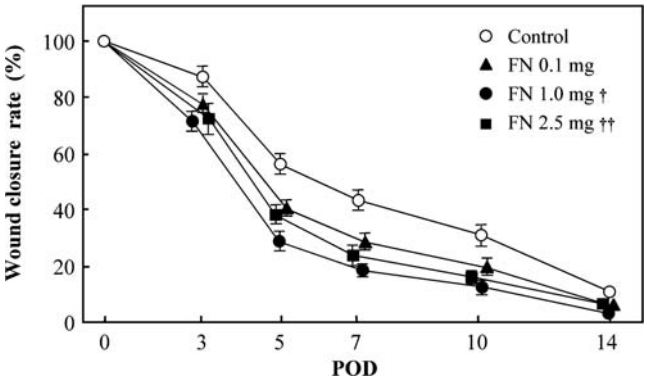

Figure 3. Wound closure in response to various doses of pFn. The wound closure rate was expressed as the percent of wound area compared with that on POD 0 (100%). Values are means ± SEM, n = 6 for each group. †$P = 0.003$ and ††$P = 0.018$ vs. control group (two-way ANOVA). Control, albumin (1 mg/rat).

Histologic Analysis. *Cellular Components.* A single topical application of pFn reduced the number of infiltrating neutrophils detected by AS-D chloroacetate esterase staining during skin wound healing, and significant differences were seen on POD 1, 3, 5, and 7 compared with the control group (Fig. 4a). Conversely, macrophage migration, identified by immunohistochemical staining, was significantly greater in the FN group than in the control group on POD 1 (Fig. 4b). The number of migrated fibroblasts expressing TGF-β1 in the subcutaneous layer after the wounding period was significantly greater in the FN group than in the control group on POD 3 and 5 (Fig. 5a). Moreover, the number of myofibroblasts (fibroblasts

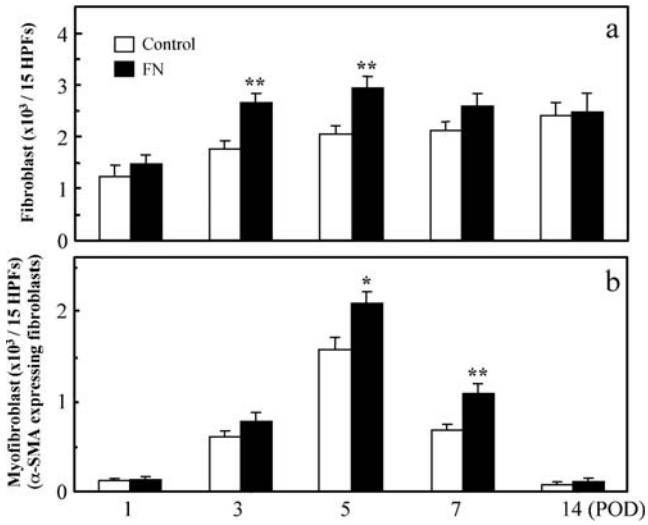

Figure 5. Effect of pFn on fibroblast and myofibroblast migration during skin wound healing. (a) Number of infiltrated fibroblasts (immunohistochemical staining with anti-human TGF-β1 rabbit polyclonal antibody). (b) Number of infiltrated myofibroblasts (immunohistochemical staining with monoclonal mouse anti–α-SMA antibody). Values are means ± SEM, n = 5 for each time point. *$P < 0.05$ and **$P < 0.01$ vs. control group (one-way ANOVA). FN, plasma fibronectin (1 mg/rat); control, albumin (1 mg/rat).

expressing α-SMA) during skin wound healing was significantly higher in the FN group than in the control group on POD 5 and 7 (Fig. 5b). Vascularization identified by von Willebrand factor was enhanced in the FN group on POD 7; however, there was no significant difference from the control group (Fig. 6a). Finally, the epithelialization area stained for cytokeratin was significantly increased after skin

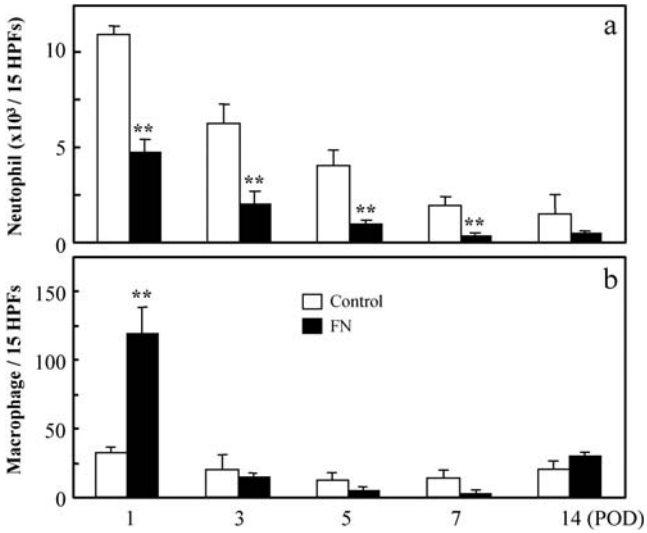

Figure 4. Effect of pFn on neutrophil infiltration and macrophage migration during skin wound healing. (a) Number of infiltrated neutrophils stained with naphthol AS-D chloroacetate esterase. (b) Number of infiltrated macrophages (immunohistochemical staining with anti-macrophage marker mouse monoclonal antibody). Values are means ± SEM, n = 5 for each time point. **$P < 0.01$ vs. control group (one-way ANOVA). FN, plasma fibronectin (1 mg/rat); control, albumin (1 mg/rat); HPFs, high power fields.

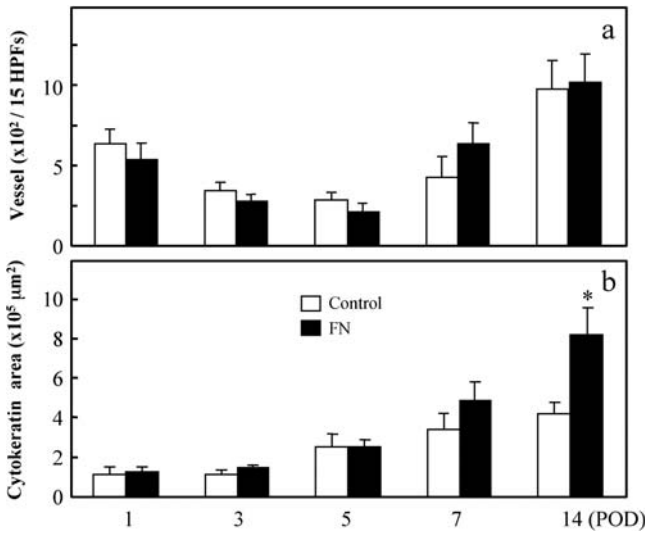

Figure 6. Effect of pFn on vascularization and epithelialization during skin wound healing. (a) Number of vessels (immunohistochemical staining with anti–von Willebrand factor rabbit polyclonal antibody). (b) Cytokeratin areas (immunohistochemical staining with anti-cytokeratin wide-spectrum screening rabbit polyclonal antibody). Values are means ± SEM, n = 5 for each time point. *$P < 0.05$ vs. control group (one-way ANOVA). FN, plasma fibronectin (1 mg/rat); control, albumin (1 mg/rat).

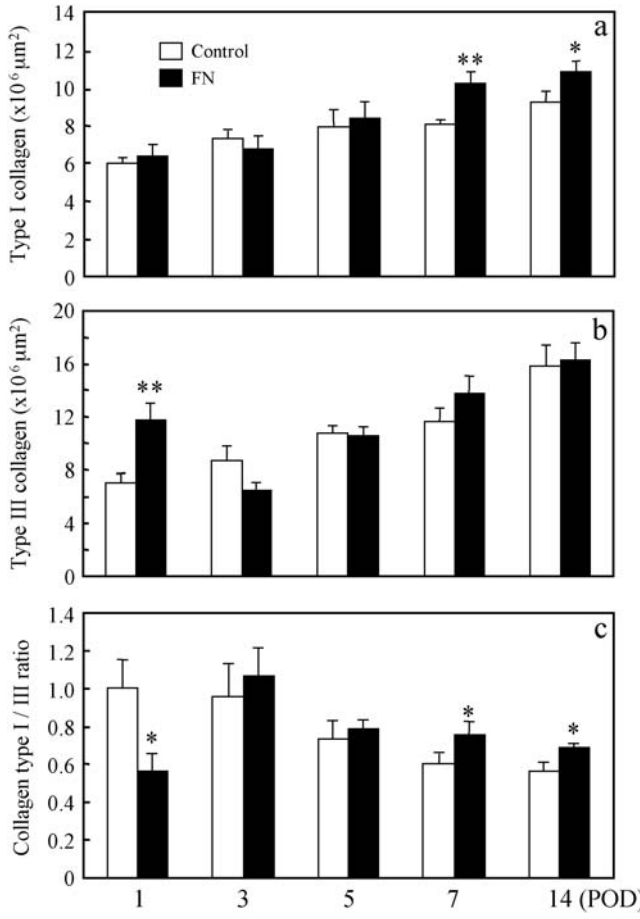

Figure 7. Effect of pFn on collagen regeneration during skin wound healing. (a) Collagen type I areas (immunohistochemical staining with anti–collagen type I goat polyclonal antibody). (b) Collagen type III areas (immunohistochemical staining with anti–collagen type III goat polyclonal antibody). (c) Collagen type I:III ratio. Values are means ± SEM, n = 5 for each time point. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group (one-way ANOVA). FN, plasma fibronectin (1 mg/rat); Control, albumin (1 mg/rat).

wounding in the FN group compared with the control group on POD 14 (Fig. 6b).

ECM Components. The collagen type I regeneration area increased during skin wound healing, and significant differences were seen on POD 7 and 14 compared with the control group (Fig. 7a). The collagen type III regeneration area was significantly wider in the FN group than in the control group on POD 1 (Fig. 7b). Finally, the collagen type I:III ratio was significantly lower on POD 1 and significantly higher on POD 7 and 14 in the FN group than in the control group (Fig. 7C). The hydroxyproline level in the skin wound sites after a single topical application of pFn was higher than in the control group, with significant differences noted on POD 1, 3, 5, and 7 (Fig. 8).

Discussion

The wound-healing process can be categorized as follows: inflammation, proliferation (formation of granulated tissue), and tissue remodeling. Re-epithelialization of

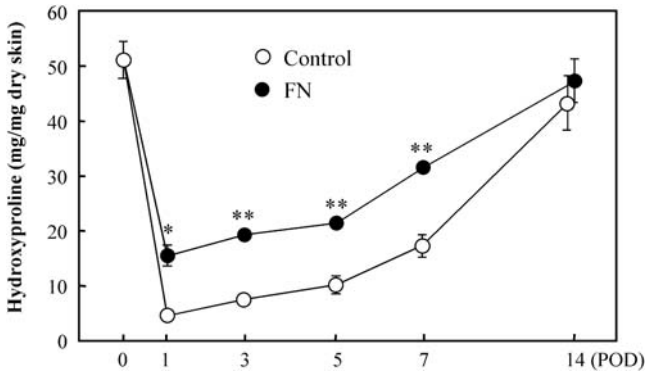

Figure 8. Effect of pFn on hydroxyproline concentration during skin wound healing. Values are means ± SEM, n = 5 for each time point. $^{*}P < 0.01$ and $^{**}P < 0.0001$ vs. control group (one-way ANOVA). Control (open circles), albumin (1 mg/rat); FN (closed circles), plasma fibronectin (1 mg/rat).

wounds begins within hours of injury and proceeds first over the margin of the residual dermis and subsequently over granulation tissue (15, 16). Injury to the skin and concomitant blood vessel disruption lead to extravasation of blood constituents, followed by platelet aggregation and blood clotting. These events initiate inflammation and set the stage for repair processes. In response to tissue loss, fibroblasts proliferate and migrate into the defect until the wound is populated by fibroblasts and ECM (17).

The Fn molecule is mainly constructed from three modular units, denoted Fn1, Fn2, and Fn3. Atomic resolution structures are now available for all three single modules, for Fn1 and Fn3 module pairs, and for the disulphide-linked joining of the Fn monomers (18). Fn promotes the spread of platelets at the site of injury; the adhesion and migration of neutrophils, monocytes, fibroblasts, and endothelial cells into the wound region; and the migration of epidermal cells through granulation tissue. At the level of matrix synthesis, Fn appears to be involved in the organization of both the granulation tissue and basement membrane (19). In some injuries, such as ischemic brain injury, extravasation of pFn occurs but cellular Fn is not expressed in the wounded tissue (20).

The inflammation response is considered to be a key process during which growth factors, including TGF-β1 and platelet-derived growth factor, are released from macrophages and platelets to initiate granulation of tissue (1, 2, 21). The macrophage both scavenges tissue debris and releases a plethora of biologically active substances that include growth factors (22). Epidermal cells located at the wound edge undergo keratinocyte activation and adopt several different phenotypes to permit migration across the wound, including the disassembly of desmosomes, which provide physical connections between the cells; retraction of intracellular tonofilaments (23); expression and/or redistribution of several integrin receptors; and formation of peripheral cytoplasmic actin filaments, which allow cell movement (24). The expression of integrin receptors on

epidermal cells allows them to interact with a variety of ECM proteins (e.g., Fn and vitronectin) and invade the fibrin clot in the wound space (25, 26). Fn and laminin-5 are present at the basal aspect of migrating keratinocytes in all stages of wound healing, serving as putative ligands for integrins (27).

Fn matrix assembly is a cell-mediated process in which soluble dimeric Fn is converted into a fibrillar network. Binding of cell surface α5β1 integrin receptors to Fn converts it to an active form that promotes fibril formation through interactions with other cell-associated Fn dimers. As Fn fibrils form on the outside of the cell, cytoplasmic domains of integrin receptors organize cytoplasmic proteins into functional complexes within. Movement of α5β1 integrins and associated proteins along stress fibers toward the cell center redistributes intracellular components into paxillin-rich focal adhesions and tensin-rich fibrillar adhesions (28). Thus, the appearance of Fn and the appropriate integrin receptors that bind Fn, fibrin, or both to fibroblasts seems to be the rate-limiting step in the formation of granulation tissue, providing a scaffold or conduit for cell migration (29–31). After migrating into wounds, fibroblasts synthesize the ECM, and following stimulation by TGF-β1, the provisional, fibrin-based ECM is gradually replaced by collagenous matrix (32, 33). Type I collagen is the most abundant type of collagen in the normal dermis (approximately 90%). Type III collagen is the collagen actively secreted by fibroblasts during the early stages of wound healing and may account for up to 30% of the collagen in a healing wound. During remodeling in a healing wound, type III collagen is replaced by type I collagen, restoring the normal dermal collagen profile (34).

TGF-β1 is one of the cytokines that is known to promote formation of granulation tissue, is expressed during active fibroplasia in wound healing, and induces cell influx in the inflammatory phase (2, 23, 35). TGF-β1–stimulated proliferation is dependent on Fn matrix assembly. Anti-Fn antibodies that block matrix assembly also inhibit the ability of TGF-β1 to stimulate fibroblast proliferation. Although both Fn and collagen are present in the matrix, only the Fn matrix is necessary for the TGF-β response (36). Alternatively, the Fn matrix may be necessary for fibroblasts to transit the cell cycle (37). Dermal fibroblasts are responsible for the synthesis of new ECM proteins, primarily type I and III collagen, that initially make up granulated tissue and form new normal tissue or scar tissue (4). In the present study, the number of infiltrating fibroblasts expressing TGF-β1 and hydroxyproline content were significantly increased in the wounds of pFn-treated rats compared with the control group. Broadley *et al*. (38) reported that the exogenous addition of TGF-β1 increased cellularity and hydroxyproline content in the wounds of diabetic rats. The deposition of hydroxyproline reflects an equivalent amount of collagen in the granulation tissue of a wound; therefore, elevated levels of plasma active TGF-β1 lead to prestimulation of monocytes, causing the rapid infiltration of cells and an increase in collagen deposition (39). To the contrary, polyclonal anti-human Fn antibodies block collagen binding to Fn and inhibit collagen accumulation in the ECM (40).

On the other hand, the myofibroblast is a key cell for the connective tissue remodeling that takes place during wound healing and fibrosis development (41). The myofibroblastic modulation of fibroblastic cells begins with the appearance of the protomyofibroblast, which has stress fibers that contain only cytoplasmic β- and γ-actins. The protomyofibroblast usually, but not necessarily always, evolves into the appearance of the differentiated myofibroblast, the most common variant of this cell, with stress fibers containing α-SMA. The switch from protomyofibroblast to differentiated fibroblast has been related to the secretion by inflammatory cells and perhaps the fibroblasts themselves of TGF-β1, the most recognized stimulator of myofibroblastic differentiation (3). The action of TGF-β1 depends on the local presence of the cellular Fn splice variant ED-A (42). Thus, myofibroblast differentiation is regulated by both cell products and ECM components.

Low doses of exogenous supplies of pFn precluded firm conclusions on the enhancement of wound healing (43, 44). Lariviere *et al*. (45) demonstrated that exogenously applied high concentrations of pFn (>20 μg/mm^2) significantly stimulates the formation of new granulation tissue and therefore can accelerate the healing process. In the present study, a relatively low dose (13 μg/mm^2) of pFn also enhanced excisional wound healing, which may have been dependent on the purity of the pFn (98%) used in this study.

In conclusion, our results indicated that pFn has beneficial effects on skin wound healing. It seems that pFn initially increases the migration of macrophages, myofibroblasts, and fibroblasts. Migrating fibroblasts then synthesize collagen, contributing to wound healing. In addition, the further release of TGF-β1 from activated fibroblasts, keratinocytes, may also contribute to the beneficial effect of Fn on wound healing. Therefore, a single application of topical pFn may have a good "cost/benefit" balance and be useful for wound treatment.

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