Overexpression of hepatocyte growth factor/scatter factor promotes vascularization and granulation tissue formation in vivo

Mitsuo Toyoda[a], Hisashi Takayama[a], Norio Horiguchi[a], Toshiyuki Otsuka[a], Toshio Fukusato[b], Glenn Merlino[c], Hitoshi Takagi[a,*], Masatomo Mori[a]

[a] *First Department of Internal Medicine, Gunma University School of Medicine, Showa-Machi 3-39-15, Maebashi 371-8511, Gunma, Japan*
[b] *Division of Diagnostic Pathology, Gunma University School of Medicine, Showa-Machi 3-39-15, Maebashi 371-8511, Gunma, Japan*
[c] *Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD 20892-4255, USA*

Received 26 August 2001; revised 25 October 2001; accepted 26 October 2001

First published online 13 November 2001

Edited by Ned Mantei

Abstract The effect of hepatocyte growth factor/scatter factor (HGF/SF) during wound healing in the skin was investigated, using HGF/SF-overexpressing transgenic mouse model. Histological analysis of HGF/SF transgenic mouse excisional wound sites revealed increased granulation tissue with marked vascularization. Northern blot analysis demonstrated that, relative to control, vascular endothelial growth factor (VEGF) expression in transgenic skin was significantly higher at baseline and was robustly up-regulated during wound healing. Elevated levels of VEGF protein were detected immunohistochemically, predominantly in endothelial cells and fibroblasts within the granulation tissue of HGF/SF transgenic skin. Serum levels of VEGF were also elevated in HGF/SF transgenic mice. Thus, results from our study suggest that HGF/SF has a significant effect on vascularization and granulation tissue formation during wound healing in vivo, involving with induction of VEGF.

Key words: Hepatocyte growth factor/scatter factor; Vascular endothelial growth factor; Wound healing; Angiogenesis; Transgenic mouse

1. Introduction

The process of cutaneous wound healing is characterized by four overlapping repair phases involving hemostasis, inflammation, proliferation, and remodeling. After injury, new tissue formation starts with re-epithelialization and is followed by granulation tissue formation. The latter process encompasses macrophage accumulation, fibroblast ingrowth, matrix deposition and angiogenesis [1]. Angiogenesis, the formation of new blood vessels from existing blood vessels, is an essential step during wound healing and is driven in part by a complex mixture of growth factors and cytokines, which are released into the area of injury. New vessels are necessary for supplying nutrients and oxygen to the cells involved in the repair process, for removal of debris and formation of granulation tissue. Of a number of cytokines involved in the wound healing process, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor (TGF) α have been detected in wound transudate and reported to contribute to the angiogenic response after injury [2–4].

Hepatocyte growth factor/scatter factor (HGF/SF) was first recognized as a highly potent hepatocyte mitogen [5,6]; however, it is now known to be a pleiotropic cytokine that helps regulate cell growth, movement, morphogenesis, development, and regeneration of various tissues [7–11]. Its receptor has been identified as the c-Met proto-oncogene product, a transmembrane tyrosine kinase that is expressed on the surface of epithelial cells [8,12]. HGF/SF is produced mainly by mesenchymal cells such as fibroblasts and smooth muscle cells [13–15] and is considered to play an important role in mesenchymal-epithelial/endothelial interactions that contribute to embryogenesis, organ regeneration, wound healing, and angiogenesis [16–19].

Previous investigations have established that HGF/SF directly stimulates proliferation and migration of cultured endothelial cells, promotes development of capillary-like structures in vitro, and stimulates blood vessel formation in Matrigel plugs and in the cornea [19–22]. More recently, HGF/SF has been shown to produce an angiogenic effect via induction of VEGF in vitro [23,24]. Although it is known that HGF/SF influences the growth of endothelial cells and keratinocytes [23–25], the precise in vivo effects of HGF/SF during skin wound healing are still poorly understood. In this study, we investigated the in vivo effects of HGF/SF on the process of wound healing using the HGF/SF-overexpressing transgenic mice as a model system.

2. Materials and methods

2.1. Animals

Transgenic mice overexpressing a mouse HGF/SF cDNA by means of the mouse metallothionein gene promoter were generated on an FVB/N background as described previously [26]. All animal studies were performed according to the guidelines for animal care and use established by Gunma University School of Medicine.

2.2. In vivo wound assay

Animals were anesthetized by inhalation of diethyl ether, and the dorsal hair was removed using a hair remover (Hair Remover, National, Osaka, Japan). Full-thickness dermal wounds (6 mm diameter)

*Corresponding author. Fax: (81)-27-220 8136.
E-mail address: htakagi@med.gunma-u.ac.jp (H. Takagi).

Abbreviations: bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; HGF/SF, hepatocyte growth factor/scatter factor; HPF, high power field; PECAM-1, platelet endothelial cell adhesion molecule-1; TGF, transforming growth factor; VEGF, vascular endothelial growth factor

0014-5793/01/$20.00
PII: S0014-5793(01)03126-X

were made on the dorsum of 7-week-old female mice with a standard biopsy punch (Maruho, Osaka). At selected time intervals, mice were sacrificed and wounds were harvested with a rim of approximately 2 mm of unwounded tissue, fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

2.3. Immunohistochemistry

For immunohistochemical analysis of VEGF, formalin-fixed and paraffin-embedded wounds were sectioned at a thickness of 10 μM. An anti-mouse VEGF monoclonal antibody (Santa Cruz Biotechnology, CA, USA) was used at a concentration of 2 μg/ml at room temperature for 1 h. After rinsing, slides were incubated with the secondary goat anti-mouse antibody for 1 h at room temperature. Slides were then rinsed and incubated with an avidin–biotin–peroxidase complex (Vectastain ABC kit, Burlingame, CA, USA) followed by 3,3′-diamino-benzidine tetrahydrochloride (Wako, Tokyo, Japan) color development. The distribution of HGF/SF protein was analyzed as described above except that anti-human HGF/SF monoclonal antibody (R&D systems, Minneapolis, MO, USA) was used at a concentration of 50 μg/ml at room temperature for 15 h and then slides were incubated with the secondary goat anti-mouse antibody. Immunolocalization of platelet endothelial cell adhesion molecule-1 (PECAM-1) was performed as described above except that frozen embedded sections were fixed in acetone for 5 min, and sections were incubated with secondary rat anti-mouse antibody. The antibody (clone MEC 13.3, Pharmingen, San Diego, CA, USA) was applied at a concentration of 10 μg/ml.

2.4. Northern blot analysis

Wounds from mice at different time points were excised and frozen immediately in liquid nitrogen. For each time point, six wounds were pooled and total RNA was prepared using TRIZOL reagent (Gibco BRL, Grand Island, NY, USA) following the instructions provided by the manufacturer. For Northern blot hybridization, 20 μg of total RNA were loaded per lane onto an agarose gel, electrophoresed and then transferred to nylon membrane. HGF/SF and c-Met transcripts were detected with a ^{32}P-labeled mouse cDNA probe generated by polymerase chain reaction (PCR) as described previously [26,27]. VEGF transcripts were detected with a ^{32}P-labeled human VEGF cDNA probe generated by PCR using the clone LA737 (kindly provided by Dr. W.J. LaRochelle) as a template, and the following set of primers: 5′-GTAAAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGAC-3′. To control for RNA loading and transfer variation, filters were routinely rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

2.5. Quantification of wound healing

The number of blood vessels was analyzed under a microscope by counting PECAM-1-positive figures within granulation tissue (area per high power field (HPF)). Estimation of neodermal formation was performed on Azan Mallory-stained sections. Using a standard ocular grid, the thickness of the intact dermis and thickness of the dermis at the center of the wound site were measured. The percentage of neodermal formation was estimated by the following formula: % neodermal formation = (thickness of dermis at the center of the wound/thickness of intact dermis) $\times 100$, as modified by Swift et al. [28].

2.6. Determination of VEGF levels in serum

Blood samples were collected and serum was separated by centrifugation, immediately frozen, and stored at −80°C. Serum was collected from five animals per time point for each genotype. Serum VEGF levels were determined using a mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the method recommended by the manufacturer.

2.7. Statistical analysis

The results are expressed as mean ± S.D. Statistical analysis was performed by repeated measure analysis of variance and subsequent Fisher's PLSD test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. HGF/SF transgene expression in the skin

Northern blot analysis showed that the 2.4 kb HGF/SF transgenic transcript was strongly expressed in the skin of transgenic mice (Fig. 1). When the c-Met receptor mRNA level was analyzed by Northern blot hybridization, no significant difference in amount of c-Met transcript was detected between HGF/SF transgenic and FVB/N control mice.

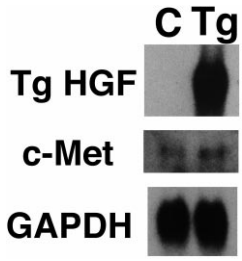

Fig. 1. Analysis of transgenic HGF/SF and endogenous c-Met transcripts in transgenic mouse skin by Northern blot hybridization. HGF/SF transgene expression and endogenous c-Met expression in skin from 7-week-old control (C) and transgenic mice (Tg) before wound induction. Transgenic HGF (Tg HGF) was strongly expressed in the skin of transgenic mice. No significant difference in the amount of c-Met transcripts was detected between HGF/SF transgenic and FVB/N control mice. Filters were rehybridized with a GAPDH probe to assess RNA loading and transfer variation.

3.2. Histological appearance of healing wounds

Microscopic examination revealed that regenerating granulation tissue from the HGF/SF transgenic mice exhibited more prominent vascularity and a highly accelerated granulation tissue formation relative to control mice. These two dramatic changes became apparent from day 3 and were obvious at day 21 of the observation period. In H&E-stained sections, HGF/SF transgenic mouse wounds demonstrated more robust dermal blood vessel formation, around which dense and thick mesenchymal matrix deposition was observed (Fig. 2B, D versus Fig. 2A, C). Examination of Azan Mallory-stained wound sections revealed dense and thick stromal tissue formation in HGF/SF transgenic mice (Fig. 2F) compared with non-transgenic littermates (Fig. 2E).

To further quantify this overt increase in blood vessels, immunohistological analysis of endothelial cells was carried out using anti-PECAM-1 antibodies. Fig. 2H shows a marked increase in the number of blood vessels in the regenerating transgenic skin compared with wild type (Fig. 2G). No significant differences were observed in capillary content prior to wounding. However, a prominent number of red blood cells were observed in granulation tissue of HGF/SF transgenic mice commencing at day 3, accompanied by the formation of new blood vessels. Quantification of blood vessels demonstrated a prolonged period of neovascularization in HGF/SF transgenic mice (Fig. 3A). On day 14, the number of blood vessels in granulation tissue of HGF/SF transgenic mice (45.3 ± 7.9 per HPF) was significantly increased compared with control mice (27.6 ± 1.7 per HPF) ($P < 0.01$). Unlike control wounds, in which a decrease in vascularization occurred with time, HGF/SF transgenic mouse wounds remained highly vascularized throughout the observation period (61.8 ± 10.7 and 13.5 ± 1.9 per HPF on day 21, in HGF/SF transgenic and control mice, respectively) ($P < 0.01$).

We also quantified the effect of HGF/SF overexpression on granulation tissue formation in our wound model (Fig. 3B). The rate of neodermal formation was calculated using the formula described in Section 2. Neodermal formation of

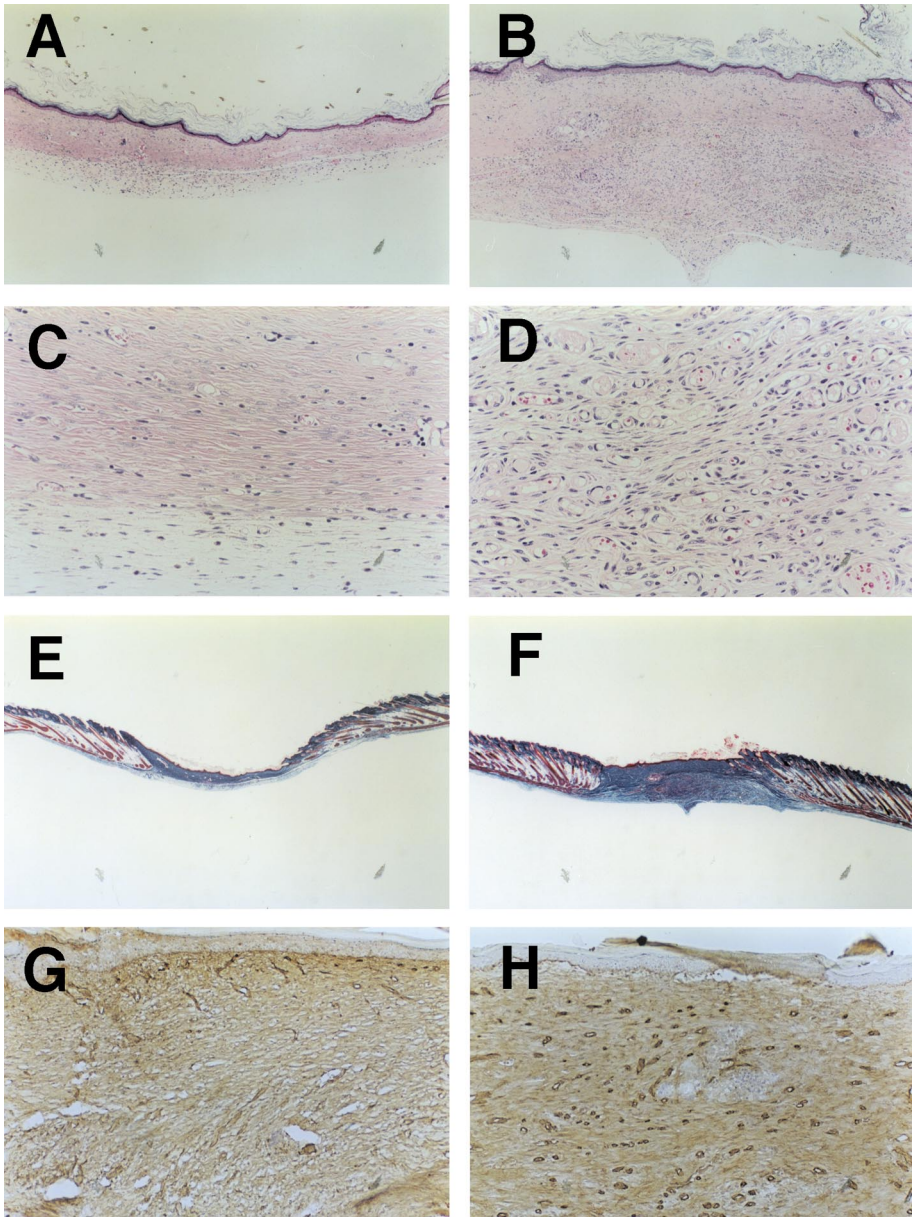

Fig. 2. Histological features during wound healing in the skin of control and HGF/SF transgenic mice (day 21). Low power view of wound sites from control (A) and HGF/SF transgenic (B) mice. Note the markedly thickened granulation tissue in the transgenic wound site (B). C and D: High power views of granulation tissue from the same animals shown in (A) and (B), respectively. The presence of markedly increased and highly vascularized granulation tissue was observed in transgenic mice. E and F: Azan Mallory-stained sections demonstrate greater deposition of collagen fibers in the HGF/SF transgenic mouse wounds (F) than in control wounds (E). G and H: PECAM-1 staining demonstrated a marked increased number of blood vessels in the granulation tissue of transgenic skin (H) than in controls (G). A–D: H&E; E and F: Azan Mallory; G and H: anti-PECAM-1 antibody. Original magnifications: A and B, ×40; C and D, ×200; E and F, ×10; G and H, ×100.

non-transgenic mice was calculated to be an average of 12.8% on day 3, 36.6% on day 7 and 56.7% on day 14. In contrast, neodermal formation in the HGF/SF transgenic mice proceeded much more rapidly with an average of 15.3, 75.2 and 97.4% on days 3, 7 and 14, respectively. Even at day 21, wounds in non-transgenic mice had not completely recovered (approximately 80% of neodermal formation).

3.3. *VEGF mRNA expression associated with HGF/SF during wound healing*

In order to determine whether the observed HGF/SF-mediated angiogenesis detected during wound healing was associated with VEGF induction, VEGF mRNA levels before and after injury were analyzed by Northern blot hybridization (Fig. 4A). Previously, VEGF mRNA transcripts of various sizes (2.7∼4.2 kb) have been reported in a number of mouse tissues [29]. In the present study, a 4.2 kb transcript was observed in both HGF/SF transgenic and non-transgenic mice, and an additional 3.7 kb transcript was observed only in the HGF/SF transgenic mice. In both genotypes, the level of 4.2 kb transcript reached a peak value on day 7, however, it was more strongly up-regulated in HGF/SF transgenic mice than in non-transgenic mice. In HGF/SF transgenic mice, the 3.7 kb transcript was observed before injury,

reached a peak level on day 3, and then decreased towards day 14.

3.4. Serum levels of VEGF during wound healing

Serum VEGF levels were measured on days 0, 7, and 14 after wound induction (Fig. 4B). Prior to injury, serum levels of VEGF in HGF/SF transgenic mice (68.1 ± 10.3 pg/ml, range 60.2–85.3 pg/ml) were already higher than those in their non-transgenic littermates (32.1 ± 9.17 pg/ml, range 18.9–42.9 pg/ml) ($P < 0.01$). At day 7, serum VEGF levels in HGF/SF transgenic mice (104.3 ± 11.4 pg/ml, range 91.1–115.6 pg/ml) were significantly elevated compared with those at baseline ($P < 0.01$), while VEGF levels of non-transgenic littermates (41.6 ± 6.90 pg/ml, range 32.3–48.2 pg/ml) exhibited no significant changes. In HGF/SF transgenic mice, serum VEGF levels declined to baseline by day 14.

3.5. Immunolocalization of HGF/SF and VEGF during wound healing

Immunohistological analysis at day 14 revealed that HGF/SF protein was detected mainly in endothelial cells and fibroblasts within the granulation tissue at a higher level in transgenic mice relative to controls (Fig. 5A versus B). The expression level of VEGF was affected by overexpression of HGF/SF; fibroblasts and endothelial cells in the granulation tissue expressed much higher levels of VEGF in HGF/SF transgenic mice than in non-transgenic littermates (Fig. 5C versus D).

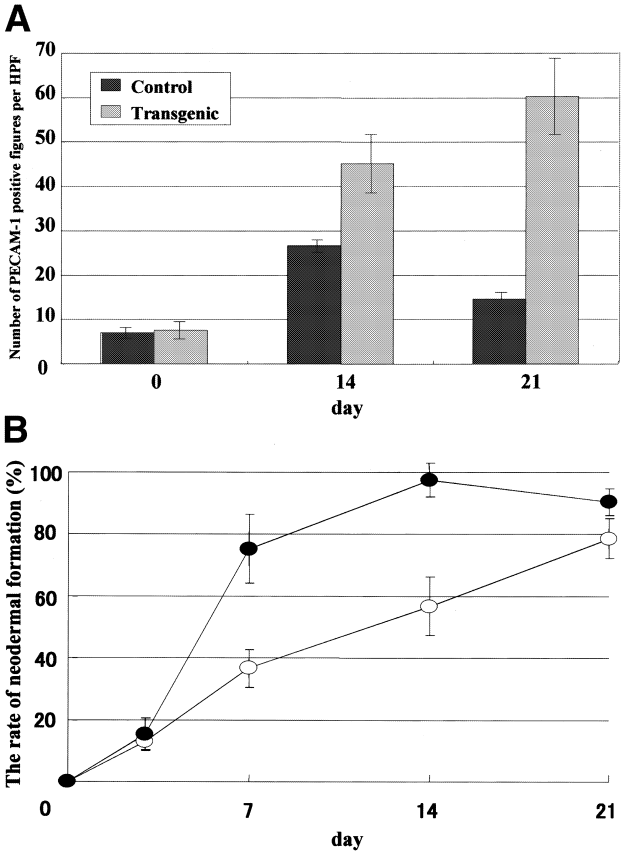

Fig. 3. A: Quantification of blood vessels on days 0, 14 and 21. At day 14, the number of blood vessels in granulation tissue from HGF/SF transgenic mice was significantly higher than in control mice ($P < 0.01$), and remained high on day 21 ($P < 0.01$). Six wounds per time point for each genotype were quantitated (area per HPF). B: Promotion of granulation tissue formation by HGF/SF overexpression. The rate of neodermal formation was calculated using the formula described in Section 2. Neodermal formation occurred much more rapidly in HGF/SF transgenic mice than in control mice. ○: control mice, ●: HGF/SF transgenic mice. Six wounds per time point for each genotype were quantitated.

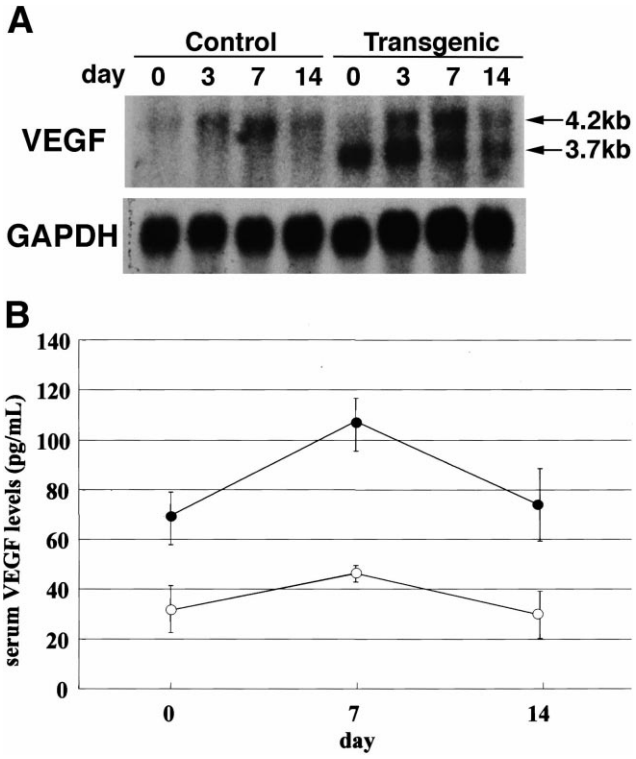

Fig. 4. A: Northern blot analysis of VEGF transcripts in mouse skin during wound healing. Transcripts of approximately 4.2 kb were observed in both HGF/SF mice and non-transgenic mice, while an additional transcript of 3.7 kb was observed only in HGF/SF transgenic mouse skin. In both genotypes, the 4.2 kb transcript peaked on day 7; however, it was more strongly up-regulated in HGF/SF transgenic mice than in controls. In HGF/SF transgenic mice, the 3.7 kb transcript was observed prior to injury, peaked on day 3, and then declined towards day 14. B: Serum levels of VEGF during wound healing. Serum VEGF levels were measured at days 0, 7 and 14. Prior to wound induction, serum VEGF in HGF/SF transgenic mice was already higher than in non-transgenic littermates ($P < 0.01$). At day 7, serum VEGF in HGF/SF transgenic mice was significantly elevated relative to baseline ($P < 0.01$), while non-transgenic littermates exhibited no significant changes. ○: control mice, ●: HGF/SF transgenic mice. Five samples per time point for each genotype were analyzed.

4. Discussion

The results of the present study demonstrate that overexpression of HGF/SF accelerates neovascularization as well as granulation tissue formation during wound healing. This is the first report, to the best of our knowledge, in which HGF/SF has been shown to promote angiogenesis involving with induction of VEGF in vivo.

The effect of growth factors on wound healing has been investigated both through administration of growth factors and by analyzing transgenic animals. TGFα, bFGF, epidermal growth factor, and keratinocyte growth factor have all been shown to promote epithelial regeneration and dermal tissue formation [30–34]. Quaglino et al. reported that TGFβ1 enhanced granulation tissue formation but not regen-

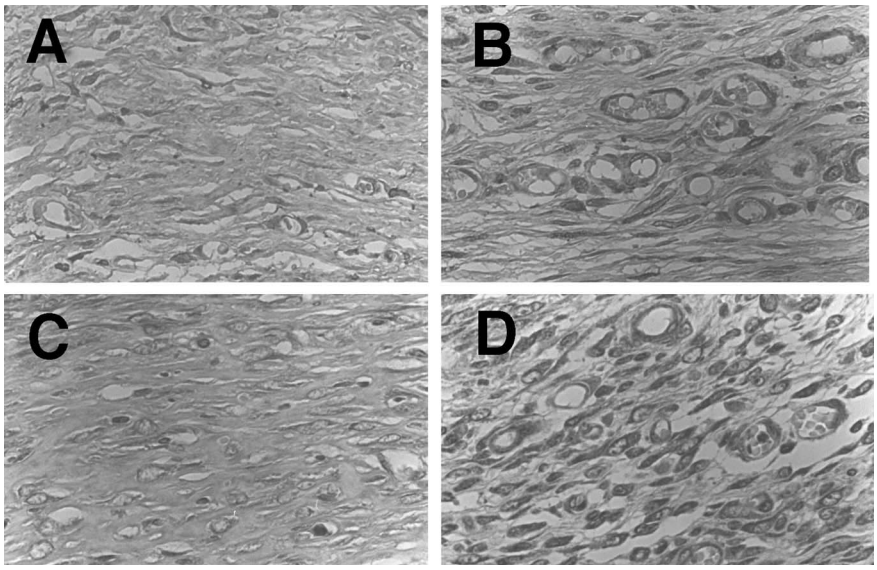

Fig. 5. Immunohistological analysis of HGF/SF and VEGF during wound healing. Sections of day 14 wounds were stained with an anti-HGF/SF (A and B) and anti-VEGF antibody (C and D). Intense HGF/SF and VEGF staining were detected in the endothelial cells and fibroblasts in granulation tissue in HGF/SF transgenic mice (B) and (D) relative to their non-transgenic littermates (A) and (C). Original magnification: ×500.

eration of the epithelia [35]. With respect to angiogenesis, an essential process of wound healing, VEGF, bFGF, and TGFα have been reported to have a potent angiogenic effect after injury [2–4]. VEGF, originally purified on the basis of its vascular permeability-enhancing activity [36], is a powerful vascular endothelial cell-specific mitogen [37,38] and acts as a mediator of both physiologic and pathologic angiogenesis [39–41]. On the other hand, HGF/SF, which was first recognized as a highly potent hepatocyte mitogen [5,6], has been identified as an angiogenic growth factor [19–22]. Recent studies have shown that HGF/SF is capable of inducing VEGF in cultured keratinocytes and endothelial cells [23,24]. Moreover, Belle et al. demonstrated that the combination of HGF/SF and VEGF produced an additive effect on proliferation of endothelial cells and a synergistic effect on endothelial migration [23]. HGF/SF thus may affect the biological behavior of endothelial cells through a combination of the direct effects of HGF/SF itself and indirect effects mediated via induction of VEGF in vitro. The observed up-regulation of VEGF in our HGF/SF-overexpressing wound model is consistent with recent in vitro studies. Since HGF/SF-dependent up-regulation of VEGF was observed at the level of both mRNA and protein, it is probable that angiogenesis in this transgenic wound healing model was due in part to VEGF induction through HGF/SF overexpression.

Although the level of VEGF expression in HGF/SF transgenic mice was already higher than in controls prior to wound induction, no overt phenotype concerning vascular bed was noted in the skin as well as other organs. Alternatively, it has been shown that intra-arterial or intra-muscular administration of VEGF may significantly augment perfusion and development of collateral vessels in a rabbit model where chronic hindlimb ischemia was created by surgical removal of the femoral artery [42]. However, the exact in vivo effect of administration of VEGF in the absence of tissue damage is not clear. It is possible that VEGF associated with HGF/SF overexpression only stimulates angiogenesis under specific pathologic conditions, such as tissue injury or hypoxia resulting from arterial occlusion. To date, several reports have demonstrated induction of VEGF by HGF/SF in various cell types, such as keratinocytes, endothelial cells, and glioma cells that express the c-Met receptor [23,24,43]. In this point Dong et al. reported that HGF/SF induces expression of VEGF through both MEK- and PI3K-dependent pathways [44].

We also demonstrated that overexpression of HGF/SF affected the generation of granulation tissue. A previous study demonstrated that HGF/SF enhanced wound closure by influencing the migratory and spreading response in cultured intestinal epithelial cells [45]. Based on the duration of wound scab formation and microscopic examination, HGF/SF overexpression did not overtly affect wound closure in the present study. However, neodermal formation was highly promoted, as judged by enhanced proliferation of endothelial cells and fibroblasts in HGF/SF transgenic mice. We suggest that proliferation of endothelial cells in transgenic mice may be due to the combined effect of HGF/SF itself and HGF/SF-induced VEGF. Since fibroblasts do not express c-Met, enhanced fibroblast proliferation in transgenic granulation tissue may be explained by a secondary effect of highly organized new blood vessels on the supply of nutrients and oxygen to the cells, rather than a direct effect of HGF/SF.

Recent studies using a rabbit model of hindlimb ischemia showed that administration of recombinant human HGF/SF was associated with a significant improvement in collateral formation and regional blood flow, and a significant reduction in muscle atrophy [23]. Overexpression of HGF/SF in our study induced rapid formation of highly vascularized granulation tissue, in which increased cell proliferation and markedly thicker collagen deposition were noted. These results imply a therapeutic utility for HGF/SF administration on skin wound sites such as deep burns or refractory skin ulcers associated with various diseases. However, we previously demonstrated that chronic exposure to high levels of HGF/SF caused several adverse effects such as renal dysfunction, intestinal disorders and, ultimately, neoplasia at multiple sites [27,46–48]. Therefore, when applied as a clinical reagent, short

term and/or local, rather than systemic, administration of HGF/SF would be desirable for treating wounds. For example, HGF/SF could be applied topically over the wound site, or injected locally at the wound edge.

In summary, we have demonstrated here that neovascularization and granulation tissue formation are accelerated under conditions of overexpression of HGF/SF. Our results suggest that HGF/SF has a significant effect on organization and vascularization of granulation tissue during wound healing in vivo, involving the induction of VEGF.

Acknowledgements: This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of the Japanese Government. The authors thank Dr. William J. LaRochelle for providing clone LA737. We also thank Dr. Osamu Ishikawa and Dr. Sachiko Akimoto for helpful discussions.

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