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Recruitment of a Prostaglandin E Receptor Subtype, EP3-Expressing Bone Marrow Cells Is Crucial in Wound-Induced Angiogenesis

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E-type prostaglandins have been reported to be proangiogenic *in vivo*. Thus, we examined prostaglandin receptor signaling relevant to wound-induced angiogenesis. Full-thickness skin wounds were created on the backs of mice, and angiogenesis in wound granulation tissues was estimated. Wound closure and re-epithelization in EP3 receptor knockout mice ($EP3^{-/-}$) were significantly delayed compared with their wild-type (WT) mice, whereas those in $EP1^{-/-}$, $EP2^{-/-}$, and $EP4^{-/-}$ were not delayed. Wound-induced angiogenesis estimated with CD31 immunohistochemistry in $EP3^{-/-}$ mice was significantly inhibited compared with that in WT mice. Immunoreactive vascular endothelial growth factor (VEGF) in wound granulation tissues in $EP3^{-/-}$ mice was markedly less than that in WT mice. Wound closure in WT mice was delayed significantly by VEGF neutralizing antibody compared with control IgG. Wound-induced angiogenesis and wound closure were significantly suppressed in $EP3^{-/-}$ bone marrow transplantation mice compared with those in WT bone marrow transplantation mice. These were accompanied with the reductions in accumulation of VEGF-expressing cells in wound granulation tissues and in mobilization of VEGF receptor 1-expressing leukocytes in peripheral circulation. These results indicate that the recruitment of EP3-expressing cells to wound granulation tissues is critical for surgical wound healing and angiogenesis via up-regulation of VEGF. ***(Am J Pathol 2006, 169:1458–1472; DOI: 10.2353/ajpath.2006.051358)***

Nonsteroidal anti-inflammatory drugs that inhibit the enzyme cyclooxygenase (COX) and suppress prostaglandin (PG) synthesis have been widely used as anti-inflammatory, antipyretic, and analgesic agents. It was frequently reported that nonsteroidal anti-inflammatory drugs delay healing of experimental gastric ulcers by arresting epithelial proliferation in the margins of ulcers, interfering with re-epithelialization, and inhibiting angiogenesis in the granulation tissue.[1–4] Although the cellular and molecular mechanisms of ulcer healing have been extensively studied,[5–9] the PG-dependent mechanisms involved in the healing of surgical wounds remain virtually unexplored.

Angiogenesis is involved in many physiological and pathological conditions, including the female reproductive cycle, embryonic development, tumor growth, metastasis, chronic inflammation, and retinopathy.[10–17] Furthermore, it is widely accepted that the wound-healing process is highly dependent on angiogenesis.[18] The angiogenic process is complex and includes local degradation of the basement membrane, endothelial cell proliferation and migration, tube and branch formation, and reconstitution of the basement membrane.[13,14] The complexity of this process implies that it is highly regulated with numerous endogenous factors.[19] It is generally accepted that the angiogenesis switch in a tissue is operated by both positive and negative factors. It is believed that the trigger of an angiogenic response represents an

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unbalanced situation of increasing angiogenic factor levels and decreasing angiogenesis inhibitor levels.[13,20,21]

PGs, including PGE_2, comprise a group of oxygenated metabolites of arachidonic acid that are produced by the sequential actions of COX and specific synthases. Two COX isoforms have been identified: COX-1 is constitutively expressed in various tissues, whereas COX-2 is induced by mitogens, cytokines, and tumor promoters. Disruption of the COX-2 gene in mice reduced the size of intestinal polyps generated by a mutation in the adenomatous polyposis (*APC*) gene. This reduction in the size of intestinal polyps was accompanied with reduced angiogenesis.[22] In this regard, endogenous PGs could be one of the contributors to angiogenesis.

For mechanistic analysis of angiogenesis *in vivo*, we have developed a sponge implantation model in which a polyurethane sponge disk implanted subcutaneously induces extensive angiogenesis in the surrounding proliferative granulation tissues.[23,24] With the use of this model, we have previously shown that angiogenesis not only occurs concomitantly with the induction of COX-2 mRNA but is also inhibited by the administration of either a nonselective nonsteroidal anti-inflammatory drug (indomethacin) or selective COX-2 inhibitors.[25] We further showed that PGE_2 topically injected into the sponge promoted angiogenesis.[25] Angiogenesis induced by either endogenous COX-2 or exogenous PGs was accompanied by increased expression of vascular endothelial growth factor (VEGF), and angiogenesis was abolished by administration of an anti-sense oligonucleotide specific for VEGF mRNA or a VEGF antibody. Reductions in angiogenesis and the development of granulation tissue were reported in proliferative inflammatory angiogenesis models in inducible PGE synthase knockout mice.[26] These results suggest that PGE_2 may mediate the angiogenic action of COX-2 *in vivo*. However, both the contribution of PGE_2 to the healing process of surgical wounds and the identity of the PG receptors responsible remain unknown.

PGs exert their biological actions by binding to specific receptors that contain seven transmembrane domains. Eight different PG receptors have been defined pharmacologically and cloned, including the PGD receptor (DP), four subtypes of PGE receptor (EP1, EP2, EP3, EP4), the PGF receptor (FP), the PGI receptor (IP), and the thromboxane (TX) receptor (TP).[17,27] Genes for each of these receptors have been disrupted, and the corresponding knockout mice have been produced.[28–37] Furthermore, with the use of the cloned receptors, agonists and antagonists highly selective for each of the four EP subtypes have been developed.[38–40]

With the use of agents acting selectively on EP receptors and of receptor knockout mice, we have now identified the EP receptor subtype responsible for facilitating wound-induced angiogenesis. Furthermore, we have transplanted EP receptor-lacking bone marrow (BM) cells into irradiated mice and have clarified the crucial roles of recruitment of EP receptor-expressing hematopoietic cells from BM on the wound-induced angiogenesis and the wound-healing processes. Our present results indicate that PGE_2-EP3 signaling on the BM-derived cells appears to be crucial for the wound-induced angiogenesis and the wound-healing processes and that the EP3 signaling pathway linked to the induction of a potent proangiogenic growth factor, VEGF, certainly has a proangiogenic action and facilitates wound closure.

Materials and Methods

Determination of Wound-Induced Angiogenesis

Female 8-week-old C57BL/6 wild-type mice (WT) and EP3 receptor knockout mice ($EP3^{-/-}$) were used for wound angiogenesis studies.[36] In some experiments, other types of EP receptor knockout mice lacking EP1, EP2, and EP4, respectively, were used for comparison.[27,36] Surgical wounds were made on the back of mice as reported previously.[41,42] After shaving the dorsal hair and cleaning the exposed skin with 70% ethanol, full-thickness excisional skin wounds of 8-mm diameter were made aseptically on either side of the dorsal midline using a 8-mm biopsy punch (Kai Industries, Tokyo, Japan). Four wounds were made on the same animal. Twelve hours after the above-mentioned surgery, after it was confirmed that the bleeding was completely stopped, the size of the wound was measured to give the day 1 result. Each wound region was digitally photographed at the fixed time, and the areas of the wounds were calculated by PhotoShop software, version 7.0 (Adobe Systems, San Jose, CA). Changes in the wound areas were expressed as the percentage of the wound areas at day 1. In some series of experiments, wounds and their surrounding area, including the scab and epithelial margins, were harvested with a fine pair of scissors in a genotype-blind manner after the mice were killed with an overdose of diethyl ether. All mice had free access to tap water and rodent chow and were housed individually with a 12-hour light/dark cycle and at a constant temperature ($25 \pm 1^{\circ}C$) and humidity ($60 \pm 5\%$). All experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Microvessel density in the wound granulation tissues was assessed as a parameter of wound-induced angiogenesis in a blind manner according to the established methods described previously.[16,43–45] The individual microvessels were counted in 10 100 μm $\times$ 100 μm fields of a wound granulation tissue, and the numbers were averaged. CD31-immunoreactive endothelial cells were stained with CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and were differentiated from other connective tissue elements. Microvessel density was expressed in terms of microvessel number per observed area (vessel number/mm^2).[41]

A COX-2 selective inhibitor, NS-398[46] or JTE522,[47] was suspended in 5% gum Arabic at a concentration of 6 mg/ml and was orally administered (0.05 ml/10 g body weight, 30 mg/kg, twice a day) to the WT mice. For vehicle control mice, distilled water with 5% gum Arabic was administered orally (0.05 ml/10 g body weight, twice a day). Aspirin was also administered to WT mice orally (30 mg/kg, twice a day). An anti-angiogenic agent, FR118487,[48,49] was administered to WT mice orally in the same manner as that of COX-2 inhibitors. The dose of

FR118487 was 6 mg/kg, twice a day. The administration of above-mentioned compounds was started from the day of wounding throughout the experimental periods.

Neutralizing antibody against mouse VEGF (IgG; Genzyme, Cambridge, MA),[16] which can block the biological activity of recombinant mouse VEGF164 and VEGF120 with the neutralizing $dose_{50}$ (100 ng/ml determined in the presence of 10 ng/ml of mouse VEGF164), was dissolved in physiological saline (14 μg/0.1 ml) and subcutaneously infused to WT mice with an Alzat miniosmotic pump (0.1 ml/7 days). For control mice, a nonimmune IgG fraction (14 μg/0.1 ml/7 days; Genzyme) was infused subcutaneously with the same osmotic pump. One day after pump implantation, full-thickness skin wounds were made. An orally active, low-molecular weight inhibitor of VEGF receptor (KDR/VEGFR 2) tyrosine kinase (ZD6474)[50] was administered to WT mice orally, in the same manner as that used for COX-2 inhibitors. The dose of ZD6474 was 50 mg/kg (twice a day).

For $EP3^{-/-}$ mice, human VEGF (Genzyme) dissolved in physiological saline (14 μg/0.1 ml) was subcutaneously infused with an Alzat miniosmotic pump (infusion rate was 0.1 ml/14 days). For vehicle-treated $EP3^{-/-}$ mice, physiological saline was infused subcutaneously with the same osmotic pump (infusion rate was 0.1 ml/14 days). One day after pump implantation, full-thickness skin wounds were made.

Murine Bone Marrow Transplantation (BMT) Model

Bone marrow (BM) cells were obtained by flushing the cavities of freshly dissected femurs, tibias, and pelves of donor male EP3 receptor knockout mice ($EP3^{-/-}$) with phosphate-buffered saline (PBS). Donor BM cells of their WT counterparts (male C57BL/6 mice, 8 weeks old) were also harvested by the same method. The flushed BM cells of each donor were dispersed by pipetting and resuspended in PBS at a density of 1×10^7 cells/ml. WT mice (male C57BL/6 mice, 8 weeks old) were lethally irradiated with 9.0 Gy using an MBR-1505R X-ray irradiator (Hitachi Medico Co., Tokyo, Japan) with a filter (copper, 0.5 mm; aluminum, 2 mm) monitoring the cumulative radiation dose. Each donor's BM mononuclear cells (2×10^6 cells) in 200 μl of PBS were transplanted via the tail vein of irradiated WT mice. After 12 weeks, peripheral blood was collected, and the smear preparation was stained with β-galactosidase to confirm the chimerism. The mice in which more than 95% of the peripheral leukocytes exhibited a positive reaction to β-galactosidase were used in the present experiment. GFP transgenic mice (a gift from Dr. M. Okabe, Genome Information Research Center, Osaka University, Osaka, Japan) with C57BL/6 background were used as WT mice to confirm the chimerism. The mice in which more than 95% of the peripheral leukocytes exhibited GFP-positive in fluorescence-activated cell sorting analysis were used in the present experiment as a control group.

Surgical Sponge Angiogenesis Model

Sponge disks (thickness, 5 mm; diameter, 1.3 cm)[23–25] were implanted under light ether anesthesia into the subcutaneous tissues of the backs of WT mice. After a fixed number of days, the granulation tissues that had formed around the sponge were taken immediately after death induced by the intraperitoneal administration of excessive doses of sodium pentobarbital. Neovascularization was assessed by measuring the concentration of hemoglobin in the granulation tissues formed around the sponge implants.[23–25] In brief, after the granulation tissues from the sponge were weighed, they were cut into several pieces with scissors. Distilled water 4 times the weight of the sample of granulation tissues was added to each sample, which was then homogenized with a Polytrone homogenizer (Kinematica GmbH, Lucerne, Switzerland). The hemoglobin concentration in the supernatant after centrifugation at $5000 \times g$ was determined using a hemoglobin assay kit (Hemoglobin B Testwako; Wako Pure Chemical Industries Ltd., Osaka, Japan). The concentrations of hemoglobin in the granulation tissues were expressed as mg/g wet tissue. Microvessel density in the sponge granulation tissues was assessed as a parameter of angiogenesis in a blind manner according to the methods described above.[16,43–45] Microvessel density was expressed in terms of microvessel number per observed area (vessel number/mm^2).

To assess the EP receptor subtype responsible for angiogenesis, EP receptor subtype-selective agonists, ONO-DI-004, ONO-AEI-257, ONO-AE-248, and ONO-AEI-329, which were specific to EP1, EP2, EP3, and EP4, respectively,[39] were topically injected (15 nmol/sponge, twice a day) into the sponges for 2 weeks. As a positive control, we administered basic fibroblast growth factor (bFGF) topically for 2 weeks (100 ng/sponge, once a day). PGE_2 (15 nmol/sponge, twice a day) was also topically injected.

Immunohistochemistry

Wound tissues from the mice at days 3 and 7 were immediately fixed with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4), dehydrated with a graded series of ethanol solutions, and embedded in paraffin. Sections (4 μm in thickness) were prepared from the paraffin-embedded tissue and were mounted on glass slides; after removal of paraffin with xylene, the slides were then placed in cold (4°C) acetone. The sections were subjected to either hematoxylin and eosin (H&E) staining or immunostaining. For immunostaining, the sections were first exposed to diluted normal horse serum and then incubated with either rabbit antiserum to mouse VEGF (Santa Cruz Biotechnology), rabbit antiserum to mouse CD31 (Santa Cruz Biotechnology),[16] or rabbit antiserum to β-galactosidase (ICN/Cappel, Aurora, OH). Immune complexes were detected with a LSAB+System-HRP kit (DakoCytomation, Carpinteria, CA).

Enzyme-Linked Immunosorbent Assay (ELISA) for VEGF

Wound granulation tissues were removed from the mice at day 3 and homogenized in PBS immediately. The supernatants (1500 × *g*, 15 minutes, at 4°C) of the homogenates were used as samples for ELISA. After the protein concentration was equalized, the VEGF level was measured with an ELISA kit (R&D Systems, Minneapolis, MN). Samples were measured in duplicate and averaged. VEGF levels were expressed as pg/mg protein. For control, the normal skin tissues were used in the same manner as the above-mentioned granulation tissues.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Transcripts encoding VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by RT-PCR analysis. Sponge granulation tissues were removed and rapidly frozen in liquid nitrogen. The frozen tissue was pulverized in a stainless steel cylinder cooled with liquid nitrogen. Total RNA was extracted from the tissue with Isogen (Wako), and cDNA was synthesized from 1 μg of total RNA with the use of an oligo-p(dT)15 primer and AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). Fifty ng of cDNA were amplified with 1 U of *Taq*DNA polymerase in a 25-μl reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxynucleoside triphosphate, and 0.6 μmol/L each of forward and reverse primers. The amplification protocol comprised 25 cycles (VEGF), or 20 cycles (GAPDH) of 45 seconds at 94°C, 60 seconds at 55°C, and 60 seconds at 72°C. The reaction mixtures were subsequently applied to a 2% agarose gel and the amplified products were stained with ethidium bromide. Primers used were as follows: 5′-AACCATGAACTTTCTGCTCTC-3′ (sense) and 5′-GTGATTTTCTGGCTTTGTTC-3′ (anti-sense) for VEGF, and 5′-CCCTTCATTGACCTCAACTACAATGGT-3′ (sense) and 5′-GAGGGGCCATCCACAGTCTTCTG-3′ (anti-sense) for GAPDH.

In separate experiments, transcripts encoding EP1, EP2, EP3, and EP4 in BM cells isolated from $EP3^{-/-}$ mice and WT mice were quantified by RT-PCR analysis. Tissue was removed and rapidly frozen in liquid nitrogen. The amplification protocol comprised 25 cycles (EP3), 30 cycles (EP1), or 40 cycles (EP2, EP4) of 45 seconds at 94°C, 60 seconds at 55°C, and 60 seconds at 72°C. Primers used were as follows: 5′-TACGAGTTACAGTACCCTGGC-3′ (sense) and 5′-CACACTAATGCCGCAAGGAGC-3′ (anti-sense) for EP1, 5′-AGCAGGACCTCTATCTCCTTG-3′ (sense) and 5′-CCACCAGGGTCTGGTTTCTTG-3′ (anti-sense) for EP2, 5′-GCTCGTGTCGCGCAGCTACCG-3′ (sense) and 5′-TGTGACAGGTACACGAGGATG-3′ (anti-sense) for EP3, and 5′-CTGGGCACCTTGTTGGTAAGC-3′ (sense) and 5′-ACCCGACAGACCGAAGAAAAG-3′ (anti-sense) for EP4.

In Situ *Hybridization*

For *in situ* hybridization, dissected wound granulation tissue was sectioned with a cryostat, and the resulting sections were fixed with 4% paraformaldehyde. Digoxigenin-labeled anti-sense and sense riboprobes for mouse EP3 mRNA were prepared by *in vitro* transcription of the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) containing mouse EP3. Sections were treated with proteinase K (10 μg/ml) and then subjected to hybridization with labeled riboprobes in hybridization solution (Novagen, Madison, WI) for 18 hours at 50°C in moistened plastic boxes. They were then exposed to RNase A (20 μg/ml) and washed extensively, and hybridized probe was detected by incubation first with alkaline phosphatase-conjugated antibodies to digoxigenin and then with 5-bromo-4-chloro-3 indolyl-phosphate and 4-nitroblue tetrazolium chloride (Roche Diagnostics, Indianapolis, IN). The specimens were finally counterstained with hematoxylin.

Flow Cytometry of VEGFR-1- and VEGFR-2-Positive Cells

Surgical wounds were made in BMT mice. At day 2, peripheral blood was collected from the tail vein to the heparinized PBS, and was centrifuged on a Lymphosepar I (specific gravity, 1.007; Immuno-Biological Laboratories, Fujioka, Japan) at 1500 × *g* for 20 minutes. The cells on the interface were used for flow cytometry. Cells (2 to 10 × 10^5) were stained in and washed with ice-cold Hanks' balanced salt solution containing 0.5% bovine serum albumin and 0.02% sodium azide. Secondary staining was performed in the same manner. After washing the stained cells were examined by flow cytometric analyses on FACSCalibur (Becton Dickinson, Mountain View, CA). Anti-mouse VEGFR-1 (Flt-1) antibody (R&D Systems) was biotinylated in our laboratory, and R-phycoerythrin (R-PE)-conjugated rat anti-mouse VEGFR-2 (Flk-1) monoclonal antibody was purchased from BD Pharmingen (San Diego, CA). The total peripheral leukocyte number was counted as the number of nucleated cells using a hemocytometer, and VEGFR-1-positive cell number and VEGFR-2-positive cell number were calculated with the results from flow cytometry and a hemocytometer. VEGFR-1-positive cell number and VEGFR-2-positive cell number were expressed in terms of cell number/ml of whole blood.

Drugs

Aspirin was kindly provided by Merck (Rahway, NJ), NS-398[46] was obtained from Cayman Chemical (Ann

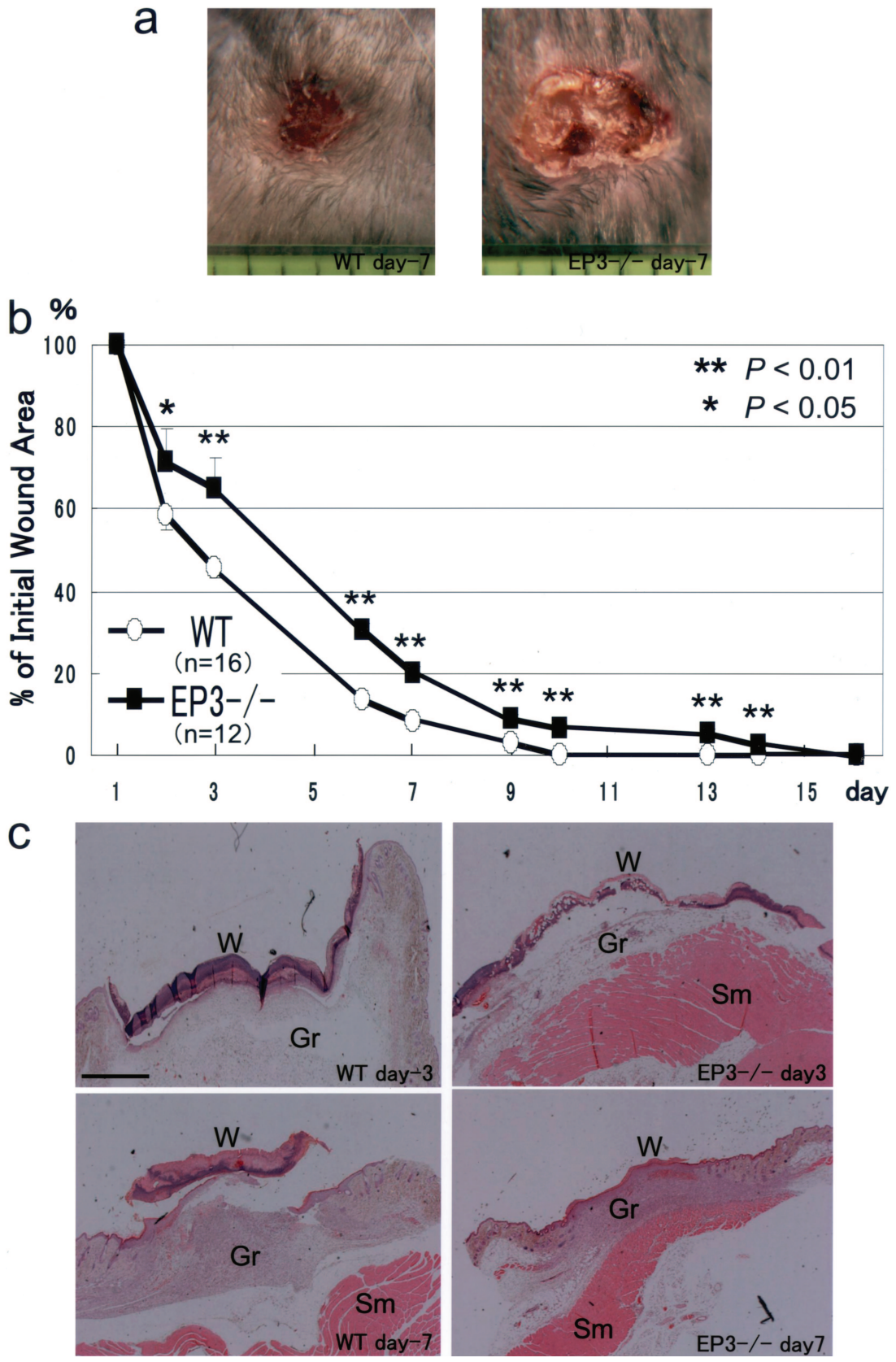

Figure 1. Delayed wound healing in EP3 receptor knockout mice. Surgical wounds were made on the backs of EP3 receptor knockout mice ($EP3^{-/-}$) and their WT counterparts, and wound closure was determined as described in Materials and Methods. **a:** Typical appearance of wounds in $EP3^{-/-}$ and WT at day 7. The original diameter of the wounds was 8 mm. One division on the scale below the wound represents 1 mm. **b:** Time course of wound closure in $EP3^{-/-}$ and WT. Data are means ± SEM for the indicated number of mice. $^{*}P < 0.05$, and $^{**}P < 0.01$ versus WT mice (analysis of variance). **c:** H&E staining was used for wound tissues including granulation tissues from $EP3^{-/-}$ and WT. Tissues were fixed at days 3 and 7. W, wound; Gr, granulation tissue; Sm, skeletal muscle. Scale bar = 1 mm.

Table 1. Wound Closure in EP Receptor Knockout Mice

Mice	Wild type ($n = 8$)	EP1$^{-/-}$ ($n = 8$)	EP2$^{-/-}$ ($n = 8$)	EP3$^{-/-}$ ($n = 8$)	EP4$^{-/-}$ ($n = 8$)
Day 2	64.6 ± 5.4	66.5 ± 4.4 (NS)	60.2 ± 5.3 (NS)	82.8±2.7†	66.0 ± 5.6 (NS)
Day 3	52.8 ± 3.2	53.1 ± 6.0 (NS)	48.2 ± 5.6 (NS)	66.9±3.4*	53.1 ± 5.0 (NS)
Day 4	38.0 ± 4.3	41.4 ± 4.4 (NS)	36.3 ± 4.1 (NS)	53.7±4.2†	40.1 ± 4.5 (NS)
Day 7	12.2 ± 2.5	13.2 ± 3.6 (NS)	9.0 ± 1.1 (NS)	36.8±4.3†	15.1 ± 2.6 (NS)

Surgical wounds were made on the backs of mice, and wound closure was determined as described in Materials and Methods. Data are means ± SEM for the indicated number of wounds (n).
NS, not significant. *$P < 0.05$, $^{\dagger}P < 0.01$ (compared with vehicle-treated mice at the same day).

Arbor, MI), and JTE-522[47] was kindly supplied by Japan Tobacco (Tokyo, Japan). EP receptor selective agonists, ONO-DI-004, ONO-AEI-257, ONO-AE-248, and ONO-AEI-329,[39] were kindly supplied from Ono Pharmaceutical Co., Osaka, Japan. An orally active, low-molecular weight inhibitor of the tyrosine kinase (ZD6474) of VEGF receptor (KDR/VEGFR 2)[50] was a gift from AstraZeneca (Cheshire, UK). An anti-angiogenic agent, FR118487, was supplied by Fujisawa Pharmaceutical Co. (Osaka, Japan).

Statistical Analysis

Data are expressed as means ± SEM. Comparisons among multiple groups were performed by factorial analysis of variance followed by Scheffé's test. Comparisons between the two groups were performed with Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

Results

Reduced Wound-Induced Angiogenesis in EP3 Receptor Knockout Mice

Full-thickness skin wounds were surgically created on the backs of WT mice, and the closure of wounds was then measured. The healing process of this mouse model was essentially the same as the process reported previously.[18,41,42] At 12 hours after wounding, all wounds were covered with a dry scab that remained adherent until day 5 in control WT mice. Three days after wounding, wound areas were reduced by >50% in control WT mice, and wounds were completely closed after 10 days. In WT mice, invasion of granulation tissue from the wound margins into the wound bed was observed 2 days after injury (data not shown). At day 3, the wound bed was completely filled with granulation tissue, and at this stage marked neovascularization was observable in WT mice.

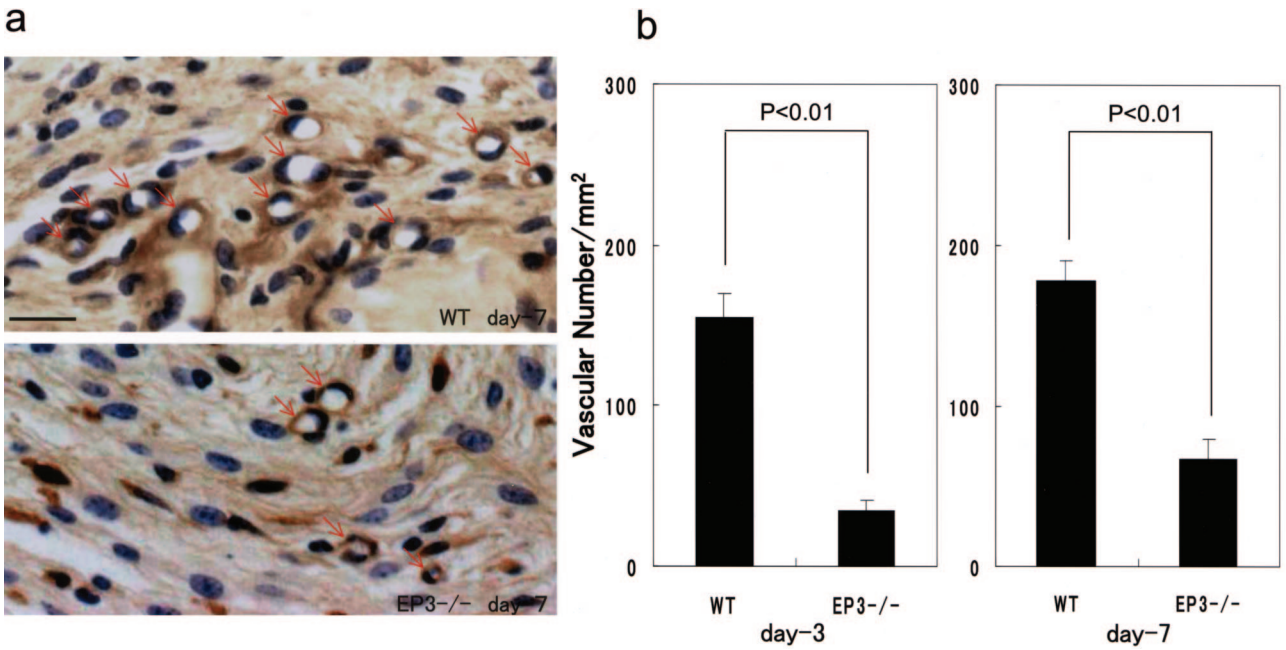

Figure 2. Angiogenesis in wound granulation tissues in EP3 receptor knockout mice. Surgical wounds were made on the backs of EP3 receptor knockout mice (EP3$^{-/-}$) and of their WT counterparts, and angiogenesis in the wound granulation tissues was determined as described in Materials and Methods. **a:** Typical results of CD31 immunostaining in the wound granulation tissues in EP3$^{-/-}$ and WT at day 3. CD-31-positive microvessels (brown stains indicated by **arrowheads**) were rich in the granulation tissues in WT but were apparently poor in EP3$^{-/-}$. **Arrowheads** in **a** indicate microvessel-like constructions. **b:** Changes in the microvessel density in the granulation tissues at days 3 and 7. After CD-31 immunohistochemistry was photographed, the microvessel density per observation field (100 μm × 100 μm) of the wound granulation tissues was counted. The microvessel density was finally expressed per mm^2, and results from 10 100 μm × 100 μm fields were averaged. Data are means ± SEM for six mice. Analysis of variance was used to test the significance of difference. Scale bar = 50 μm.

Table 2. Effects of an Anti-Angiogenic Agent and COX Inhibitors on Wound Closure in Wild-Type Mice

Compounds	Vehicle ($n = 12$)	FR-118487 ($n = 8$)	Aspirin ($n = 12$)	NS-398 ($n = 8$)	JTE-522 ($n = 8$)
Day 2	60.4 ± 4.5	79.8 ± 6.8†	79.5 ± 4.2†	77.8 ± 7.8†	77.7 ± 7.8†
Day 3	48.1 ± 4.5	62.6 ± 6.2*	60.6 ± 3.4*	64.0 ± 4.8†	65.4 ± 5.6†
Day 4	38.6 ± 4.0	50.9 ± 4.6 (NS)	51.4 ± 3.4*	56.8 ± 4.8†	60.1 ± 3.7†

Surgical wounds were made on the backs of mice, and wound closure was determined as described in Materials and Methods. FR118787 was administered daily (6 mg/kg p.o., twice a day). A nonselective COX inhibitor, aspirin (30 mg/kg p.o., twice a day), and COX-2 selective inhibitors NS-398 (30 mg/kg p.o., twice a day) and JTE-522 (30 mg/kg p.o., twice a day) were administered daily. For vehicle control mice, a vehicle solution (5% gum Arabic) was given to mice daily. Data are means ± SEM for the indicated number of wounds (n).

NS, not significant. *$P < 0.05$, †$P < 0.01$ (compared with vehicle-treated mice at the same day).

To evaluate the roles of endogenous PGE_2 and EP receptor signaling in wound-dependent angiogenesis and wound closure, skin wounds were created in four kinds of EP receptor knockout mice and WT mice, and the angiogenesis and the healing process were estimated to last for 16 days. As shown in Figure 1, a and b, wound closure in $EP3^{-/-}$ mice was significantly delayed compared with that in WT mice. When compared with the wound closure processes in $EP3^{-/-}$ mice, there is no delay in closure of the wounds in $EP1^{-/-}$ mice, $EP2^{-/-}$ mice, and $EP4^{-/-}$ mice (Table 1). Histological examination revealed that the development of granulation tissue under the scab is poor in $EP3^{-/-}$ mice at day 3 compared with that in WT mice (Figure 1c). In an immunohistochemical study using CD31 antibody, the microvessel density in $EP3^{-/-}$ mice at days 3 and 7 was significantly less than that in WT mice (Figure 2, a and b). These results suggested that angiogenesis in wound granulation tissues and wound closure process were dependent on the endogenous PGE_2 and EP3 receptor signaling. As shown in Table 2, the initial closure of wounds in the WT mice treated with an anti-angiogenic agent, FR-118487, was delayed significantly, suggesting that this wound healing model was dependent on the angiogenesis. When WT mice were treated with a nonselective COX inhibitor, aspirin, the initial closure of wounds was delayed significantly compared that in WT mice with vehicle solution (Table 2). This delay was also observed in WT mice treated either with NS-398 or with JTE-522, and the effects of these COX-2-selective inhibitors were essentially the same as that of aspirin (Table 2). These suggested that COX-2-derived prostaglandin E_2 facilitated the wound healing through EP3 signaling in this model.

Identification of EP Receptor Signaling to Facilitate Angiogenesis in Sponge Implantation Model

When circular sponge disks were implanted into the mouse subcutaneous tissues, the granulation tissues around the implants, which were constituted mainly of infiltrated macrophages/fibroblasts with collagen fibers, were developed. Newly developed capillaries and more extensive vascularization were evident in areas of encapsulated granulation tissues. In this model, angiogenesis was quantified by the determination of hemoglobin concentrations and microvessel density in the granuloma tissues.[24,25] Because the angiogenesis during the granulation tissue formation in this model may mimic the wound-induced angiogenesis, we evaluated EP receptor signaling relevant to angiogenesis in sponge granulation tissues. In the present experiment, the authentic proangiogenic activity was confirmed using topical injections of basic fibroblast growth factor (bFGF) (Table 3). To identify the PGE_2 receptor mediating the proangiogenic action, we topically injected recently developed EP agonists that are highly selective for each subtype. Neither the EP1 agonist ONO-DI-004 nor the EP2 agonist ONO-AEI-257, nor the EP4 agonist ONO-AEI-329 significantly enhanced angiogenesis (Table 3). In contrast, the EP3 agonist ONO-AE-248 markedly increased the extent of angiogenesis in sponge model (Table 3). The effect of ONO-AE-248 was the same as that of PGE_2, when the same dose of PGE_2 was administered (Table 3). These results suggested that angiogenesis during granulation tissue development in this model is also dependent on the PGE_2-EP3 receptor signaling.

Table 3. Proangiogenic Activity of Selective EP Receptor Agonists in Sponge Angiogenesis Model

Compound	Vehicle	ONO-DI-004 (EP1 agonist)	ONO-AEI-257 (EP2 agonist)	ONO-AE-248 (EP3 agonist)	ONO-AEI-329 (EP4 agonist)	PGE_2	bFGF
Angiogenesis (Hb mg/g tissue)	1.3 ± 0.3 (12)	1.0 ± 0.2 (8)	2.0 ± 0.4 (7)	3.6 ± 0.6 (9)*	1.4 ± 0.2 (6)	3.8 ± 0.4 (6)*	4.3 ± 0.3 (8)*
Angiogenesis [microvessel number (/mm^2)]	87 ± 15 (6)	81 ± 10 (6)	132 ± 23 (6)	254 ± 32 (6)*	94 ± 20 (6)	250 ± 30 (6)*	

EP subtype-selective agonists or PGE_2 were topically injected to the sponge implants for 2 weeks (15 nmol/sponge, twice a day). bFGF was also topically injected to the sponge implants as a positive control for 2 weeks (100 ng/sponge, once a day). Angiogenesis was estimated by the Hb (hemoglobin) contents, and the neovascularized microvessel number per mm^2 of observation field in granulation tissues.

*$P < 0.05$, compared with vehicle-treated sponges.

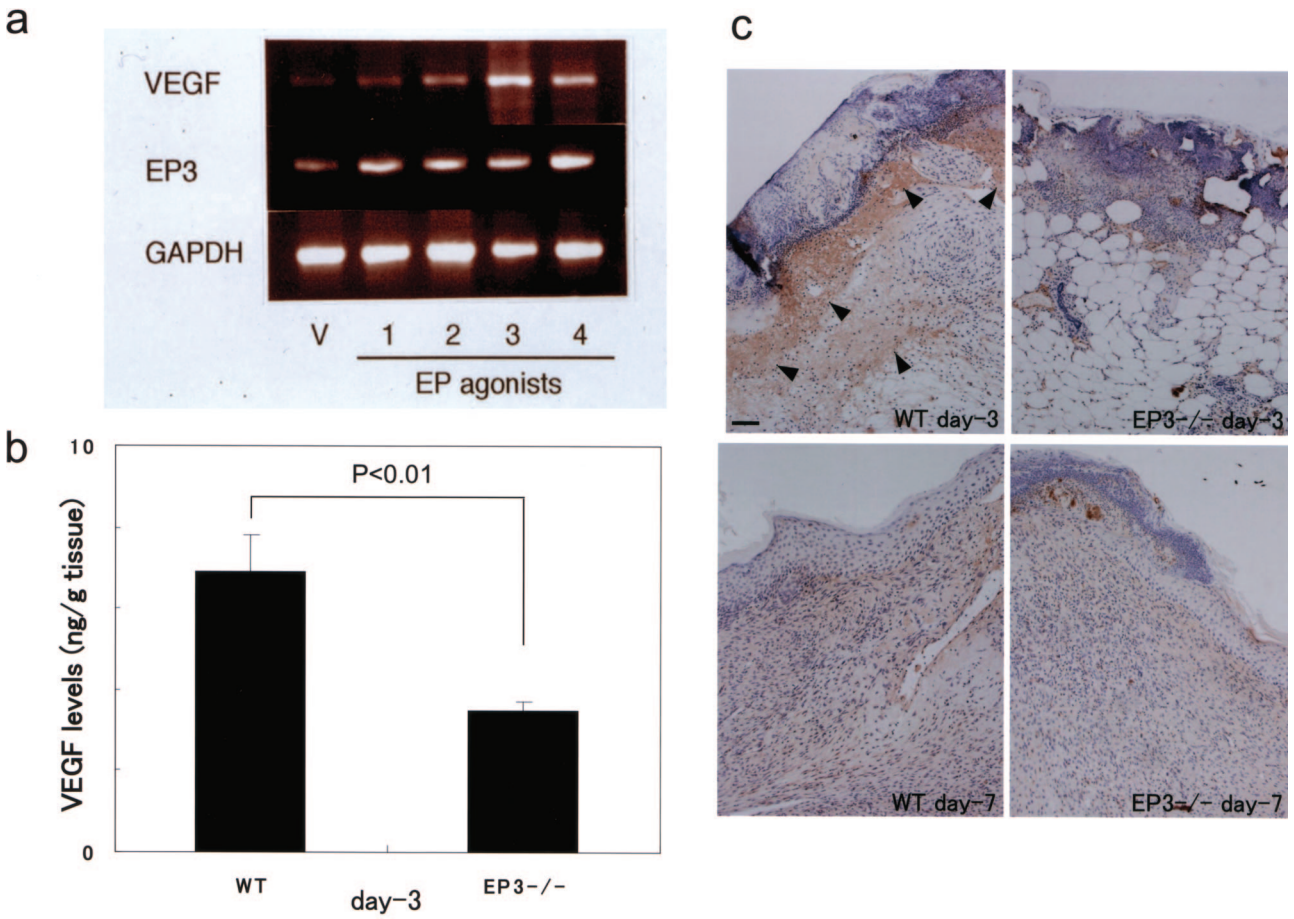

Figure 3. Expression of VEGF in sponge granulation tissues in WT mice stimulated with selective EP agonists and in wound granulation tissues in EP3 receptor knockout mice. **a:** VEGF expressions in sponge granulation tissues in WT mice stimulated with selective EP agonists for 2 weeks. EP receptor subtype-selective agonists, ONO-DI-004, ONO-AEI-257, ONO-AE-248, and ONO-AEI-329, which were specific to EP1, EP2, EP3, and EP4, respectively,[18] were topically injected (15 nmol/sponge, twice a day) into the sponges implanted in the subcutaneous tissues of WT mice. RT-PCR was performed as described in the text. **b:** Immunoreactive VEGF levels, determined by ELISA, in the wound granulation tissues in EP3$^{-/-}$ mice and WT mice at day 3. VEGF protein was not detected in normal skin tissue in WT mice (data not shown). VEGF expression was detected in wound tissue in WT mice (left) but was reduced in EP3$^{-/-}$ mice (right). To test the significance of difference, *t*-test was used. **c:** Typical results of VEGF immunostaining in the wound granulation tissues in EP3$^{-/-}$ and WT at days 3 and 7. VEGF-positive cells were markedly accumulated in the wound granulation tissues in WT mice at day 3 (brown stains indicated by **arrowheads**). Scale bar = 100 μm.

Expression of Vascular Endothelial Growth Factor in Wound Granulation Tissues

To study the EP3 signaling-dependent downstream molecules, we examined the expression of a potent proangiogenic factor, vascular endothelial growth factor (VEGF) in wound tissues. RT-PCR analysis revealed that the expression of VEGF in sponge granulation tissues was markedly up-regulated with topical injections of EP3 agonist (Figure 3a). Immunohistochemical study with mouse VEGF antibody clarified that the VEGF-expressing cells localized mainly in granulation tissues under the wounds in WT mice (Figure 3b). In an immunohistochemical analysis, immunoreactive VEGF was also detectable in wound granulation tissues in EP3$^{-/-}$ mice, but the number of VEGF-expressing cells in EP3$^{-/-}$ mice were markedly less than that in WT mice at day 3 (Figure 3c). The difference of VEGF expression between EP3$^{-/-}$ mice and WT mice was not so obvious in late stage of wound healing (day 7) (Figure 3c). ELISA for VEGF also revealed that the immunoreactive VEGF level at day 3 was significantly reduced in the wound granulation tissues in EP3$^{-/-}$ mice compared with WT mice (Figure 3b). When VEGF was continuously infused with miniosmotic pumps into the subcutaneous tissues of EP3$^{-/-}$ mice and the wound-healing process was determined, the wound-healing process in EP3$^{-/-}$ mice was facilitated (Table 5). Wound healing in VEGF-treated EP3$^{-/-}$ was essentially the same as that observed in WT mice (Table 1, results from WT). These results suggested that the endogenous PGE_2 and EP3 receptor signaling induced VEGF in the wound tissues and that EP3 signaling is a major determinant of VEGF induction.

Role of Vascular Endothelial Growth Factor in Wound Closure Process

To estimate the contribution of VEGF to the wound-healing processes, neutralizing antibody against VEGF was continuously infused to the subcutaneous tissues of WT mice. With this treatment, wound healing was delayed significantly compared with control IgG infusion, and the time course of delay was almost the same as that observed in EP3$^{-/-}$ mice (Table 4). The same was true in WT mice

Table 4. Effects of a VEGF-Neutralizing Antibody and a VEGF Receptor Kinase Inhibitor on Wound Closure in Wild-Type Mice

Treatment	Control IgG (n = 8)	VEGF antibody (n = 8)	Vehicle (n = 12)	ZD-6474 (n = 12)
Day 2	58.8 ± 5.9	89.2 ± 5.5*	64.6 ± 3.9	82.1 ± 4.6‡
Day 3	43.9 ± 4.2	78.6 ± 6.3*	49.1 ± 3.8	61.7 ± 4.1†
Day 4	36.3 ± 3.2	57.0 ± 6.0*	41.0 ± 3.1	55.5 ± 3.3‡

Surgical wounds were made on the backs of mice, and wound closure was determined as described in Materials and Methods. An anti-VEGF antibody or control IgG was subcutaneously infused with a mini-osmotic pump, and 1 day after the pump implantation, surgical wounds were made. Either ZD6474 (50 mg/kg, twice a day), a VEGF receptor kinase inhibitor, or vehicle control solution was orally administered. Data are means ± SEM for the indicated number of wounds (n).

*$P < 0.01$ (compared with control IgG-treated mice at the same day).

†$P < 0.05$, ‡$P < 0.01$ (compared with vehicle-treated mice at the same day).

receiving an orally active low-molecular weight inhibitor of VEGF receptor (KDR/VEGFR 2) tyrosine kinase ZD6474 (Table 4). The magnitude of inhibition of ZD6474 in wound healing was not different from that of neutralizing antibody (Table 4). These results indicated that VEGF contributed to the closure of the surgical wounds in this model and suggested that up-regulation of VEGF may explain the proangiogenic response in wound tissues via the endogenous PGE_2 and EP3 receptor signaling.

Role of Recruited Bone Marrow Cells Expressing EP3 in Wound-Induced Angiogenesis

Recent results have suggested that recruitment of hematopoietic cells from the BM showed crucial roles in wound healing and angiogenesis.[41,42,51] Finally, we examined the role of recruited BM cells expressing EP3 in wound-induced angiogenesis and wound closure. Selective knockdown of EP3 by recruitment of genetically modified BM cells lacking EP3 receptors was performed by transplantation of BM cells from EP3$^{-/-}$ mice. In WT mice transplanted with BM cells from EP3$^{-/-}$ mice, wound closure and wound-induced angiogenesis were markedly suppressed compared with those in mice transplanted with WT BM cells (Figure 4, a and b). When we performed RT-PCR to detect the mRNA for four subtypes of EP receptors in the BM cells isolated from WT mice and EP3$^{-/-}$ mice, EP3 mRNA was expressed in BM cells from WT mice but was not detected in EP3$^{-/-}$ mice (Figure 3c). Other subtypes of EP, such as EP1, EP2, and EP4, were detected both in WT mice and in EP3$^{-/-}$ mice (Figure 3c). Immunohistochemical study revealed that β-galactosidase-positive cells were accumulated in the wound granulation tissues in WT mice transplanted with BM cells from EP3$^{-/-}$ mice (Figure 5, b and d), suggesting that transplanted EP3$^{-/-}$ cells were certainly recruited into the wound granulation tissues. WT mice receiving WT BM cells did not show β-galactosidase-positive stains in the wound tissues (Figure 5, a and c), by contrast, EP3-positive cells accumulated in the granulation tissues when estimated with EP3 *in situ* hybridization (Figure 5e). The uninjured lesions around the wounds introduced in WT mice transplanted with BM cells from EP3$^{-/-}$ mice did not exhibit β-galactosidase (Figure 5, b and d; asterisks), indicating that recruitment of BM cells into the wound granulation tissues was restricted. VEGF

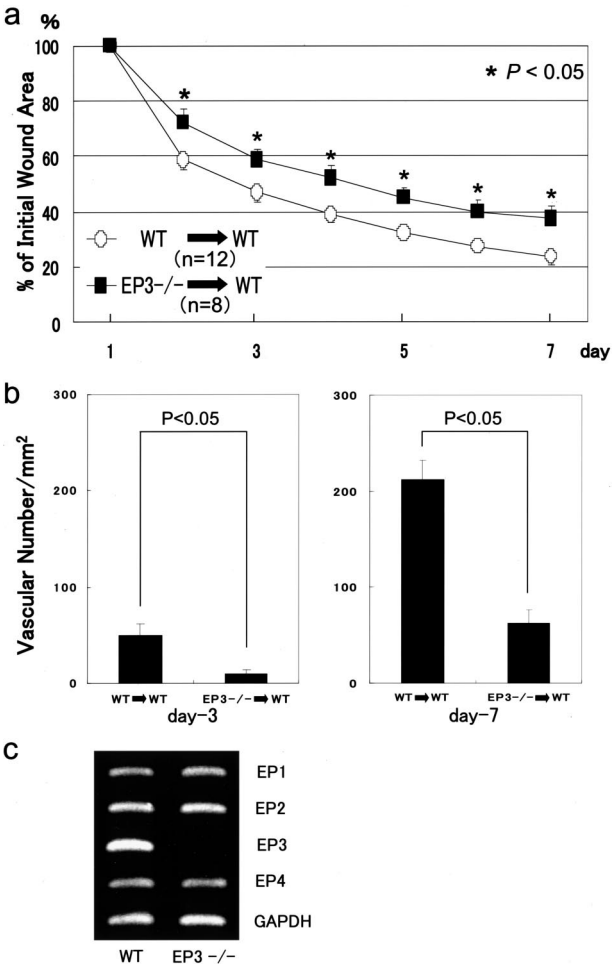

Figure 4. Time course of wound healing and angiogenesis in mice transplanted with BM cells from EP3 receptor knockout mice. A lethal dose of radiation was given to WT mice, and BM cells either from WT mice or from EP3$^{-/-}$ mice were injected into the tail vein under light ether anesthesia (WT→WT or EP3$^{-/-}$→WT). Surgical wounds were made on the backs of the mice, and wound closure was determined as described in Materials and Methods. **a:** Time course of wound closure in WT mice transplanted with BM cells either from EP3$^{-/-}$ or from WT mice. Data are means ± SEM for the indicated number of mice. *$P < 0.05$ versus WT→WT mice (analysis of variance). **b:** The changes in the microvessel density in the wound granulation tissues in WT mice transplanted with BM cells either from EP3$^{-/-}$ or from WT mice. After CD31 immunohistochemistry was photographed, the number of vessels in the observed area was counted and expressed per mm^2 observed. Data are means ± SEM for the indicated number of mice. $P < 0.05$ versus WT→WT (analysis of variance). **c:** Expressions of EP receptor subtypes in BM cells isolated from EP3$^{-/-}$ mice and WT mice. RT-PCR was performed as described in the text.

expression was seen mainly in granulation tissue in WT mice receiving WT BM cells (Figure 5, f and h), whereas marked accumulation of VEGF-positive cells was not seen in mice implanted with $EP3^{-/-}$ cells (Figure 5, g and i).

During wound closure processes in BMT mice, we determined circulating leukocyte numbers expressing a hematopoietic cell marker, VEGFR-1, and an endothelial precursor cell marker, VEGFR-2, at day 3. As shown in Table 5, the total number of circulating leukocytes was not different between WT mice receiving WT BM cells and $EP3^{-/-}$ BM cells before wounds were made. The same was true in VEGFR-1-positive cells and VEGFR-2-positive cells in the peripheral circulation (Table 6). Three days after the wounds were created, VEGFR-1-positive cell number and the total number of circulating leukocytes were increased in mice transplanted with WT BM cells. By contrast, the increase in VEGFR-1-positive cells and the total circulating leukocyte number was significantly blunted in mice transplanted with $EP3^{-/-}$ BM cells. The circulating cell numbers of VEGFR-2-positive cells were not increased in mice with wounds receiving $EP3^{-/-}$ BM cells or WT BM cells.

Discussion

Angiogenesis, the formation of new blood vessels from the pre-existent microvasculature, is an essential component of normal wound repair. Immediately after injury, it allows delivery of oxygen, nutrients, and inflammatory cells to the site of injury. It also assists in the development of granulation tissue formation and ultimately wound closure. Both angiogenic agonists and antagonists are identified at various stages of the wound repair process,[52,53] suggesting a dynamic balance of stimulators and inhibitors that favor either vascular growth or regression.[54] Anti-angiogenic agents may delay the healing of wounds. In the present experiment, we showed that the anti-angiogenic agent FR118487 inhibits significantly the initial closure of surgical wounds. FR118487 is a chemical modification of FR111142, which was isolated from the fermentation products of *Scolecobasidium arenarium* F-2015.[55] This agent was reported to inhibit choroidal neovascularization, and this effect was attributable to the inhibition of endothelial cell proliferation.[56] The mechanism of action of FR118487 in the inhibition of angiogenesis was not fully clarified, but FR118487, like TNP-470 of the fumagillin family, is considered to block endothelial cell-cycle progression in the late G_1 phase through the activation of the p53 pathway, causing an accumulation of the G_1 cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$.[57] Thus, FR118487 may cause endothelial cell-cycle inhibition similar to that mediated by p53 and p21WF1/AIP1 *in vivo*.[57] This compound was not very toxic, which allowed us to use a high dose of it.[48,49] With the use of this compound, we showed that the initial wound closure in the present model was dependent on angiogenesis.

It has been previously reported that E-type PGs have a proangiogenic activity in corneal tests[58] and in the chorioallantoic membrane (CAM) technique.[59] Furthermore, Form and Auerbach[60] reported that PGE_2 strongly induced angiogenesis on the CAM of 8-day-old chicken embryos, but PGA_2, PGF_2, and a derivative of TXA_2 did not.[60] Another report described that the endothelial migration was mediated by COX-2.[61] This experiment was performed *in vitro* using confluent monolayer endothelial cells stimulated with PMA, and the authors also reported that corneal angiogenesis was suppressed with COX-2 inhibitor, suggesting the involvement of COX-2 products *in vivo*. In the present *in vivo* experiment, COX-2 selective inhibitors NS-398 and JTE-522 delayed the healing of surgical wounds (Table 2). These effects were the same as that of the nonselective COX inhibitor aspirin (Table 2). Thus, because we used sufficient doses of these COX inhibitors, COX-2-derived PGs have a significant role in wound healing. It was reported that ulcer healing process was delayed with a COX-2 inhibitor.[62] Thus, the present results suggested that both ulcer and wound healings are COX-2-dependent. We did not estimate vascular density in the wound tissues under COX inhibition, but, as mentioned above, the delay of healing may have been a result of inhibition of angiogenesis. In our previous experiment, angiogenesis in the sponge implantation model was reduced with either COX-2 selective inhibitors or the nonselective COX inhibitor indomethacin to the same extent. Thus, the same may be true in this wound-healing model. Because the reductions in angiogenesis and in the development of granulation tissues which were induced by the subcutaneous implantation of cotton threads were also reported in mice that lack inducible PGE synthase,[26] the relevant PG generated in the site of angiogenesis and wound healing may be PGE_2. In fact, an EP agonist has a proangiogenic activity in a sponge model, as shown here (Table 3).

If angiogenesis and wound healing are enhanced by PGE_2, the identification of a relevant receptor and the development of pharmacological tools to modify the receptor signaling of PGE_2 are critical for controlling angiogenesis and wound healing. The quantitative sponge angiogenesis model adopted in the present study showed the responsible EP subtype to be EP3 (Figure 1 and Table 1). In our preliminary experiment, in which EP receptor subtypes expressed in normal subcutaneous tissues were identified, EP3 was detectable predominantly in the subcutaneous tissues, although we could not detect EP2 but were able to detect EP1 and EP4 faintly by RT-PCR analysis (data not shown). The expression profile of EP receptors is quite a reasonable one for explaining the proangiogenic activity of EP3 agonist in the sponge model (Table 3). To confirm the roles of EP3 signaling in response to endogenous PGE_2, we used $EP3^{-/-}$ mice and assessed the wound-healing processes (Figure 1). As shown in the present study, not only wound healing but also angiogenesis in wound tissues is delayed and suppressed in $EP3^{-/-}$ mice. Judging from the time course of the changes in closure of wounds in $EP3^{-/-}$ mice (Figure 1), the delay of the initial wound closure was almost the same as that under COX-2 inhibition. This also suggested that the responsible PG is

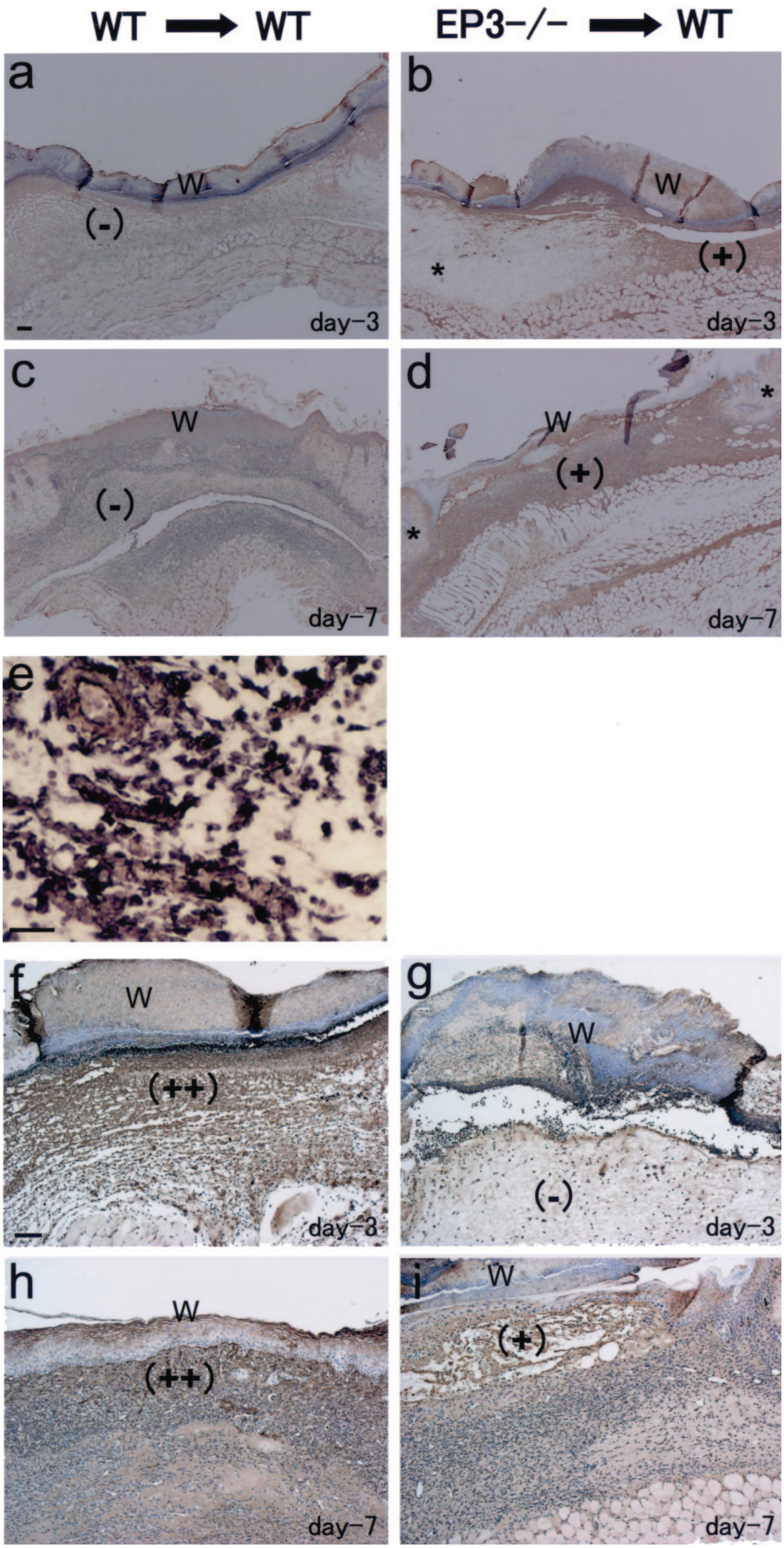
WT → WT
EP3−/− → WT
a
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Table 5. Effects of Continuous Subcutaneous Infusion of VEGF on Wound Closure in $EP3^{-/-}$ Mice

Treatment	Vehicle (n = 6)	VEGF (n = 6)
Day 2	84.5 ± 3.3	60.8 ± 2.6*
Day 3	69.6 ± 4.0	42.5 ± 2.1*
Day 4	57.0 ± 4.9	30.8 ± 2.5*
Day 7	39.7 ± 5.1	12.9 ± 1.0*

Surgical wounds were made on the backs of $EP3^{-/-}$ mice, and wound closure was determined as described in Materials and Methods. VEGF was subcutaneously infused (1 μg/day) with a mini-osmotic pump, and 1 day after the pump implantation, surgical wounds were made. Vehicle solution (physiological saline) was infused with the same pump. Data are means ± SEM for the indicated number of wounds (n).
*$P < 0.01$ (compared with vehicle-treated mice at the same day).

PGE_2 and that PGE_2-EP3 receptor signaling is the predominant type that is functionally active.

To identify the EP3-dependent downstream molecules, we performed a preliminary *in vivo* experiment in which the effects of topical injections of the neutralizing antibodies against several growth factors and against a cytokine were tested in the sponge implantation model. The antibodies against epithelial growth factor (EGF), transforming growth factor-β, and interleukin-1 did not reduce angiogenesis at all, but by contrast, the VEGF antibody markedly suppressed the angiogenic reaction (data not shown). Such an experiment implied that VEGF was a major responsible factor for angiogenesis and that VEGF also plays a crucial role in wound healing. In the wound tissues in WT mice, VEGF was detectable, although the expression of VEGF mRNA was not detectable in normal subcutaneous tissues in WT mice (Figure 3). VEGF immunoreactive levels in the wound in $EP3^{-/-}$ mice were reduced compared with those in WT mice (Figure 3), suggesting that VEGF is one element responsible for EP3 signaling. We previously reported that PGE_2 induces VEGF together with angiogenesis through the activation of adenylate cyclase/protein kinase A signaling pathway in sponge model.[63] It was reported that EP3 receptor has several splicing variants.[64] Some are linked to the inhibition of adenylate cyclase and others to its activation. One of the EP3 splicing variants,[64,65] which may be related to the elevation in intracellular cAMP levels, may enhance wound healing and angiogenesis. We sum that VEGF neutralizing antibody or VEGF receptor kinase inhibitor suppressed the process of wound healing (Table 4), and the efficacy of these treatments was the same as that in COX-2 inhibition or in EP3 receptor knockout. This suggested that VEGF is a major downstream molecule of EP3 receptor signaling.

It is widely accepted that recruitment of BM hematopoietic cells to the wound granulation tissues is crucial for wound healing processes.[41,42,51] Because EP3 signaling in the granulation tissues was important in wound healing and angiogenesis (Figures 1 and 2), the accumulation of EP3-expressing cells from BM may contribute in the present surgical wound-healing model. To clarify this possibility, we replaced BM cells with those from EP3 knockout mice to knock down EP3 receptor signaling in BM derived cells (Figures 4 and 5). In $EP3^{-/-}$ mice, EP3 gene was replaced with LacZ, so the EP3-null cells are expected to express the LacZ gene product β-galactosidase. Immunohistochemical examination with the use of antibody against β-galactosidase can reveal the recruitment of EP3-null cells into the tissues in question. BMT of WT mice with the BM cells from $EP3^{-/-}$ mice resulted in the delay in wound healing together with reduced angiogenesis (Figure 4). This was accompanied with infiltration of β-galactosidase-positive cells into the wound granulation tissues, although β-galactosidase-positive cells were not seen in the intact subcutaneous tissues around repairing wounds in $EP3^{-/-}$ mice (Figure 5). These results suggested that wound tissues may selectively induce recruitment of EP3-expressing cells and that these accumulated EP3-expressing cells secreted VEGF and enhanced wound healing and angiogenesis.

Furthermore, as shown in Figures 1 and 2, the delay of wound healing in $EP3^{-/-}$ mice was accompanied by reduced formation of wound granulation tissues. This

Table 6. VEGFR-1- and VEGFR-2-Positive Cells in Peripheral Blood during Wound Healing in Bone Marrow-Transplanted Mice

Wound	BMT	Total leukocyte no. ($\times 10^5$ cells/ml)	% of VEGFR-1-positive cells	% of VEGFR-2-positive cells	Number of VEGFR-1-positive cells ($\times 10^5$ cells/ml)	Number of VEGFR-2-positive cells ($\times 10^5$ cells/ml)
+	WT→WT	170 ± 11*†	20.7 ± 2.4	0.46 ± 0.10	35.2 ± 2.1*†	0.78 ± 0.12
+	EP3→WT	145 ± 8	19.3 ± 2.0	0.54 ± 0.11	28.0 ± 1.1	0.78 ± 0.10
−	WT→WT	121 ± 7	16.7 ± 1.7	0.60 ± 0.08	20.2 ± 1.0	0.73 ± 0.09
−	EP3→WT	130 ± 9	18.0 ± 1.4	0.48 ± 0.12	23.4 ± 2.0	0.63 ± 0.13

After wound generation, peripheral blood was collected at day 3, and total leukocyte number (no.) and % of VEGFR-1- or of VEGFR-2-positive cells were determined as described in Materials and Methods. VEGFR-1, vascular endothelial growth factor receptor-1; VEGFR-2, vascular endothelial growth factor receptor-2; BMT, bone marrow transplantation; WT→WT, irradiated wild-type (WT) mice receiving bone marrow cells from WT mice; EP3→WT, irradiated WT mice receiving bone marrow cells from $EP3^{-/-}$ mice. Results were mean ± SEM from six mice.
*$P < 0.05$, compared with EP3→WT with wounds; †$P < 0.05$, compared with EP3→WT without wounds.

Figure 5. Typical results of immunostainings of β-galactosidase (**a–d**) and VEGF (**f–i**) and of EP3 *in situ* hybridization (**e**) in the wound granulation tissues in WT mice transplanted with BM cells either from $EP3^{-/-}$ mice ($EP3^{-/-}$→WT) or from WT mice (WT→WT) at day 3 (**a**, **b**, **e–g**) and at day 7 (**c**, **d**, **h**, and **i**). β-Galactosidase-positive cells accumulated in the wound granulation tissues in WT mice transplanted with BM cells from $EP3^{-/-}$ mice (**b** and **d**, ++). WT mice receiving WT BM cells did not show β-galactosidase-positive stains in the wound tissues (**a** and **c**). The uninjured lesions around the wounds introduced in WT mice transplanted with BM cells from $EP3^{-/-}$ mice did not exhibit β-galactosidase (**b** and **d**, **asterisks**). EP3 mRNA was detected in granulation tissues just beneath the wound in WT mice receiving WT BM cells (**e**, high magnitude). VEGF immunoreactivity was mainly seen in granulation tissues in WT mice receiving WT BM cells (**f** and **h**), whereas no marked accumulation of VEGF-positive cells was seen in WT mice implanted with $EP3^{-/-}$ cells (**g** and **i**). W indicates surgical wound in recovery stage. Scale bars = 100 μm (**a**, **f**); 25 μm (**e**).

poor degree of granulation tissue formation may reflect the marked suppression of recruitment of BM cells in EP3$^{-/-}$ mice. It was widely accepted that VEGF[66] mediates the angiogenic switch[10,14,67,68] by interacting with two tyrosine kinase receptors, VEGF receptor-1 (VEGFR1, Flt-1)[69] and VEGFR2 (Flk-1, KDR).[70,71] Although VEGFR2 regulates proliferation and survival of endothelial cells and one of the functions of VEGFR1 is thought to be the modulation of angiogenesis, the contribution of two receptors to angiogenesis during wound healing is not clear at present. VEGFR1-deficient mice have vascular malformations suggesting that VEGFR1 has a role in vascular remodeling.[69,72,73] As shown here, the process whereby mobilization of VEGFR1-positive cells from BM may be modulated by EP3 receptor signaling (Table 5). In a mouse tumor model, both VEGFR1 and VEGFR2 signaling was reported to be necessary to block tumor angiogenesis.[74] The numbers of VEGFR-2-positive cells were not changed in this wound model. The peripheral number of leukocytes may be determined by the rates in mobilization from BM and in the accumulation of cells to the wound granulation tissues. Therefore, the kinetics of recruitment of VEGFR-2-positive cells may be differently regulated in tumor models and in wound models. Present results suggested the importance of recruitment of VEGFR-1-positive cells during wound healing. The effects of VEGF or other stimulators to mobilize the BM cells may be affected by EP3-signaling expressing in BM cells, because the EP3$^{-/-}$ cells were rich in the mouse BM after transplantation of EP3$^{-/-}$ cells. COX-2 knockout mice experience delayed hematological recovery after 5-fluorouracil treatment, even though the basal hematopoiesis is normal.[75] Furthermore, PGE_2 directly stimulates the *in vitro* proliferation of burst-forming units of granulocyte-macrophage progenitors from CD34$^+$ cells.[76] The chemokine macrophage inflammatory protein-1 promotes the recruitment of monocytes into the peritoneal cavity of mice and their subsequent differentiation into macrophages, and these events are mediated indirectly by PGE_2.[77] Thus, the present result may reflect an unexplored role of EP3 receptor signaling in response to PGE_2 in the differentiation and migration of mononuclear cells.

In conclusion, as discussed above, the PGE_2-EP3 signaling appears critical for surgical wound healing and angiogenesis in the wound tissues. EP3 signaling on the cells recruited into the wound granulation tissues was relevant to the induction of a potent proangiogenic growth factor, VEGF. Up-regulated VEGF certainly enhances the wound healing processes, as assessed by inhibition of VEGF activity and VEGF receptor signaling. Thus, the recruitment of EP3-expressing cells to wound granulation tissues appears critical for surgical wound healing and angiogenesis. A highly selective EP3 agonist is therefore expected to enhance the wound-healing process and will become a novel therapeutic tool for surgical wounds. The results presented here neatly provide an answer to the question of how nonsteroidal anti-inflammatory drugs delay wound healing.

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