P-selectin glycoprotein ligand-1 contributes to wound healing predominantly as a P-selectin ligand and partly as an E-selectin ligand

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Abbreviations: PSGL-1, P-selectin glycoprotein ligand-1; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; ICAM-1, intercellular adhesion molecule-1; mAb, monoclonal antibody; L-selectin$^{-/-}$, L-selectin-deficient; RT-PCR, reverse transcriptase-polymerase chain reaction; IL,

interleukin; TNF, tumor necrosis factor; H&E, hematoxylin and eosin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Abstract

Cell adhesion molecules are critical to wound healing through leukocyte recruitment. Although P-selectin glycoprotein ligand-1 (PSGL-1) regulates leukocyte rolling by binding P-selectin but also binding E- and L-selectins with lower affinity, little is known about a role of PSGL-1 in wound healing. To clarify a role of PSGL-1 and its interaction with E- and P-selectins in wound healing, we investigated cutaneous wound healing in PSGL-1-deficient (PSGL-1$^{-/-}$) mice in comparison with E-selectin$^{-/-}$ mice, P-selectin$^{-/-}$ mice, and P-selectin$^{-/-}$ mice treated with anti-E-selectin antibody. PSGL-1 deficiency inhibited early wound healing, which was accompanied by decreased inflammatory cell infiltration and growth factor expression. By contrast, E-selectin deficiency did not affect wound healing. In general, inhibitory effect of PSGL-1 deficiency on wound healing was similar to that of P-selectin deficiency either alone or with E-selectin blockade. However, early granulation tissue formation, late angiogenesis, and early infiltration of neutrophils and macrophages in PSGL-1$^{-/-}$ mice was inhibited beyond inhibition in P-selectin$^{-/-}$ mice, but to a similar level of inhibition in P-selectin$^{-/-}$ mice with E-selectin blockade. These results suggest that PSGL-1 contributes to wound healing predominantly as a P-selectin ligand and partly as an E-selectin ligand by mediating infiltration of inflammatory cells.

Introduction

The healing of cutaneous wounds consists of three phases: 1) an inflammatory phase which consists of platelet aggregation and recruitment of inflammatory cells; 2) a proliferative phase which involves the migration and proliferation of keratinocytes, fibroblasts, and endothelial cells; and 3) a long remodeling phase (Singer and Clark, 1999; Werner and Grose, 2003). Skin wound healing is a complex biological event as a result of the interplay of resident and infiltrating cells, including neutrophils, macrophages, and mast cells (Gillitzer and Goebeler, 2001). Infiltrating neutrophils form a first line of defense against infection and are also a source of proinflammatory cytokines (Hart, 2002). Macrophages also regulate wound healing by antimicrobial function, wound debridement, and production of various growth factors, such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, heparin binding epidermal growth factor, and TGF-α (Park and Barbul, 2004; Singer and Clark, 1999; Hart, 2002; Tsirogianni *et al.*, 2006; Werner and Grose, 2003). Mast cells are also considered an important source of mediators during wound healing and are detected at higher frequencies than in non-injured skin (Hebda *et al.*, 1993; Trautmann *et al.*, 2000). Thus, immune cells play a critical role in the wound healing process.

The recruitment of immune cells into wound sites is a multistep process that is highly regulated by multiple cell-surface adhesion molecules (Ley *et al.*, 2007). Leukocytes first tether and roll on vascular endothelial cells, before they are activated to adhere firmly and subsequently immigrate into the extravascular space. The selectin family cooperates to support leukocyte tethering and rolling along inflamed vascular walls and consists of three cell-surface molecules expressed by leukocytes (L-selectin), vascular endothelium (E- and P-selectins), and platelets (P-selectin) (Ley and Kansas, 2004). P-selectin is rapidly expressed on the endothelial cell surface upon degranulation of Weibel-Palade bodies, and its synthesis

can be further increased by cytokines (Petri *et al.*, 2008). E-selectin is expressed only after a delay, as it is up-regulated by cytokines and requires de novo synthesis (Petri *et al.*, 2008). By contrast, L-selectin is constitutively expressed on most leukocytes (Petri *et al.*, 2008). Three selectins have partially overlapping function for leukocyte rolling (Robinson *et al.*, 1999; Ley and Kansas, 2004) . P-selectin supports leukocyte rolling along postcapillary venules at the earliest time of inflammation by interacting with its ligand P-selectin glycoprotein ligand-1 (PSGL-1), a mucin-like glycoprotein expressed by all subsets of leukocytes (Norman *et al.*, 2000; McEver and Cummings, 1997; Ley and Kansas, 2004). PSGL-1 has been also shown to bind E- and L-selectins, although their affinities are lower than P-selectin (Yang *et al.*, 1999; Asa *et al.*, 1995; Guyer *et al.*, 1996; Sako *et al.*, 1995).

Previous studies have shown critical roles of cell adhesion molecules in wound healing (Nagaoka *et al.*, 2000; Subramaniam *et al.*, 1997; Yukami *et al.*, 2007; Tsirogianni *et al.*, 2006). The loss of intercellular adhesion molecule-1 (ICAM-1) inhibits wound healing, while L-selectin deficiency alone dose not affect it (Nagaoka *et al.*, 2000). However, the loss of both L-selectin and ICAM-1 delays wound healing than loss of ICAM-1 alone, suggesting that L-selectin plays some roles in absence of ICAM-1 (Nagaoka *et al.*, 2000). Like L-selectin-deficient (L-selectin$^{-/-}$) mice, E-selectin$^{-/-}$ or P-selectin$^{-/-}$ mice exhibit normal wound healing (Subramaniam *et al.*, 1997). However, combined E- and P-selectin blockade or loss delays wound healing (Subramaniam *et al.*, 1997; Yukami *et al.*, 2007). These results indicate that skin wound healing is regulated cooperatively by selectins and ICAM-1. However, a role of PSGL-1 in the wound-healing process was not investigated previously. Furthermore, in vitro and in vivo studies have shown that PSGL-1 binds all selectins with different affinities; however, the interaction of PSGL-1 with P- or E-selectin during wound healing remained unknown. In this study, we examined the wound-healing process using PSGL-1$^{-/-}$ mice in comparison with E-selectin$^{-/-}$ mice, P-selectin$^{-/-}$ mice, and P-selectin$^{-/-}$ mice

treated with anti-E-selectin monoclonal antibody (mAb).

Results

Macroscopic examination of wound healing

The areas of open wounds were measured 3 and 7 days after wounding to assess macroscopic healing defects (Figures 1 and 2A). The areas of open wounds were similar between wild type and E-selectin$^{-/-}$ mice on both days 3 and 7. On day 3, the open wound area was larger in P-selectin$^{-/-}$ mice ($p<0.0001$ and $p<0.005$), PSGL-1$^{-/-}$ mice ($p<0.0001$ and $p<0.0001$), and P-selectin$^{-/-}$ mice administrated with anti-E-selectin mAb ($p<0.005$ and $p<0.05$) than in wild type and E-selectin$^{-/-}$ mice, respectively. However, on day 7, such difference was no longer observed. There was no significant difference between P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade on days 3 and 7. Therefore, the loss of PSGL-1 expression delayed macroscopic wound healing at the early time point, while inhibitory effect of PSGL-1 deficiency was similar to that of P-selectin deficiency either alone or with E-selectin blockade.

Microscopic examination of wound healing

Three microscopic parameters for wound healing were evaluated: 1) epithelial gap that is the distance between the migrating edges of keratinocytes (Figure 2B), 2) area of granulation tissue (Figure 2C), and 3) vessel density which represents angiogenesis (Figure 2D). On both days 3 and 7, E-selectin deficiency did not affect any of these microscopic parameters. On day 3, P-selectin$^{-/-}$ mice showed larger epithelial gap and lower vessel density relative to wild type mice; there was no significant difference in epithelial gap and vessel density between P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade. By contrast, on day 3, P-selectin$^{-/-}$ mice had normal area of granulation tissue; therefore, area of granulation tissue was significantly smaller in PSGL-1$^{-/-}$ mice and P-selectin$^{-/-}$ mice with E-selectin blockade compared to P-selectin$^{-/-}$ mice. On day 7, the loss

of P-selectin only decreased vessel density relative to wild type mice. However, when compared with P-selectin$^{-/-}$ mice, vessel density was significantly smaller in PSGL-1$^{-/-}$ mice and P-selectin$^{-/-}$ mice with E-selectin blockade.

On day 3, PSGL-1$^{-/-}$ mice and P-selectin$^{-/-}$ mice with E-selectin blockade exhibited larger epithelial gap, smaller granulation tissue area, and lower vessel density compared to wild type mice; there was no significant deference between these two groups. On day 7, such significant difference remained for granulation tissue area and vessel density but not epithelial gap; like day 3, there was no significant deference between these two groups. Therefore, early granulation tissue formation and late angiogenesis in PSGL-1$^{-/-}$ mice was inhibited beyond the inhibition associated with P-selectin deficiency, but to a similar level of the inhibition observed in P-selectin$^{-/-}$ mice with E-selectin blockade.

Infiltration of inflammatory cells

Numbers of neutrophils that migrated outside the blood vessels were assessed in the wound tissues by hematoxylin and eosin (H&E) staining (Figures 3A and 4A). Neutrophils are the first to appear in significant numbers immediately after wounding, followed by macrophages. Furthermore, by day 1, the large number of neutrophils precluded microscopic quantitation. Therefore, we counted the neutrophil number after early 1 and 4 hours. The loss of E-selectin expression did not affect neutrophil numbers at 1 and 4 hours after wounding. At 1 hour after wounding, neutrophil numbers were reduced in P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade relative to wild type and E-selectin$^{-/-}$ mice. Furthermore, neutrophil numbers were reduced in PSGL-1$^{-/-}$ mice and P-selectin$^{-/-}$ mice with E-selectin blockade compared to P-selectin$^{-/-}$ mice. Similar results were obtained at 4 hours after injury.

Macrophage infiltration was assessed by immunohistochemistry using anti-F4/80 mAb

(Figures 3B and 4B). Macrophage numbers were similar between E-selectin$^{-/-}$ and wild type mice on days 3 and 7. Like neutrophil numbers, macrophage numbers decreased in P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade relative to wild type and E-selectin$^{-/-}$ mice on day 3. Moreover, macrophage numbers were lower in PSGL-1$^{-/-}$ mice and P-selectin$^{-/-}$ mice with E-selectin blockade than P-selectin$^{-/-}$ mice. On day 7, such difference was no longer detected.

Mast cell infiltration was assessed by toluidine blue staining (Figures 3C and 4C). E-selectin deficiency did not affect mast cell numbers on days 3 and 7. On day 3, mast cell numbers were significantly reduced in P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade relative to wild type mice. Unlike neutrophil and macrophage numbers, there was no significant difference in mast cell numbers between P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade. Similar results were obtained on day 7, except that significant difference between P-selectin$^{-/-}$ and wild type mice was no longer observed. Thus, either P-selectin or PSGL-1 loss inhibited early inflammatory responses until day 3, except for reduced mast cell numbers in PSGL-1$^{-/-}$ mice on day 7. Furthermore, PSGL-1 deficiency inhibited early infiltration of neutrophils and macrophages more strongly than P-selectin deficiency, but to a similar level of P-selectin$^{-/-}$ mice with E-selectin blockade.

Growth factor mRNA expression in the wounded skin tissue

Expression of bFGF, interleukin (IL)-6, IL-10, TGF-β, PDGF, and tumor necrosis factor (TNF)-α mRNA in the wounded skin tissue was examined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR; Figure 5). There was no significant difference in all cytokines examined in this study between E-selectin$^{-/-}$ and wild type mice. By contrast, mRNA levels of all cytokines examined in this study were significantly reduced

in P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade relative to wild type mice on both days 3 and 7, except that bFGF mRNA levels were similar between all groups on day 7. There was no significant difference in all cytokines examined in this study between P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade, except that P-selectin$^{-/-}$ mice with E-selectin blockade exhibited lower IL-6 mRNA levels than P-selectin$^{-/-}$ mice on day 3.

Discussion

Loss or blockade of PSGL-1 reduces various inflammatory conditions, such as ischemic reperfusion injury, peritonitis, and vasculitis (Hayward *et al.*, 1999; Scalia *et al.*, 1999; Yanaba *et al.*, 2004; Borges *et al.*, 1997). By contrast, PSGL-1 is not required for the development of experimental autoimmune encephalomyelitis (Engelhardt *et al.*, 2005). Thus, contribution of PSGL-1 to the inflammatory process varies according to the tissue site of inflammation and/or the nature of the inflammatory stimuli. In this study, PSGL-1 deficiency inhibited macroscopic wound healing, keratinocyte migration, granulation tissue formation, and angiogenesis on day 3. This inhibitory effect of PSGL-1 loss on the early wound-healing process was associated with reduced infiltration of neutrophils, macrophages, and mast cells and decreased expression of various growth factors. In contrast, macroscopic wound healing and epithelial gap were not affected by PSGL-1 loss on day 7, which could be explained in part by normal macrophage infiltration and bFGF expression on day 7, since macrophages is one of the important sources for bFGF (Hart, 2002; Singer and Clark, 1999; Tsirogianni *et al.*, 2006). However, granulation tissue formation and angiogenesis were inhibited by PSGL-1 deficiency on day 7, indicating that PSGL-1 also regulates granulation tissue formation, especially angiogenesis, at the later time point. The results of this study indicate that PSGL-1 regulates the wound-healing process, especially early phase, by mediating infiltration of inflammatory cells that produce various growth factors.

In vitro studies have shown that PSGL-1 binds P-selectin, but also binds E- and L-selectins with lower affinity (Asa *et al.*, 1995; Guyer *et al.*, 1996; Sako *et al.*, 1995; Yang *et al.*, 1999) . A previous study has revealed that L-selectin plays minor roles during wound healing (Nagaoka *et al.*, 2000). However, in this study, would healing was suppressed in PSGL-1$^{-/-}$ mice to a similar level of P-selectin$^{-/-}$ mice with E-selectin blockade, suggesting that L-selectin may not function as a PSGL-1 ligand during wound healing. Although many

studies have shown that the PSGL-1 and P-selectin interaction is predominant (Sako *et al.*, 1995; Yang *et al.*, 1999), it is sill controversial whether PSGL-1 also interacts with E-selectin. PSGL-1$^{-/-}$ mice showed defects in P-selectin-mediated, but not E-selectin-mediated rolling, bringing the relevance of PSGL-1 as an E-selectin ligand into question (Yang *et al.*, 1999). In contrast, another study has revealed that the E-selectin-PSGL-1 interaction supports slow rolling (Norman *et al.*, 2000). Furthermore, a recent study using another line of PSGL-1$^{-/-}$ mice have demonstrated that PSGL-1 has an important function in tethering flowing leukocytes to E-selectin (Xia *et al.*, 2002). In this study, there was generally no significant difference in wound healing, leukocyte accumulation, and growth factor expression between P-selectin$^{-/-}$ and PSGL-1$^{-/-}$ mice, suggesting that the PSGL-1 and P-selectin interaction is also predominant in wound healing. However, early granulation tissue formation and late angiogenesis in PSGL-1$^{-/-}$ mice were inhibited beyond the inhibition associated with P-selectin deficiency, but to a similar level of the inhibition observed in P-selectin$^{-/-}$ mice with E-selectin blockade, suggesting that PSGL-1 may interact with E-selectin in some aspects of wound healing. This is also true of neutrophil and early macrophage infiltration. However, we did not examine wound healing using mice lacking both P-selectin and E-selectin, since these mice are susceptible to skin infection that could affect the wound healing (Frenette *et al.*, 1996) (Bullard *et al.*, 1996). Furthermore, no direct functional or mechanistic results were presented in this study to prove the direct interaction between PSGL-1 and E-selectin. Nonetheless, the results of this study suggest that the PSGL-1 and P-selectin interaction may primarily regulate the wound-healing process, while E-selectin may also contribute to some aspects of wound healing as a ligand of PSGL-1.

In this study, P-selectin$^{-/-}$ mice exhibited impaired wound healing and reduced infiltration of neutrophils, macrophages, and mast cells, especially at the early phase. However, a previous study using P-selectin$^{-/-}$ mice showed no defects of wound healing with

only reduced neutrophil infiltration at 1 hour after wounding (Subramaniam *et al.*, 1997). This discrepancy may be due to the difference in genetic background: C57BL/6 background was used in this study, while mixed 129/sv x C57BL/6 background was used in the previous study. Many studies have shown that the difference between C57BL/6 and mixed 129/sv x C57BL/6 background affects phenotypes of mice. For example, $C1q^{-/-}$ mice have an increased susceptibility to nephrotoxic nephritis but this is seen on mixed 129/sv x C57BL/6 background but not C57BL/6 background (Robson *et al.*, 2003). These findings suggest that 129/sv genetic background also affects the wound-healing process mediated by P-selectin.

In the current study, mRNA expression of IL-6 IL-10, TNF-α, TGF-β, and PDGF was reduced in P-selectin$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-selectin$^{-/-}$ mice with E-selectin blockade on day 7, while only bFGF expression was normal in these mutant mice. Nonetheless, macroscopic wound healing and keratinocyte migration were not impaired in these mutant mice on day 7, suggesting that bFGF plays the most important role in wound healing. Consistent with this notion, application with bFGF but not PDGF or TGF-β normalized the delayed wound repair in mice lacking both L-selectin and ICAM-1 (Nagaoka *et al.*, 2000). In summary, the results of this study indicate that PSGL-1 significantly regulates the early wound-healing process by mediating early recruitment of leukocytes, especially macrophages that produce various growth factors, especially bFGF. Furthermore, our results suggest that PSGL-1 may function mainly as a P-selectin ligand and partly as an E-selection ligand during wound healing.

Materials and Methods

Mice

P-selectin$^{-/-}$ (Frenette *et al.*, 1996), E-selectin$^{-/-}$ (Frenette *et al.*, 1996), and PSGL-1$^{-/-}$ (Yang *et al.*, 1999) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were healthy, fertile, and did not display evidence of infection or disease. All mice were backcrossed between 5 to 10 generations onto the C57BL/6 genetic background. Mice used for experiments were 7 to 12 weeks old. Age-matched wild type littermates (Jackson Laboratory) were used as controls. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of Nagasaki University Graduate School of Biomedical Sciences.

Wounding and macroscopic examination

Mice were anesthetized with diethyl ether and their backs were shaved and wiped with 70% alcohol. Full-thickness excisional wounds were made by picking up a fold of skin and using a disposable sterile 6-mm biopsy punch (Maruho, Osaka, Japan) to punch through the two layers of skin on one flank, resulting in generation of two wounds at the same time, as described (Subramaniam *et al.*, 1997). Similarly, two other wounds were made on the other side of flank. After wounds were covered with an occlusive dressing (Tegaderm®, 3M Canada, London, USA), mice were caged individually. At 3 and 7 days after wounding, mice were anesthetized, and areas of open wounds were measured by tracing the wound openings onto a transparency. Any signs suggestive for local infection were not detected in the wounded skin. After distorted wounds were excluded, one wound was randomly selected in each mouse for the analysis. For macroscopic analysis of wound closure, 14 mice were used in each group. For a blocking study, mAbs to E-selectin (10E9.6, rat IgG2a, 30 μg per mouse; BD PharMingen, San Diego, CA) (Bosse and Vestweber, 1994) were injected

intravenously before wounding and on day 3. This was the mAb concentration required to inhibit E-selectin-dependent leukocyte rolling in vivo as described previously (Kunkel *et al.*, 1996).

Histological examination and immunohistochemistry

After the mice were sacrificed, wounds were harvested with a 2-mm rim of unwounded skin tissue. The wounds were cut into halves laterally; one-half of the samples was fixed in 3.5% paraformaldehyde and then paraffin embedded, and the remaining of each wound was frozen with liquid nitrogen for mRNA analysis. Six-mm paraffin sections were stained with H&E, toluidine blue staining, or immunostaining.

For immunostaining, deparafinized sections were treated with endogenous peroxidase blocking reagent (DAKO Cytomation A/S, Copenhagen, Denmark) and proteinase K (DAKO Cytomation A/S) for 6 minutes at room temperature. Sections were then incubated with rat mAbs specific for macrophages (F4/80; American Type Culture Collection, Rockville, MD). Rat IgG (Southern Biotechnology Associates Inc., Birmingham, AI) was used as a control for nonspecific staining. Sections were incubated sequentially with a biotinylated rabbit anti-rat IgG as secondary Ab (Vectastain ABC method, Vector Laboratories, Burlingame, CA), then horseradish peroxidase-conjugated avidin-biotin complexes. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and then counterstained with methyl green.

The number of neutrophils determined with H&E staining was counted in the entire section outside the blood vessels at 1 and 4 hours after wounding. The number of mast cells was counted with toluidine blue staining in the entire section at 3 and 7 days after wounding. Numbers of F4/80-positive macrophages were determined by counting in 9 high-power fields (0.07 mm^2, magnification, ×400) in the wound bed per section. Among the 9 fields, 6 fields

were selected from both edges of the wound bed, and the remaining 3 fields were chosen from the middle of it. For analysis of neutrophils, macrophages, and mast cells, 10 mice were used in each group. The epithelial gap, which represents distance between the leading edge of migrating keratinocytes, was measured in H&E-stained sections of wounds. We identified the area that consists of newly formed capillaries and the collection of fibroblasts and macrophages as granulation tissue. Wound sections were visualized by the color monitor (PVM-14M4J, OLYMPUS, Tokyo, Japan) with the charge-coupled device camera (CS-900, OLYMPUS). Then, the area of granulation tissue or vessel density was gated and measured by the video micrometer system (VM-60, OLYMPUS). Using the free-hand tool of Photoshop (Version 7.0, Adobe systems, Tokyo, Japan), vessel density determined with H&E staining was measured in the whole wound bed areas and was expressed as a percentage of the whole wound bed areas. For measurement of epithelial gap, granulation tissue area, and vessel density, 14 mice were used in each group.

RNA isolation and real-time RT-PCR

Total RNAs were extracted from frozen wounded skin samples using Qiagen RNeasy spin columns (QIAGEN, Crawley, UK) and subsequently were reverse transcribed to cDNA using Ready-To-Go RT-PCR Beads (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's protocols. Real-time quantitative RT-PCR was performed using the TaqMan® system (Applied Biosystems, Foster City, CA) on ABI Prism 7300 Sequence Detector (Applied Biosystems) according to the manufacturer's instructions. TaqMan probes and primers for bFGF, IL-6, IL-10, TGF-β1, PDGF, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. Relative expression of PCR products was determined using the $\Delta\Delta C_T$ method. Briefly, each set of samples was normalized using the difference in threshold cycle (C_T) between the target gene and

housekeeping gene (GAPDH): $\Delta C_T = (C_{T\ target\ gene} - C_{T\ GAPDH})$. Relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ calibrator}$. Each sample was examined in duplicate and the mean C_T was used in the equation. For real-time RT-PCR analysis of growth factor mRNA expression, 10 mice were used in each group.

Statistical analysis

The Mann-Whitney test was used for determining the level of significance of differences between samples and Bonferroni's test was used for multiple comparisons. A P value <0.05 was considered statistically significant.

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Figure legends

Figure 1: Macroscopic wound closure in wild type mice, E-selectin (E-sel)$^{-/-}$ mice, P-selectin (P-sel)$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-sel$^{-/-}$ mice treated with anti-E-selectin mAb (P-sel$^{-/-}$ + anti-E) at 3 and 7 days after wounding. Representative photographs of open wounds in wild type and mutant mice at 3 and 7 days after wounding are shown. Full-thickness cutaneous wounds were made using 6-mm biopsy punch.

Figure 2: Macroscopic and microscopic wound healing parameters in wild type mice, E-selectin (E-sel)$^{-/-}$ mice, P-selectin (P-sel)$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-sel$^{-/-}$ mice treated with anti-E-selectin mAb (P-sel$^{-/-}$ + anti-E) at 3 and 7 days after wounding. (A) The microscopic area of open wound was measured at 3 and 7 days after wounding by tracing of the wound openings onto a transparency. The distance between the migrating edges of keratinocytes under the eschar (epithelial gap; B) and the area of granulation tissue (C) were both microscopically measured in the tissue sections. (D) Vessel density was microscopically measured in the whole wound bed areas and was expressed as a percentage of the whole wound bed areas. All values represent the mean ± SEM. These results were obtained from 14 mice in each group.

Figure 3: Representative inflammatory cell recruitment in the wounded skin from wild type mice, E-selectin (E-sel)$^{-/-}$ mice, P-selectin (P-sel)$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-sel$^{-/-}$ mice treated with anti-E-selectin mAb (P-sel$^{-/-}$ + anti-E). (A) Neutrophils were detected in H&E-stained sections at 1 and 4 hours after wounding. Arrows show representative neutrophils. Bars: 100μm. (B) Macrophages were detected in anti-F4/80 Ab-stained sections at 3 and 7 days after wounding. Bars: 50μm. (C) Mast cells were detected in toluidine blue-stained sections at 3 and 7 days after wounding. Arrows show mast cells. Bars: 50μm.

Figure 4: Inflammatory cell recruitment in the wounded skin from wild type mice, E-selectin (E-sel)$^{-/-}$ mice, P-selectin (P-sel)$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-sel$^{-/-}$ mice treated with anti-E-selectin mAb (P-sel$^{-/-}$ + anti-E). (A) Numbers of neutrophils per section were counted in entire H&E-stained sections outside the blood vessels under the microscope. (B) Numbers of anti-F4/80 Ab-positive macrophages per field (0.07 mm^2) were also counted. (C) Numbers of mast cells per section were counted in entire toluidine blue-stained sections. All values represent the mean ± SEM. These results were obtained from 10 mice in each group.

Figure 5: Growth factor mRNA expression in the wounded skin tissue from wild type mice, E-selectin (E-sel)$^{-/-}$ mice, P-selectin (P-sel)$^{-/-}$ mice, PSGL-1$^{-/-}$ mice, and P-sel$^{-/-}$ mice treated with anti-E-selectin mAb (P-sel$^{-/-}$ + anti-E) at 3 and 7 days after wounding. Relative mRNA expression of IL-6, IL-10, TNF-α, TGF-β, PDGF, and bFGF was quantified by real-time RT-PCR and normalized relative to endogenous GAPDH levels. All values represent the mean ± SEM. These results were obtained from 10 mice in each group.

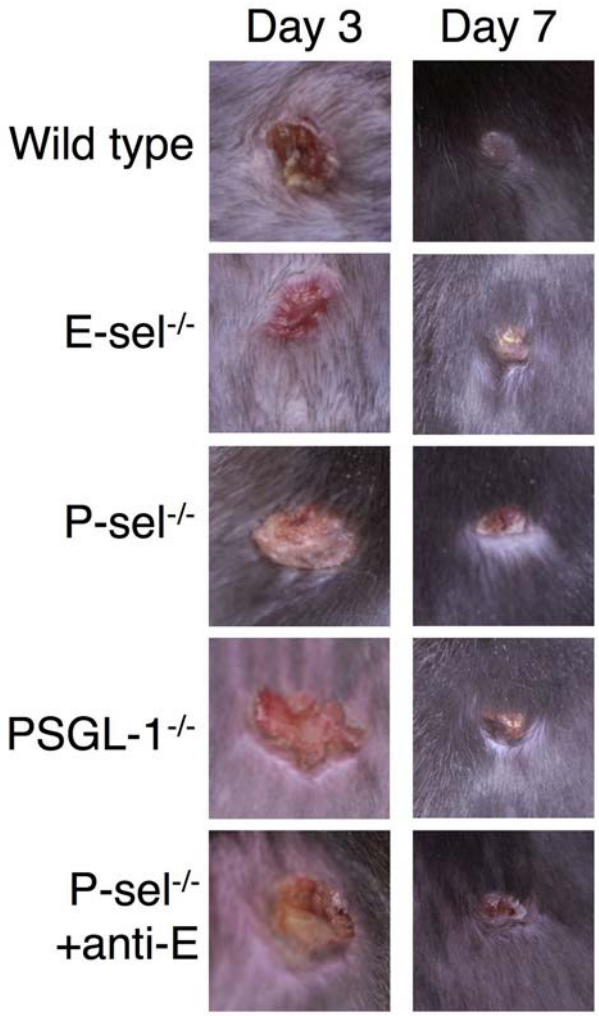

Figure 1
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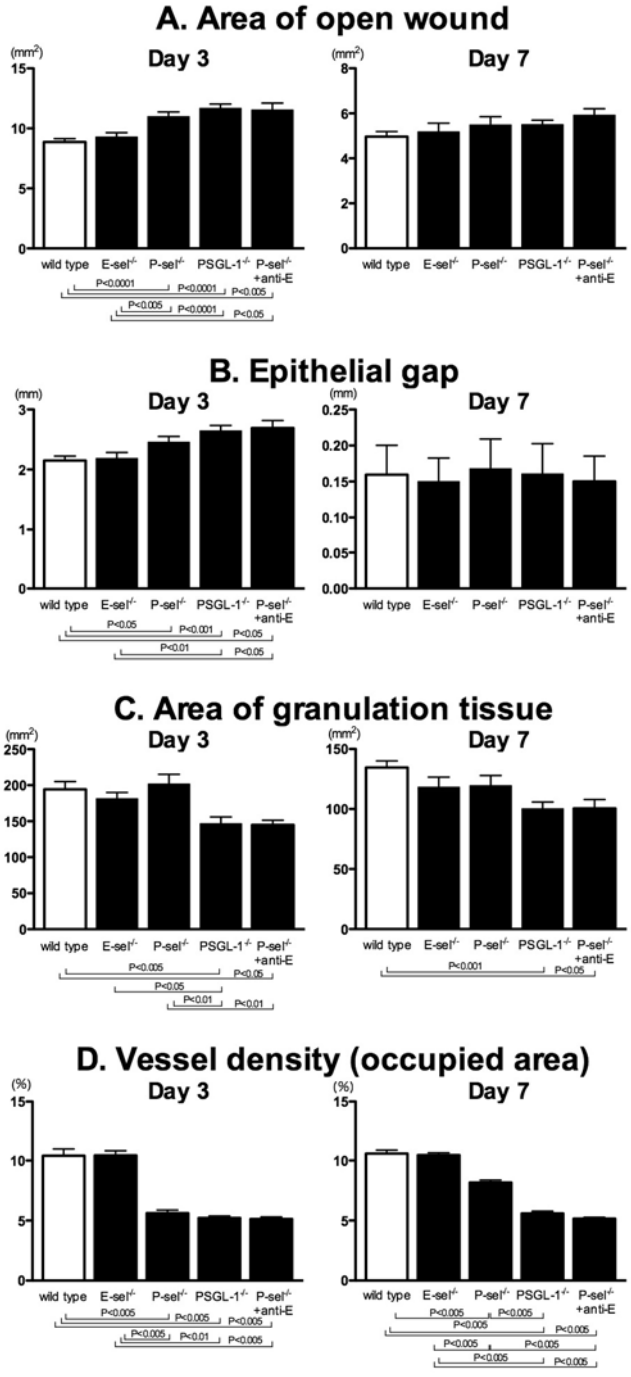

Figure 2
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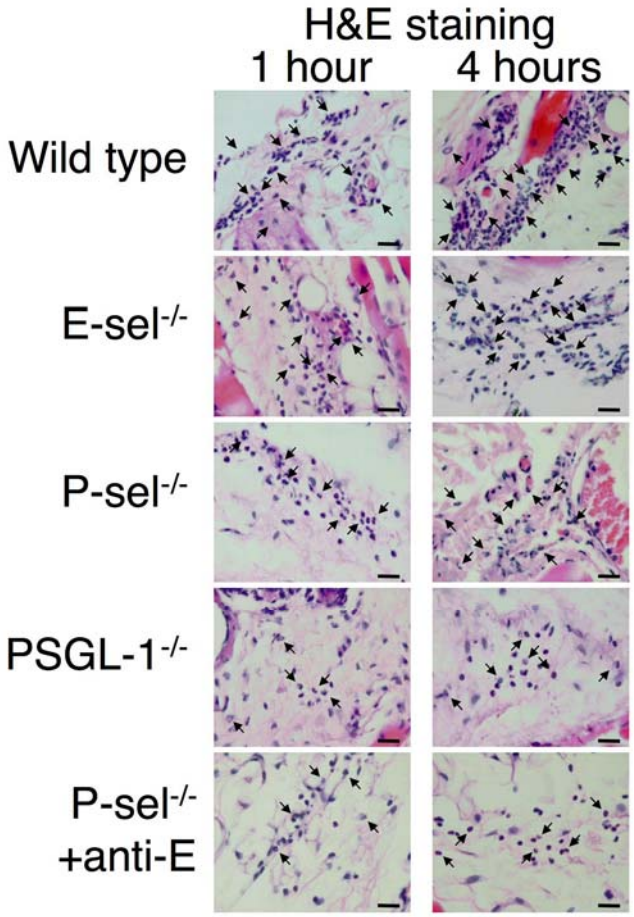

Figure 3A
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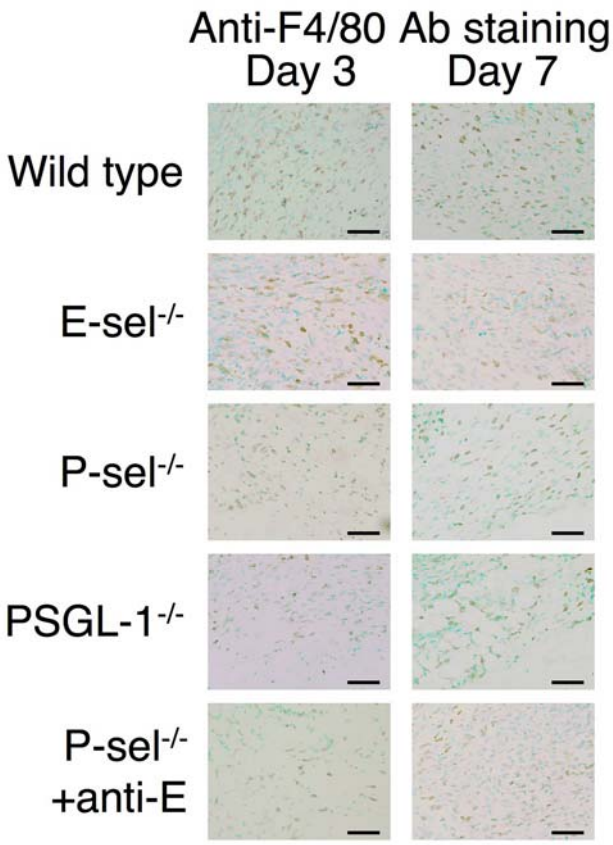

Figure 3B
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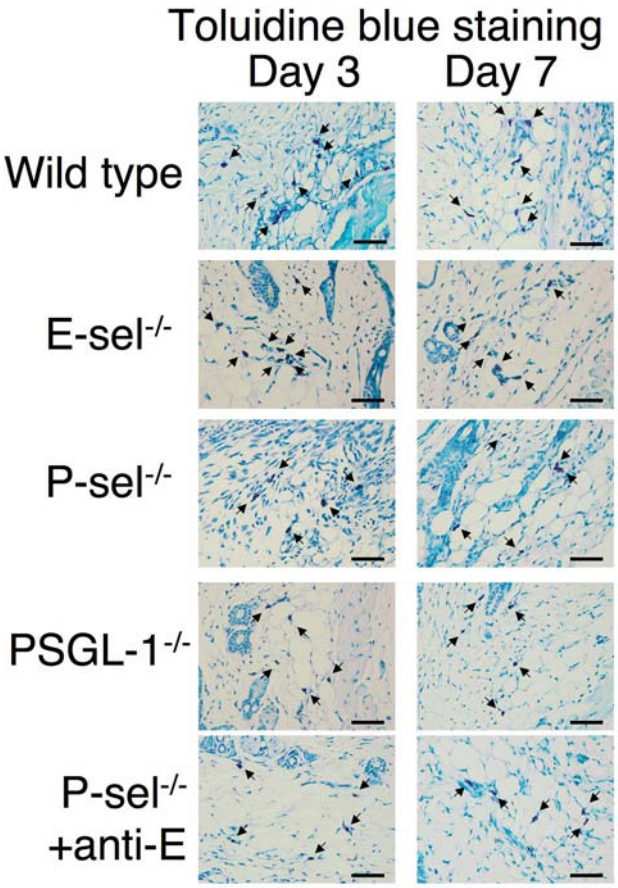

Figure 3C
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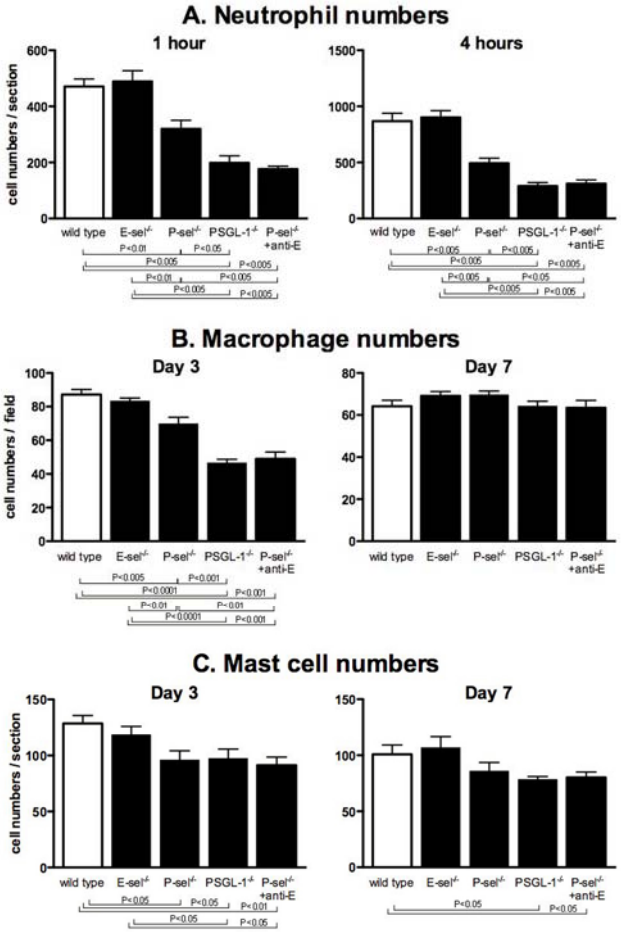

Figure 4
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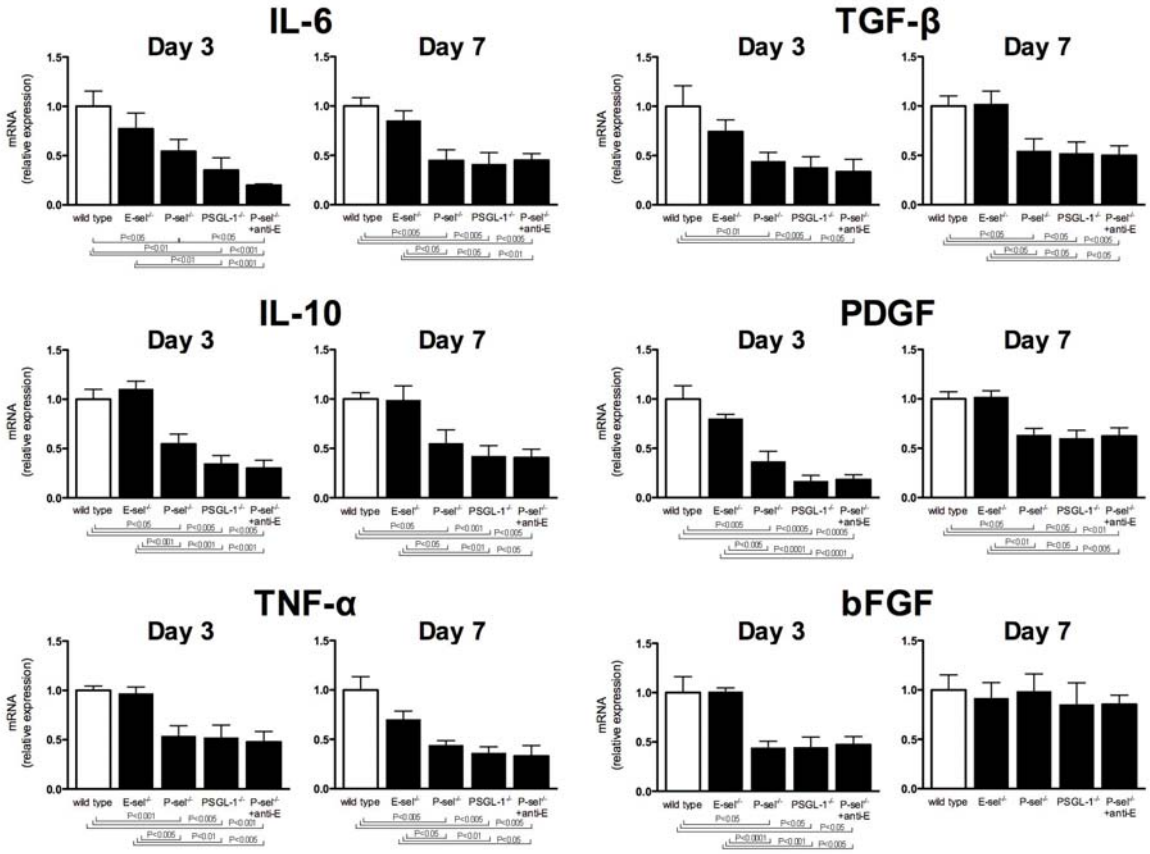

Figure 5
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