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Possible Platelet-Derived Growth Factor Involvement on Helium–Neon Laser Stimulated Wound Healing in Rats

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The role of platelet-derived growth factor (PDGF) during helium–neon (He–Ne) laser (6 mW, 7.01 J/cm^2)-stimulated wound healing in rats was investigated. Two square skin defects were produced, one on either side of the dorsal midline of each rat. PDGF was applied to the defect on one side, and these defects healed slightly more rapidly than the defects on the control sides, with a statistical significance ($p < 0.01$). Next, the production of PDGF on the laser-treated sides at days 1 and 2 was significantly ($p < 0.05$) higher than that on the non-laser-treated sides, the difference being approx. 2-fold (from 1.79 to 3.20 ng/mg protein at day 1, and from 1.43 to 3.15 ng/mg protein at day 2). Using an antibody which could neutralize PDGF activity, wound healing on the antiboby-applied sides was found to be significantly ($p < 0.01$) slower than that on the control sides. Moreover using the antibody following laser irradiation partially suppressed the laser-stimulated effect, with a statistical significance ($p < 0.01$). These results suggest that He–Ne laser stimulated the production of PDGF during wound healing in rats *in vivo*, and that PDGF plays a role in laser-stimulated wound healing.

Keywords: Biostimulation; Low-energy laser; Wound healing; He–Ne Laser;
Platelet-derived growth factor; PDGF; Rat skin wounds

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INTRODUCTION

Lasers were introduced into the medical field soon after they were invented. The helium–neon (He–Ne) laser is one of a number of low-energy types of laser, which are said to evoke the effects of 'biostimulation' (Mester *et al.*, 1985). The cellular mechanisms of the effects of low-energy lasers are poorly understood. In addition, *in vitro* studies have provided inconsistent results concerning the effects of He–Ne lasers on cell cultures (Martin *et al.*, 1988; Colver and Priestley, 1989; Hein *et al.*, 1992). Moreover, relatively little is known concerning the effect of lasers at the biochemical level. As for wound healing, a considerable amount of conflicting literature exists concerning the effects of low-energy lasers. Although a few negative results have been reported (Colver and Priestley, 1989; Hein *et al.*, 1992; Braekt *et al.*, 1991; Jongsma *et al.*, 1983; Hunter *et al.*, 1984), several studies have suggested the enhancement of wound healing, both in a human study (Mester *et al.*, 1985) and in experimental animal models (Martin *et al.*, 1988; Mester *et al.*, 1971; Mester and Jaszsagi-Nagy, 1973; Abergel *et al.*, 1987). During our previous research, using an experimental animal model, we found evidence that certain aspects of wound healing may be favorably influenced by lasers (Kimura *et al.*, 1991). It has been reported that several growth factors are also concerned with wound healing (Lynch *et al.*, 1989). However, there has been no report concerning the relationship between growth factor-stimulated wound healing and laser-stimulated wound healing except our report (Kimura *et al.*, 1993). We previously suggested that He–Ne laser stimulated the enhancement of transforming growth factor-β (TGF-β) activity (Kimura *et al.*, 1993), which is one of the growth factors concerned with wound healing (Lynch *et al.*, 1989).

In the experiments reported here, therefore, we examined whether or not laser irradiation at 632.8 nm stimulated the production of platelet-derived growth factor (PDGF), which is yet the another of growth factors concerned with wound healing (Lynch *et al.*, 1989), since PDGF is a potent mitogen, chemotactic agent, and a stimulator of protein synthesis for cells of a mesenchymal origin (Antonidades and Owen, 1984; Ross, 1987).

MATERIALS AND METHODS

Animals

After approval by our Institutional Animal Care and Use Committee, a total of 50 healthy male Wistar strain rats, initially 250–350 g in body weight and 11–13 weeks old, were used throughout the experiments. They were given water and food (MF, Oriental Yeast CO. Ltd., Tokyo, Japan) *ad libitum*. The animals were shaved closely on the dorsum. Operations were performed with the animals under anesthesia by intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, IL, USA). Two full-thickness square skin defects were produced with surgical scissors, one on either side of the dorsal midline in each rat. The two defects had a constant depth of 5 mm and covered almost the same surface area within a range of 120–140 mm^2. One side was used as the control side, and the other as the experimental side. The wounds were left open without any dressing. The animals were kept in wire cages without bedding, so that the wounds remained, while not strictly sterile, at least in a clean condition.

PDGF Application

The PDGF which we obtained from Biomedical Technologies Inc. (Stoughton, MA, USA) was PDGF B–B dimer, and it was recombinant human PDGF. After being dissolved in sterilized phosphate buffer saline (PBS), it (100 ng) was applied to one side of the wound areas every day from the operative day to day 4, by external applications to 7 rats. The same dose of bovine serum albumin (BSA) was applied to the control sides.

Laser Irradiation

One wound on one side of the dorsum of each rat in the laser irradiation group experiment was irradiated by He–Ne laser (Soft Laser 632 ®, Worldwide Lasers Industry, Geneva, Switzerland) at a wavelength of 632.8 nm and a maximum power output of 6 mW. The beam diameter was set at 0.4 mm. This wound was irradiated

under anesthesia every day at days 0, 1, and 2, from a distance of 2–3 mm, for a period of 30 min ($7.01 \, J/cm^2$). The spot size was approximately 2 mm. During the irradiation, the laser was fixed over the wound surface.

Measurement of Wound Areas

Wound areas were measured at scheduled days using a Nexus Qube image analysis processor (Nexus Inc., Tokyo, Japan) by a technician who was not informed of the true nature and purpose of these experiments. Thus, the measurement of wound areas was kept blind. Where there was the presence of a scab on both sides of the rat, the size of the scab was assessed. However, in cases where a scab was present on only one side, this was removed, and only the wound area itself was assessed. For the purposes of statistical analysis, all data were expressed as the mean of six determinations and standard deviations. Each figure shows data from one representative rat, with each experiment using at least 4 rats. All rats survived the entire course of the experiments. The significances between data points and the controls at scheduled days were determined using Student's t-test.

Measurement of PDGF Production after Laser Irradiation

After laser irradiation, wound tissues from 24 rats were excised from the wound areas, and were homogenized in cold buffer containing 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, and 1 mM di-isopropyl-fluorophosphate with Polytron ® (Kinematica, Geneva, Switzerland). Measurements of the PDGF production were carried out according to the enzyme-linked immunosorbent assay (ELISA) method (Engvall and Perlman, 1971). The primary antibody was obtained from R & D Systems (Minneapolis, MN, USA), and this was rabbit IgG fraction, which was reacted with PDGF A–B and B–B dimers. The secondary antibody was obtained from R & D Systems, and this was biotin-conjugated anti-rabbit IgG, which was produced in goats. In addition, we used alkaline phosphatase-conjugated avidin, which was also obtained from R & D Systems. P-nitrophenylphosphate was used as an enzyme substrate.

The protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as the standard. The significances between data points and the controls at indicated days were determined using Student's t-test.

Histological Preparation

The tissue samples were excised from the dorsum of 4 rats, fixed in 10% neutral formalin overnight at 4°C and were then processed into paraffin blocks for routine hematoxylin and eosin (H.E.)-staining and for immunohistochemically stained microscopic sections. The immunohistochemical staining procedure was the same as the ELISA method we used, namely the indirect method, using avidin–biotin–alkaline phosphatase. The Enzyme Substrate Kit II (Vector Laboratories Inc., Burlingme, CA, USA) was used as a substrate. The healthy dorsal skin of a rat was used as a negative control.

Suppression of PDGF Activity by Antibody

The PDGF-neutralizing antibody (R & D Systems) had specificity for neutralization of PDGF (mainly A–B dimer) activity and was dissolved in sterilized PBS. The antibody (50 µg) was applied to one wound area every day from the operative day to day 2 by external applications, and BSA (50 µg) in sterilized PBS was similarly applied to the control side for 15 rats. Wound areas were measured at scheduled days. The significances between data points and the controls at scheduled days were determined using Student's t-test.

RESULTS

PDGF Application Effect

Figure 1 shows the process of wound healing, from the operative day to day 4. The PDGF-applied side appeared almost the same as the control side. Next, the wound areas were measured, and then plotted (Fig. 2). At day 4 the wound area on the side to which PDGF had been applied was found to be significantly different from that on the control side. But at other days, we observed no significant difference.
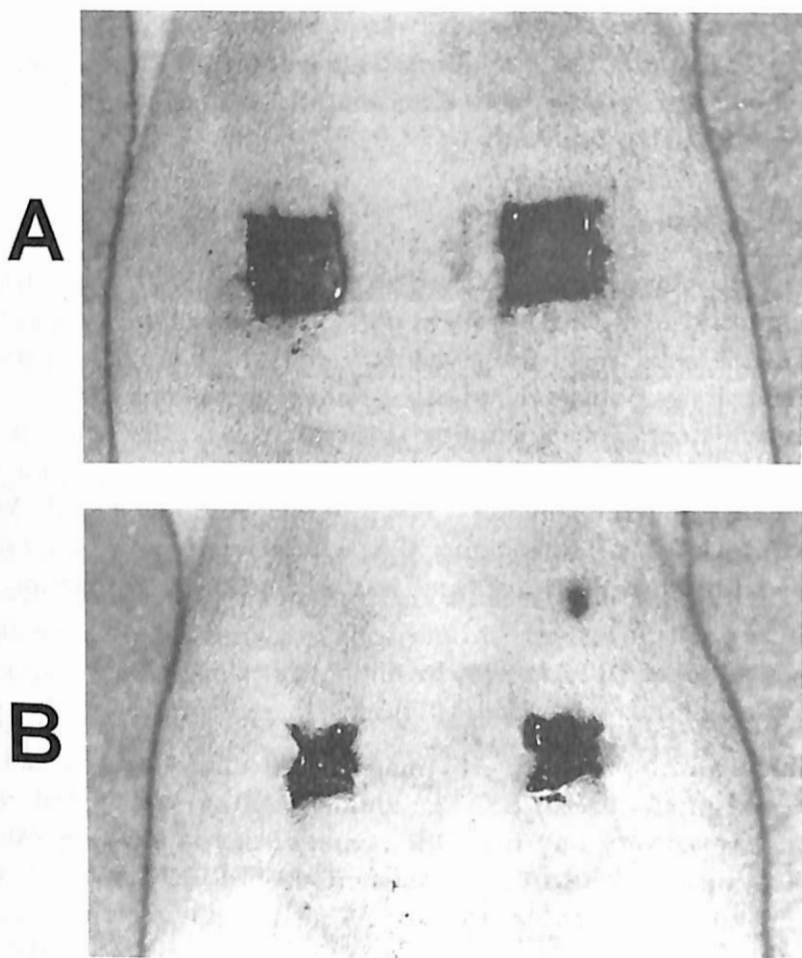
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FIGURE 1 Photographs of wounds on the operative day (A), and 4 days later (B). The right side was applied with PDGF (100 ng) every day from the operative day to day 4, and the left side, the contralateral side, was applied with the same dose of BSA.

As this difference was not convincing, in order to make a more accurate judgement we examined the tissues histologically. Figure 3 shows the results at day 7. The granulation tissue (vertical thickness, 1.5 mm), epidermal ratio (42.2%) and connective tissue (percentage of wound area, 16.7%) on the PDGF-applied sides were more extensive than those on the control sides (1.0 mm, 33.3%, 11.1%, respectively), but there was no clear difference in neovascular development (16, 14/0.5 mm^2, respectively). This demonstrated histologically, that the PDGF-applied sides had healed more rapidly than
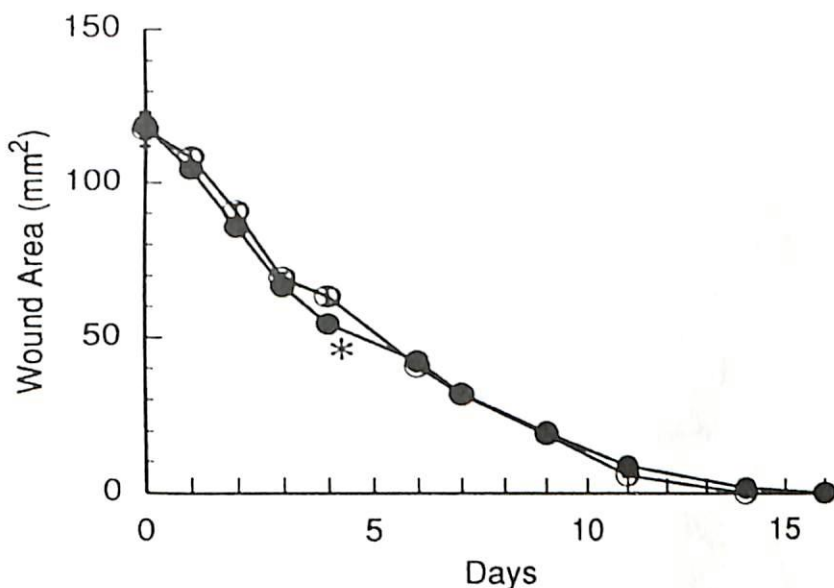
FIGURE 2   Effects of PDGF on wound healing. The wound areas were measured and then plotted. Data are expressed as the mean of six determinations. Bars represent the standard deviation. The data are from one representative rat, where at least four rats were used. ○, control; ●, PDGF-applied side. * shows a significant difference ($p < 0.01$).

the control sides. The histological results at days 2 and 9 were the same (data not shown).

Measurement of PDGF Production after Laser Irradiation

Figure 4 shows the results of PDGF production. At operative day just after laser irradiation, laser stimulated the PDGF production, but this was not significant difference. The PDGF production on the laser-treated side was significantly greater than that on the control side at days 1 and 2, but we observed no significant difference at other days.

Localization of PDGF

Figure 5 shows the results at day 2. Judging from immunohisto-chemical staining studies, the localization of PDGF was much greater in the granulation tissues, particularly in the subsurface zone. The
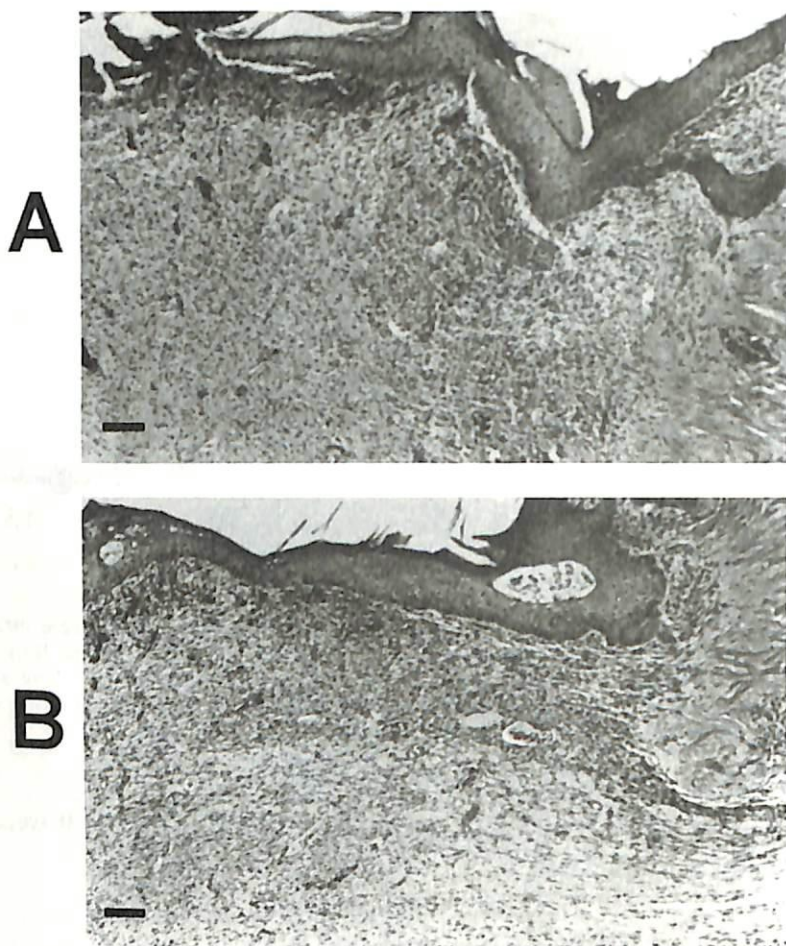
FIGURE 3 Histological section of H.E.-stained tissues on wound areas at day 7.
(A) is the control side, (B) is the PDGF-applied side. Granulation tissue in (B) was
much more extensive than in (A), and the epidermal ratio and connective tissues were
also greater in (B). Magnification is ×16, bar = 0.1 mm.

strength of staining on the laser-treated sides appears to be slightly
greater than that on the control sides.

Suppression of PDGF Activity by Antibody

Figure 6 shows the process of wound healing, from the operative
day to day 9. Wound areas at scheduled days were then measured
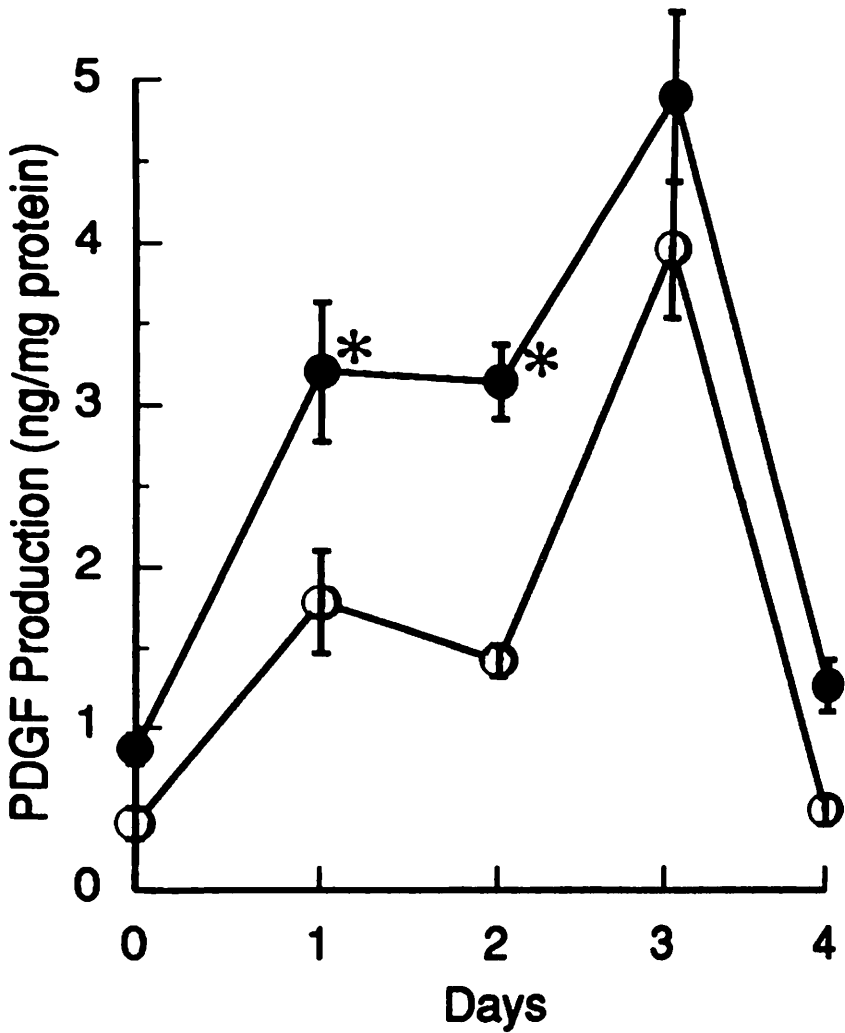and plotted (Fig. 7), and significant difference between the control

FIGURE 4   Enhancement of PDGF production by lasers. The PDGF production was measured on indicated days. Data are expressed as the mean of six sets of rats. Bars represent the standard error of the mean. O, control; ●, laser-treated side. * shows a significant difference ($p < 0.05$).

sides and the sides to which antibody had been applied could only be recognized 7–9 days later. In order to make a more accurate judgement, we examined the tissues histologically. Figure 8 shows the results at day 14. The connective tissue (percentage of wound area, 66.7%) on the antibody-applied sides was much less extensive than that (77.8%) on the control sides, but there were no clear
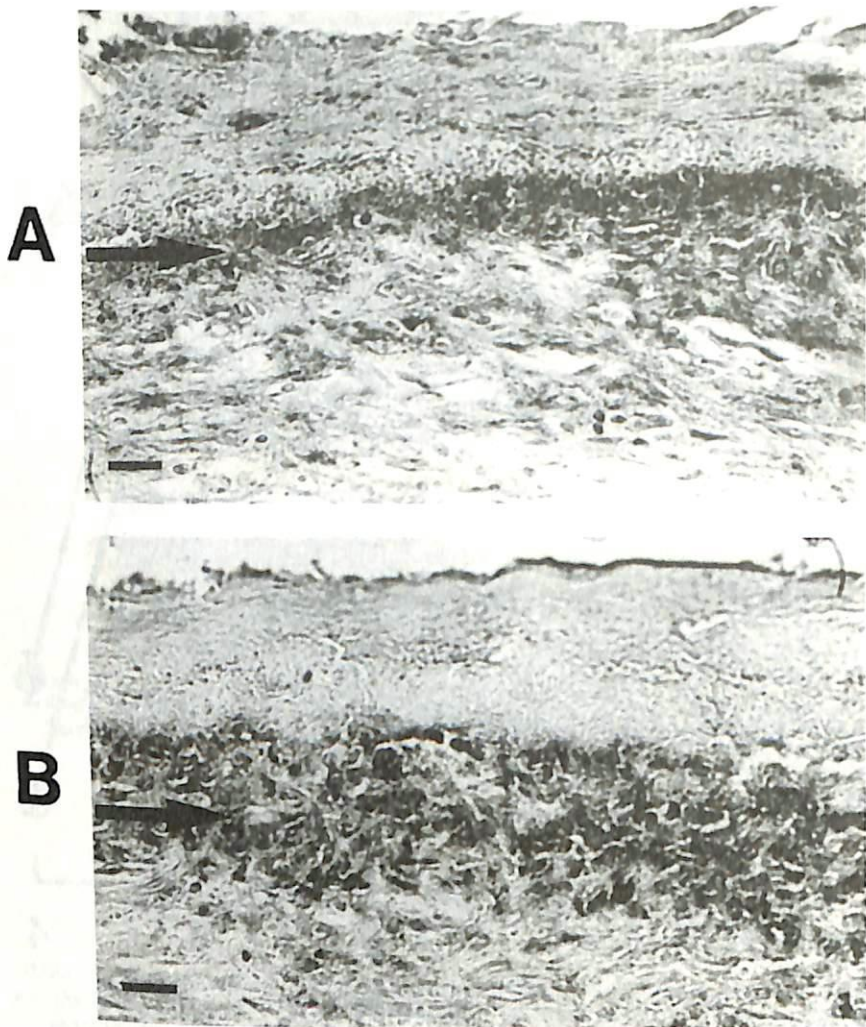
FIGURE 5 Immunohistochemical localization of PDGF (arrow) in the wound area at day 2. The wound area was irradiated by lasers every day. (A) is the control side and (B) is the laser-treated side. Magnification is × 80, bar = 25 μm.

differences in epidermal ratio (100%, each) or neovascular development (11, 10/0.5 mm², respectively) between the two sides. Healing on the antibody-applied side was slightly delayed compared to that on the control side. We obtained similar results at day 21, but there was no difference at day 4 between the control and antibody-applied side (data not shown).
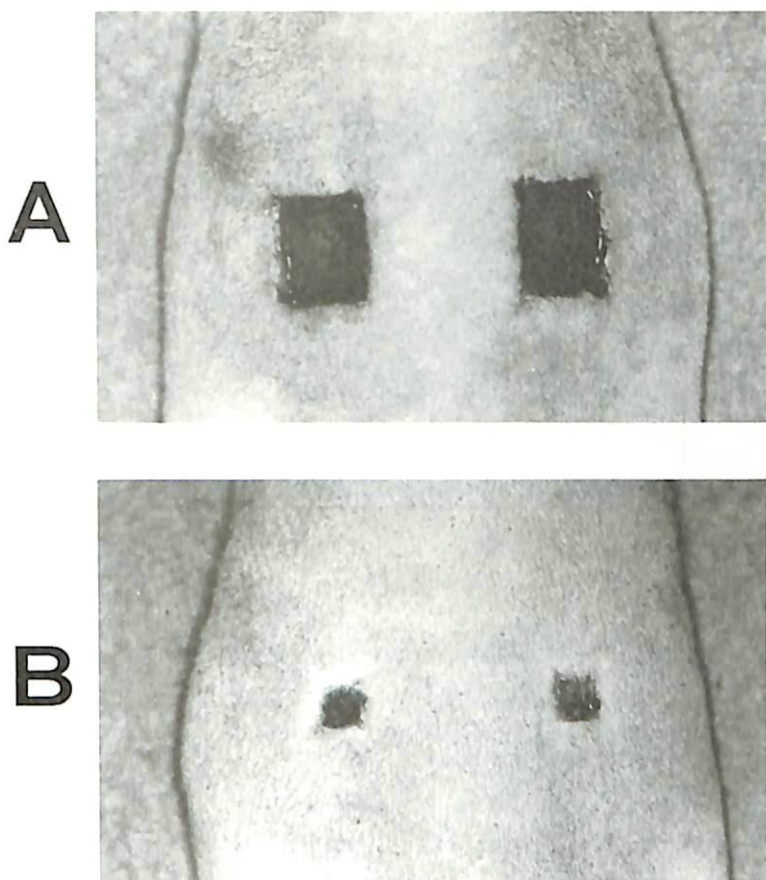
FIGURE 6   Photographs of wounds on operative day (A) and 9 days later (B). The right side was applied with PDGF antibody (50 µg) every day from the operative day to day 2, and the left side was applied with the same dose of BSA.

Suppression of PDGF Activity by Antibody after Laser Irradiation

Figure 9 shows the process of wound healing, from the operative day to days 2 and 9. The neutralizing antibody brought a slight delay in the wound healing at days 9–11, which was similar to that seen when the antibody alone was applied. However at day 2, a laser-stimulated healing effect was observed. Next, wound areas were measured and plotted (Fig. 10), and significant differences between the control sides and the sides to which lasers had been irradiated and antibody had then been applied, were recognized at days 2 and days 9–11. In order to make a more accurate judgement, we examined the
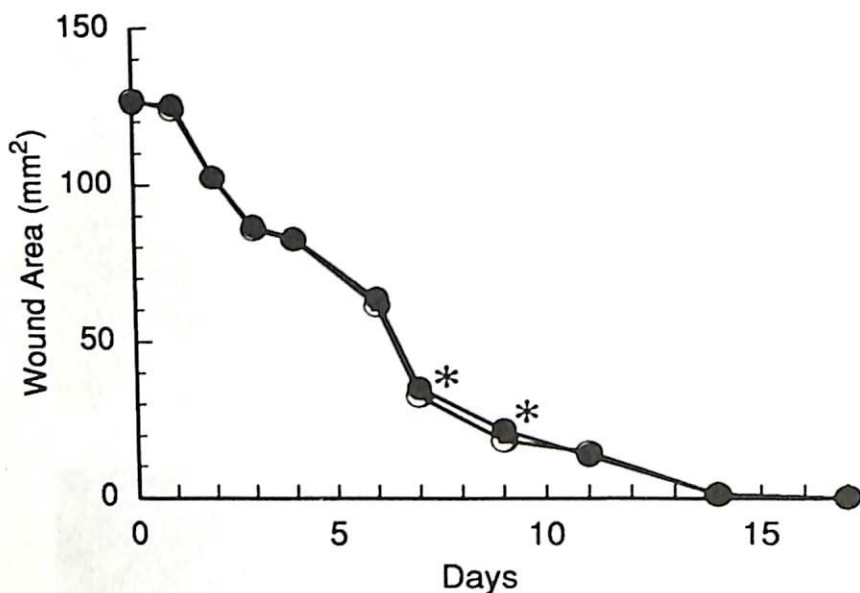
FIGURE 7   Effects of the neutralizing antibody on wound healing. The wound areas were measured and then plotted. Data are expressed as the mean of six determinations. Bars represent the standard deviation. The data are from one representative rat, where at least four rats were used. ○, control; ●, antibody-applied side. * shows a significant difference ($p < 0.01$).

tissues histologically. Figure 11 shows the results at day 7. The connective tissue (percentage of wound area, 13.3%), epidermal ratio (22.2%) and neovascular development ($17/0.5 \, mm^2$) on the laser-irradiated and antibody-applied side were remarkably more extensive than those (2.2%, 4.1%, $8/0.5 \, mm^2$, respectively) on the control side. Similar results were obtained at days 19, 21 and 26 (data not shown).

DISCUSSION

In this study, we suggested the involvement of PDGF in the enhancement of wound healing by He–Ne lasers. Low-energy lasers have been noted to stimulate wound healing by enhanced collagen synthesis (Lyons *et al.*, 1987), and by activation of some enzymes (Mester, 1980). However, relatively little is known concerning the effects of lasers at the biochemical level. In particular, details about the effects of growth factors have not yet been reported except in
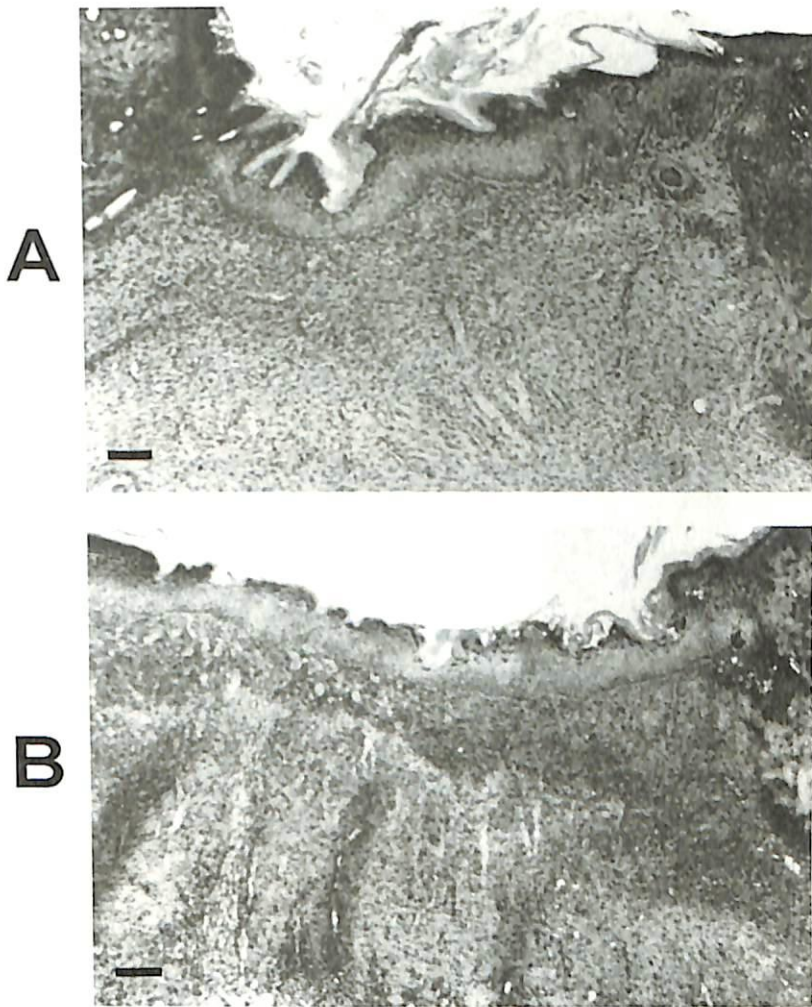
FIGURE 8   Histological section of H.E.-stained tissues on wound area at day 14. (A) is the control side, (B) is the PDGF antibody-applied side. Connective tissue in (B) was less than in (A). Magnification is ×16, bar = 0.1 mm.

our previous report (Kimura *et al.*, 1993). Since other growth factors may affect laser-stimulated wound healing, we examined the involvement of PDGF in laser-stimulated wound healing in this study. It had previously been reported that PDGF stimulates the growth of connective tissue cells *in vitro* and that it may play a role in wound repair (Ross *et al.*, 1974; Scher *et al.*, 1979). In measuring the PDGF production involved in wound healing, we obtained a significantly
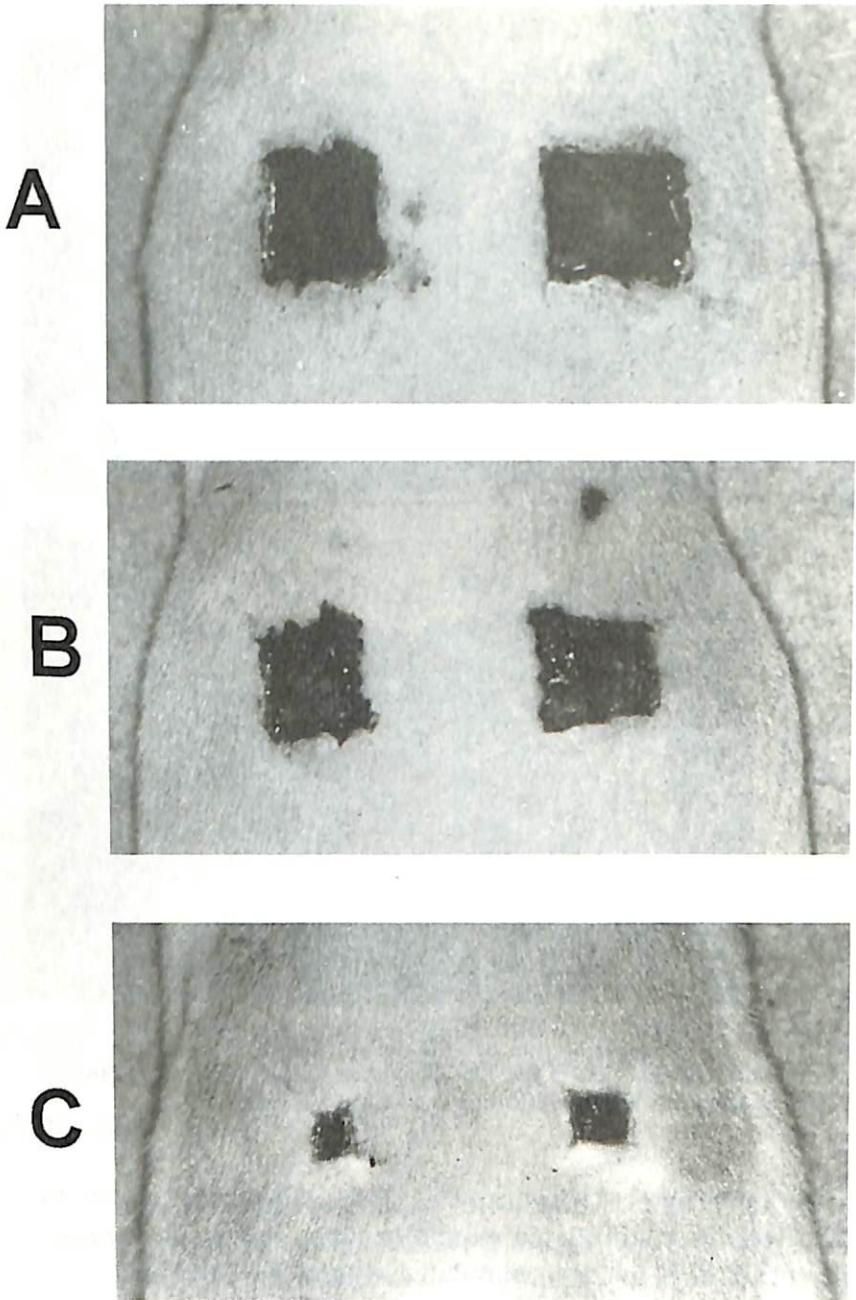
FIGURE 9   Photographs of wounds on operative day (A), 2 days later (B), and 9 days later (C). The right side was irradiated with laser and applied with PDGF antibody (50 µg) every day from the operative day to day 2, and the left side was applied with the same dose of BSA.
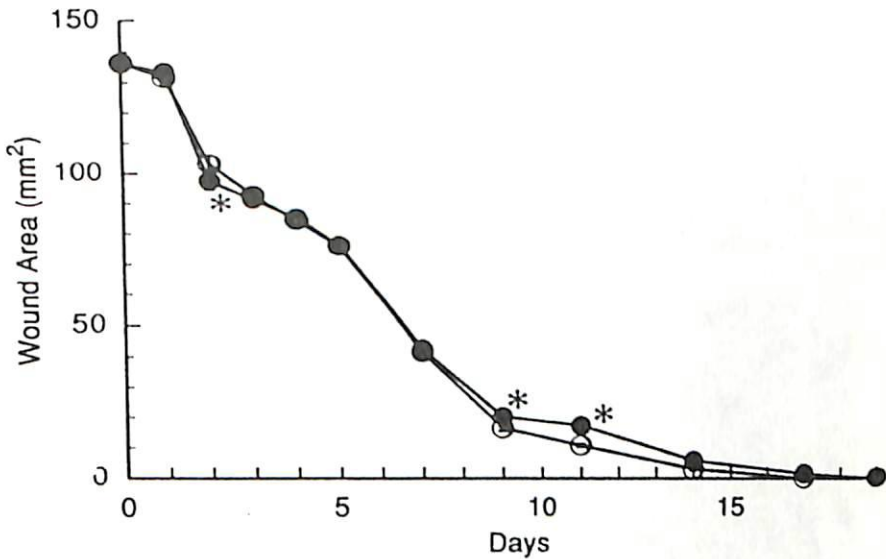
FIGURE 10   Effects of neutralizing antibody, following laser irradiation, on wound healing. The wound areas were measured and then plotted. Data are expressed as the mean of six determinations. Bars represent the standard deviation. The data are from one representative rat, where at least four rats were used. O, control; ●, antibody following laser irradiation. * shows a significant difference ($p < 0.01$).

different result between controls and laser-treated sides at days 1 and 2, but at not day 3 which was peak. This suggests that laser stimulated early healing stage only. From immunohistochemical analysis, most of the PDGF was found to be in the granulation tissue, especially in the subsurface zone of the granulation tissue. It is therefore possible for external applications of PDGF-neutralizing antibody to suppress PDGF activity. However, the suppression of PDGF activity was incomplete. This may have been due an inadequate dose of PDGF antibody, or to physical blockage of the surface zone, for example, by a clot. Nevertheless, we were able to observe a partial suppression of the laser-stimulated effect by the antibody. These results suggest that lasers stimulate the enhancement of PDGF production and that PDGF plays a role in laser-stimulated wound healing. However, the manner through which lasers stimulate the enhancement of PDGF activity remains unknown, and further studies are needed to clarify this point. In addition, further studies on the effects of lasers on wound healing at the biochemical level, especially studies concerning growth factors, should also be undertaken, since
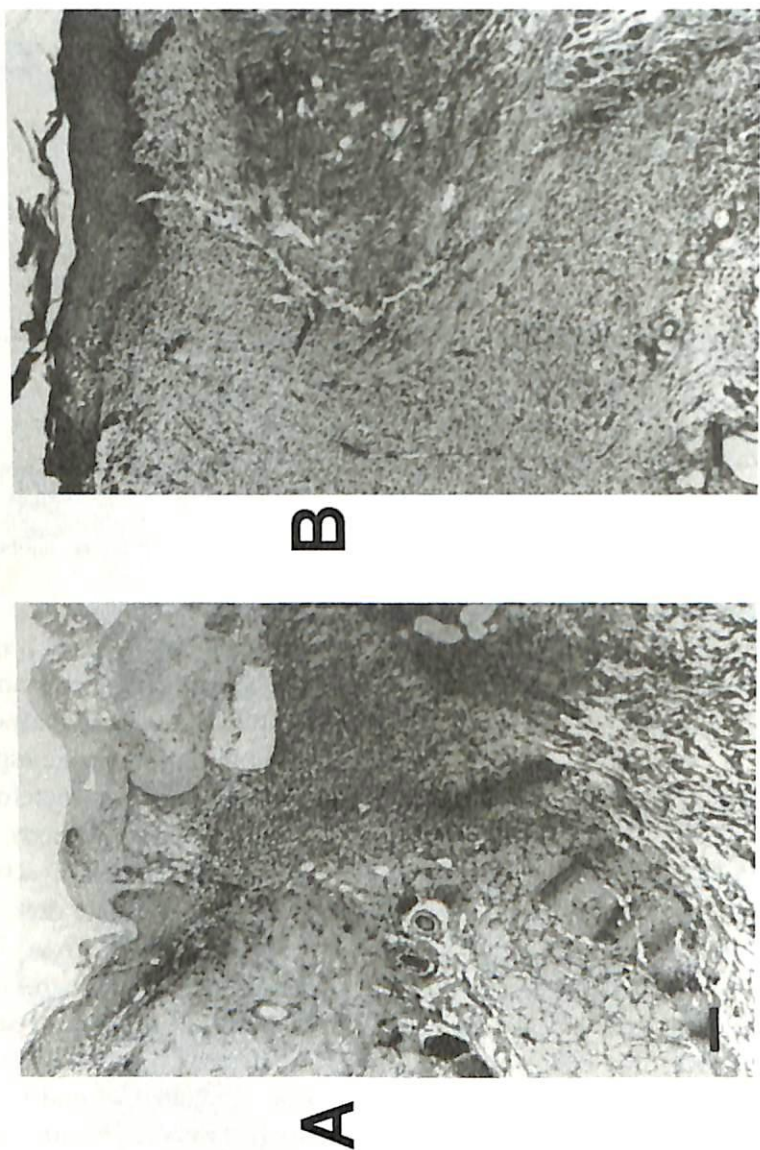
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FIGURE 11 Histological section of H.E.-stained tissues on wound areas at day 7. (A) is the control side. (B) is the laser-irradiated and antibody-applied side. Connective tissue in (B) was greater than in (A). Magnification is ×16, bar = 0.1 mm.

it is possible that other growth factors may also be involved in laser-stimulated wound healing. Some researchers believe that low-energy laser irradiation has a systemic effect (Braverman *et al.*, 1989), in particular the irradiation produced by the argon laser (Kana *et al.*, 1981). We cannot refute this, but feel that the local effect produced by the laser may well be of far greater significance than any resulting systemic effect, as judged by the results obtained in this research and by previous reports (Kimura *et al.*, 1991 and 1993).

Acknowledgements

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