Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo

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Balazs, Louisa, Juraj Okolicany, Mike Ferrebee, Betsy Tolley, and Gabor Tigyi. Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo. Am J Physiol Regulatory Integrative Comp Physiol 280: R466–R472, 2001.—The lipid mediator lysophosphatidic acid (LPA) regulates cell proliferation and enhances cell motility in vitro, both of which are important events in wound healing. To evaluate the effects of LPA in vivo, it was applied to a full-thickness wound of rat skin. LPA in micromolar concentrations, or solvent, was applied daily. Animals were killed at 1, 3, 6, and 9 days after wounding and processed for histological evaluation, including hematoxylin-eosin staining and histochemical markers for macrophage-histiocytes, proliferating cells, and capillary endothelial cells. LPA treatment accelerated wound closing and increased neoepithelial thickness. Cytological evaluation showed no evidence for a secondary inflammation-mediated injury, infection, or increased keloid formation. Whereas LPA caused only a modest dose-dependent increase in proliferating cells, a marked increase in the immigration of histiocyte-macrophage cells was observed as early as day 1. The peaks of several cytological features and immunohistological markers preceded those of the untreated side. Our data suggest that exogenously applied LPA in this model promotes healing and that macrophage-histiocytes are the primary LPA-responsive cells in vivo.

MATERIALS AND METHODS

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the University of Tennessee, and animals were maintained in an animal facility accredited by the American Association for Accreditation of Laboratory Animal Care. Adult Sprague-Dawley rats weighing 319–482 g were purchased from Harlan Bioproducts (Indianapolis, IN). Rats were housed two to a cage in a room with controlled temperature and humidity with a 12:12-h light-dark cycle and were maintained on a standard diet with food and water ad libitum. Animals were divided into treatment groups, each consisting of five rats, and assigned a code number.

Rats were anesthetized with intraperitoneal injection of ketamine and xylazine (87/13 mg/kg). The back was shaved, and two full-thickness circular skin wounds of 1.9-cm diameter were cut 4 cm caudal to the ears and placed symmetrically on either side of the midline 2 cm apart (Fig. 1). A Teflon wound chamber with a 2.2-cm inner diameter (14) centered around the wound was glued into the edge of the skin with Histoacryl (Fisher Scientific, Atlanta, GA) and then sewn.
infiltration. The intensity of these cellular features was scored on a scale of 0–3 according to the following criteria: 0, no detectable presence; 1, mild; 2, moderate; and 3, extensive presence of the cytological feature. For semiquantitative evaluation of the cytological features, an index was calculated from the means of the scores given to the treated wounds and compared with those assigned to the contralateral control wounds. Student’s t-test for paired variables was used to determine whether the index was significantly different as a result of the treatment. Healing of the wounds also was evaluated by measuring the largest diameter between the wound edges (wound gap) and the average thickness of the neoeithelium on either side (epithelial thickness).

Proliferating cells were identified by indirect immunoperoxidase staining using a mouse monoclonal antibody to BrdU (Sigma) according to an established protocol (27). A horseradish peroxidase (HRPO)-labeled anti-mouse (goat) secondary antibody (rat serum protein-absorbed, Sigma) was used with 3,3’-diaminobenzidine substrate (DAB, Sigma). Cells of the macrophage-histiocyte lineage were stained with the ED2 (Serotec, Raleigh, NC) rat anti-mouse monoclonal antibody (2) using the same secondary antibody. Capillary endothelial cells were stained with biotin-labeled *Griffonia simpliciflora* lectin I isolectin (GSL-B4) (10) (Vector Laboratories, Burlingame, CA) and HRPO-labeled streptavidin (Vector Laboratories) using DAB for substrate.

The sections were assigned a four-digit code number at random; thus the pathologist was unaware of the treatment dose and length when evaluating the sections. Cells bearing each marker were counted in 10 randomly selected microscopic fields along the cross-section of the wound at ×400 magnification.

Statistical analysis. Variables, BrdU-, ED2-, and GSL-B4-positive cell count/microscopic field, expressed as differences between treated and control sides and ratios, were analyzed by two-way ANOVA with dose and length of treatment as factors of interest. The pooled between animal error term was used as the estimated variance in the denominator of F-tests for preplanned contrasts. First, a series of simple F-tests was used to determine whether the mean difference (or ratio) for a specific dose and time combination was equal to zero. Second, the mean differences (or ratios) for each dose were compared across the three lengths of treatments.

RESULTS

The animal preparation and the schematic drawing of the positioned wound chamber are shown in Fig. 1. The wound chamber permitted the daily administration of the treatment under sterile conditions and anesthesia. The gap between the wound edges was measured along the largest diameter in carefully embedded and oriented sections (Fig. 2A). The wound gap was smaller on the treated side throughout the experiment. After a 1-day treatment, the wound gap was ~3 mm less than in the control side; however, this difference diminished for day 3. On day 6, the treated wounds were significantly smaller in diameter than the controls, which amounted to an average difference as much as 4 mm. This trend continued to the end of the experiment; however, on day 9 the difference in wound gap was not statistically significant due to the extensive healing on the control side (Fig. 2C). Similar trends were seen in all three dose groups. However, although the closing of the wound gap showed a dose-
dependent trend, this trend was not significant (data not shown). The maximum thickness of the neoeplithelium at the wound edges showed a steady increase with time (Fig. 2B) and was statistically significant from day 3 onward. Again, no statistically significant effect of the dose applied was observed (data not shown).

Cytological analysis of the granulation tissue (Fig. 3) showed several changes attributable to LPA treatment. The cellularity index showed a marked increase at day 1, which was statistically significant in all treatment groups (Fig. 3A and data not shown). In the day 3 and day 6 samples, no statistically significant differences were found in the cellularity index. However, there was a significant decrease in the cellularity index in the day 9 samples, suggesting that the inflammatory component was already subsiding in the granulation tissue. The perivascular infiltration index was significantly increased in the day 1 sample (Fig. 3B), followed by a gradual decrease in the later samples. In contrast, the most pronounced perivascular infiltrates were seen in the day 3 sample in the control side, and this index remained above that of the treated side throughout the experiment. Fibroblast cells (Fig. 3C) showed a statistically significant increase in the treated wounds from day 3 until the end of the study. Infiltration by polymorphonuclear leukocytes is one of the earliest events in the granulation tissue in this model (13). Whereas there was a significant increase in the intensity of granulocytic infiltration on day 1 (Fig. 3D), this index subsided below that of the control for the remainder of the experiment. Similar trends were seen in each dose group without statistically significant dose-dependent changes in the intensity of the individual markers (data not shown).

Proliferating cells were identified by quantifying the number of DNA-synthesizing cells using BrdU labeling for each treatment group (Fig. 4). For this, the mean number of BrdU-labeled nuclei per microscopic field was calculated. There was a higher number of BrdU-labeled nuclei in the LPA-treated side (Fig. 4A). However, the increase in the number of labeled nuclei was only significant for the 60 pmol/mm² treatment group after 3 and 6 days of treatment, respectively. The LPA-elicited increase in BrdU labeling was more apparent when the ratio of positive cell nuclei was calculated by dividing the mean number of labeled cells per microscopic field on the treated side for each animal by that of the contralateral side (Fig. 4B). This method of analysis unmasked the differences between the treated and control wounds by eliminating the individual differences in the number of labeled cells between the animals. ANOVA showed that there was a statistically significant increase in the proportion of BrdU-labeled cells after 1 and 3 days of treatment for each dose. Only the 60 pmol/mm² group showed a significant increase in BrdU labeling after 6 days of treatment, whereas this group had a significantly lower proportion of labeled cells on day 9 compared with the control side. Groups with the two lower doses showed a lower proportion of labeled cells on the treated side after 9 days of treatment; however, this difference was not statistically significant.

No statistically significant differences were found in the number or the ratio of GSL-B4 positive vessels for either group up to day 6 (Fig. 5). The only statistically significant difference detected was in the 60 pmol/mm² group after 9 days of treatment, whereas the groups receiving treatment with the two lower doses of LPA showed no significant difference even after 9 days.

Immunohistological staining for cells bearing the ED2 marker for histiocyte-macrophage lineage revealed major effects of LPA treatment (Fig. 6). LPA treatment increased the number of ED2-positive cells in the wound during the first 3 days of the treatment for each dose compared with the control side (Fig. 6A). The maximal differences were found after 3 days of
treatment. Interestingly, the ED2-positive cell count rapidly subsided and fell below that of the control on days 6 and 9. The ratio of ED2 marker-positive cells between the treated and control sides showed a marked increase as early as day 1 (Fig. 6B). However, after 6 days of treatment, the labeling ratio reversed, indicating that there was a relative abundance of ED2 marker-positive cells in the control side.

Analysis of the dose-response relationship after a 1-day treatment for the BrdU-labeled cell nuclei showed no statistically significant increase between the 6 and 19.8 pmol/mm² group (Fig. 4). However, the ratio of BrdU labeling in the 60 pmol/mm² group was statistically significant compared with either the 6 or 19.8 pmol/mm² group. In those samples collected after 3, 6, and 9 days of LPA treatment, no significant dose-dependent differences were detected. The labeling of the GSL marker-positive capillary endothelial cells showed no significant LPA-elicited changes after a 1-day treatment in either treatment group (Fig. 5). Similarly, no statistically significant dose-dependent differences were detected even after longer treatment.

In contrast, there was a dose-dependent increase in the ratio of ED2-positive cells after a 1-day treatment with LPA (Fig. 6). Despite the clear trend of a dose-dependent increase in the ED2-labeling ratio, only the differences between the highest dose and the lower two doses were statistically significant, whereas the difference between the lower two doses was not significant.

DISCUSSION

In vitro assays have identified LPA as a lipid mediator with mitogenic, chemotactic (for a review, see Ref. 6), and matrix metalloprotease-inducing (17) activities, all of which are important elements of wound healing. LPA is generated from activated platelets (3) and growth factor-stimulated fibroblasts (5) that are present in every wound. Therefore, on the basis of the in vitro results, several investigators have speculated that LPA could be involved in wound healing (9, 11, 16, 23). Despite this mounting evidence from in vitro studies, the in vivo effects of LPA during wound healing are completely unknown. The first of the two published in...
vivo studies investigating the effects of LPA in mice by Piazza et al. (16) was limited to topical application of LPA to the intact skin without wounding. The other, more recent report by Sturm et al. (21) focused on the epithelial response in a chemical-induced model of colitis. Therefore, the primary goal of our present study was to evaluate the effects of LPA in a wound model of the rat skin.

The measurement of the wound closure indicated by the decreasing size of the wound gap showed two effects. In the day 1 specimen, a marked decrease was observed in the wound diameter in each dose group (e.g., Fig. 2A). Because cytological evaluation did not reveal any neoeipithelialization after 24 h (Fig. 2B), we speculate that this effect was due to the LPA-induced contraction of the wound and was transient in its nature since the day 3 diameter increased and showed no significant differences. LPA has been shown to elicit the contraction of smooth muscle in several organs (22, 24, 25). In addition to smooth muscle contraction, LPA causes actin polymerization (18) leading to cell contraction and rounding (8, 23) that could also contribute to the macroscopic phenomenon of wound contraction.

Analysis of the cytological features of the wound-granulation tissue revealed that the density of the cellular infiltration was markedly increased in the LPA-treated wound, but only in the day 1 samples (Fig. 3A). Because LPA promotes blood coagulation through platelet aggregation (20), the increased cellularity is unlikely to be due to extensive bleeding into the wounds. In contrast, because of the chemotactic effect of LPA on macrophages (29) and other types of cells (15), we speculate that the increased cellularity was due to an LPA-induced increase in the chemotactic migration of white blood cells into the wound area and also to an LPA-induced secondary release of cytokines such as tumor necrosis factor-α (16). The increases seen in the ED2-positive macrophages (Fig. 6) and the polymorphonuclear leukocyte index (Fig. 3D) tend to support this hypothesis. To what extent the immigration of inflammatory cells into the wound area is a direct or indirect effect of LPA treatment remains an open question and subject of further investigation. The fact that the polymorphonuclear leukocyte index was only increased in the day 1 specimen and was below that of the control for the remainder of the experiment tends to suggest that LPA may not have a direct effect on this type of cell, which has been shown for human peripheral polymorphonuclear leukocytes (7). Specific LPA antagonists will be necessary to elucidate whether the polymorphonuclear leukocyte response is due to a direct or a secondary response elicited by an LPA-triggered release of chemotactic-chemoattractive factors. In vitro LPA induced the expression of adhesion molecules recognized by polymorphonuclear leukocytes (19), which might provide a molecular mechanism for the polymorphonuclear leukocyte component of the response. It is important to note that the cytological evaluation showed no evidence for a secondary inflammation-mediated injury, infection, or keloid formation.

We focused our study on markers of cells proliferation, capillary neogenesis, macrophage-histiocyte lineage, polymorphonuclear leukocytes, and epithelialization. Capillary neogenesis was selected because a related mediator, sphingosine-1-phosphate, has been shown to promote angiogenesis in vitro (28) and in vivo.
The inclusion of macrophage-histiocyte cells in this study was prompted by a previous report by Zhou et al. (29) concerning the haptotactic effect of LPA on macrophages in vitro and also because of the crucial role of these cells in orchestrating the formation of granulation tissue through the release of cytokines, chemokines, and other growth factors, which are essential to the recruitment and coordination of the cellular response during healing.

The time-course analysis was important to enable some degree of the distinction of the early, presumably direct, effects of LPA expected to manifest after a 1-day treatment from the later effects, which might be due to secondary responses mediated by cells activated by LPA. In this regard, we limit our discussion to the effects observed after a 24-h treatment separately from those seen at the later time points. The most prominent observation in the wound tissue collected after 1 day of treatment was a dose-dependent increase in the ED2-positive macrophage-histiocyte cells (Fig. 6A). There were on average 88 more ED2 marker-bearing macrophages per microscopic field compared with the control in the wounds of the animals receiving the highest dose (Fig. 6A). Even in the lowest dose group, we found an average of 10 macrophages more compared with the control. This observation suggests that macrophages are a highly and rapidly responding cell type to LPA in vivo.

We found only a very moderate, but nevertheless significant, increase in the number of BrdU-positive proliferating cells in the LPA-treated wounds. This was most apparent in the 60 pmol/mm² group, in which on average 14 more cells were counted compared with the contralateral vehicle-treated wounds. The BrdU-positive nuclei, particularly after day 3, were often those of cells with fibroblast-like morphological features and capillaries. On day 1, virtually none of the polymorphonuclear cells was positive with BrdU. In adjacent sections, we found that only a small proportion of the ED2-labeled cells were BrdU positive, suggesting that the majority of macrophages migrated into the wound area rather than proliferated in loco. There were no statistically significant effects in the GSL marker-bearing capillary endothelial cells compared with the control side. This observation underlines an interesting difference between the in vivo effects of LPA compared with sphingosine-1-phosphate (12).

It is important to note that the kinetics of the cellular response appears to show a uniform pattern. The cytological indexes (Fig. 3) showed a shift to the earlier time points compared with that of the controls. For example, the perivascular infiltration index (Fig. 3B) or the polymorphonuclear infiltration index (Fig. 3D) peaked on day 3 in the control samples and subsequently followed a diminishing trend, whereas in the treated side it peaked at day 1 and then assumed a similar diminishing trend as seen in the control but at a slightly accelerated rate. The ratio of ED2-labeled cells (Fig. 6B) was higher than one after 1 and 3 days of treatment and then dropped below one, indicating that the cellular response had already begun diminishing on the treated side. A similar but prolonged increase in the BrdU labeling ratio was seen (Fig. 4), indicating that cell proliferation and regeneration (Fig. 2) was more protracted on the control side. Because the intensity of the cytological and immunohistological markers at their respective peaks did not exceed that of the control but rather, preceded that seen in the controls, we speculate that the overall effect of topical LPA treatment promotes the healing process by shifting the onset of the cellular responses to an earlier time, presumably through the recruitment of macrophages. However, a thorough kinetic analysis that would provide decisive evidence for the accelerated pace of the healing response after LPA treatment will have to be addressed in a future study. The present data, however, provide the first direct evidence for the positive effect of LPA on wound healing and describe many of the cellular responses affected. Perhaps the most surprising, but not completely unexpected (29), result from these experiments was the robust, dose dependent, and early macrophage-histiocyte response elicited by LPA. An early activation of macrophages by LPA could well play a key role in setting up a host of secondary responses through the production of cytokines and growth factors, which is required for the normal healing process. Therefore, our studies identify macrophages as a physiologically important LPA-responsive cell type in vivo. Clearly, many more studies will have to be performed in the future to understand the complexity of cellular responses triggered by the endogenous stimulus-coupled production as well as the exogenous delivery of LPA.

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