Activation of Wound Healing in Aged Rats by Altering the Cellular Mitogenic Potential

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We previously showed that lysophosphatidic acid (LPA) and an adenylyl cyclase inhibitor (ACI) stimulate mitogenic activation of senescent human diploid fibroblasts. Because the modulation of cell proliferation may affect wound healing in aged organisms, we studied the effects of LPA and ACI on in vivo skin wound healing in aged Fisher 344 rats. We found that, in aged rats, wound healing improved in animals treated with LPA and/or ACI (relative to untreated controls), as assessed by histological analysis of reepithelialization and immunostaining for proliferating cell nuclear antigen. The age-dependent activation of mitogenic responses by LPA and ACI was confirmed in other cell types. Taken together, our findings suggest that the activation of mitogenic potential in senescent cells by LPA and/or ACI may translate into enhanced in vivo wound healing and tissue regeneration in aged animals.

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MOST studies on age-dependent differences in wound healing have reported that wound healing is slower in aged cells, animals, and people than in young ones (1–4). Studies have suggested that the higher numbers of senescent cells in aged skin than in the young skin might slow down or impair cellular replication, which is required for tissue regeneration (5,6). However, a precise understanding of the underlying mechanisms in age-delayed wound healing remains elusive. Therefore, the question as to the role of cellular aging in age-dependent impairment of wound healing is pertinent.

Following injury, fibroblasts rapidly proliferate and migrate to the wound site, where they participate in healing. Therefore, modulating the proliferation and migration of fibroblasts might be an effective strategy for regulating wound healing. In vivo wound healing can be promoted by topical application of receptor tyrosine kinase (RTK) agonists such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (7,8), as well as lysophosphatidic acid (LPA) (9). We have previously reported that LPA induces proliferation of senescent human diploid fibroblasts (HDFs) (10), which are hypo-responsive to PDGF (11) or EGF (12,13). We therefore hypothesized that LPA might more effectively stimulate wound healing than PDGF or EGF in aged organisms. In addition, because the ability of adenosine 3’5’-cyclic monophosphate (cAMP) to inhibit cell proliferation and migration is well established (1), we hypothesized that modulating cellular cAMP levels might alter the proliferation of senescent cells. As expected, abrogation of cAMP synthesis by the adenylyl cyclase inhibitor (ACI) SQ22536 (15) resulted in increased proliferation of senescent HDFs (through inhibition of the catalytic activities of AMP-activated protein kinase α and p53) (16).

Therefore, in this study, an extension of our work using an in vitro fibroblast model, we examined the effects of LPA and/or ACI on in vivo wound healing in aged Fisher 344 rats. LPA- and/or ACI-induced proliferation and migration of senescent cells were confirmed in vitro in other skin cells with roles in wound healing, including skin fibroblasts from aged donors, skin keratinocytes, and endothelial cells.

Materials and Methods

Materials

The following reagents and instruments were used in this study: Dulbecco’s modified Eagle’s medium (DMEM), LPA, eosin, hematoxylin, and peroxidase-labeled anti-mouse (goat) secondary antibody (rat serum protein adsorbed) (Sigma Chemical Co., St. Louis, MO); fetal bovine serum (FBS), penicillin, and streptomycin (Gibco/BRL Life Technologies Inc., Carlsbad, CA); SQ22536 (Calbiochem, San Diego, CA); [3H]-thymidine (Amersham Pharmacia Biotech,
Little Chalfont, Buckinghamshire, UK; methoxyflurane (Metophane; Smith Kline Beecham, Philadelphia, PA); trypsin and Zymed Picture Plus Kit (Zymed, South San Francisco, CA); anti-proliferating cell nuclear antigen (PCNA) (NCL-PCNA) antibody (Novocastra, Newcastle upon Tyne, UK); anti-PCNA monoclonal IgG, Protein Block (Serum-Free), and avidin–biotin peroxidase complex (LSAB) kit (Dako, Glostrup, Denmark); an electric diamond bar (Korea Science Academy, Busan, Korea); and Permount and Histopaque (Fisher Scientific, Atlanta, GA).

Animals
Wound healing experiments were performed in both young (average age 6.5 months, \( n = 9 \)) and aged (average age 25 months, \( n = 9 \)) male Fisher 344 rats, which were purchased from Samtaco BioKorea (Seoul, Korea) and housed (two per cage) in a room with controlled temperature and humidity under a 12 hour:12 hour light–dark cycle. The rats were maintained on a standard diet with food and water ad libitum in an animal facility accredited by the Korean Association for Accreditation of Laboratory Animal Care. The animal protocol was reviewed and approved by the Animal Care and Use Committee of Seoul National University.

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords during full-term delivery, as described previously (17). Human foreskin fibroblasts and keratinocytes were isolated from the foreskins of neonates, as described by Boyce and Ham (18). The skin fibroblasts used in the present study were similarly obtained from the epidermis of skin samples from three young (aged 23 ± 2 years) and three elderly (64 ± 5 years) donors. The use of tissue samples was approved by the ethics committee of Seoul National University. Fibroblasts and HUVECs were maintained in DMEM supplemented with 10% FBS and antibiotics, whereas the keratinocytes were maintained in keratinocyte growth medium containing antibiotics. Cells from the early stages of culture were compared with senescent cells (population doubling: <25 vs 65–70 for fibroblasts, and <5 vs 10–12 for HUVECs). Senescent cells were identified by morphological changes, enhanced β-galactosidase activity, and reduced rate of proliferation (19). Prior to LPA or ACI treatment, the cells were grown for 2 days to 60%–70% confluence, and then serum starved to quiescence through incubation for 2 days in either serum-free DMEM containing 0.1% bovine serum albumin (BSA; fibroblasts and HUVECs) or keratinocyte basal medium.

Measurement of DNA Synthesis
Quiescent young cells and senescent cells were pretreated with either vehicle or ACI for 2 hours, and then treated with LPA in the presence of \( ^{3} \text{H} \)-thymidine (0.5 μCi/mL) for 36 hours. After removal of the radiolabeled thymidine by washing four times with phosphate-buffered saline (PBS), DNA was precipitated with 10% trichloroacetic acid and disrupted in 0.5 N NaOH. The radioactivity in the neutralized lysates was counted in a liquid scintillation cocktail.

In vitro Wound Healing Assay
Basal and LPA-induced migrations were assessed based on the ability of cells to move into an acellular area, as described previously (20). Cells were plated to a 10-cm plastic dish and grown to 70%–80% confluence in culture medium that contained 10% FBS. After incubation for 2 days in serum-free medium supplemented with 0.1% BSA to induce quiescence, the cells were pretreated with vehicle or ACI (300 μM) for 2 hours at 37°C. An area was denuded using a yellow tip (1 mm wide), and the cells were incubated at 37°C with or without LPA (30 μM). Photographs were taken at 6, 12, 18, 24, 30, 36, 42, 60, and 72 hours, and the numbers of cells that had migrated to the acellular area were counted.

Skin Wounding and Preparation for Immunohistochemistry
Rats were anesthetized by intraperitoneal injection of ketamine and xylazine (87 and 13 mg/kg, respectively). Skin wounding was performed as described previously (9). The back was shaved, and using a dermabrasion technique, four second-degree, partial-thickness, circular wounds (each 1 cm in diameter) were made using a diamond bar, 4 cm caudal to the ears and 1 cm either side of the midline. A Teflon wound chamber (inner diameter 1.2 cm) was centered over the wound, glued onto the edge of the skin with Histoacryl, and then sewn into place with surgical nylon suture (Ethicon 4-0). A sterile glass filter (GF-C; Whatman, Maidstone, Kent, UK) was placed at the bottom of the wound to protect against mechanical trauma resulting from the daily treatments and to ensure even spreading of the applied solutions. This work was performed by a single plastic surgeon. LPA, ACI, and a mixture of LPA and ACI were dissolved in isotonic saline at concentrations of 30 μM, and 200-μL aliquots (corresponding to 30 pmol/mm²) were applied twice daily to the wounds of animals that had been anesthetized by methoxyflurane inhalation. The contralateral wounds were treated with 200 μL of isotonic saline as a control.

On postoperative Days 2, 4, and 7, groups of three animals were anesthetized with ketamine and xylazine. The animals were killed by intracardiac perfusion with 4% (wt/vol) paraformaldehyde in PBS (pH 7.4). A 5-mm-thick section of tissue (removed by cutting along the dorsoventral axis of the wound) was fixed overnight in ice-cold 10% paraformaldehyde and embedded in paraffin.

Histology and Immunohistochemistry
Serial sections (4 μm thickness) were cut, mounted on microslides, and processed for hematoxylin–eosin (H&E)
and immunohistochemical staining. The tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. Antigen retrieval was achieved by heating the tissue sections in 10 mM citrate buffer (pH 6.0) in a 700-W domestic microwave oven for 15 minutes. The slides were treated with hydrogen peroxide (3%) to block endogenous peroxidase activity and then washed in 0.05 M tris-buffered saline buffer (pH 7.6). Nonspecific binding sites were blocked through incubation in 5% skim milk for 1 hour at room temperature. The slides were then incubated at 4°C overnight with a primary anti-PCNA monoclonal antibody, diluted 1:2,000 in blocking buffer (Protein Block, serum-Free). Signals were developed by the avidin–biotin peroxidase complex method using an LSAB kit. The slides were counterstained with hematoxylin, dehydrated in a graded series of ethanol solutions, cleared with xylene, and mounted using Permount.

**Statistical Analysis**

Statistical significance was determined by one-way analysis of variance using the statistics program SAS 9.1 for Windows Version 12.0 (SAS Institute Inc., Cary, NC). Post hoc testing was performed using Duncan’s multiple comparison tests. Data are presented as means ± standard deviations with the data relating to individual treatments being labeled “a–d.” Values not sharing a letter are significantly different (p < 0.001).

Student’s t tests were performed using Excel 2007 (Microsoft, Redmond, WA). Data are presented as the mean ± standard deviation. A p value <0.05 was considered to be statistically significant.

**Results**

**Modulation of Wound Healing in Aged Fisher 344 Rats by LPA and ACI**

Because LPA and/or ACI alter the rate of proliferation of senescent skin HDFs (16), we hypothesized that they might also modulate wound healing in aged organisms in vivo. In this study, the wound healing responses to LPA and/or ACI were examined in both young and aged male Fisher 344 rats (n = 9 per group). LPA (30 μM), ACI (300 μM), and a mixture of LPA and ACI were applied to each wound chamber twice a day.
WOUND HEALING IN AGED RATS AND CELLULAR MITOGENIC POTENTIAL

On postoperative Days 2, 4, and 7, groups of three rats were killed. Wound tissue sections were then prepared as described in the Materials and Methods section. Reepithelialization and cell proliferation following wounding were examined by H&E staining (Figure 1) and immunostaining for PCNA (Figure 2), respectively. Wound healing was quantitatively evaluated by measuring the average thickness of the neoepithelium (Figure 1i and j), as well as by calculating the percentages of PCNA-positive proliferating cells (Figure 2i and j) at different time points after wounding.

Reepithelialization on Day 4 postwounding, as visualized by H&E staining (Figure 1a–h), epithelial thickness was greater in LPA- or LPA + ACI–treated rats than in untreated controls (p < 0.05). However, epithelial thickness in ACI-treated young rats was lower than that in untreated controls (Figure 1a–d and i). In aged rats, epithelial thickness was significantly higher in LPA-, ACI-, and LPA + ACI–treated animals than in untreated controls (p < 0.05) (Figure 1e–h and j). Our data suggest that wound healing was enhanced by LPA in both young and aged rats, but that ACI produced different effects in rats of different ages, inhibiting and stimulating healing in young and aged animals, respectively.

PCNA staining in young rats was increased by treatment with LPA or LPA + ACI on postoperative Days 2 and 4 (Figure 2b and d), as compared with levels in untreated controls (Figure 2a). PCNA staining was very weak in untreated aged rats (Figure 2e), although it increased significantly after treatment with LPA and/or ACI (Figure 2f–h). Numbers of PCNA-positive cells within defined areas of wounds were significantly increased by treatment with LPA and/or ACI (Figure 2i and j), especially in aged animals (Figure 2j). These data suggest that reducing the cAMP levels through treatment with ACI may be beneficial for wound healing in aged rats.

Proliferation of Senescent Nonfibroblast Cells Through Alteration of Cellular Mitogenic Potential

Because LPA and ACI alter the rate of proliferation in senescent HDFs, we attempted to confirm their effects on DNA synthesis and proliferation using other types of senescent or...
aged cells. To provide evidence that these agents also affect in vivo–aged fibroblasts, we performed the same experiments using skin fibroblasts isolated from elderly human donors (referred to as “elderly” and to be compared with in vitro–senescent fibroblasts). Because they are also known to participate in wound healing, we wanted to confirm our hypothesis using keratinocytes and endothelial cells. HUVEC was chosen because it is a model endothelial cell type. Senescent or aged human skin keratinocytes and HUVECs were referred to as “senescent” due to their senescent characteristics in vitro.

Rates of DNA synthesis were determined by measuring \[^{3}\text{H}^\text{H}\]-thymidine incorporation into DNA (Figure 3a–c). Because basal and thymidine incorporation is much lower in senescent cells (100–200 counts per minute [cpm]) than in young cells (1,000–8,000 cpm), comparing fold-changes in thymidine incorporation between young and senescent cells was more appropriate than raw counts.

DNA synthesis increased significantly \((p < 0.001)\) following treatment with LPA in both young and senescent or elderly cells (Figure 3a–c). However, ACI differentially affects DNA synthesis in young and senescent cells. Basal and LPA-stimulated DNA synthesis in young cells decreased significantly \((p < 0.001)\) following treatment with ACI (Figure 3a–c). In contrast, ACI significantly enhanced \((p < 0.001)\) basal and LPA-stimulated DNA synthesis in the various senescent cells (Figure 3a–c).

We also confirmed that numbers of elderly or senescent cells were further increased by treatment with ACI, alone or in combination with LPA, albeit to different degrees depending on the specific cell type (Figure 3d–f). When young cells were cotreated with ACI, the mitogenic effect of LPA was reduced (Figure 3d–f). These results strongly suggest that treatment with LPA and ACI increases DNA synthesis in, and proliferation of, various senescent cells, including fibroblasts, keratinocytes, and endothelial cells, in contrast to its effects on the equivalent young cells.

**Effects of Altering Cellular cAMP Levels on the Migration of Senescent Cells**

Because the migration of cells from the adjacent intact tissue to the wounded area is an important process during wound healing, we determined the effects of LPA and ACI on rates of cell migration in an in vitro wound healing assay. Cell monolayers of young and senescent HDFs were serum starved and denuded by scraping with a sharp pipette tip. After treating the wounded cell monolayers with 30 \(\mu\)M LPA in the presence or absence of ACI, we counted the numbers of cells that migrated into the wound area within 72 hours (Figure 4a and b).

As shown in Figure 4a, young cells migrated continuously even in the absence of serum (control). Treatment with LPA enhanced this migration \((p < 0.05; \text{Student’s } t\text{ test})\) (Figure 4c). In contrast, senescent cells migrated slowly in the absence of serum. LPA significantly increased the...
numbers of senescent cells that had migrated within 72 hours ($p < 0.001$; Duncan’s multiple comparison test) (Figure 4d). The migration of young cells was unaltered by ACI treatment (Figure 4c), whereas ACI increased the migration of senescent cells ($p < 0.001$) (Figure 4d). Treatment with both ACI and LPA dramatically enhanced senescent cell migration ($p < 0.001$) (Figure 4d). These data suggest that the migration of senescent cells may be induced by LPA and further enhanced by blocking cAMP synthesis.

**DISCUSSION**

Because the healing of skin wounds in animals has been shown to be accelerated by licking of the wound sites, presumably due to the presence of growth factors such as EGF in the saliva, the topical use of EGF-enriched artificial saliva has been considered for use in wounded patients (8). The use of PDGF, another RTK agonist and a potent mitogen, has also been proposed as a therapeutic solution for problem wounds (7). However, RTK agonists may not be effective at promoting healing in aged animals and humans due to their reduced levels of growth factor receptors and/or the failure of RTK-stimulated signal transduction as a result of the age-associated increase in the level of caveolin-1 (11,12).

LPA is known to promote in vivo wound healing (9). As a phospholipid mitogen present in serum, plasma, aqueous humor, and other biological fluids, it is secreted by several cell types, including platelets, fibroblasts, and cancer cells, in different pathophysiological contexts (eg, aggregation, injury, and cancer) (21). Previously, we suggested that LPA might be suitable for promoting wound healing in aged humans and animals, based on the maintenance of G-protein–coupled LPA receptor expression and the consequent efficiency of signal transduction and cell proliferation in senescent cells (10). In the present study, LPA was shown to be effective in inducing DNA synthesis in, and proliferation and migration of, senescent cells, resulting in the efficient healing of wounds in aged Fisher 344 rats.

LPA increases cAMP accumulation in senescent cells, but decreases cAMP levels in young cells (10). The increase in cAMP levels in senescent cells treated with LPA resulted from reduced inhibition of adenylyl cyclase (AC) by G$i\alpha$, and protein kinase C-dependent stimulation of AC isoforms 2, 4, and 6 (22). Because cAMP has been suggested to inhibit cell proliferation, we postulated that the LPA-induced increases in cellular cAMP levels might have inhibitory effects on the proliferation of senescent cells, and therefore, that the modulation of the cAMP levels would influence the rates of DNA synthesis and cellular proliferation in senescent cells. Indeed, we observed that ACI enhanced the mitogenic responsiveness of these cells to LPA (16). Although we initially tested three ACIs, SQ22536, 2′,5′-dideoxyadenosine, and 2′,5′-dideoxyadenosine 3′-triphosphate, we found SQ22536 to be the most effective (data not shown).
Our findings are in line with the previous observation that ACI reversed suppression of mitogenesis by tissue inhibitor of metalloproteinases-2 (which implicates cAMP as a second messenger) (23). In addition, increased protein kinase A (PKA) activity coincided with a significant decrease in the cellular proliferative potential (14). The senescence-associated increase in cAMP accumulation caused by LPA results in the activation of PKA (24), which reduces the rates of DNA synthesis and cell proliferation by inhibiting the Raf/ERK pathway (25–27). The role of cAMP in regulating mitogenic responses of senescent cells suggests that modulation of cAMP is a potential tool for modulating the aging process and associated degenerative changes. LPA is reported to induce cell migration by activating Rho through the heterotrimeric GTPases G12 and G13 in neuronal cells (28) and G13 in Swiss 3T3 cells (29) and fibroblast cell lines derived from wild-type and Gqq/Gxi1-null mice (30). Our results show that LPA significantly enhances the migration of both young and senescent HDFs, suggesting that LPA induces Rho activation in young and senescent cells.

Agents such as dibutyryl cAMP, forskolin, prostaglandin E2, and isoproterenol that increase cellular cAMP levels also inhibit the migration of fibroblasts by activating PKA (31). The cAMP/PKA pathway has been shown to inhibit both basal (unstimulated) and agonist-induced migration of smooth muscle cells (32–36). The inhibitory effect of cAMP may be effected by the inhibition of RhoA (37–39). In young HDFs, LPA may strongly activate Rho by inhibiting PKA, whereas in senescent cells, LPA activates Rho to a limited degree through activation of PKA, thereby reducing migration (24).

The cross talk between the cAMP/PKA and RhoA signaling pathways also has significant effects on morphologic and cytoskeletal characteristics, migration, and anchorage-independent growth of cancer cells (39). Thus, the modulation of cAMP by ACI may alter the rate of migration of senescent cells. As expected, cotreatment with ACI and LPA increased the migration of senescent cells to a greater extent than treatment with LPA alone. When ACI was applied (with or without LPA) to wounded rats, their wounds healed more rapidly. We observed that old cells continued to proliferate after extended periods of treatment with these compounds. The effects of LPA and ACI were more evident in aged rats than in young rats, suggesting that cotreatment with ACI and LPA is the optimum option for stimulating healing in aged animals.

In summary, adjusting cellular cAMP levels may enhance LPA-induced cellular proliferation and migration not only of fibroblasts but also of other skin cells, which may lead to the activation of wound healing in aged animals in vivo. Our findings may contribute to the development of novel therapeutic strategies for combating delayed wound healing in aged humans.

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