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Pharmacological Mobilization of Endogenous Stem Cells Significantly Promotes Skin Regeneration after Full-Thickness Excision: The Synergistic Activity of AMD3100 and Tacrolimus

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Stem cell therapy has shown promise in treating a variety of pathologies including skin wounds, but practical applications remain elusive. Here, we demonstrate that endogenous stem cell mobilization produced by AMD3100 and low-dose tacrolimus is able to reduce by 25% the time of complete healing of full-thickness wounds created by surgical excision. Equally important, healing was accompanied by reduced scar formation and regeneration of hair follicles. Searching for mechanisms, we found that AMD3100 combined with low-dose tacrolimus mobilized increased number of lineage-negative c-Kit+, CD34+, and CD133+ stem cells. Low-dose tacrolimus also increased the number of SDF-1-bearing macrophages in the wound sites amplifying the "pull" of mobilized stem cells into the wound. Lineage tracing demonstrated the critical role of CD133 stem cells in enhanced capillary and hair follicle neogenesis, contributing to more rapid and perfect healing. Our findings offer a significant therapeutic approach to wound healing and tissue regeneration.

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INTRODUCTION

Every year, in the United States, more than 1.25 million people suffer burns and 6.5 million have chronic skin ulcers caused by pressure, venous stasis, or diabetes mellitus (Singer and Clark, 1999). The treatment of these conditions remains imperfect and expensive. There is hope that stem cell therapy may prove beneficial as it has been increasingly well established that stem cells have an important role in wound healing. For example, artificial skin substitutes have been shown to be more effective when stem cells are incorporated into these membranes. The quality of burn wound healing improves (Leonardi *et al.*, 2012), reducing scar formation and reestablishing the skin appendages (Mansilla *et al.*, 2010; Tamai *et al.*, 2011; Huang and Burd, 2012). The treatment of a chronic static diabetic ulcer has been improved by using the patient's bone marrow (BM) mesenchymal stem cells (MSCs) in combination with autologous skin fibroblasts embedded on a biodegradable collagen membrane (Coladerm; Vojtassák *et al.*, 2006). BM cells from both embryonic and postnatal sources have been shown to repair genetic defects in collagen synthesis and basement membrane defects, thereby promoting skin wound healing (Chino *et al.*, 2008; Tolar *et al.*, 2009; Fujita *et al.*, 2010). Recently, a clinical trial found that allogeneic whole-BM transplantation in humans suffering from a blistering skin disorder caused by the lack of Col 7 resulted in the restoration of skin integrity and Col 7 expression in basement membranes (Wagner *et al.*, 2010).

Many basic cellular studies have also emphasized the plastic relationships between skin and BM. Fibroblast-like cells in the dermis having hematopoietic and mesenchymal lineages are derived from BM, and the number of these cells increases after skin wounding (Fathke *et al.*, 2004; Ishii *et al.*, 2005). Donor cells have replaced some keratinocytes after BM transplantation and have persisted in the epidermis for at least 3 years (Körbling *et al.*, 2002). BM cells contribute to fetal skin development as infusion of green fluorescent protein (GFP) BM cells *in utero* in mice led to the accumulation of GFP-positive cells in the developing dermis, particularly in association with developing hair follicles (Chino *et al.*, 2008).

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Abbreviations: BM, bone marrow; CD133+/C-L, CD133 positive-Cre-nuclear(n)LacZ; GFP, green fluorescent protein; HGF, hepatocyte growth factor; MSC, mesenchymal stem cell; SDF-1, stromal cell–derived factor-1

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These abundant studies document the importance of BM stem cells in wound healing and raise the tantalizing possibility that cellular processes can be harnessed to develop practical therapeutic protocols to treat large full-thickness burns and massive soft tissue injuries, which demand immediate therapy. The promise of improved wound repair by harnessing stem cells is testified by the existence of 90 clinical trials using MSC-based therapies listed in the NIH registry (Cerqueira *et al.*, 2012).

To avoid the preparation of endogenous stem cells, which is expensive and time-consuming, we proposed in these studies to mobilize endogenous stem cells pharmacologically with AMD3100 and tacrolimus. AMD3100 has been shown to drive endogenous stem cells from the BM to the bloodstream in both animals and man (Hendrix *et al.*, 2000; Liles *et al.*, 2003; Devine *et al.*, 2004; Broxmeyer *et al.*, 2005; Hisada *et al.*, 2012). Tacrolimus in low dosages proved to have synergistic effects (Okabayashi *et al.*, 2011), and combination treatment promised a simple, safe, and rapid means of presenting stem cells to injured areas. Here, we test this hypothesis in the healing of full-thickness skin wounds in mice and rats. On finding that this treatment was able to reduce the time for complete healing by 25%, we characterized stem/progenitor cells and related cytokines/growth factors in the wound and substantiated the important role of mobilized CD133+ stem cells in angiogenesis and hair follicle regeneration in wound areas using lineage tracing.

RESULTS

AMD3100 plus low-dose tacrolimus accelerated wound healing after full-thickness skin excision

Four full-thickness wounds were generated by 5 mm diameter circular excisions on the shaved back of a wild-type C57/B6 mouse (Figure 1a). Each wound site was photographed digitally at the indicated time intervals, and wound areas were calculated using Adobe Photoshop software. Changes in wound areas over time were expressed as the percentage of the initial wound areas (Figure 1b). Wounded mice were divided randomly into four experimental groups as follows and received subcutaneous injections of saline or drugs immediately after wounding until complete healing: (1) control group treated with saline; (2) tacrolimus group treated daily with low-dose ($0.1\,mg\,kg^{-1}$); (3) AMD3100 group treated every other day ($1.0\,mg\,kg^{-1}$); and (4) combination group given low-dose tacrolimus and AMD3100. All wound evaluations were double blinded.

Wounds reached complete closure on day 12 after surgery in group 1 ($n=6$; Figure 1c and d), which is consistent with the known healing kinetics in this established model (Shinozaki *et al.*, 2009; Mack *et al.*, 2012). The six animals treated with tacrolimus or AMD3100 alone exhibited significantly, but only moderate, faster healing compared with the saline control group (Figure 1d) as wounds reached complete closure at day 11. The healing time was reduced to 9 days or by 25% in the group four mice treated with AMD3100 plus low-dose tacrolimus. Digital images showed that treatment with dual drug therapy had significant effects reducing the size of the skin defect as soon as day 5 (Figure 1d), which was the start of the re-epithelialization phase of wound healing. Macroscopically, minimal ulceration and early epithelial ingrowth were observed at the skin boarders on post-wounding day 5 in the dual drug–treated group, whereas wounds in the other three groups showed little, if any, epithelialization and continued ulceration (Figure 1c and h). We repeated these studies in rats and found that rats receiving dual drug therapy also showed an equivalent effect, significantly reducing the time for complete healing from 18 to 13 days or by 28% (Supplementary Figure S1 online).

Mouse skin is mobile, and contraction accounts for a large part of wound closure. To deter this mechanism, we performed the excisional wound–splinting model in which a splinting ring is bonded tightly to the skin around the wound (Figure 1e). The wound therefore heals through granulation and re-epithelialization, a process similar to the healing of most human skin defects. We found that wound repair was accelerated in the splinted wounds treated with low-dose tacrolimus or AMD3100 monotherapy compared with the control (saline) group, whereas the most accelerated healing was found in animals receiving dual treatment (Figure 1f and g). Thus, the therapeutic effects of AMD3100 plus low-dose tacrolimus were primarily on skin wound epithelialization. Other groups of mice were treated with high-dose AMD3100 ($5.0\,mg\,kg^{-1}$) or tacrolimus ($1.0\,mg\,kg^{-1}$) and we found that high-dose tacrolimus impeded skin wound healing, whereas increased dosage of AMD3100 showed no significant difference (Supplementary Figure S2 online).

AMD3100 plus low-dose tacrolimus ameliorated scar formation and promoted hair follicle regeneration

Dermal wound repair commences with the arrest of hemorrhage, followed by an inflammatory response, formation of granulation tissue within the wound space, fibrosis, and re-epithelization of the wound culminating in the production of a scar.

Histologically, the re-epithelialized wound in the control groups on day 15 showed a disorganized epidermis, with blurring of the boundary between the epidermis and the dermis (Figure 2b). There were few hair follicles in the group 1, 2, and 3 mice and the collagen was abundant and disorganized (Figure 2c), which is in agreement with the results of published studies (Devine *et al.*, 2004). By contrast, the dual drug–treated animals had a thin and well-organized epidermis with well-formed hair follicles and much better organized collagen (Figure 2b and c; lower panels). The most striking finding was that hair appeared only in the re-epithelialized wound in the dual drug–treated animals after 15 days (Figure 2a and d). Not surprisingly, the number of hair follicles in the tissue sections of re-epithelialized wound was significantly higher in the dual drug–treated animals compared with the control groups (Figure 2e). We found that dual drug treatment also stimulated hair follicle neogenesis and reduced scarring in rats (Supplementary Figure S3 online). Thus, the combination of low-dose tacrolimus and AMD3100 improved wound healing by promoting both re-epithelialization and differentiation of skin components.