In Vitro Reconstructed Human Skin Equivalents for Autologous Transplantation in Burns, Chronic Ulcers, and Pigment Defects

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Background: Cultured epidermal and dermal cells reconstitute normal skin structure and retain biochemical and molecular characteristics of the original donor site. Application of in vitro engineered skin on a well-prepared wound bed allows to permanently regenerate full-thickness wounds because of the presence of epidermal stem cells in culture. Further, cultured cells produce growth factors and components of extracellular matrix that facilitate the wound healing process.

The Problem: There is a need to develop and optimize both structure and functions of the in vitro reconstructed human skin equivalents. Should skin equivalents be different for the treatment of chronic ulcers, burns, or white skin spots in vitiligo? How to prepare fully autologous and easy-to-monitor cultures that form ready-to-apply skin sheets?

Basic/Clinical Science Advances: Interference between different human skin cells in cultures grown on a proper substrate controls cell growth, differentiation, cytokine production, and distinct cellular functions. This should be taken into consideration during preparation of skin equivalents targeting various skin pathologies.

Clinical Care Relevance: In acute wounds with an increased risk of scar formation and in chronic nonhealed ulcers as well as in skin pigment disorders where clinical efficacy of conventional therapies is unsatisfactory, autotransplantation of properly in vitro engineered skin equivalents could substantially improve and accelerate closure of skin defects and restore esthetical appearance of normal skin.

Conclusion: In vitro reconstructed fully autologous and functional skin equivalents engineered for different skin defects could be considered as safe and efficient cellular medicinal products for individualized therapy.

BACKGROUND

During the past 30 years, several experimental and commercial strategies have been developed to support wound healing by providing proper substitution for serious skin defects.1,2 The in vitro reconstructed skin equivalents for autotransplantation should ideally substitute major functions of damaged skin, cover large damaged/defective areas, and additionally elicit regeneration response from the wound bed without causing inflammation or rejection. For safety reasons, in vitro reconstructed skin equivalents should not contain xenobiotics and cells of non-human origin, and they should be continuously monitored through an appropriate quality assurance system as per requirement from regulatory bodies. From the esthetic point
of view, these autologous skin substitutes should be durable, elastic, and pigmented to resemble natural skin. Indications for skin substitutes are burns, chronic wounds (leg ulcers), pigment defects, plastic, aesthetic, dental surgery, and defects in oral mucosa. The novel, efficient, and safe cellular products manufactured, delivered, and applied in accord with good practices (laboratory, manufacturer, and clinical ones) will start a new era of individualized cell-based therapeutic approach in the management of severe chronic and acute skin defects with high morbidity, risk of invalidity, great social/economical impact, and heavy psychological burden.5

TARGET ARTICLES

CLINICAL PROBLEM ADDRESSED
Chronic ulceration of the lower leg is a frequent condition. Prevalence numbers range from 1% in the adult population to 3%–5% in the population over 65 years of age. In western countries, the incidence of chronic leg ulceration is rising as a result of the ageing population and of the increased risk factors for atherosclerotic occlusion, such as smoking, obesity, and diabetes. Among these patients the amputation rate has been reported to be 15–70 times that of the general population. Because of the indolent, resistant, or recurrent nature of chronic wounds, a wide variety of treatments exist and new wound management techniques are continuously being developed.7

Healing of third-degree burn wounds is accompanied by severe scar formation. These scars are characterized by excessive extracellular matrix (ECM) deposition, altered collagen remodeling, and contraction. The presence of abundant inflammatory mediators mediates the migration of fibroblast-like cells to the wound area. These peculiar fibroblasts (myofibroblasts) produce high levels of different components of the ECM, first of all collagen. In normal wound healing the myofibroblasts disappear during the remodeling phase, whereas in hypertrophic scars the myofibroblasts remain present and actively produce collagen. Because of the disfiguring and mobility-limiting scars, patients have to undergo many surgeries to release the limitations. 

There are several options for the treatment of vitiligo, a skin pathology characterized by the appearance of depigmented spots. Physical (phototherapy) and medical therapies (corticosteroids, calcineurin inhibitors, vitamin D analogs, and antioxidants) are usually utilized in active vitiligo with scarce success. Repigmentation is initiated by proliferation, migration, and activation of melanocytes still present in the hair follicles, either in the margins or within the depigmented spots. Advanced cellular therapies, such as autotransplantation of isolated melanocytes or in vitro engineered skin equivalents, are emerging for stable vitiligo, when medical therapies failed to induce repigmentation.9,10

RELEVANT BASIC SCIENCE CONTEXT
During the research and development of innovative cell-based skin products, several important basic science aspects concerning (1) cell-to-cell interactions in the cultures, (2) effects of cell origin and culture conditions on the molecular mechanisms of cellular senescence, (3) differentiation, and (4) prosurvival/death alternatives, (5) clonal evolution of skin cells in cultures, and (6) stem cell characteristics are essential for the future success of clinical application. Distinct genetic manipulations with proteins involved in the keratinocyte senescence and programmed death allowed to increase proliferation of cultured keratinocytes and decrease their differentiation (effect of immortalization) without, however, tumor transformation of the cells.7

EXPERIMENTAL MODEL OR MATERIAL—ADVANTAGES AND LIMITATIONS
Skin cell cultures grown on the biologically compatible material are simultaneously the experimental model and the final product in the tissue engineering process. As an experimental model, individual cultures or cocultures of different skin
cells (keratinocytes, fibroblasts, and melanocytes) allow to easily manipulate cultural conditions, substrates-scaffolds for cell growth, ratio of cocultured cells, addition of biologically active factors, or elimination of foreign molecules and cells.7,9,14 Cellular behavior, the histological and molecular characteristics of reconstructed skin, as well as its possible contamination with microbial and viral material could be effectively monitored at selected time points. Serious limitations of the model are low reproducibility, high cost, and time-consuming procedures for its maintenance and quality control.

**DISCUSSION OF FINDINGS AND RELEVANT LITERATURE**

Normal human skin cells can be isolated by a small skin biopsy and serially propagated in vitro.1,7,8,16,17 Keratinocytes reconstitute sheets of stratified epidermis, the upper layer of the skin, with a physiological melanocyte-to-keratinocyte ratio. Fibroblasts may be amplified in a large quantity and may be cocultured with keratinocytes if an appropriate biological substrate is used. Interaction between keratinocytes and fibroblasts is crucial for the full regeneration of a functional epidermis.18 Both keratinocytes and fibroblasts contribute to protein deposition and to the reconstitution of the basal membrane. Keratinocyte- and fibroblast-derived cytokines and growth factors are of utmost importance in regulating wound healing. Thus, keratinocyte-derived cytokines inhibit synthesis of ECM by fibroblasts, fibroblast-mediated graft contraction, and scar formation.7,8,18 On the other hand, fibroblasts exert profound effects on keratinocyte proliferation and differentiation.18 Cultured keratinocytes are essential to maintain melanocyte survival and functions through production of growth factors targeting melanocytes and cytokines regulating melanogenesis.9 Of note, keratinocytes isolated from the depigmented spots of vitiligo patients or patients with inherited piebald trait exhibit limited capacity to control a ratio of melanocyte survival/death in cocultures.9,11,12

Epidermis needs to constantly replace damaged or dead cells throughout the life. Typically, continuous replacement is maintained because of the presence of stem cells. Normally, in human epidermis at least 10% of basal cells are stem cells.1,7,13,15 If the in vitro engineered skin contains stem cells, it enables long survival of the transplant. Several burn patients treated with the in vitro reconstructed skin have been followed up for more than 20 years. Although numerous stem cell markers now exist, it is rather difficult to a priori identify stem cells in culture. Only a posteriori analyses of cell clones, regenerative capacity, and “life span” of the cultures provide some hints on the presence of functional stem cells.7,11,14

For the in vitro tissue engineering, matrices are developed to support cultured cells and promote their differentiation and proliferation toward the formation of a new tissue. Substrates-scaffolds enabling proper wound coverage should have some essential characteristics that include (1) being easy to handle and apply to the wound site; (2) provide appropriate barrier function; (3) be readily adherent; (4) have appropriate physical and mechanical properties; (5) undergo controlled degradation; (6) be sterile, nontoxic, and nonantigenic; (7) evoke minimal inflammatory reactivity; (8) incorporate into the host with minimal pain and low risk of further scarring; (9) facilitate angiogenesis; and (10) being cost effective.1,7,14 The fibrin matrix is a homogeneous and transparent gel where cell growth may be easily monitored. When keratinocyte-containing fibrin gels are applied onto the wounds, fibrin is quickly degraded, allowing cells to take on the wound bed. Accurate quality controls have demonstrated that keratinocytes’ ability to form the epidermal stem cell–containing clones, growth rate, and long-term proliferative potential are not affected by fibrin substrate.14 When applied to a properly prepared wound bed, the fibrin adhesive fixes the cells to the wound and allows better in-growth.

Accepted practice for third-degree burns is to accurately debride the burned tissue, provide a dermal equivalent, and, once this has become well vascularized, provide an autologous epidermal layer.8,19 The most commonly used production of graftable epidermis relies on the presence of lethally irradiated murine 3T3-J2 fibroblasts.17 The utilization of nonirradiated autologous human fibroblasts shows excellent results in terms of unaltered keratinocyte behavior, proliferative potential, cytokine production, and stem cell presence.14

In chronic ulcers, cultured cells are mainly considered to act as “biological factories” by stimulating the patient’s own wound repair mechanism: (1) production of growth factors and ECM and (2) reepithelialization coming from the edge of the wound.1,2,6,16 The lack of dermis is, however, a critical factor for cell engraftment. A better approach would be to supply the clean and preliminary revascularized ulcer with both (1) ready-to-be-vascularized dermis consisting of biocompatible, porous, and oxygen and growth factors penetrable three-dimensional matrix plus autologous fi-
broblasts and (2) autologous cultured keratinocytes, which could be taken and proliferated.\textsuperscript{7,14}

In the physiological coculture of keratinocytes and melanocytes, (1) keratinocytes regulate melanocyte growth and differentiation by a variety of growth and survival factors for melanocytes; (2) keratinocytes regulate the physiological melanocyte-to-keratinocyte ratio; and (3) melanocytes organize themselves into the basal layer of the cultured epidermis and develop dendrite “bridges” to transfer melanosomes into basal keratinocytes. When autologous cultured epidermal sheets are grafted onto a well-prepared erbium-YAG (yttrium, aluminum, garnet) laser-receiving bed, it facilitates stable repigmentation of the achromatic areas in 90% of vitiligo\textsuperscript{10} and piebaldism\textsuperscript{20} patients.

**INNOVATION**

The most innovative aspect of the target articles by Guerra’s group\textsuperscript{7,9,14} is that the classical procedure of human skin equivalents preparation in the in vitro conditions described by Rheinwald and Green 35 years ago,\textsuperscript{17} with the obligatory use of lethally irradiated murine fibroblasts as a feeder layer V, is substituted by another one, where autologous human nonirradiated fibroblasts perfectly maintained normal growth, differentiation, and staminality (the presence of stem cells) of cultivated keratinocytes.\textsuperscript{14} Skin equivalents manufactured without cells and molecules of animal origin would better meet international safety requirements for cell-based medicinal products.

**SUMMARY ILLUSTRATION**

The figure opposite shows the standard operations for the in vitro reconstruction and autotransplantation of skin equivalents to (1) third-degree burns (left column)—subconfluent primary keratinocyte cultures are expanded on fibrin gel, and layers of confluent keratinocytes (secondary cultures) grown on fibrin are applied on the top of dermal reconstruct substituting a soft tissue defect. Biopsy as small as 1 cm\textsuperscript{2} gives skin equivalents to cover large burn areas (more than 1 m\textsuperscript{2}), which usually heal without excessive scar formation; (2) chronic ulcers (middle column)—subconfluent primary keratinocytes are layered on the autologous fibroblasts expanded within a fibrin gel. Completely autologous equivalents having architecture of normal skin are transferred to properly prepared ulcer bed; (3) vitiligo or piebaldism stable pigment defect (right column)—confluent primary keratinocyte + melanocyte cultures (primary cultures) obtained from the biopsy of pigmented parts of the skin are serially expanded (secondary cultures) and keratinocyte/melanocyte ratio as well as melanin production by cultivated melanocytes are thoroughly controlled. The in vitro reconstructed pigmented skin equivalents cover large surfaces of depigmented skin preliminary slightly ablated (deepithelialized) by erbium-YAG laser (for details, see refs.\textsuperscript{10,20}).

**CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS**

The technology of in vitro tissue engineering has been shown to be feasible and several products have been successfully marketed. There is still a great need for the development of new advanced products with higher clinical impact.

Translation of research from the bench to bedside requires long-term rather than short-term quality controls of cell-based products. Biological “drugs” for the somatic cell therapy must be thoroughly evaluated before proposing them for human use either in clinical trials or as commercial products. In accord with current regulations, products based on manipulated human cells for cell therapy are considered as medicinal preparations to be manufactured in compliance with good manufacturing practice, which considerably increases cost of the production.

**TAKE HOME MESSAGE**

**Basic science advances**

- Interference between different human skin cells in cultures grown on a substrate mimicking ECM influences cell growth, differentiation, cytokine production, and several other cellular functions.

**Clinical science advances**

- Distinct procedures of the in vitro reconstruction of skin equivalents containing different cells and an appropriate scaffold lead to differentiated production of cell-based medicinal preparations targeting either acute or chronic wounds or pigment skin defects.

**Relevance to clinical care**

- In acute wounds with an increased risk of scar formation and in chronic nonhealed ulcers as well as in skin pigment disorders with unmet therapeutic needs, autotransplantation of purposefully in vitro engineered skin equivalents substantially improves and accelerates wound healing and restores esthetical appearance of normal skin.
The speed with which regulatory mechanisms will be developed and implemented is a critical factor for advanced cell-based drugs and therapies. Financing the high costs of engineered tissue production is always a challenge. Moreover, the cost of autologous cells/skin equivalents is very high when compared with allogenic products. However, there are many advantages in terms of safety and efficacy in the usage of completely autologous cellular medicinal products; therefore, thorough cost/efficacy analysis should be carried out to prove their market viability.

FUTURE DEVELOPMENTS

Tissue-engineered skin should include all the skin appendages (hair follicles, sweat glands, and sensory organs) and layers (epidermis and dermis),
with rapid development of functional vascular and nerve network and scar-free integration with the surrounding host tissue. Such a construct should allow the skin to fulfill its many normal functions: barrier formation, pigmentary defense against ultraviolet irradiation, thermoregulation, and mechanical and aesthetic functions. Considerable focus should be placed on multipotent adult stem cells, such as bone marrow– or adipose tissue–derived mesenchymal stem cells or hair bulge stem cells, which could accelerate wound repair or even reconstitute the wound bed. Moreover, next-generation skin equivalents will be constructed on smart scaffolds carefully engineered to release signaling molecules, growth and differentiation factors, and special proteins to facilitate cell migration and adhesion. Properly designed clinical trials on safety and efficacy of stem cell–bearing skin substitutes engineered on advanced biomaterials should be carried out.

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AUTHOR DISCLOSURE STATEMENT

The authors have no competing interests. This article was not written by any writer other than the authors.

REFERENCES


Macrophage Heterogeneity and Wound Healing

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Background: Macrophages (Mφs) participate in host defense by orchestrating inflammation, immune responses, and wound healing. In response to microenvironmental stimuli, Mφs adopt either a classically activated (M1) pro-inflammatory phenotype or an alternatively activated (M2) wound healing phenotype.

The Problem: M1 Mφs are induced by products of pathogenic agents and interferon-γ. M2 Mφs are induced in response to interleukin-4 (IL-4) and IL-13. However, recent studies show that there are IL-4/IL-13-independent pathways that induce phenotypes resembling M2 Mφs. Further characterization is necessary to elucidate the different activating agents and functional characteristics of these M2-like Mφs.

Basic/Clincial Science Advances: Unstimulated Mφs express low levels of inflammatory cytokines and growth factors such as tumor necrosis factor-α (TNF-α), IL-12, and vascular endothelial growth factor (VEGF). Stimulation of Mφs with Toll-like receptor agonists, such as lipopolysaccharide, induces M1 activation, characterized by production of TNF-α and IL-12. In the inflammatory environment, the retaliatory metabolite adenosine rapidly accumulates extracellularly as an adenosine triphosphate breakdown product. Costimulation of Mφs with Toll-like receptor (TLR) agonists and adenosine switches Mφs from an M1 to a novel M2-like phenotype by upregulating IL-10 and (VEGF) and downregulating TNF-α and IL-12 in an adenosine A2A receptor-dependent and IL-4/IL-13-independent manner, as depicted in Fig. 1.

Clinical Care Relevance: M2-like Mφs and their induction pathways present attractive potential targets for pharmacological regulation of wound healing and inflammatory diseases.

Conclusion: Switching from an M1 into an M2-like Mφ phenotype can occur in an adenosine A2A receptor-dependent and IL-4/IL-13-independent manner and may be critical to wound healing.

BACKGROUND

Macrophages (Mφs) play a pivotal role in host defense through orchestration of inflammation and wound healing. While resting Mφs are relatively quiescent, activation yields polarized phenotypes based on location and microenvironmental influences. These include the presence of activating agents, cytokines, hypoxia, and ischemia, which in turn regulate expression of numerous inducible genes that may be involved in the development of Mφ subtypes. Although categorization can be blurred by assorted combinations of activating agents, Mφs are typically described as classically activated (M1) or alternatively activated (M2). Based on the nature of activating stimuli, Mφs may switch activation state to other phenotypes.1–4

M1 Mφs promote inflammatory processes during injury and infection...
Abbreviations and Acronyms (continued)

iNOS = inducible nitric oxide synthase
LPS = lipopolysaccharide
M1 = classically activated
M2 = alternatively activated
Mφ = macrophage
NO = nitric oxide
PAMP = pathogen-associated molecular pattern
TGF-β = transforming growth factor-β
Th1 = CD4 + T helper 1
TLR = Toll-like receptor
TNF-α = tumor necrosis factor-α
VEGF = vascular endothelial growth factor
Ym1/Ym2 = chitinase 3-like 3 (CHI3L3)/chitinase 3-like 4 (CHI3L4)

that are vital for intracellular pathogen removal. These Mφs are induced by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), damage-associated molecular patterns (DAMPs), such as those derived from mitochondria of apoptotic neutrophils, and interferon-γ (IFN-γ) (produced by activated CD4+ T helper 1 [Th1], CD8+ T cytotoxic 1, and natural killer cells) to enhance pro-inflammatory responses through production of cytokines (e.g., interleukin-1 [IL-1], IL-6, IL-12, and tumor necrosis factor-α [TNF-α]) and inflammatory mediators (e.g., nitric oxide [NO] through inducible NO synthase [iNOS]), and to increase phagocytic and antigen presenting activity. In contrast, M2 Mφs are critical for induction of angiogenesis, tissue remodeling, and repair. M2 Mφs participate in resolving inflammation through production of anti-inflammatory cytokines, and growth and angiogenic factors, and by phagocytosis and elimination of debris.1,2,4-7

CLINICAL PROBLEM ADDRESSED

Because of their role in regulating inflammation and repair, modulation of M1 and M2 Mφs and their downstream effects may provide therapeutic benefits. Characterization of M2 Mφ subtypes and mechanisms by which they are induced may provide targets for therapy in various diseases. Because of their wound healing and anti-inflammatory properties, induction of M2 phenotypes may enhance angiogenesis and granulation tissue formation in wound healing. On the other hand, maintenance of an M1 phenotype may promote inflammation and host defense, while reducing excessive granulation tissue formation. Angiogenesis in heart disease and tumor growth also involves Mφs, and may be regulated by manipulation of M1/M2 polarization.7-12

RELEVANT BASIC SCIENCE CONTEXT

Mφs participate in the regulation of inflammation and wound repair by secretion of cytokines, chemokines, and growth factors and by phagocytosis. Two main categories of activated Mφs have been described: pro-inflammatory (M1) Mφs and anti-inflammatory/wound healing (M2) Mφs. M2 Mφs are defined as Mφs activated by IL-4 and IL-13 through the IL-4 receptor-α, and are characterized by upregulated expression of IL-10, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) and low expression of TNF-α and IL-12.1,4 M2 Mφs also express cell surface and intracellular markers, which are listed in Table 1. Recent studies, however, have shown that IL-4 and IL-13 are not essential for induction of the M2-like phenotype and that mouse wounds lacking IL-4/IL-13 still contain Mφs with M1 and M2-like characteristics.2 While numerous pathways, including recognition and phagocytosis of apoptotic cells (see chapter by Roy in this volume), may mediate Mφ activation, we set out to define one subtype of M2-like Mφs that is induced by costimulation of Mφs with Toll-like receptor (TLR) and adenosine A2A receptor (A2A R) agonists.13,14

Both TLRs and A2A Rs may be expressed on Mφs and are important regulators of inflammation and repair. Elucidation of signaling from these receptors may lead to approaches to enhance or block downstream effects through regulation of Mφ mediators (intracellular or secreted) or through the use of receptor agonists or antagonists. For example, we have shown that LPS activation of TLR4 induces TNF-α expression, which is down-regulated by adenosine receptor agonists, whereas costimulation of TLR4 and A2A R upregulates IL-10 and VEGF expression.14 The switch to an
M2 phenotype favors angiogenesis and granulation tissue formation in wound healing. Knowledge of the signaling pathways involved in this process should provide an understanding of the phenotypic switches occurring in Mφs and yield potential therapeutic targets for enhancing wound healing or for diminishing abnormal wound healing. Although Mφs have varying roles that are dependent on activation by agents in the surrounding milieu, a clear understanding of how to manipulate and switch these phenotypic profiles may provide potential benefits.

EXPERIMENTAL MODEL OR MATERIAL—ADVANTAGES AND LIMITATIONS

There are limitations to consider in the characterization of M2 Mφ gene expression. These include varying expression patterns when comparing models in different species and variable effects of the inflammatory milieu surrounding Mφs. Although M1/M2 Mφ polarization occurs in humans, studies of M2 Mφ marker expression have been predominantly restricted to mice. Human homologs have not always been found, giving rise to questions of identification, and indicating the need for further elucidation of M1/M2 Mφ polarization in the human system. It is critical to recognize that changes in the Mφ background influence Mφ activation. While in vitro models enable identification of activation factors, exclusive focus on Mφs in culture may overlook interactions with other cell types.

A limited focus on cytokines (such as IL-4/IL-13) in regulating Mφ activation may also result in effects of nonimmune-dependent metabolites, such as adenosine, being overlooked. Recent studies indicate IL-4/IL-13-independent M2-like Mφ activation, and our studies show that adenosine, through A2AR induction by TLR agonists, mediates an M1 to M2-like switch. The characteristics of activated Mφs also vary with time to result in early and late markers. Early wound Mφs show higher levels of TNF-α expression due to M1 activation, which diminishes with time, whereas later wound Mφs express elevated VEGF as a result of switching from an M1 to an M2-like phenotype. This switch is driven in a temporally orchestrated manner that depends upon changes in the microenvironment of the wound (see Discussion of Findings and Relevant Literature section).

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Mφs orchestrate inflammation, tissue repair, and immune responses by phagocytosis and destruction of foreign organisms through recognition of PAMPs and DAMPs, by production of cytokines and growth factors, and by antigen presentation. Tissues contain heterogeneous populations of Mφ, which have the capacity to dramatically change their phenotype as a result of differentiated plasticity as well as microenvironmental tissue- and immune-specific influences.

Upon exposure to stimuli, Mφs develop phagocytic and secretory phenotypes oriented for specific functional activities. Two general categories of activated Mφs have been defined: M1 and M2. The M1 phenotype is driven by Th1 cytokines, such as IFN-γ, and TLR agonists to induce a pro-inflammatory and cytotoxic response against intracellular pathogens and certain transformed and cancer cells. These M1 Mφs predominate in the early wound environment and secrete high levels of pro-inflammatory cytokines, low levels of anti-inflammatory cytokines, and high levels of NO (through iNOS induction) (see Table 1). An M1 phenotype may be induced by PAMPs associated with exogenous or commensal organisms, by endogenous TLR agonists, or by DAMPs. Failure of the innate immune response to eliminate these stimuli might result in the persistent induction of M1 Mφs. M2 Mφs can be induced in a Th2-dependent manner and display expression patterns associated with anti-inflammatory effects, Th1 response inhibition, and wound healing promotion. In contrast, M2 Mφs express low levels

TARGET ARTICLES
of pro-inflammatory mediators and higher levels of anti-inflammatory mediators and fibrogenic and angiogenic factors (see Table 1 and Fig. 1). M2 MΦs recruit a specific group of cells in their coordination of immune responses through secretion of chemokines that attract monocytes, basophils, memory T cells, Th2 cells, and eosinophils. Some M2 MΦ markers are listed in Table 1.1,4,8 In addition to their central role in immune responses, M2 MΦs also play a role in some pathological processes, including vulnerability to pathogens due to dampening of Th1 responses by M2 MΦs, allergic responses involving upregulated M2 gene expression in asthma, tumor progression assisted by tumor-associated M2-like MΦs, and fibroproliferative complications of infection and inflammation.7–12

M2 MΦs that are activated by IL-4 and IL-13 (produced by activated CD4+ Th2 and CD8+ Tc2 cells, natural killer cells, basophils, mast cells, and eosinophils) are designated as M2 and are associated with allergic and antiparasitic responses. These MΦs show decreased production of IL-1β and IL-8 and decreased induction of oxygen radicals through the respiratory burst, and express elevated levels of CD206 (MR1/manose receptor, class C type I) and CD204 (SR-A/scavenger receptor, class A type I) scavenger receptor-1 (MSRI).1,4,17,18 Further characterization of MΦs has demonstrated other types of M2-like MΦs involved in wound healing that are not dependent on IL-4/IL-13 induction, and M2 MΦs have been subclassified based on inducing agents and subsequent expression patterns. M2 MΦ subcategories include the more commonly investigated M2a MΦ (activated by IL-4 or IL-13), M2b MΦ (induced by immune complexes [ICs] and IL-1β or TLR agonists), and M2c MΦ (stimulated by IL-10, TGF-β, or glucocorticoids).1 It should be emphasized that there are M2 MΦs that are not associated with a Th2 immune response or IL-4/IL-13 activation, as seen in M2b and M2c MΦs. Further, these activated MΦs show overlapping and nonoverlapping expression marker patterns. Albina and colleagues recently described a dynamic M2 MΦ phenotype that is not dependent on IL-4 or IL-13 activation. In IL-4 receptor-α KO mice and in the presence of the IL-13Rα2 decoy receptor, activation occurs in spite of inhibition of IL-13-dependent phosphorylation of downstream STAT6 and the absence of IL-4 or IL-13 in the wound environment, and these mice exhibit MΦs with both M1 and M2 expression patterns. Wound MΦs in early phases of repair are more M1-like with elevated expression of TNF-α and IL-6 and less TGF-β, whereas those in later phases are more M2 like, with less pro-inflammatory cytokines, no induction of iNOS, and elevated markers of alternative activation, including CD206, β-glucan receptor (dectin-1), arylsulfatase-1 (Arg1), and chitinase 3-like 3 (Ym1).2

In contrast to the commonly described M2 MΦs induced by IL-4 or IL-13, we have demonstrated a new type of M2-like MΦ that is induced by phenotypic switching of M1 MΦs in an IL-4/IL-13-independent manner. This novel mechanism of MΦ phenotypic switching to produce an M2-like MΦ requires initial stimulation of IL-4, which strongly induces A2AR expression, followed by ligation of A2ARs by adenosine. This induction of A2ARs plays a key role in switching pro-inflammatory M1 to an angiogenic M2-like phenotype. We have previously shown that while the TLR4 agonist LPS (as well as agonists of TLR2, 7, 9) strongly induce TNF-α and IL-12 expression in MΦs, adenosine present in the microenvironment (as a metabolite formed from the breakdown of adenosine triphosphate) strongly inhibits this induction while simultaneously upregulating IL-10 and VEGF expression.13,14 Black et al. described upregulated Arg1 expression and activity and no effect on expression of other M2 markers (found in inflammatory zone-1 [Fiz1]) and Ym in MΦs treated with LPS and A2AR agonists.19 Thus, to define M2 MΦs in greater depth, we suggest designating these TLR- and A2AR-induced M2-like MΦs as M2d MΦs. A key predictor of this proposed pathway is that these M2d MΦs should express elevated levels of A2ARs. We are currently investigating this hypothesis in detail (Figure 1).

INNOVATION

We propose the redefinition of M2 MΦs to include a new subtype that is induced by switching of M1 MΦs into M2-like MΦs through activation of TLRs and A2ARs. We suggest that these MΦs be called M2d MΦs. These newly described M2d MΦs express elevated VEGF and IL-10 and diminished TNF-α and IL-12.13 Analysis of the influence of multiple costimulators on MΦs allows for the integrated analysis of pathways downstream of these receptors and the resulting phenotypic MΦ profiles. This analysis may more accurately mimic true micro-environmental conditions, where adenosine accumulation occurs in inflammatory, ischemic, and hypoxic settings, leading to phenotype switching from pro-inflammatory M1 MΦs to wound healing, anti-inflammatory M2d MΦs in a temporally defined manner. Singular activation of these recep-
Table 1. Phenotypic profiles of activated macrophages

<table>
<thead>
<tr>
<th>Inducing agents</th>
<th>M1</th>
<th>M2a, M2b, M2c, M2d</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IFN-γ, TLR agonists (e.g., LPS)</td>
<td>M2a: IL-4, IL-13</td>
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<tr>
<td></td>
<td></td>
<td>M2b: IL-1β, TGF-β</td>
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<tr>
<td>High expression</td>
<td>TNF-α, IL-12, IL-1β, IL-6, IL-23, iNOS, CCL3</td>
<td>CD206, CD204, dectin-1, IL-10, TGF-β, Arg1, Ym1, Ym2, Fizz1, CCL18</td>
</tr>
<tr>
<td>Low expression</td>
<td>CD206, IL-10, TGF-β</td>
<td>TNF-α, IL-12, IL-1β, IL-6, IL-23, iNOS</td>
</tr>
<tr>
<td>General phenotypic profile</td>
<td>Inflammatory Th1 response</td>
<td>Anti-inflammatory, wound healing Th2 response</td>
</tr>
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Shown in the table is a partial list of activating agents and subsequent marker expression patterns in M1 and M2 Mφs. Note that some markers may be specific to certain Mφ types or may overlap in some profiles.

FIGURE 1. Pathways of Mφ activation. Nonactivated Mφs are induced to express activated phenotypes based on specific inducing agents, and to exhibit expression patterns of markers that may overlap with those of other phenotypic profiles. Note that the aim of this figure is to provide a general representation of M2 Mφ expression. Not all agents are included, and expression profiles for different M2 Mφ subtypes vary based on inducing agents.

tors may not show expression patterns seen in costimulation models. Identification of this new IL-4/IL-13-independent and A2AR-dependent M2d Mφ shows that Mφ activation and its role in wound healing is a complex event involving numerous microenvironmental stimuli.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

The observation that both classical and alternatively activated Mφ phenotypes are found in wounds of mice where IL-4 and IL-13 signaling is absent raises the question of the origin of these heterogeneous Mφ populations. Our discovery of the A2AR-dependent switch of M1 Mφs to an M2-like phenotype provides one possible mechanism for this heterogeneity, and provides a simple, temporally regulated pathway for the development of M2d Mφs. Mφs are initially activated to an M1 phenotype by PAMPs and DAMPs, which also induce A2AR expression. When (and if) extracellular adenosine levels rise as a result of adenosine triphosphate breakdown in response to cell stress, inflammation, hypoxia, or ischemia, signaling through the elevated A2ARs switches the M1 Mφs into the M2d
phenotype, which then participates in the promotion of angiogenesis and repair. Future studies will determine the contribution of this pathway to aberrant wound healing, fibrosis in disease, and cancer development. Analysis of signaling pathways that mediate the M1/M2d switch will provide focus for the development of pharmaceutical approaches to regulating this switch.

FUTURE DEVELOPMENTS
The discovery of a novel A2AR-dependent M2 MΦ subtype (M2d) that is induced independently of IL-4/IL-13 provides a potential target for modulating inflammation and wound healing by pharmacological regulation of the M1/M2d switch. Our investigation continues with characterization of M2d MΦs and signaling pathways that mediate their induction. Subsequent studies will examine MΦ phenotypes in wound healing, chronic inflammatory, and tumor models, to determine when and where the M2d phenotype is expressed. While most studies of this M1/M2d switch are in murine MΦs, human monocytes also undergo a similar LPS/A2AR-mediated phenotypic switch in vitro. Future studies should characterize human monocytes/MΦs in wound healing, chronic inflammatory, and tumor models, to determine when and where the M2d phenotype is expressed. While most studies of this M1/M2d switch are in murine MΦs, human monocytes also undergo a similar LPS/A2AR-mediated phenotypic switch in vitro. Future studies should characterize human monocytes/MΦs and the role of the A2AR-mediated switch in regulating their properties. These studies will help phenotype these M2 MΦs and will allow to the testing of agonists and antagonists of signaling pathways involved in acquisition of these phenotypes, with the aim of developing therapeutics for their regulation. Maintenance of an M1 phenotype might promote clearance of pathogenic organisms and wound debridement; promotion of an M2 phenotype might enhance angiogenesis and granulation tissue formation, whereas reverting M2 MΦs to an M1 phenotype could diminish excessive granulation and scarring.

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REFERENCES


Sustained Release and Dual Delivery Strategies for Platelet-Derived Growth Factor

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Background: Strategies for improving impaired wound healing are clinically relevant areas of investigation. Many of the consequences from failed healing may be caused by altered intracellular signaling, which implies that administration of signaling molecules to sites of tissue repair is a viable strategy for improving healing.

The Problem: Local delivery of recombinant human platelet-derived growth factor BB (PDGF-BB) from a carboxymethylcellulose gel is a Food and Drug Administration–approved therapy for treatment of diabetic foot ulcers. Recent studies have aimed to overcome the limitations of the bolus release of growth factor from topical gels by designing drug delivery systems and scaffolds capable of sustained release and dual delivery with other factors and cells.

Basic/Clinical Science Advances: Dual delivery of PDGF-BB and transforming growth factor-beta was reported to increase the tensile strength of incisional wounds relative to no treatment and treatment with a single growth factor at very low dosages (<5 μg cm⁻²). Another study reported that dual delivery of fibroblasts and PDGF-BB enhanced ingrowth of granulation tissue and wound resurfacing relative to delivery of fibroblasts alone. Other studies cited in this article show that the sustained release of PDGF-BB enhances wound healing relative to the bolus release associated with topical gels.

Clinical Care Relevance: Alternative delivery approaches that achieve predictable wound healing at lower PDGF-BB doses are anticipated to reduce the need for repeated administration associated with a high-concentration bolus release.

Conclusion: Sustained local delivery of PDGF-BB, dual delivery of PDGF-BB and transforming growth factor beta, and dual delivery of fibroblasts and PDGF-BB are promising approaches for improving the healing of impaired wounds relative to currently available therapies.

BACKGROUND

Wound healing is a complex series of events regulated by intercellular communication via cytokines and growth factors. Many of the consequences from failed healing may be caused by altered intercellular signaling, which implies that administration of signaling molecules to sites of tissue repair is a viable strategy for improving healing. Local delivery of recombinant human platelet-derived growth factor BB (PDGF-BB) has been shown to enhance the healing of cutaneous defects under...
impaired healing conditions. In recent years, several studies have aimed to overcome the limitations of the bolus release of growth factor from topical gels by designing drug delivery systems and scaffolds capable of sustained release. The target articles reviewed in this article investigate the strategies for improving the efficacy of locally delivered PDGF-BB by a sustained release delivery system, codelivery of another factor associated with wound healing, or codelivery with fibroblasts.

TARGET ARTICLES

CLINICAL PROBLEM ADDRESSED
Regranex™, a carboxymethylcellulose gel incorporating 100 μg cm⁻³ PDGF-BB, is a Food and Drug Administration (FDA)–approved therapy for treating foot ulcers. Because of the bolus release of growth factor, the gel must be applied at least daily at high concentrations. A retrospective study found a fivefold increased risk of cancer mortality in patients exposed to three or more tubes of Regranex compared with patients who had not been exposed. Although this is a controversial unpublished report, it nevertheless prompted FDA to mandate a black box warning on the product label. Sustained release of PDGF-BB is anticipated to reduce the amount of drug delivered, thereby reducing the frequency of complications and improving patient outcomes.

RELEVANT BASIC SCIENCE CONTEXT
Implanting or injecting a biodegradable, biocompatible scaffold into a tissue defect is an established approach for regenerating tissue and restoring its architecture. By adding an appropriate growth factor(s), the scaffold can also function as a local delivery system in addition to providing mechanical support. The scaffold must be biocompatible, support the infiltration of cells and new tissue, degrade to nontoxic decomposition products at a rate comparable to that of new tissue ingrowth, and release biologics at a time scale that is relevant to the biology of the wound site. PDGF is a useful growth factor for wound healing because of its chemotactic and mitogenic properties for a wide variety of cell types, as well as the fact that it is a cofactor with vascular endothelial growth factor (VEGF) for angiogenesis. PDGF-BB, the most widely investigated isoform for tissue regeneration, has been reported to stimulate the formation of new tissue in both healthy and diabetic rats. It is desirable to lower the dose of PDGF to reduce the frequency of complications and improve patient outcomes. Several approaches have been taken to improve the safety and efficacy of PDGF-BB, including development of sustained release vehicles, codelivery with other growth factors associated with wound healing (such as transforming growth factor-beta [TGF-β]), and codelivery with fibroblasts. The effects of each of these strategies on wound healing in preclinical models are reviewed in this article.

EXPERIMENTAL MODEL OR MATERIAL—ADVANTAGES AND LIMITATIONS
In one of the reviewed studies, the effects of PDGF and TGF-β, delivered either alone or in combination, on the healing of 6-cm incisional wounds were investigated in a cyclophosphamide-induced impaired wound healing model in rats. The delivery system comprised two 0.7 x 15 mm ethylene-vinyl acetate copolymer rods that have been shown to achieve sustained release of growth factors. The doses were 3.0 μg cm⁻³ PDGF-BB and/or 2.0 μg cm⁻³ TGF-β. The effects of dual drug delivery on healing were evaluated using histology and biomechanical testing on days 4, 7, and 14 post-implantation.

In another study, the effects of dual delivery of PDGF-BB and fibroblasts on the healing of full-thickness biopsy wounds in a rabbit ear model were investigated. The delivery system comprised four fibrinogen–thrombin formulations of fibrin sealant (FS) combined with fibroblasts and PDGF-BB. The dose of PDGF-BB was 3 mg in a 7 x 0.27 mm full-thickness wound (~300,000 μg cm⁻³). The extent of wound resurfacing and ingrowth of granulation tissue were measured histologically on day 7 post-injection.
In the sustained release approach, the effects of PDGF-BB delivered from a biodegradable polyurethane scaffold on the healing of full-thickness excisional wounds in rats were investigated. PDGF-BB doses of 31.6 and 316 \( \mu \)g cm\(^{-3} \) were tested. Ingrowth of new granulation tissue and scaffold degradation were measured by histomorphometry on days 3, 7, and 14 postimplantation.

**DISCUSSION OF FINDINGS AND RELEVANT LITERATURE**

The results from the three target reports show that the efficacy of locally delivered PDGF-BB can be enhanced by (a) sustained release up to day 7, (b) codelivery with another factor, such as TGF-\( \beta \), or (c) codelivery with fibroblasts. In a study investigating the effects of PDGF-BB released from biodegradable polyurethane scaffolds, PDGF-BB (31.6 \( \mu \)g cm\(^{-3} \)) was observed to accelerate cellular infiltration, ingrowth of new tissue, and scaffold degradation relative to blank scaffold controls at days 3, 7, and 14 in a rat excisional wound model. Interestingly, no differences were observed at an order of magnitude higher dose (316 \( \mu \)g cm\(^{-3} \)). These observations are consistent with a previous study reporting that sustained release of PDGF-BB from poly(lactic-co-glycolic) acid microspheres embedded in poly(lactic acid) nanofibrous scaffolds implanted subcutaneously in rats increased the formation of new tissue at days 7 and 14 relative to a bolus release of PDGF-BB. Interestingly, in the nanofiber scaffolds, dose-dependent effects on tissue ingrowth were observed when the PDGF-BB dose was increased from 31 to 310 \( \mu \)g cm\(^{-3} \). Further, at a low dose, ingrowth of new tissue was significantly higher for the slow-release scaffolds (day 1 burst release <5%) compared with the fast-release scaffolds (day 1 burst release = 30%). Surprisingly, for the polyurethane scaffolds characterized by a day 1 burst release of 60%, formation of new granulation tissue was significantly higher relative to the empty scaffold controls as early as day 3, compared with day 7 for the nanofiber scaffolds with linear release of PDGF-BB.

In the PDGF-BB/TGF-\( \beta \) dual-delivery study, cyclophosphamide treatment was found to significantly decrease the tensile strength at break of 6-mm incisional wounds relative to untreated controls at all time points, thus validating the impaired healing model. Interestingly, delivery of either PDGF-BB or TGF-\( \beta \) alone did not significantly increase the tensile strength of the wounds at all time points and, in fact, resulted in a decrease in wound strength relative to the control (no growth factor treatment). However, the combination of PDGF-BB (3 \( \mu \)g cm\(^{-3} \)) and TGF-\( \beta \) (2 \( \mu \)g cm\(^{-3} \)) was observed to significantly increase wound strength at days 7 and 14. The ethylene-vinyl acetate copolymer delivery system exhibited a linear release of growth factor with no burst. In another study investigating the effects of PDGF-BB delivered from a fibrin gel on the healing of flexor digitorum profundus tendons in dogs, PDGF-BB (3.3 \( \mu \)g cm\(^{-3} \)) was observed to significantly improve functional properties, but not tensile properties. The failure to improve mechanical properties was attributed to suboptimal PDGF-BB dose or release kinetics. Taken together, these results suggest that the failure of PDGF-BB to improve the tensile strength of incisional wounds at a dose of \( \sim \) 3 \( \mu \)g cm\(^{-3} \) could be attributed to a suboptimal dose and/or release kinetics. The incisional wound study demonstrates that enhanced healing can be achieved at a relatively low dose of PDGF-BB through codelivery of another factor associated with wound healing, which is conjectured to more closely mimic the *in vivo* wound healing environment.

Another approach to improving the efficacy of PDGF is codelivery with fibroblasts. Delivery of fibroblasts from FS was reported to increase both the area of granulation tissue as well as the percentage of wound coverage at day 7 postinjection. Interestingly, incorporation of fibroblasts in the FS was observed to elicit migration of the epithelium over the material, as well as incorporation of granulation tissue within the material. The authors conjectured that secretion of cytokines by fibroblasts delivered in the FS altered the behavior of and/or protease secretion by the host cells, thereby modifying the chemical composition of the FS and accelerating infiltration of granulation tissue. The authors further speculated that this enhanced integration of the FS could lead to more effective delivery of therapeutic agents as well as less scarring. Addition of PDGF-BB to the fibrinogen component with fibroblasts significantly increased both granulation area and surface coverage relative to delivery of fibroblasts alone. These results are consistent with another study reporting that delivery of a plasmid encoding for VEGF from a fibrin gel increased VEGF-A protein expression, tissue perfusion, and flap survival in a rat model at 7 days postimplantation. In the rabbit ear study, the effects of PDGF-BB alone were not investigated and so it is difficult to identify the relative contributions of the fibroblasts and PDGF-BB. Further, the dose of PDGF-BB was estimated to be \( \sim \) 300,000 \( \mu \)g cm\(^{-3} \), which is substantially higher than that used clinically or in other preclinical
studies. Taken together, these studies suggest that delivery of cells in combination with PDGF-BB enhances wound healing and is a potentially effective approach for improving the efficacy of PDGF for the healing of cutaneous defects.

INNOVATION
Several significant innovations have been reported, which contribute to substantially moving the field forward. Sustained release of PDGF-BB from biodegradable polymeric scaffolds has been shown to enhance the healing of cutaneous wounds relative to a bolus release of the growth factor. Second, codelivery of PDGF-BB with TGF-β enhances the healing of incisional wounds at doses of 2 orders of magnitude lower than that incorporated in FDA-approved topical gels. These results show that dual delivery of growth factors enhances healing relative to delivery of one factor alone. In another study, dual delivery of fibroblasts and PDGF-BB has been reported to significantly increase the area of granulation tissue and wound resurfacing. Taken together, these studies suggest that the sustained release and dual-delivery approaches improve wound healing outcomes by more accurately mimicking the in vivo microenvironment, potentially providing the significant benefit of requiring a lower dose of growth factor.

SUMMARY ILLUSTRATION
(A) Tensile strengths required to break 6-cm incisional wounds in rats at days 4, 7, and 14 postwounding (n = 4 per time point) are shown. Dorsal skin specimens were tested for tensile breaking strength. Error bars represent standard deviations. Groups were found to be significantly different by two-way analysis of variance (p < 0.0001). For each time point, the Tukey post hoc multiple comparison test was used to evaluate differences among groups (p < 0.05). On days 7 and 14, delivery of either PDGF-BB or TGF-β significantly reduced wound strength, whereas dual delivery significantly increased wound strength relative to the control.1 (B) Granulation tissue area measured in 7-mm full-thickness excisional wounds in rabbit ears on day 7 postoperation is shown. Wounds were treated with one of four FS formulations alone or FS with embedded rabbit dermal fibroblasts. Inclusion of dermal fibroblasts (3 × 10^6 cells/wound) in the fibrinogen component of the sealant increased the measured area of granulation tissue for formulations 2–4, although no differences were observed between formulations. Granulation tissue formation was dramatically enhanced by addition of PDGF-BB as tested with formulation 4. Data are shown as mean ± SEM. Background lines indicate saline control levels (n = 12). *p < 0.05 and **p < 0.01 versus FS alone for each formulation by two-way t-test. †Versus FS + cell groups by one-way factorial analysis of variance with Tukey’s highly significantly different test.3 (C) Histology and histomorphometric analysis of biodegradable polyurethane scaffolds with and without PDGF-BB implanted in 6-mm excisional wounds in Sprague–Dawley rats was performed. Implants were harvested on days 3, 7, and 14 postimplantation,
embedded in paraffin, cut into thin (~5 μm) sections, and stained with trichrome. In the representative histological sections (left), the white, red, and green sections represent the presence of polymer, tissue, and collagen deposition, respectively. The area percentages of polymer and new granulation tissue within the implants at each time point are shown in the plots on the right. Polyurethane scaffolds incorporating PDGF-BB showed significantly higher percentages of new tissue area and lower percentages of scaffold area relative to the control at each time point.²

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS
It is important to consider that the studies reviewed in this article were performed using varying dosages of PDGF-BB. Although the FS incorporating fibroblasts exhibited enhanced healing when PDGF-BB was delivered with fibroblasts, the dose of PDGF-BB was considerably higher than that used clinically and the effects of PDGF-BB independent of fibroblasts were not evaluated. Future studies investigating the independent contributions of fibroblasts and PDGF-BB at lower doses would be of interest.

FUTURE DEVELOPMENTS
Although topical gels incorporating PDGF-BB are an FDA-approved therapy for treatment of diabetic foot ulcers, there are concerns regarding complications and patient morbidity due to the bolus release of growth factor, which necessitates a high dose and repeated applications. A recent
study has shown that sustained release of PDGF-BB for at least 7 days enhances healing relative to a bolus release, suggesting that effective wound healing can be achieved at lower dosages in a sustained release delivery system. A potentially useful therapy is envisioned to comprise a scaffold/delivery system that delivers both autologous cells as well as PDGF-BB. The dual-delivery approach is anticipated to yield a device that improves wound healing at a substantially lower dose than that can be achieved through delivery of PDGF-BB alone.

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The author has nothing to disclose.

REFERENCES
REVIEW DIGEST

Management of Scars with Lasers

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Background: Cutaneous scars can cause functional impairment, discomfort, and significant aesthetic disfigurement.

The Problem: Choice of laser modality depends on scar characteristics, location, and previous treatments. Hypertrophic scars and keloids respond best to 585-nm pulsed-dye laser irradiation, whereas atrophic scars (e.g., acne or surgical scars, striae distensae) require laser skin resurfacing. Deep atrophic scars, scars on nonfacial sites, and those in darker skin are traditionally more difficult to treat.

Basic/Clinical Science Advances: The 585-nm pulsed-dye laser selectively targets cutaneous blood vessels, modulates transforming growth factor-β1 expression, and induces regression in hypertrophic scars and keloids. Laser skin resurfacing is useful for blending scar texture with the surrounding skin. Ablative and nonablative lasers have essentially been replaced by newer, fractional laser devices. This latter technology delivers energy to tissue in microscopic columns in a grid pattern, leaving intervening islands of untreated skin to promote rapid healing. Shorter recovery times have improved the safety profile of laser skin resurfacing.

Relevance to Clinical Care: Optimized treatment protocols and fractional laser technology have led to improved safety and efficacy for the treatment of scars in sensitive or delicate body locations and in patients with darker skin types.

Conclusion: Advances in laser technology have improved the prognosis for virtually all types of cutaneous scars.

BACKGROUND

SCARRING IS A COMMON SEQUELA OF CUTANEOUS INJURY that can cause obvious physical disfigurement, functional impairment, pain, and dysesthesia. Patients are usually most impacted by the psychological burden and stigma associated with the appearance of their scars. Although it is not possible to remove a scar completely with any therapy, advances in laser technology have improved treatment outcomes for all types of scarring.

The earliest attempts to treat scars with continuous-wave argon, neodymium:yttrium–aluminum–garnet, and carbon dioxide (CO2) laser devices resulted in a high risk of tissue necrosis and scar recurrence. In the 1980s, pulsed laser systems were developed for selective absorption of laser light by target chromophores (e.g., hemoglobin, melanin, water) in the skin to induce temperature-controlled, target-specific injury without damage to surrounding healthy tissue. By the early 1990s, the first series of clinical studies showed sustained improvements in erythematous, hypertrophic scars and keloids using the pulsed-dye laser (PDL), a refined vascular laser system that is widely used today. Treatment of atrophic scars has evolved from traditional pulsed CO2 and erbium laser systems to safer ablative and non-

Abbreviations and Acronyms

CO2 = carbon dioxide
PDL = pulsed-dye laser
TGF-β1 = transforming growth factor-β1

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ablative fractional technologies. These lasers limit
delivery of energy in microscopic columns within is-
lands of nontreated skin, thereby maximizing clin-
ical efficacy and improving the recovery time and
side-effect profile of laser skin resurfacing.7–10

TARGET ARTICLES
1. Alster TS and Zaulyanov-Scanlon L: Laser
scar revision: a review. Dermatol Surg 2007; 33:
131.
2. Sobanko JF and Alster TS: Laser treat-
ment for scars and wounds. G Ital Dermatol
Venerol 2009; 144: 583.
3. Tanzi EL and Alster TS: Skin resurfacing:
ablative lasers, chemical peels, and dermabra-
sion. In: Fitzpatrick’s Dermatology in General

CLINICAL PROBLEM ADDRESSED
Characterization of the scar type and location are
essential to selection of the appropriate treatment
modality and protocol (see Summary Illustration
section).

Hypertrophic scars and keloids (Fig. 1A, B)
present as red, thickened, firm scars that occur
when collagen deposition is exuberant and exceeds
collagen breakdown during the remodeling phase of
wound healing. Hypertrophic scars remain confined
to the area of original injury and may slowly im-
prove over time, whereas keloids continue to grow
beyond the site of the original injury and tend to
cause obvious disfigurement. Histologically, keloids
can be distinguished from hypertrophic scars by a
unique pattern of thickened, hyalinized collagen
arranged in whorls, which is attributed to an in-
herited alteration in fibroblast response to stimuli
and continued production of excessive collagen.11

Atrophic scars (Fig. 2A, B) appear thinner than
the surrounding skin because of inadequate colli-
gen replacement in the scarred areas. Atrophic
scars typically follow inflammatory skin diseases
such as acne or varicella and present as indentations
in the skin, wrinkled areas resembling “cigare-
ette paper,” or bulges where the subcutaneous fat
herniates through the thinned dermis. Acne scars
are commonly atrophic and are best classified ac-

RELEVANT BASIC SCIENCE CONTEXT
Modern lasers are based on the theory of selective
photothermolysis, which describes the use of
specific laser wavelengths to effect a controlled,
temperature-mediated change in a specific cutaneous

Figure 1. Keloid before (A) and after pulsed-dye laser treatment (B).
target. The three target chromophores in skin include hemoglobin, melanin, and water. The thermal energy applied to the cutaneous chromophore is limited so that the target is heated/destroyed, but transfer of energy to surrounding tissue is. Parameters can be calculated based on the size/shape and absorption characteristics of each target. In 2004, the concept of fractional delivery in a novel beam pattern was described. The regular, pixilated pattern of the laser beam is delivered in a grid where the energy is limited to regularly spaced microthermal zones or photocoagulated columns within areas of untreated

Figure 2. Atrophic acne scars before (A) and after fractional carbon dioxide laser skin resurfacing (B).

Figure 3. Striae distensae on the thigh before (A) and after nonablative fractional 1550-nm laser resurfacing (B). Note subtle improvement in skin color and texture.
skin. Fractional ablative systems vaporize tissue in the treated microthermal zones, whereas fractional nonablative resurfacing systems deliver enough heat to denature collagen and cause cell necrosis, but the treated tissue remains intact.16

EXPERIMENTAL MODEL OF MATERIAL—ADVANTAGES AND LIMITATIONS

Although lasers have been used for decades, molecular effects following laser irradiation of scar tissue are only just beginning to be understood. Recent work by Kuo et al. provides evidence that the 585-nm PDL alters signaling pathways involved with keloid formation by suppressing activator protein-1 (AP-1) expression via the mitogen-activated protein kinase pathway, thus reducing proliferation of fibroblasts and inducing keloid regression via fibroblast apoptosis during the remodeling phase of wound healing.17–19 PDL irradiation also selectively reduces scar vasculature and increases mast cells and mediators within the scar, but it is not clear if these effects have any role in scar improvement following treatment.2,4 Other studies have demonstrated laser- and light-induced modulation of TGF-β. Arany et al. demonstrated that low-power 904-nm laser irradiation activates latent TGF-β1 in early wounds in vivo.20 Infrared light irradiation also upregulates TGF-β1 in cultured human fibroblasts and may speed wound healing following multiple exposures.21 It is unclear what role these effects will play in the clinical management of scars with lasers.

The action of skin resurfacing lasers varies according to the device, wavelength, pulse energy, and density, with the overall goal being reinitiation of the wound healing response in a controlled fashion to induce collagen remodeling. In experimental models of normal skin, depth of penetration and width of microthermal zones increases in a fairly predictable pattern with increasing energy.16 It is not clear how these models correlate with scar tissue.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Compared with older destructive methods (e.g., dermabrasion, deep chemical peels), modern laser systems are more precise and controlled and represent a superior method of initiating the wound remodeling process to improve scars. Management varies considerably depending upon scar type, location, and patient characteristics.

Management of scars with lasers

Hypertrophic scars/keloids. The vascular PDL remains the first-line laser for treatment of hypertrophic scars and keloids. Two decades of study demonstrates remarkable improvements in scar vascularity, color, height, pliability, texture, and symptomatology after one to several treatments using low energy densities and short pulse durations.2–6 Studies have confirmed the 585 nm wavelength as the superior wavelength for this application.22 Following PDL irradiation, there is selective heating of cutaneous blood vessels. Short pulses confine the heating to the intended target,2–6 but no clinical differences have been demonstrated between 0.45 and 1.5 ms pulse durations.23 Treatment parameters used should be adjusted according to the scar type, location, and previous treatment response. Despite abundant literature available on the subject, determining the appropriate settings comes with experience. In general, fluences are decreased for patients with darker skin phototypes and in sensitive scar-prone areas such as the neck and chest. Immediate mild-to-

Figure 4. Prescar at 1 month after trauma to upper lip (A) and after pulsed-dye laser therapy (B).
moderate purpura is typically produced, along with transient erythema and edema. After treatment, the skin is cooled and protected from the sun. Purpura resolves in 7–10 days. The patient can be reevaluated and treated at 6–8-week intervals depending upon response. Adjunctive use of intralesional corticosteroids, 5-fluorouracil, or surgical debulking should be considered for extremely thick (>1 mm), nodular, or rapidly proliferative scars. Use of adjunctive therapy should be considered early for these large/aggressive scars because laser energy will not penetrate the lesion. Facial acne scars with and without hypertrophic and erythematous components should be considered for laser resurfacing combined with 585-nm PDL irradiation.

**Prescars.** The 585-nm PDL can also be used to treat early scars (prescars) within the first few weeks after wounding. Studies confirm that scars treated early will ultimately heal more favorably than if left untreated\(^8,13–15\) and new fractional laser technology, but further study is warranted to validate this approach.

The goal of treatment for atrophic surgical and acne scars is to soften the scar borders and blend the texture with the surrounding skin. Modern laser systems are superior to older modalities (e.g., dermabrasion, deep chemical peels) because they allow precise, controlled heating or vaporization of tissue to stimulate neo-collagenesis, scar remodeling, and skin tightening. The 1,064- and 1,450-nm devices have essentially replaced earlier ablative devices.

**Atrophic scars and striae.** Early pink/red striae respond to low-energy, short-pulse PDL treatment, with clinical improvements in skin color and texture.\(^8\) Older white striae can be treated with fractional laser technology, but further study is warranted to validate this approach.

The goal of treatment for atrophic surgical and acne scars is to soften the scar borders and blend the texture with the surrounding skin. Modern laser systems are superior to older modalities (e.g., dermabrasion, deep chemical peels) because they allow precise, controlled heating or vaporization of tissue to stimulate neo-collagenesis, scar remodeling, and skin tightening. The 1,064- and 1,450-nm devices have essentially replaced earlier ablative devices.

- **Lasers selectively alter dermal collagen to induce collagen remodeling in unfavorable scar tissue.**
- **Fractional delivery of laser energy in a grid pattern allows for faster, safer (and potentially more effective) treatment of scars.** This concept represents a major advance in laser technology over the last 2 decades.

**Clinical science advances**

- **The 585-nm PDL remains the preferred laser treatment for hypertrophic scars, keloids, new surgical scars, and erythematous striae distensae.** PDL treatment protocols have been refined to optimize efficacy, safety, and predictability.
- **Traditional ablative and nonablative resurfacing lasers have been essentially replaced by newer, fractional devices.**
- **Fractional laser skin resurfacing is a safe and effective treatment for atrophic and hypopigmented surgical and acne scars in facial and nonfacial sites.** The 1,550-nm and 1,600-nm devices are currently the most widely used for this application.

**Relevance to clinical care**

- **The prognosis for all types of scars is changing rapidly with the development of new fractional laser technology.**
- **Successful management of scars with laser depends upon proper patient selection, realistic expectations, and appropriate pre- and postoperative management.**
- **Operator experience with selecting parameters and familiarity with available evidence-based literature is essential to successful outcomes and prevention of complications.**
scar resurfacing. Emerging studies demonstrate efficacy for various types of acne, surgical, and burn scars.\textsuperscript{27,28} The ablative wavelengths vaporize microscopic columns of tissue and induce surrounding areas of collagen denaturation. For more fibrotic moderate-to-deep boxcar, atrophic, and icepick scars, these ablative devices have the potential to effect a greater change than nonablative resurfacing because of their superior ability to tighten the skin and stimulate collagen remodeling. They do, however, have a greater risk of complications.\textsuperscript{29} Anesthesia requirements vary according to treatment area—for isolated scars, local anesthesia is sufficient, whereas a full facial treatment for acne scars would require nerve blockade and systemic agents for pain and anxiety. Recovery times involve 5–7 days of serosanguinous discharge during the reepithelialization process, followed by a week or more of erythema.

**INNOVATION**

Fractional technology can be applied to virtually any laser or optical device. The operator can manipulate the energy density (or % coverage), width of the microscopic beams, and energy/depth of delivery. This technologic advance enhances the safety of many skin resurfacing procedures, allowing treatment of scars not only on facial skin, but also on nonfacial sites and in patients with darker skin types. With repeat treatments, even at nonablative wavelengths, the clinical efficacy is similar to that of traditional ablative resurfacing.

**CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS**

It is important to note that many of the laser devices discussed in this article can themselves cause dyspigmentation and scarring. Appropriate patient selection and operator experience are crucial variables that ultimately determine treatment efficacy, patient satisfaction, and overall outcome. The risk of complications with fractional resurfacing is lower than with traditional ablative modalities, but serious complications have been reported.\textsuperscript{29} Hypertrophic scars, ectropion formation, dyspigmentation, and viral or bacterial infection are rare; however, side effects such as acne, contact dermatitis, and prolonged erythema are occasionally observed. As many laser technologies are relatively new, delayed reactions may also be discovered.

Prior to treatment, a complete discussion of these risks must be weighed against the patient’s goals and expectations. The consultation must include a documented discussion of the patient’s prognosis, projected number of treatments, and understanding of medical/social factors that may impact their ability to tolerate the procedures and associated recovery. The provider should pay specific attention to skin phototype, concurrent infection or inflammatory skin disorders, medication use, and other conditions that would impact healing and present a contraindication to treatment (such as previous radiation). The provider must also be familiar with, and able to communicate, evidence-based literature so that patients understand and accept circumstances where new devices are being used without studies of long-term follow-up.

**FUTURE DEVELOPMENTS**

Studies are needed to evaluate the safety and efficacy of new laser technologies for the treatment of difficult-to-treat scars such as scars on
nonfacial sites, burn scars, striae distensae, and those in darker skin types. Unlike the face, skin on the neck, trunk, and extremities has relatively few adnexal structures and less capacity to replace the epidermis following resurfacing procedures. Fractional technology reduces the postoperative risk of prolonged healing, infection, scarring, and pigmentary alterations. Appropriate treatment protocols and safety assessment for these new applications have yet to be characterized.

**SUMMARY ILLUSTRATION**

Relating scar type to preferred laser treatments modality.

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### REFERENCES


