

equal, or greater, clinical relevance.<sup>5</sup> Thus far, a majority of therapeutic agents proposed for treatment of neurodegenerative disease are secreted trophic factors such as insulin-like growth factor 1 and glial cell line–derived neurotrophic factor.<sup>29</sup> Because most trophic factors are typically secreted by glia, it is easy to envision that rAAV-mediated overexpression of trophic factors in these supporting cells would result in significant improvement of cell rescue, similar to or better than that seen when neurons themselves have been targeted.

Although the neuronal transduction in the adult brain was limited in scope in the Foust *et al.* study,<sup>5</sup> the ubiquitous astrocytic transduction holds similar promise to that of astrocytic infection of the spinal cord. Trophic factors have been implicated as potential therapeutic agents aimed at slowing disease progression in neurodegenerative diseases such as Parkinson's disease. In the case of disorders that require more targeted delivery such as Parkinson's disease, the use of i.v. AAV9 might still be advantageous if tissue-specific promoters are included in the therapeutic construct, as pointed out correctly by Foust *et al.*<sup>5</sup> Although brain neurosurgery is relatively safe, systemic delivery across the BBB remains an even safer alternative to direct CNS injections.

Going forward, widespread protein expression in the CNS via the use of rAAV9 needs a proof of principle in a rodent model of a neurodegenerative disorder that can be treated with ectopic gene expression in astrocytes. Moreover, replication of these results in other laboratories as well as in larger species will also constitute the next important hurdle for the method described by Foust *et al.*

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## Bioengineered Human Skin: Working the Bugs Out

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**T**he bacterial contamination of wounds is an important global health-care

issue that is bound to grow as the population ages. Bacterial wound infections increase morbidity and mortality and are of considerable socioeconomic impact. The risk of wound infection increases as disturbances in local conditions favor bacterial growth rather than host defense. This can lead to impaired wound healing, resulting on the one hand in rising treatment costs and on the other hand in a traumatic and potentially life-threatening condition for the patient.<sup>1</sup> The current clinical gold standard for treating wound infection is antibiotic therapy. However,

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the benefit of systemically delivered antibiotics to advance wound healing is still unclear.<sup>2</sup> Moreover, the emergence of bacterial resistance to antibiotics is a growing threat, and this has increased the urgency of finding novel means of combating pathogenic assault. A growing number of studies indicate potential clinical applications of host defense peptides (HDPs) for skin antimicrobial therapy.<sup>3-8</sup> In this issue, Thomas-Virnig *et al.* report the potential for engineered keratinocytes that over-express human HDPs to enhance wound healing by counteracting the growth of microbial pathogens.<sup>9</sup>

### HOST DEFENSE PEPTIDES

Parallel evolution of hosts and pathogens has led to a diversity of multifaceted survival mechanisms that are either encoded in the germ line (innate immunity) or acquired through adaptation to specific antigens (adaptive immunity). Among the numerous defense mechanisms, HDPs are important evolutionarily conserved elements of innate immunity. They are found in organisms as diverse as insects and humans. Salient examples include the cecropins (insects), magainins (amphibians), and defensins (mammals). They are generally small peptides (12–50 amino acids) that contain at least two positively charged (arginine or lysine) residues. Predominantly membrane active, these peptides exhibit a high positive net charge and can adopt amphipathic conformations. This amphipathic design, consisting of spatially separated hydrophobic and charged regions, permits intercalation of the peptide into microbial membranes. Their unique mechanism of action and ability (in many cases) to kill organisms make them attractive prototypes for antibacterial drug development. Recent developments highlight the importance of vertebrate epithelial cells as sites of antibiotic peptide production and suggest a critical role for this system in human health and disease. Several reviews on HDPs have already been published.<sup>3,10,11</sup>

The importance of this innate system as a check on infection is evident when one considers that most bacteria have generation times of 20–30 minutes, whereas the mounting of a specific adaptive immune response may take days or weeks. Although many cationic peptides

demonstrate direct antimicrobial activity against bacteria, fungi, eukaryotic parasites, and viruses,<sup>3,7,10-12</sup> it has also been established that many also have a key modulatory role in the innate immune response and form an important link between the innate and adaptive immune responses.<sup>13</sup> Owing to their multiple functions, they are considered promising agents for new therapeutic approaches in infectious diseases and wound healing.<sup>3,4,6,10,11,14-18</sup>

The emergence of bacterial resistance to antibiotics increased the urgency of exploring alternative means of combating pathogenic assault. HDPs are rapidly emerging as attractive candidates for antimicrobial treatment. Different therapies that effect wound repair have been proposed over the past few decades. Although HDP therapy was initially focused on antimicrobial activity, it can be applied equally well to the local, temporary treatment of acquired diseases, including impaired wound healing and tissue repair.

Thomas-Virnig *et al.* elegantly investigated the potential therapeutic role of genetically engineered NIKS skin substitute tissue expressing hCAP-18 (NIKShCAP-18), which significantly counteracted bacterial infection by a multidrug-resistant nosocomial bacterial strain in a mouse model of infected third-degree cutaneous burn wounds.<sup>9</sup> Cathelicidins are a family of HDPs found in most mammalian species. They consist of an N-terminal cathelin domain, which is highly conserved throughout species, and a variable C-terminal peptide. This propeptide is activated extracellularly after proteolytic cleavage.

The only known member of this family thus far is hCAP-18, an 18-kDa human cationic antimicrobial protein that is produced mainly by leukocytes and epithelial and mucosal cells. Its cationic C-terminal 37-amino acid domain, LL-37, displays broad antimicrobial activity mediated through direct interaction with and disruption of the microbial cell membrane. The enzyme responsible for cleavage of the proprotein in neutrophils is serine proteinase 3. In skin, the serine proteases kallikrein 5 and 7 were recently reported to mediate alternative processing of hCAP-18 generating several novel peptide fragments, suggesting that peptide profiles may differ among tissues and

biological conditions.<sup>19</sup> This opens a potential new area of research, in that their functional profile may differ. Interestingly, both the proprotein, hCAP-18, and the mature processed form of LL-37 are constitutively produced in sweat.<sup>19</sup> The unprocessed proprotein does not have antimicrobial activity<sup>20</sup> until it is cleaved by proteinase 3.

In addition to being antimicrobial, LL-37 is implicated in diverse biological processes, including chemotaxis, cytokine production, histamine release, and angiogenesis.<sup>18,21,22</sup> Furthermore, hCAP-18/LL-37 is highly expressed during normal wound healing,<sup>23,24</sup> displays growth factor-like properties enhancing the *in vitro* proliferation and migration of human keratinocytes, and is involved in re-epithelialization of wounds.<sup>24,25</sup> LL-37 is upregulated at the messenger RNA and protein levels upon exposure to bacteria or by sterile incision.<sup>26</sup> Expression is also increased by various growth factors that are involved in wound healing, such as insulin-like growth factor. Consistent with this, LL-37 has been demonstrated to be involved in the re-epithelialization of skin wounds. Using a noninflammatory *ex vivo* wound healing model of organ-cultured human skin, upregulation of LL-37 expression has been demonstrated in the wound area, and antibodies specific to LL-37 have been shown to inhibit the re-epithelialization process in a concentration-dependent manner.<sup>27</sup> Because LL-37 is probably not found at concentrations sufficient for antimicrobial activity,<sup>26</sup> it is possible that one of the primary functions of LL-37 in the skin is to promote re-epithelialization. None of the clinically used antibiotics demonstrates these interesting features.

As a result of the alarming increase of multidrug-resistant bacteria, the new experimental approach of Thomas-Virnig *et al.* with hCAP-18/LL-37 has considerable clinical relevance. The investigators demonstrated a two-log reduction in the growth of a clinical isolate of multidrug-resistant *Acinetobacter baumannii*. This well-conducted study provides strong evidence that the antimicrobial peptide hCAP-18/LL-37 may reduce the risk of infection from pathogenic bacteria, including multidrug-resistant nosocomial strains, and thereby actively enhance

wound healing. Although this study did not show evidence of better wound healing in addition to reduced bacterial growth, as Thomas-Virnig *et al.* note, HDPs may also act to amplify the endogenous innate immune response to invading microbes. Moreover, there is evidence that HDPs such as hCAP-18/LL-37 exhibit wound healing properties distinct from their antimicrobial function, such as the stimulation of keratinocyte growth and/or migration and the stimulation of wound vascularization.<sup>3,4,10,11,21,22,24,26–29</sup>

Thomas-Virnig and colleagues' study of enhanced production of an HDP using a genetically modified tissue substitute offers a feasible mechanism for cutaneous gene therapy to improve wound healing and may provide a major advantage in the ability to deliver a composite graft that aims to replicate the physiological function and anatomical architecture of skin. The skin is the site of many debilitating diseases for which effective therapy is currently unavailable. Gene therapy in the skin requires different gene transfer strategies depending on the therapeutic effect sought. Two major applications can be easily distinguished: (i) permanent correction of genetic disorders and (ii) transient treatment or improvement of pathological conditions.

The skin is an attractive target for HDP therapy for many reasons. The predominant cells of the skin—fibroblasts and keratinocytes—are harvested easily, and protocols exist for their successful culture.<sup>30</sup> This situation not only enables testing of skin cells *in vitro* but also highlights their availability for use as vehicles in *ex vivo* protocols, because such cells can readily be transplanted back to a host. The superficial location of the skin makes it simple to monitor the behavior of a genetically modified skin area for any reactions and, if necessary, to remove it in case of an adverse reaction to the transgene product. The skin is also accessible to direct HDP transfer by many techniques, including injection and topical application, avoiding unnecessary systemic delivery.

The skin allows (i) easy collection of the gene-targetable cells, (ii) efficient *in vitro* gene transfer (using viral vectors), (iii) expansion of the modified cell population (either selected/enriched or not), and (iv) grafting of genetically altered

cells to the donor individual using the technique described by Thomas-Virnig *et al.* Diseases that could be treated using this approach can be divided into three major categories: (i) disorders that affect the skin and whose molecular defect has already been characterized, (ii) disorders that—whether or not they affect the skin—may improve when the skin is converted into a source of certain proteins, and (iii) disorders that do not affect the skin but may improve when the skin is converted into a metabolic sink.

However, differences in important skin features, such as epidermal thickness, epidermal kinetics, and gene transfer efficiencies between animal and human tissue, have limited the use of HDP therapy. Thomas-Virnig *et al.* nicely circumvented this problem by using a human-skin substitute grafted onto immunodeficient mice (athymic nude mice). Their approach is particularly attractive because the regenerated human skin on these mice could preserve clinical, histological, molecular, and functional properties of the donor skin (either normal or diseased).

For cutaneous HDP therapy aimed at the treatment of genetic skin disorders such as psoriasis, retention of pathological defects *in vivo* provides a valuable system for testing the efficacy and safety of strategies. It is interesting to note that keratinocyte gene transfer has also been explored as a treatment modality for nondermatological conditions. Considerable progress has recently been made toward the treatment of systemic diseases caused by insufficient amounts of a protein in the circulation, using the epidermis as a “bioreactor” for producing therapeutic protein.

The concept of delivery of HDPs by a skin substitute is a new development. Combinations of HDPs or their sequential use may be the future answer to accelerating wound healing processes, and with the advent of their delivery by a skin substitute, these HDPs may become increasingly important. Developments in matrix components and tissue engineering technology may offer promise for the future. The development of slow-release matrices may prolong the delivery of their potential antimicrobial and wound healing properties.

Viral vectors are the original and most established technology for gene delivery.

A wide range of applications have been developed, and many virus-mediated gene transfer models are successful. The production of viral vectors, however, is costly and time-consuming, transfection efficacy is variable, and the risk of local or systemic infections that may lead to fatal outcomes remains a concern. Thomas-Virnig and colleagues' nonviral approach using sustained delivery of hCAP-18 to the wound bed from temporarily applied human-skin-substitute tissue should also circumvent viral gene transfer issues such as the preexisting immunity exhibited by much of the human population toward adenoviruses. Other positive attributes of their approach include elimination of the risk of viral infection and avoidance of the higher cost of vector production.<sup>31</sup> The transient nature of gene expression is also a benefit in wound healing applications. On the negative side, some nonviral gene transfer methods tend to be nonspecific and highly variable within the levels of gene expression reported.

#### THE USE OF SKIN SUBSTITUTES TO DELIVER HDPs

The problem then will be establishing which of the many HDPs identified as having potential therapeutic benefit should be the ones of choice. This question highlights the need for a database of HDP functions in wound healing to facilitate further study. The numerous therapeutic options for delivery of HDPs by a bioengineered skin substitute include anti-infection, anti-endotoxin, immunomodulation, chemotaxis, cell proliferation and differentiation, wound healing, angiogenesis, influencing initiation and polarization of adaptive immunity, induction of gene expression, and enhancement of protein secretion functions.

Recent advances in cell biology have identified a variety of molecules—specifically, growth factors and their receptors—that are critically involved in directing cell functions during soft-tissue organogenesis. The study by Thomas-Virnig *et al.* indicates that delivery of HDPs via a skin substitute is a promising therapeutic tool for modifying this repair microenvironment. Skin with inherently poor or pathologically altered healing potential would thus benefit from a therapeutic bioengineered skin approach that could augment healing capacity while minimizing infection.

## A PARADIGM SHIFT

HDP research is becoming an area of great importance. Delivery of HDPs by a bioengineered skin substitute has evolved from a purely experimental scientific endeavor to a clinically pertinent treatment for the skin. In wound healing, there remain challenges in the selection of optimal target cells, development of sequential therapeutic methods, and identification of factors that may be detrimental to the introduction of a genetically modified skin substitute.

It is probable that the only way to answer many questions that still arise about therapeutic delivery of HDPs by a skin substitute will be through clinical trials. The following steps have been achieved/developed: (i) culture and targeting of human epidermal stem cells, (ii) surgical techniques that allow the grafting of large skin areas without scarring, and (iii) sustained transgene expression *in vivo* in preclinical models. Now is the time to validate these steps in a clinical context. Implementation of a phase I/II clinical trial of HDP therapy in selected individuals suffering from psoriasis may shed light on important issues, mainly, the persistence of transgene expression at therapeutic levels and host tolerance to the gene product. The focus in the development of HDPs for clinical applications has been on small HDPs containing the biologically active core of the endogenous molecules, thereby limiting related toxicity components, improving efficacy, and lowering the cost of goods. Only a modest number of HDPs have been tested in clinical-efficacy trials, and they demonstrated potential in both preclinical and clinical trials.<sup>24–27</sup>

Thomas-Virnic and co-workers' study indicates that delivery of HDPs via skin substitute is a promising therapeutic tool for modifying the repair microenvironment and may lead to a paradigm shift in the management of skin pathologies. Future basic and clinical research will tell if and when this new powerful "biological

weapon" will become part of the health professionals' armamentarium. Further research of HDPs will improve our knowledge of their involvement in the recognition and neutralization of pathogens, which will support the development of new anti-infective therapeutic approaches. HDP immunomodulating functions will become clearer with additional research on structure–function analyses to elucidate their mechanisms of action. Most important, future research must take advantage of and build on the diverse nature of HDPs and adhere to physiologically relevant conditions, ultimately validating, *in vivo*, their beneficial functions.

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