Tissue Engineering for Cutaneous Wounds

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Skin, the largest organ in the body, protects against toxins and microorganisms in the environment and serves to prevent dehydration of all non-aquatic animals. Immune surveillance, sensory detection, and self-healing are other critical functions of the skin. Loss of skin integrity because of injury or illness may result acutely in substantial physiologic imbalance and ultimately in significant disability or even death. It is estimated that, in 1992, there were 35.2 million cases of significant skin loss (US data) that required major therapeutic intervention. Of these, approximately 7 million wounds become chronic. Regardless of the specific advanced wound care product, the ideal goal would be to regenerate tissues such that both the structural and functional properties of the wounded tissue are restored to the levels before injury. The advent of tissue-engineered skin replacements revolutionized the therapeutic potential for recalcitrant wounds and for wounds that are not amenable to primary closure. This article will introduce the reader to the field of tissue engineering, briefly review tissue-engineered skin replacement from a historical perspective and then review current state-of-the-art concepts from our vantage point.

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Introduction

The problem: a failure to heal. Skin, the largest organ in the body, protects against toxins and microorganisms in the environment and serves to prevent dehydration of all non-aquatic animals. Immune surveillance, sensory detection, and self-healing are other critical functions of the skin. Loss of skin integrity because of injury or illness may result acutely in substantial physiologic imbalance and ultimately in significant disability or even death. It is estimated that, in 1992, there were 35.2 million cases of significant skin loss (US data) that required major therapeutic intervention (1993). Of these, approximately seven million wounds become chronic.

The most common single cause of significant skin loss is thermal injury, which accounts for approximately one million hospital emergency visits per year in the US (2005a). Other causes of skin loss include trauma and chronic ulcerations secondary to diabetes mellitus, pressure, and venous stasis. In a recent fact sheet published by the Center for Disease Control and Prevention, the estimated prevalence of diabetes in the US population is 7% or 21 million individuals (2005b). Of these up to 10% (2 million) have chronic diabetic ulcers, many of which (~82,000) eventually necessitate amputation (Ehrenreich and Ruszczak, 2006). According to Ehrenreich and Ruszczak (2006), the Medicare cost for amputations alone in this population was $1.5 billion in 1995. Approximately 600,000 patients suffer from venous ulcers at an average cost of $9,685 per patient (Olin *et al.*, 1999). Another 1.4 million people in the US have pressure ulcers. The total treatment costs for these two group has been estimated to be as high as $8 billion annually (Supp and Boyce, 2005). In 2003, a survey estimated the US market for advanced wound care products, including biological and synthetic dressings, to be greater than $1.7 billion and predicted significant increase as the population ages, becoming more susceptible to underlying causes of chronic wounds (2003; Grinnell and Lamke, 1984). The quality of life of patients with chronic wounds can be extremely poor, thus adding indirect costs to the burden of cutaneous ulcers. Therefore, the total cost of chronic wounds is difficult to assess.

Over the past three decades, there have been extraordinary advances in our understanding of the cellular and molecular processes involved in acute wound healing and in the pathobiology of chronic wounds (Singer and Clark, 1999; Mustoe, 2004; Ghosh and Clark, 2007). A brief overview of these physiologic and pathologic processes is presented in Figure 1. This increased knowledge base has led to wound care innovations that have facilitated more

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Abbreviations: ECM, extracellular matrix; RGD, arginine–glycine–asparatic acid; rPDGF-BB, recombinant platelet-derived growth factor-BB

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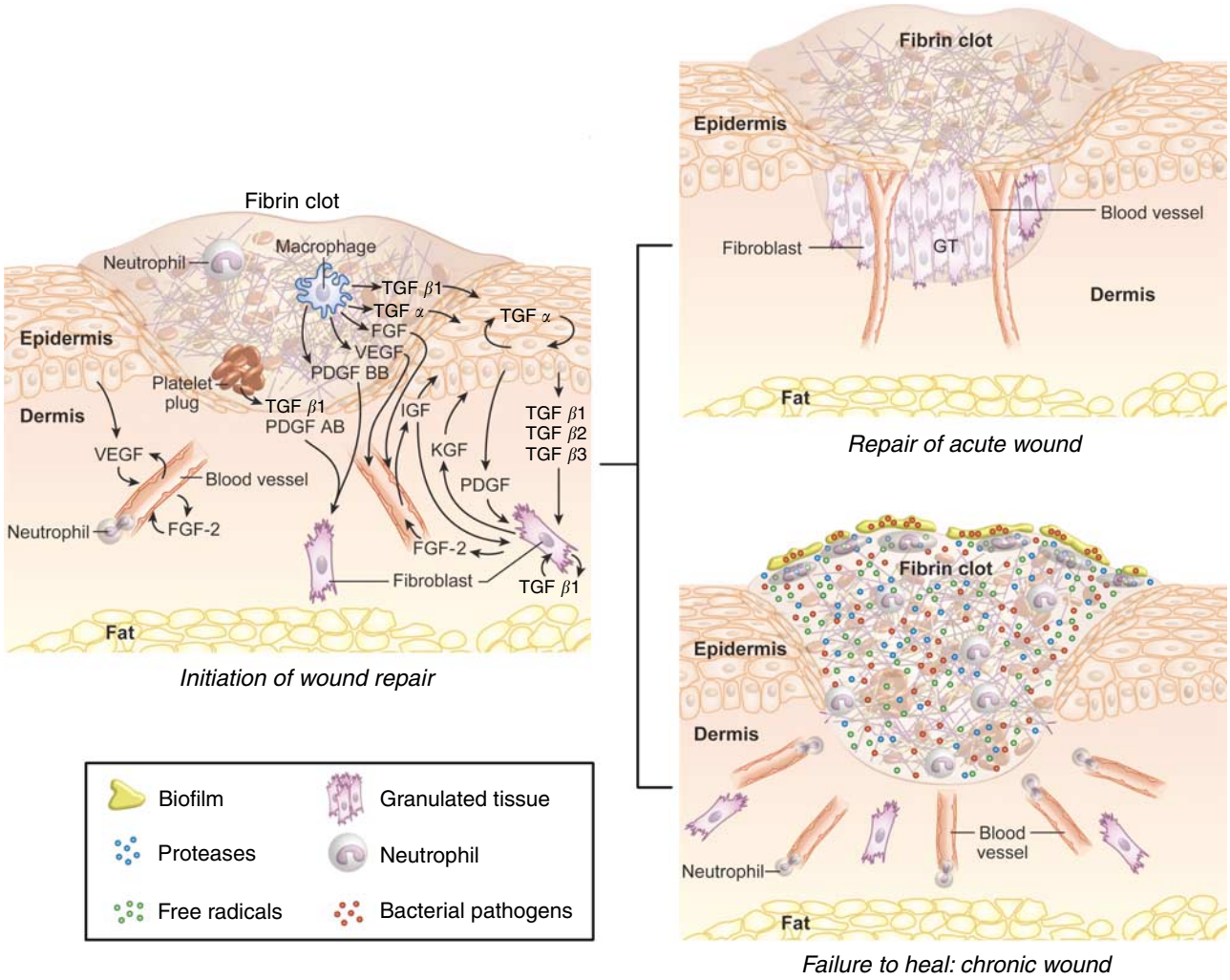

Figure 1. Tissue injury results in an acute wound healing response under normal physiologic conditions but may fail when underlying pathobiology or microbial invasion interfere with the healing process, thereby creating a chronic wound. (Left panel) Tissue injury precipitates blood clotting, platelet aggregation, and migration of leukocytes, including neutrophils and macrophages, to the site of injury. Initially, the blood clot is composed of fibrin and fibronectin, which provide a scaffold for cell migration and aggregated platelets, which release growth factors into the surrounding tissue. By 3 days the clot has synerzied (contracted) and accumulated numerous neutrophils, which phagocytose and kill microorganisms; and macrophages, which produce and secrete growth factors into the wound environment. Although epidermal migration along the interface between the clot and surrounding normal tissue begins within 24 h after injury, no tissue cells have invaded the clot even by 3 days. Nevertheless by 3 days, fibroblasts and endothelial cells in the periwound stroma are activated to express the appropriate integrins (ECM receptors) for migrating on fibrin and fibronectin, and to secrete growth factors. Epidermal cells also participate in this cytokine network of growth factor release at 3 days. (Upper right panel) Under normal physiologic conditions the wound continues to heal at 5 days with an ingrowth of granulation tissue composed of fibroblasts, additional macrophages, and neovasculature. The migrating epidermis now changes its course and migrates over the newly forming tissue. Proteases restricted to the leading edge of migrating tissue cells are critical for their invasion of the clot and ingrowth into the wound. These proteases include urokinase type-plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and matrix metalloprotineases 1, 2, 3, 9, and 13 (MMP 1, 2, 3, 9, and 13). The latter include collagenases (MMP 1 and 13), gelatinases (MMP 2 and 9), and stromolysin (MMP 3). Many growth factors released by platelets and secreted by macrophages during the first phase of healing have been sequestered in the provisional matrix and stimulate tissue cells as they move into the wound. To these are added growth factors now secreted by tissue cells themselves including epidermal cells, fibroblasts, and endothelial cells as well as macrophages. (Lower Right Panel) When underlying pathobiology or microorganism invasion interrupts the wound healing process, a failure to heal occurs often leading to a chronic wound (ulcer). Underlying pathobiology known to interfere with acute wound healing includes venous insufficiency that results in fluid transudation and fibrin cuffing of venules secondary to high hydrostatic pressure in the venous system; diabetes mellitus that results in high glucose and both cell and ECM dysfunction from non-enzymatic glycation; arterial occlusion or high external pressure that results in tissue hypoxia and cell dysfunction or death. Bacteria colonizing the wound often produce a biofilm composed of a wide variety of polysaccharides. The biofilm protects these colonies of mixed microorganisms, as it is relatively impervious to phagocytic cells and impermeable to antibiotics. Frustrated phagocytes release a plethora of proteases and toxic oxygen radials into the wound milieu making a bad situation worse as these agents destroy tissue cells, extracellular matrix, and growth factors in the wound. Not surprisingly, such chronic wounds lack epidermal migration and ingrowth of granulation tissue.

rapid closure of ulcer and normal wounds, better functional and more aesthetic scars, and decreased incidence of keloids. As tumor microenvironments have many similarities to wound healing (Dvorak, 1986), better understanding of wound healing (Ashcroft *et al.*, 2003) has led to advances in tumor therapy (Basu *et al.*, 2006). Several specific products for wound treatment that germinated from our increased understanding of fundamental processes underlying wound healing have reached the market place for second-line therapy of recalcitrant ulcers (Singer and Clark, 1999; Ehrenreich and Ruszczak, 2006). These products include recombinant growth factor, platelet-derived growth factor-BB (rPDGF-BB) (Regranex, Ortho-McNeil), and several skin substitutes (Dermagraft and TransCyte, Advanced Tissue Sciences; Apligraf, Organogenesis; Integra Matrix Wound Dressings, Integra LifeSciences Holding; OrCel, Ortec International; AlloDerm, LifeCell). Most of these interventions demonstrate a 25% increase in closure or healing rates in chronic wounds (Ehrenreich and Ruszczak, 2006). Unfortunately, rPDGF-BB must be applied daily forcing patients to change their bandages daily and two companies producing skin substitutes have undergone bankruptcy and reorganization. Regardless of the specific advanced wound care product, the ideal goal would be to regenerate tissues such that both the structural and functional properties of the wounded tissue are restored to the levels before injury.

Embryonic wounds, in contrast to wounds in children and adults, undergo regeneration during the first and second trimester and scarless repair early in the third trimester (Redd *et al.*, 2004). Morphogenetic cues from these embryonic phenotypes could be utilized to develop engineered constructs capable of tissue regeneration. In particular, as cellular response to biological stimuli depends on the geometry and mechanical strength of extracellular matrix (ECM) (Engler *et al.*, 2006; Vogel and Sheetz, 2006), the therapeutic success of tissue-engineered constructs will depend not only on their bioactivity but also on their mechanical properties.

A possible solution: tissue engineering. The advent of tissue-engineered skin replacements revolutionized the therapeutic potential for recalcitrant wounds and for wounds that are not amenable to primary closure. This article will introduce the reader to the field of tissue engineering, briefly review tissue-engineered skin replacement from a historical perspective, and then review current state-of-the-art concepts from our vantage point.

Tissue engineering integrates many fields of science and engineering in order to design, develop, and test tissue replacement for traumatically lost or disease-damaged tissue. As such, tissue engineering relies on the expertise of scientists and engineers from multiple backgrounds, including, but not limited to, molecular and cell biology, physiology, chemistry, physics, materials science, applied mathematics, biomedical engineering, mechanical engineering, and chemical engineering. These individuals work across a broad temporal-spatial scale from nanoseconds to years and from nanometers to meters. Together they develop not only the biomaterials or organotypic assemblages for implantation but also the manufacturing processes necessary to synthesize biomaterials as well as bioreactors required to grow large quantities of genes, cells, or organotypic tissue.

Two major approaches, an *in vitro* and an *in vivo*, have been utilized to develop engineered tissue. The *in vitro* method has received the most attention from the lay-press as it is this approach that attempts to create organs in tissue culture or bioreactors for implantation and replacement of diseased or damaged tissue. In contrast, the *in vivo* approach attempts to create an acellular biomaterial that contains clues conductive for tissue cell recruitment into the biomaterial and inductive of cell differentiation to form the needed tissue. Regardless of whether they contain cells or not, many fabrications engineered for implantation contain a bioactive and a biopolymer backbone (Figure 2). Bioactives are selected to stimulate tissue cells to migrate and proliferate, and ultimately to differentiate. Biopolymers typically provide mechanical support for cell migration and proliferation. However, when prepared from natural ECM, these scaffolds and hydrogels may provide additional biological stimuli to support cell and tissue function (Lutolf and Hubbell, 2005). Importantly, the engineered bioactive-biopolymer construct and tissue cells, whether preloaded or host-derived, must interact to initiate a dynamic reciprocity for further tissue development (Nelson and Bissell, 2005). Ideally, tissue-engineered skin replacements should facilitate faster healing and promote the development of a new tissue that bears a close structural and functional resemblance to the uninjured host tissue.

Historical perspective of tissue-engineered skin replacement

Composite synthetic or biological dressings are often used to speed wound repair and improve the quality of healing in chronic or burn wounds (Robson *et al.*, 1973; Gokoo and Burhop, 1993; Purna and Babu, 2000). Although effective, these dressings do not offer permanent treatment. Eventually, a split-thickness autograft or allograft may be required to achieve complete healing. However, tissue harvest and transplantation are accompanied by undesirable consequences such as the risk and expense of surgery, donor site morbidity, and rejection of the transplanted tissue. To remedy these problems, the goal of tissue engineering has been to substitute tissue-engineered skin replacements for autografts or allografts. The first step toward this end was to substitute split-thickness autografts with autologous epidermal sheets grown from small punch biopsies.

Autologous and allogenic epidermal replacement. Cultured autologous epidermal sheets have been used to facilitate repair of both epidermal and partial thickness wounds. Autologous epidermal sheets were first used to cover burn wounds (O'Conner *et al.*, 1981), and burns have remained the major clinical target for both autolo-

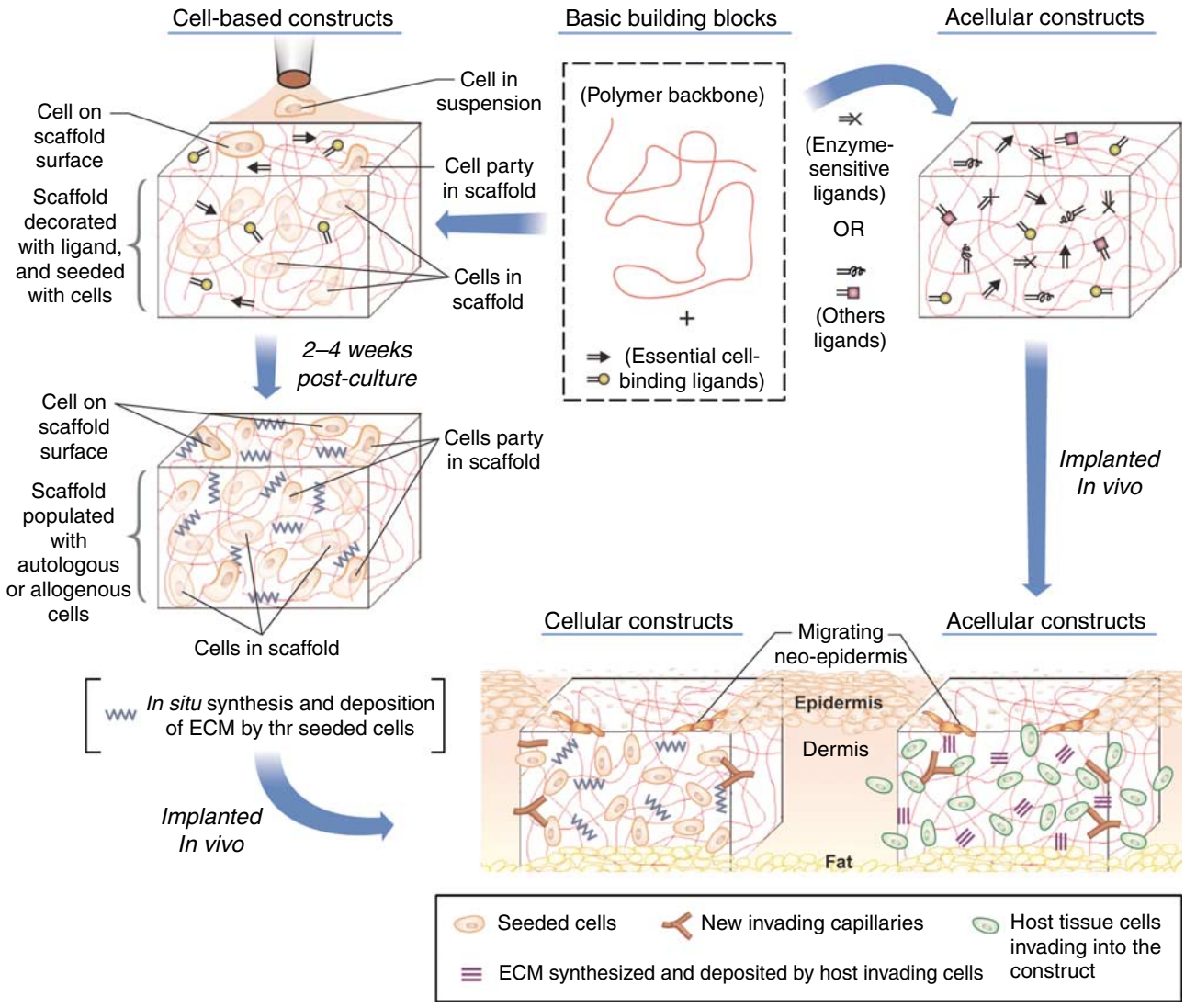

Figure 2. The basic building blocks of a tissue-engineered construct are a biopolymer, one or more biomimetics, and perhaps cells. The biopolymer must be biocompatible and biodegradable. Biomimetics are selected to add function to the biopolymer. The bioactive function may be cell-binding activity, growth factor activity, or growth factor-binding activity, or enzymatic activity or enzyme-binding activity. Cells added exogenously to the engineered biopolymer may be used to induce a functioning tissue-substitute for transplantation (left schemata). These cells may be stem cells or genetically engineered cells. When an acellular biopolymer is implanted, enough information must be available within the engineered construct to support endogenous tissue cell ingrowth and appropriate differentiation for tissue formation (right schemata).

gous and allogenic epidermal replacement (Ehrenreich and Ruszczak, 2006). Implantation of epidermal grafts cultured from a small skin biopsy was made possible when tissue culture techniques were perfected to grow epidermal cells in large quantities (Rheinwald and Green, 1975; Green *et al.*, 1979). Subsequently, there has been extensive experience with cultured epidermal grafts for the treatment of burns as well as other acute and chronic wounds (Gallico *et al.*, 1984; Odessey, 1992; Munster, 1996). Bioreactor design, patterned after the breakthroughs in epidermal cell tissue culture techniques, has allowed this technology to reach the marketplace as Epicel™ (Genzyme Tissue Repair). Autologous epidermal sheets serve as permanent wound coverage as the host does not reject them, and yield a reasonable cosmetic result. Disadvantages do exist and include the 2–3 week time interval required before sufficient quantities of keratinocytes become available and the large costs estimated at $13,000 per 1% total body surface area covered (Rue *et al.*, 1993). Furthermore, graft-take can vary widely secondary to wound preparation and its intrinsic status, patient underlying disease, and operator experience.

Cultured epidermal sheet allografts were developed to overcome the necessity to biopsy each patient and then wait 2 to 3 weeks between epidermal harvest and autograft product. With the utilization of techniques for culturing epidermal sheets, epidermal cells from both cadavers and unrelated adult donors have been used for the treatment of burns (Madden *et al.*, 1986), donor sites of skin grafts (Thivolet *et al.*, 1986), and chronic leg ulcers (Leigh

et al., 1987). The source for most current allografts is neonatal foreskin keratinocytes, which are more responsive to mitogens than adult (cadaver) cells (Phillips, 1998). Such allografts promote accelerated healing and effect pain relief in a variety of acute and chronic skin ulcers without any evidence of immunological rejection; however, the keratinocytes within these allografts are replaced within a few weeks by ingrowth of recipient cells. To facilitate mass allograft production and wide availability, cryopreserved allografts were developed. These frozen constructs gave comparable results to fresh allografts (Teepe *et al.*, 1990; De Luca *et al.*, 1992).

Although cultured epidermal autographs and allografts can be used successfully to cover partial thickness burns, they fail to produce a satisfactory response in deeper burns and other full-thickness wounds (Williamson *et al.*, 1995). The primary reasons include lack of mechanical strength and susceptibility to contractures. As an alternative, keratinocyte delivery systems were developed by which cells are delivered to the injury site via a biodegradable scaffold. For example, Laserskin, produced by FIDIA Advanced Biopolymer, Italy, is used to deliver keratinocytes via a chemically modified hyaluronan membrane (Campoccia *et al.*, 1998), perforated with micron-sized holes that allow cells to grow to confluence. Alternatively keratinocytes can be delivered to wounds intermixed with fibrin sealant as a spray (Grant *et al.*, 2002). However, this may not prove to be an optimal delivery system as keratinocytes lack receptors for fibrin or fibrinogen (Kubo *et al.*, 2001).

Engineered Dermal Constructs. While cultured epidermal sheets do enhance healing, especially of burn wounds, they lack a dermal component that, if present, might prevent wound contraction and provide greater mechanical stability. Cadaver skin allografts containing both epidermis and dermis have been used for many years, but provide only temporary coverage because of host rejection. However, allografts can be chemically treated to remove immunogenic cellular elements, for example, Alloderm (Life Cell Corporation, Woodlands, TX). Such ''decellularized'' allografts have been effectively used alone or in combination with cultured autologous keratinocytes for closure of burns and chronic wounds (Cuono *et al.*, 1986).

In 1981, a composite of bovine collagen and chondroitin-6-sulfate from shark cartilage, with an outer silicone covering, was engineered as an organotypic dermis for skin grafting (Burke *et al.*, 1981). After wound placement, the acellular composite recruited host dermal fibroblasts and was degraded during cell invasion. The silicone sheet was removed 2–3 weeks after placement, and the wound covered with an epidermal sheet autograft. This organotypic dermis material has been successful in treating burns (Heimbach *et al.*, 1988) and has received FDA approval for this indication (Integra, LifeSciences Corporation, Plainsboro, NJ). This material, however, must be avoided in patients who have developed allergic reactions to bovine products.

Another acellular implant called Transcyte (Dermagraft-TC) was produced by Advanced Tissue Sciences Inc. (ATS, La Jolla, CA). This composite consisted of an inner nylon mesh in which human foreskin fibroblasts were embedded and an outer silicone layer to limit evaporation. Fibroblasts were allowed to synthesize and secrete ECM material, such as collagen, fibronectin, and glycosaminoglycans; and cytokines, including growth factors. After a few weeks the cells were disrupted by freeze–thawing to create the final product. Transcyte was successful as a temporary wound coverage after excision of the eschar from burn wounds (Purdue, 1997) and approved by the FDA for this indication. ATS also produced a cellular composite, called Dermagraft. In this construct, human foreskin fibroblasts were cultured in a biodegradable polyglactin mesh and then cryo-preserved so that they remained viable. Dermagraft had limited success in the treatment of diabetic foot ulcers (Gentzkow *et al.*, 1996). As Dermagraft did not appear to stimulate immune rejection, it was first viewed as a dermal substitute (Marston *et al.*, 2003). However, the human foreskin fibroblasts implanted with this material die within a few weeks after implantation; therefore, the product more likely acts as a delivery vehicle for growth factors and ECM produced by the fibroblasts while they were extant (Ehrenreich and Ruszczak, 2006). Unfortunately, ATS filed for bankruptcy in 2002 (Bouchle, 2002) was acquired by Smith and Nephew and then was closed. Although Transcyte and Dermagraft are currently off the market, these technologies have been licensed to Advanced BioHealing for further production and marketing. Problems with these products that attributed to their demise included difficulties in quality control and expense (Ehrenreich and Ruszczak, 2006). In addition, Dermagraft was demanding to store and complex to use. Furthermore, reports on the clinical use of Dermagraft are relatively scarce compared to other dermal-like composites such as Alloderm and Integra (Ehrenreich and Ruszczak, 2006).

Porcine small intestinal submucosa acellular collagen matrix (Oasis, Cook Biotech Inc., West Lafayette, IN) and an acellular xenogeneic collagen matrix (E-Z-Derm) are also available and have relatively long shelf lives. A recent randomized clinical trial with small intestinal submucosa in 120 patients with venous leg ulcers demonstrated significantly increased healing at 12 weeks (55 versus 34%) in patients receiving small intestinal submucosa dressing weekly plus compression versus patients receiving compression alone (Mostow *et al.*, 2005). Although swines appear relatively resistant to prion disease (Wells *et al.*, 2003), possibly secondary to more inherent structural stability of their prion protein (PrPC) (Lysek *et al.*, 2005), prion disease and porcine retroviruses are a concern that needs to be addressed in xenograft products (Alisky, 2004).

Engineered skin substitutes. Full thickness wounds involve the loss of skin epidermis and dermis. To treat wounds of this depth, a bilayered composite composed of a contracted collagen lattice containing dermal fibroblasts

(Bell *et al.*, 1979) and an overlying epidermal sheet was designed (Bell *et al.*, 1981). Subsequently, a modification of this organotypic skin substitute utilizing type I bovine collagen and live allogeneic human neonatal foreskin fibroblasts and keratinocytes was developed (Apligraf, Organogenesis, Canton, MA) and marketed (Norvartis, Zurich, Switzerland). It provided benefit in surgical wounds (Eaglstein *et al.*, 1995) and venous ulcers (Sabolinski *et al.*, 1996) and is FDA approved for the latter. In a large multicenter trial this product resulted in approximately 25% accelerated healing of chronic non-healing venous stasis ulcers when compared to standard compressive therapy (Falanga *et al.*, 1998). Signs of wound infection, however, were observed in 29% of patients receiving Apligraf versus 14% in patients receiving standard of care. Apligraf does not result in immunologic rejection (Eaglstein *et al.*, 1999); however, donor cells do not remain viable beyond 4–8 weeks. Although Apligraf was first marketed as an organotypic skin substitute, the lack of long-term viable cells counters this claim. It is now believed that Apligraf works by delivering growth factors and ECM to the wound bed (Ehrenreich and Ruszczak, 2006). The product is provided in a 75-mm tissue culture dish and has a shelf life of 5–10 days. Thus, shipping must be closely coordinated with the patient's clinic visit. Apligraf costs approximately $30 per square cm, because this product like Dermagraft has a high cost to produce, maintain and transport (Bouchle, 2002). Organogenesis filed for bankruptcy in 2002 after being unable to provide these complex organotypic constructs at the marketed wholesale cost (Bouchle, 2002). After undergoing reorganization, Organogenesis is now back in business selling Apligraf, as well as other advanced wound care products.

Several other composite skin substitutes combining dermal and epidermal elements have been developed. A composite cultured skin substitute (OrCel, Ortec International Inc., New York, NY) is composed of both neonatal keratinocytes and fibroblasts embedded on opposite sides of bilayered bovine type I collagen. This product is currently being evaluated in clinical trials for the treatment of burns, in patients with epidermolysis bullosa and in split-thickness donor sites (Still *et al.*, 2003).

More extensive comparative data of the various tissue-engineered biologic dressings has been published elsewhere (Ehrenreich and Ruszczak, 2006).

Tissue-engineered skin replacement: state-of-the-art

Although increased healing rates of burn and/or chronic wounds can be observed with current engineered constructs, several intrinsic shortcomings limit their use: (a) epidermal grafts are fragile and therefore difficult to handle; (b) cell-populated matrices used in ''skin substitutes'' are not readily scalable for manufacturing and are difficult to store and transport; (c) autografts require creation of fresh wounds; (d) allografts or xenografts may induce immune rejection; and (e) skin substitutes, allografts, or xenografts may carry infectious agents including prions (Nunery, 2001). In part because of the latter possibility, it has been recently recommended that informed consent be obtained from all patients before the implantation of such biological material (Enoch *et al.*, 2005). Moreover, skin substitutes promote the healing of chronic leg ulcers only about 25% over patients receiving standard of care (Falanga *et al.*, 1998). These limitations suggest that further improvements are needed to insure that tissue-engineered constructs are less complex, more cost effective and user friendly, and carry minimal risk of infection.

To develop acellular, cost effective, user-friendly constructs based on non-animal products, cues should be taken from embryogenesis, morphogenesis, and the acute wound healing process (Lutolf and Hubbell, 2005). As tissue cells themselves are the primary source of various ECM molecules that facilitate and synchronize tissue repair, any acellular product must, therefore, be conductive to rapidly recruit host tissue cells and inductive to stimulate invading cells to proliferate, synthesize new ECM, and, if required, differentiate. The continuum of cell–ECM interactions over time is essential for tissue formation and has been called dynamic reciprocity (Nelson and Bissell, 2005).

Although synthetic and natural biopolymers have both been used experimentally to design tissue-engineered constructs for skin wounds and other uses (Lutolf and Hubbell, 2005), tissue-engineered products on the market for skin wounds have almost exclusively been based on natural, mostly animal-derived, biopolymers (Ehrenreich and Ruszczak, 2006). The reasons for this include biocompatibility, intrinsic cellular signals, and appropriate mechanical properties. Nevertheless, mechanical properties of synthetic biopolymers can be rigorously controlled. Therefore, such materials have been used *in vitro* to demonstrate the fundamental effects of viscoelastic properties on animal cells (Discher *et al.*, 2005) and human mesenchymal stem cells from bone marrow (Engler *et al.*, 2006). Recently, the authors have developed a system in which the biopolymer and bioactive are derivative of human materials, which are synthesized in genetically engineered bacteria, and whose viscoelastic and biomimetic properties can be independently and rigorously controlled (Ghosh *et al.*, 2006b). Using these ''natural'' building blocks for tissue engineering, we have begun to perform *in vitro* studies on human adult cell adaptation to variations of ''natural'' biomimetics and biopolymers (Ghosh *et al.*, 2006a), as well as *in vivo* testing in animal wound-healing models (Ghosh *et al.*, 2006b).

For the past several decades, many engineered skin constructs have utilized collagen as a scaffolding material for cell seeding (Balasubramani *et al.*, 2001). Collagen's popularity can be attributed to its abundance in skin, its recognition by cell surface receptors, and its ability to crosslink and thereby impart appropriate mechanical strength to the tissue (Ruszczak, 2003). Collagen, however, appears during the later stages of wound healing after fibroblasts have invaded and filled the wound space (Welch *et al.*, 1990). Thus, a scaffold constructed mainly of collagen may not be optimal for tissue

cell migration. In the acute wound-healing response, fibroblasts migrate into a fibrin/fibronectin-rich clot and then synthesize and secrete hyaluronan and fibronectin as a second provisional matrix that in turn promotes even more robust migration (Ghosh and Clark, 2007). Hyaluronan also appears coincident with tissue cell migration during embryogenesis and morphogenesis (Toole *et al.*, 2005).

Hyaluronan, a non-sulfated glycosaminoglycan, especially prominent in umbilical cord, vitreous humor, and articular cartilage, occurs in abundance in the normal adult epidermis and dermis (Sakai *et al.*, 2000; Bourguignon *et al.*, 2006). During wound repair, it serves multiple important functions, ranging from regulating inflammation to promoting fibroblast migration and proliferation (Chen and Abatangelo, 1999). Importantly, hyaluronan has been implicated in the regeneration and scarless healing of fetal wounds, perhaps owing to its ability to modulate inflammation (Wisniewski and Vilcek, 1997) and collagen deposition (Longaker *et al.*, 1991). Similar to synthetic polymers, hyaluronan can be chemically modified to create a variety of stable derivatives (Prestwich *et al.*, 1998). An additional advantage of hyaluronan is the capability to engineer its synthesis in bacteria, thereby avoiding the need to extract it from animals (Hoshi *et al.*, 2004). By offering the advantages of both natural and synthetic materials, hyaluronan may be close to an ideal biopolymer for tissue engineering. Indeed, chemically modified hyaluronan scaffolds have been successfully used for various tissue engineering applications, including wound repair (Campoccia *et al.*, 1998; Kirker *et al.*, 2002; Ghosh *et al.*, 2006b). With particular importance to wound repair, chemically modified hyaluronan can be intermolecularly crosslinked to impart increased resistance to hyaluronidase

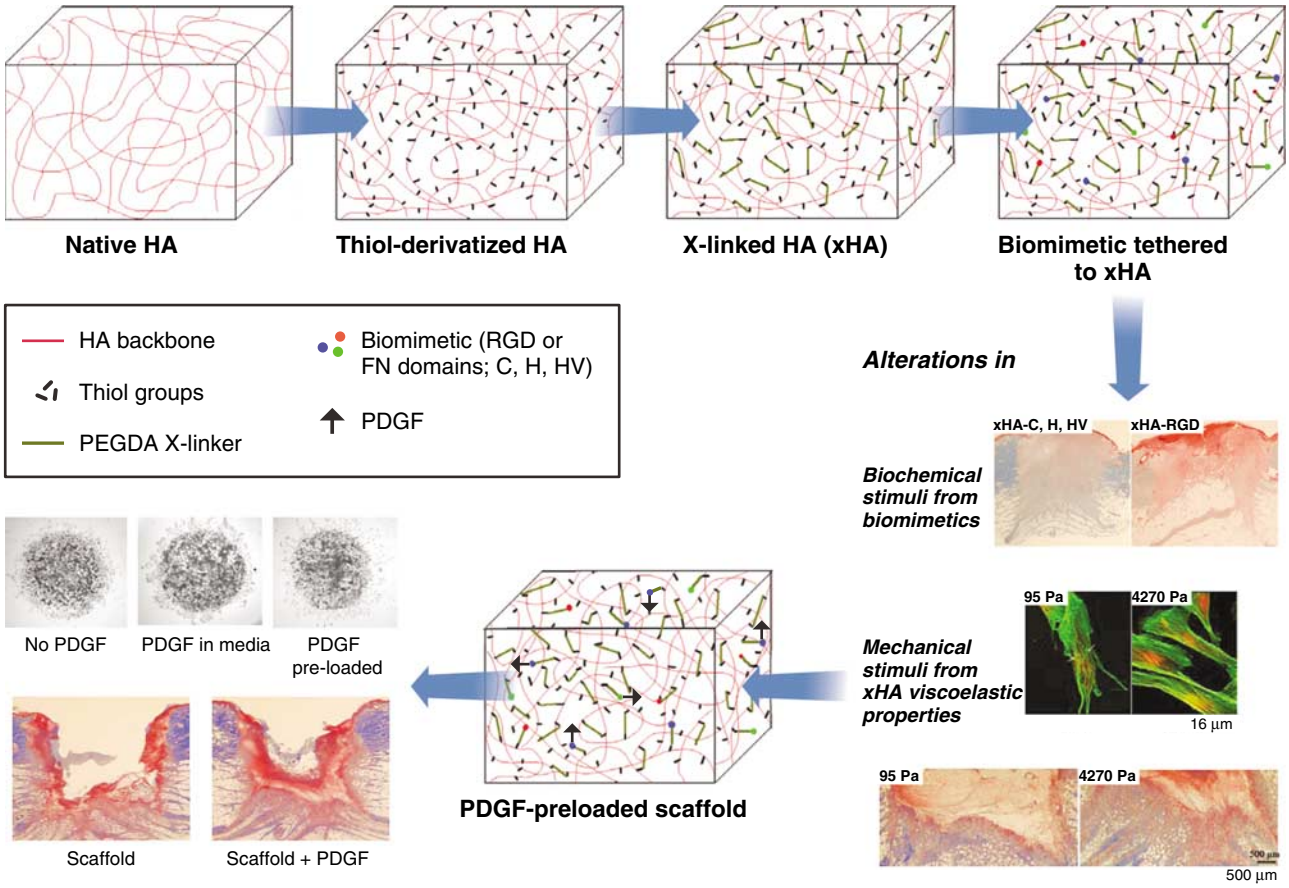

Figure 3. Example of an acellular, functionalized, tissue-engineered biopolymer for acceleration of cutaneous wound healing. Thiol-derivatized Hyaluronan (HA-DTPH) was synthesized as previously described (Shu *et al.*, 2002). Homobifunctional poly(ethylene)glycol (PEG) derivatives were added to HA-DTPH to intermolecularly crosslink hyaluronan (xHA) and also to tether cys-tagged fibronectin functional domains (C, H, HV) to the xHA (Biomimetic tethered to xHa) (Ghosh *et al.*, 2006b). Biochemical stimuli from biomimetics: xHA tethered with C, H, and HV was fully permissive for healing of reinjured porcine cutaneous wounds while xHA tethered with RGD peptides inhibited healing. Mechanical stimuli from xHA viscoelastic properties: Less stiff xHA (95 Pascals) failed to support full spreading of cultured fibroblasts, which showed buckling of actin bundles, while stiffer xHA (4270 Pa) supported robust spreading of fibroblasts, which showed tense actin bundles. Less stiff xHA tethered with C, H, and HV (95 Pa) did not enhance healing 4-day wounds while stiffer constructs (4270 Pa) increased granulation tissue formation by 75% at 4 days. When PDGF was preloaded in the stiffer xHA constructs tethered with C, H, HV (PDGF-preloaded scaffold), the healing of 4-day wounds was further increased to almost double the normal healing rate as judged by re-epithelialization, granulation tissue formation, and angiogenesis. In addition, xHA tethered with C, H, and HV and preloaded with PDGF promoted fibroblast migration *in vitro* to the same extent as PDGF in solution.

digestion and to create a hydrogel with viscoelastic properties intermediate between a fibrin clot and normal dermis (Ghosh *et al.*, 2005, 2006b).

Our laboratory used the morphogenetic clues outlined in the preceding paragraph to select hyaluronan as a platform for the design, creation, and testing of crosslinked biopolymers tethered with bioactives to enhance acute or chronic cutaneous wounds (Figure 3). As an example of a rationally designed material to facilitate restoration or replacement of injured or lost tissue, we will take the reader through the steps of this process. As fibroblast migration is the rate-limiting step in granulation tissue formation (McClain *et al.*, 1996), a bioactive was sought that optimally supports migration of activated fibroblasts. Fibronectin was a favorable candidate for this role as (a) it appears together with hyaluronan at times of cell migration during embryogenesis, morphogenesis, and wound repair (Ghosh and Clark, 2007); (b) fibroblasts invade hyaluronan/fibronectin gels approximately four-fold better than fibrin/fibronectin gels (Greiling and Clark, unpublished observation); (c) fibronectin is required for fibroblast transmigration from a collagen to fibrin matrix (Greiling and Clark, 1997); and (d) fibronectin is absent in chronic wounds (Herrick *et al.*, 1992), where it is produced normally (Herrick *et al.*, 1996) but eliminated rapidly by abundant proteases (Grinnell *et al.*, 1992; Grinnell and Zhu, 1996). Although fibronectin appears to be an ideal bioactive for use in hyaluronan gels, its stability in the proteolytic environment of chronic wounds is a major concern.

An alternative biomimetic for use in engineered wound healing materials is arginine–glycine–asparatic acid (RGD), a proteolytically stable peptide sequence from the 10th fibronectin type III repeat that supports mesenchymal cell attachment to surfaces (Pierschbacher and Ruoslahti, 1984). The RGD sequence, also present in a variety of other ECM molecules, is recognized by transmembrane integrin receptors of multiple cell types, including dermal and epidermal tissue cells (Pfaff, 1997). In fact, RGD has been widely used to promote cell attachment and spreading in various tissue engineering applications, in general (Hersel *et al.*, 2003), and wound healing applications in particular (Pierschbacher *et al.*, 1994). Previously, we observed that a biomaterial consisting of RGDS (arg–gly–asp–ser) peptides tethered to hyaluronan hydrogels supports NIH 3T3 fibroblast functions *in vitro* (Shu *et al.*, 2004) and, when seeded with 3T3 fibroblasts and implanted in nude mice, produces granulation-like tissue in 4 weeks. Although RGD-modified hyaluronan hydrogels possessed great inductive properties for mouse 3T3 cells, they did not support human adult dermal fibroblast functions nor demonstrate the conductive properties (ability to support fibroblast growth into the wound) required of an acellular scaffold in porcine wounds (Ghosh *et al.*, 2006b).

To create an acellular matrix with both inductive and conductive properties, three fibronectin functional domains, $FNIII_{(8-11)}$, $FNIII_{(12-15)}$, and $FNIII_{(12-V-15)}$, were selected as the bioactive As they are required for optimal human adult dermal fibroblast migration in both two-dimensional and three-dimensional systems *in vitro* (Clark *et al.*, 2003). When hyaluronan hydrogels were tethered with the three fibronectin functional domains, the resulting construct supported human adult dermal fibroblast functions *in vitro* and promoted wound healing *in vivo* (Ghosh *et al.*, 2006b). The crosslinked hyaluronan hydrogel possesses several positive characteristics. as it is formulated at room temperature and physiological pH, cells or additional bioactives can be incorporated in the construct without denaturation. Rapid curing (<10 min) allows *in situ* gelation after tissue injection by any route. The hydrogel's viscoelastic properties lie in the range between a fibrin clot and normal human dermis. Likewise, the bioactive fibronectin domains have several positive attributes. They are based on human plasma fibronectin and thus immunocompatible. They can be expressed in bacteria without loss of function as post-translational modification is not necessary for fibronectin function. Therefore, their production is cost-effective and carries minimal risks of viral or prion infection. Importantly, the hyaluronan–fibronectin domain-engineered biopolymer can be formulated into multiple physical forms including hydrogels that can be preformed or formed *in situ*, lyophilized scaffolds, and electrospun nanofibrous networks (Ji *et al.*, 2006) that may more closely mimic natural ECM architecture.

With similar objectives, several other groups have also developed ''intelligent'' scaffolds for tissue repair (Rosso *et al.*, 2005). Their approaches commonly employed synthetic materials to build scaffolds that allowed great flexibility during formulation. To impart bioactivity, these scaffolds contain biomimetics that can be recognized by tissue cells. One potential advantage of natural-derived materials over synthetic materials is that degradation of natural ECM can occur concomitant with cell invasion as it does normally during granulation tissue formation. To elicit a similar response in synthetic materials, protease-sensitive sequences can be incorporated within the scaffold that are cleaved upon contact with cell-secreted proteolytic enzymes (Gobin and West, 2002; Lutolf *et al.*, 2003).

Traditionally, biopolymers were thought to provide passive mechanical support to tissue-engineered composites. This view arose from the belief that cells respond to biological, rather than mechanical, signals. However, reports over the past few years demonstrate that mechanical forces can also govern cell and tissue phenotype (Huang and Ingber, 1999; Geiger *et al.*, 2001; Riveline *et al.*, 2001; Ingber, 2003). In fact, cells use an active tactile sensing mechanism to feel and respond to substrate mechanics (Lo *et al.*, 2000; Discher *et al.*, 2005; Vogel and Sheetz, 2006). Importantly, dermal fibroblast response to substrate mechanics includes alteration in gene expression that can eventually lead to differential ECM synthesis or their phenotypic transformation into myofibroblasts (Chiquet *et al.*, 2003; Hinz, 2006). Furthermore, circulating human bone marrow-derived mesenchymal stem cells, which can populate and enhance cutaneous wound healing

(Badiavas *et al*., 2003; Chapel *et al*., 2003), are exquisitely sensitive to ECM mechanical properties, leading to different differentiated phenotypes depending on matrix stiffness (Engler *et al*., 2006). As these processes are critical during wound healing, tissue engineering approaches for wound repair would require optimization of both biological and mechanical effectors.

Acellular tissue-engineered constructs discussed thus far utilize biopolymers to provide mechanical support for tissue ingrowth, and biomimetics to induce key cell functions. The primary goal of this approach is to mimic the attributes of the wound provisional matrix that is conductive for parenchymal cell migration and inductive of the appropriate differentiation for new tissue formation. However, a fibrin clot is not only composed of a fibrin/fibronectin scaffold and an array of clotting and fibrinolytic enzymes, but also of multiple growth factors that had been released during platelet aggregation (Mosesson, 2005). Growth factors play a crucial role in the healing response where they function to stimulate cell migration, proliferation, and differentiation. Growth factor deficiency often leads to impaired healing (Crowe *et al*., 2000; Peters *et al*., 2005). As a result, several groups have investigated the use of tissue-engineered constructs for local growth factor delivery (Richardson *et al*., 2001; Cai *et al*., 2005).

It is important to note that in spite of growth factor release from platelets and injured cells immediately after wounding, a 3-day lag time occurs before granulation tissue begins (McClain *et al*., 1996). This fact suggests that growth factors may bind the clot and retain functional activity. Such 'solid-state' chemical biology is supported by data that basic fibroblast growth factor and vascular endothelial growth factor bind to fibrin and retain their biological activity (Sahni and Francis, 2000; Sahni *et al*., 2004) and that IGF and vascular endothelial growth factor bind to fibronectin and retain their bioactivity (Gui and Murphy, 2001; Wijelath *et al*., 2002). Furthermore, studies from our laboratory have shown that PDGF, when preloaded onto hyaluronan hydrogels containing specific domains of fibronectin, retains its activity at a level typically observed with a much higher concentration in solution (Ghosh and Clark, unpublished observations). Although the finding was counterintuitive from the vantage of glycosaminoglycans, since heparin, rather than hyaluronan, has been demonstrated to bind a variety of growth factors (Klagsbrun, 1990**;** Shirakata *et al*., 2005), it is consonant with the ability of fibronectin to bind other growth factors. Regardless of the mechanism employed, by incorporating appropriate growth factor-binding materials, a tissue-engineered composite can be used as a growth factor repository, which accentuates cell functions through the bioactivity of bound or released growth factors as well as for mechanical properties attributable to the biopolymer backbone and for conductive and inductive activity attributable to other tethered biomimetics (Figure 3).

Conclusion

Wound healing is an integrated biological response consisting of a dynamic reciprocity among cells, ECM and growth factors that reconstitutes tissue after injury. Vigorous cellular activities observed during wound repair are similar to those occurring during embryogenesis and morphogenesis indicating the enormous complexity of this physiological reparative process. That complexity may also explain why, despite over two decades of intense research and development, medical research has still not identified an ''ideal'' therapy. However, a better appreciation of how viscoelastic and geometric properties of natural and synthetic biopolymers effect cell function and multicellular organization, how external biochemical signals interface with the cell membrane in context of the pericellular matrix, and how signals from these physicochemical events are transduced by a solid-state, yet dynamic and integrative, organization of signal transduction proteins in the cytoplasm, is beginning to open new horizons, which should ultimately translate into novel ''break-through'' wound healing therapeutics.

CONFLICT OF INTEREST

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