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MEETING REVIEW

Wound healing from a cellular stress response perspective

Bindi M. Doshi · George A. Perdrizet · Lawrence E. Hightower

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Abstract This meeting review highlights areas of mutual interest to investigators in the cellular stress response field and to those carrying out wound-healing research. Inflammation, perhaps the major unifying theme of this meeting, is an essential component of the adult wound response and understanding the control of inflammation is a common interest shared with researchers of the cellular stress response. The particular interest of the authors of this review is in chronic non-healing wounds that frequently occur in patients with major illnesses such as diabetes and diseases of the blood vessels. This orientation has undoubtedly influenced the selection of topics. It is fair to say that the authors were often surprised and certainly impressed with the overlapping interests and possibilities for collaboration among investigators of these two research areas.

Keywords Wound healing · Cellular stress response · Inflammation · TGF-β · Cytoprotection · Exercise · Hyperbaric oxygen

Introduction

The 21st Annual Symposium on Advanced Wound Care (SAWC) and Wound Healing Society (WHS) meeting was held at the San Diego Convention Center, April 24–27, 2008. This was the second joint meeting of the SAWC and the WHS. The sessions covered a broad range of topics including clinical presentations on best practices for wound care and management, new equipment for monitoring wounds and stimulating the healing process, complications of diseases such as diabetes and peripheral artery disease, and the latest basic research including the use of stem cells and systems biology approaches. There were 330 poster presentations and 24 oral presentations of selected abstracts in the categories of case studies, clinical research, informational/educational reports and laboratory research. The wound care field has a strong industry component and there were many companies at the trade exhibition marketing wound care products.

Wound healing is a complex multi-phase tissue-level response to damage (stress) and certainly must be included on the list of research problems having a high degree of biological complexity. It is useful to separate the processes of fetal and adult wound healing. The former process, described as scarless wound healing, is predominantly a regenerative process whereby injured tissues still in a developmental mode can essentially re-grow and restore wounds to nearly normal tissue organization and appearance. In contrast, evolution of the wound repair process in adults has been driven by inhibition of microbial pathogens in damaged tissue in order to allow a rapid and sturdy repair response that emphasizes functionality and re-establishment of homeostasis. The resulting repair is often a fibroblastic scar in place of epithelium and endothelium. Inflammation is an essential component of the adult wound response and understanding the control of inflammation is a common interest shared with researchers of the cellular stress response (Coelho et al. 2008). The latter response has both pro-inflammatory properties associated with extracellular chaperones and an anti-inflammatory state known histori-

B. M. Doshi · L. E. Hightower (✉)
Department of Molecular and Cell Biology,
The University of Connecticut,
Storrs, CT 06269, USA
e-mail: lawrence.hightower@uconn.edu

G. A. Perdrizet
Hartford Hospital,
Hartford, CT 06169, USA

cally as thermotolerance and more recently as cytoprotection. The attendant process of stress conditioning involves the deliberate preconditioning of patients to stimulate acquisition of protection prior to surgery and other medical procedures that risk tissue damage. Additional common ground includes gene regulation by TGF-β and roles of stress responses in diseases like diabetes (Calabrese et al. 2007; Najemnikova et al. 2007), which have a large impact on wound healing as well.

TGF-β

A symposium on cytokines and growth factors was held in memory of Dr. Anita B. Roberts. Anita received her doctorate in biochemistry from the University of Wisconsin in 1968 and joined the National Cancer Institute in 1976. She later became chief of the Laboratory of Cell Regulation and Carcinogenesis. She is credited with discovering transforming growth factor β (TGF-β), and she pioneered early work characterizing this protein. She explored the role TGF-β has in cancer as well as in wound healing. Her research interests included TGF-β signal transduction mechanisms involved in wound repair and tumorigenesis. Dr. Roberts passed away in 2006 from gastric cancer.

Several speakers who knew Anita personally commented on how enthusiastically she shared information and reagents to help move research on TGF-β ahead as quickly as possible.

On a personal note, one of my (L.E.H.) graduate students, Dr. Ivone Takenaka, showed the link between the cellular stress response and TGF-β1 as part of her Ph.D. thesis (Takenaka and Hightower 1992; Takenaka and Hightower 1993). She showed that *hsp70* and *hsp90* gene expression is up-regulated in cultured chicken embryo cells by TGF-β. Ivone found evidence of posttranscriptional regulation by a mechanism involving a nuclear event(s) such as increased half-lives of nuclear RNA transcripts, processing, or transport into the cytoplasm. Early in this project, Dr. Michael Sporn invited me to talk about our work at NCI, and I had the opportunity to meet Anita and other members of the Sporn group. Evidence of a role for TGF-β in wound responses was still a very recent discovery (Sporn et al. 1987). Subsequently, they were very helpful to us with discussions and gifts of reagents, as they were to so many others.

TGFβ has a major role in wound healing and scarring. Mark Ferguson (Manchester University and Renovo) described one of the problems in wound healing, restriction in movement and growth. Embryos younger than E16 heal without a scar. After this time point, the collagen has a different organization. The levels of TGFβ-1 and TGFβ-2 are low in fetal development and higher in adults and TGFβ-3 is higher in fetal development and lower in adults. When TGFβ-3 was added to adult wounds, scarring was reduced, and in a TGFβ-3 knockout model, fetal mice healed from wounds with a scar. TGFβ-1 and -2 have a role in organizing actin whereas TGFβ-3 increases migration and is involved in filopodia formation. Hsp27 is the heat shock protein implicated in interactions with actin and cell movement. It would be interesting to know whether or not TGFβ-3 affects Hsp27 gene expression and/or localization within human cells, and if there are changes in cell movement.

Oxygen, more or less

The speakers in a pre-conference session entitled "Supplemental Oxygen Therapy" addressed multiple aspects of oxygen therapy as it relates to experimental and clinical models of wound healing. A review of the biochemical basis for oxygen and redox sensing was presented by Chandan Sen (Ohio State University) along with evidence that hypoxia set points within a tissue are heterogeneous and changeable based on ambient tissue oxygen tension. Periannan Kuppusamy (Ohio State University) introduced early work on the use of electron paramagnetic resonance imaging which employs a new nanocrystalline oxygen sensor (LiNc-BuO, OxyChip) to monitor tissue oxygen tensions non-invasively and in real time. Harriet Hopf (University of Utah) reviewed the clinical evidence supporting the use of hyperbaric oxygen therapy in the treatment of diabetic foot ulcers. Omaida Velazquez (University of Pennsylvania) presented animal studies of the effect of hyperbaric oxygen in the mobilization and homing of endothelial progenitor cells during wound healing in a diabetic mouse model. The final presentation was by Thomas Mustoe (Northwestern University) who focused on the problem of tissue ischemia and wound healing. He emphasized the unique ability of skin to tolerate brief periods of ischemia in contrast to the more susceptible subcutaneous fat and skeletal muscle tissues. Given the national interest in the problem of pressure ulceration of skin and deeper tissues in hospitalized patients, Dr. Mustoe has begun to look at how serial exposures to acute ischemia in a rodent model alter gene expression as compared to a single exposure. Dr. Mustoe has reported previously on the changes in heat shock gene expression (HSP70, HSP90, and HO-1) during acute wounding and ischemia (Mustoe et al. 2006; Tandara et al. 2006). Most recently, this group has demonstrated the attenuation of acute gene expression for several genes involved in cytoprotection and responses to ischemia, including HSP70, HSP90, HIF-1α, HO-1 and VEGF mRNAs. The mechanism and implications of the attenuated gene expression following a second acute ischemic event are unclear at this time. Attenuated stress gene expression

may reflect a failed response or, alternatively, an adaptive response by the cell to the acute ischemia insult.

During the main conference, R. C. Fang (Northwestern University) presented studies of in vitro hypoxia/reoxygenation roles of heat shock transcription factor (HSF1). HSF1 null fibroblasts were more susceptible to hypoxia/reoxygenation-induced cell death. An increase in reactive oxygen species was observed. These cells were also more susceptible to hydrogen peroxide-induced cell death. The expression levels of phosphorylated protein kinase B (AKT), a serine/threonine kinase, were decreased in knockout cells. When the AKT pathway was inhibited, there was an increase in cell death in wild type as well as in knockout cells. AKT signaling pathways are involved in cell proliferation, apoptosis, angiogenesis and diabetes. Knockout cells showed greater cyclooxygenase (COX) levels. When a COX inhibitor was used, there was an increase in cell death in the knockout cells. It was concluded that HSF1 is needed for cytoprotection after hypoxia/reoxygenation stress.

Harriet Hopf (University of Utah) discussed the use of hyperbaric oxygen treatment (HBOT) to improve healing of diabetic foot ulcers. These types of ulcers do not heal well and while HBO treatment is not meant to replace current therapies, it may help heal the wounds sooner. These wounds do not heal well because of neuropathy, lowered resistance to infection, impaired microvascular perfusion, and abnormal response to injury or inflammation. The effects of HBOT are beneficial. It corrects hypoxia and controls excessive inflammation. Pharmacologic doses of oxygen increase growth factors and induce angiogenesis. Increased numbers of PDGF receptors are present in HBO-treated cells. The numbers of amputations decrease with treatment and patient outcomes are improved following HBOT.

Diabetes is of interest to the cellular stress response community as well. Habich and Burkart (2006) recently reviewed evidence for heat shock proteins in beta-cell-directed immunity and pathogenesis of type 1 diabetes. Hsp70, a marker for cytoprotection, is up-regulated in beta cells subjected to inflammation and may provide these cells with protection. Hsp60, released extracellularly from beta cells, may function as an immunoregulator via interactions with innate immune cells. Interestingly, islets from diabetes-prone animals had altered stress responses and were more susceptible to mediators of inflammation. HBOT of human subjects up-regulates Hsp70 levels in blood lymphocytes (Dennog et al. 1999) and HBOT of a mouse neuroblastoma cell line increases expression of Hsp70 (Shyu et al. 2004). There is also evidence that HBOT is immunosuppressive in a mouse model of cell-mediated immunity (Hansbrough et al. 1980). Perhaps an effect of HBOT that promotes healing of diabetic ulcers is the establishment of a transient anti-inflammatory state of cytoprotection in the wounded tissue. Tissue-level cytoprotection has been described as an anti-inflammatory state (Hightower et al. 2000), and it was shown recently by Li et al. (2007) that preconditioning primary cultures of mouse spinal cord neurons with HBO induces protection against oxidative injury. In addition, pretreating patients with HBO before cardiopulmonary bypass surgery appeared to reduce their inflammatory response (Alex et al. 2005).

Gerald and coworkers (Vanderbilt University) presented a poster entitled “Molecular Imaging-Assisted Optimization for Laser Preconditioning for Wound Repair Enhancement”. Based on a review of the cytoprotection literature as it relates to thermal induction of HSP70, the authors hypothesized that laser pretreatment of skin to induce HSP70 would lead to improved dermal wound healing as measured by wound burst strength. Prior work from this group describes the laser thermal doses used to obtain dermal HSP70 expression (Wilmink et al. 2006). A single treatment (43°C×15 min), 12 h before wounding resulted in significantly stronger tissue repair compared to the untreated animals. No effect was seen in a diabetic mouse model. Associated with the improved healing was an augmentation in tissue perfusion measured 12 h following the local heat shock. This work supports the concept that HSP70 present at a local level may enhance wound healing (Kovalchin et al. 2006). Local extracellular heat shock proteins may exert a proinflammatory effect within the wounded tissue and would be expected to enhance healing (and scarring). The outcome of systemic heat shock prior to wound healing may impair systemic inflammation and thereby retard wound healing. The net outcome within a wounded tissue will always reflect a complex relationship between systemic and local factors. This is an example of the potential interaction of extracellular heat shock proteins functioning as proinflammatory cytokines and intracellular heat shock proteins as contributors to the anti-inflammatory, cytoprotected state.

George Perdrizet (Hartford Hospital and University of Connecticut) presented a poster entitled “Physiologic Basis for HBO_2-Preconditioing: What Every Wound Care Clinician Needs to Know.” The potential for improving outcomes following invasive surgical and medical interventions by prior induction of the cell stress response has been amply documented in the experimental biology literature. Most recently, protection of the brain during open-heart surgery and cardiopulmonary bypass was achieved by the pretreatment of patients with hyperbaric oxygen at 2.4 ATA, for 60 min at 6, 12, and 24 h prior to surgery. This treatment resulted in significant protection of neurocognitive function at 3 and 6 months following surgery (Alex et al. 2005). The mechanism of this observed protection is unknown. Treated patients did have elevated stress proteins measured in peripheral blood during and immediately following surgery. The ability of hyperbaric oxygen to cytoprotect heart, brain, spinal cord, and liver against acute ischemia–

reperfusion injury has been previously documented (Chen et al. 1998; Dong et al. 2002; Yu et al. 2005; Cabigas et al. 2006; Nie et al. 2006; Mori et al. 2007; Qin et al. 2007). Furthermore, pretreatment of human lymphocytes in vitro with hyperbaric oxygen induces stress proteins and confers protection against DNA damage by reactive oxygen species (Rothfuss et al. 1998; Shinkai et al. 2004). HBOT may represent a clinically effective and well tolerated method by which to test the Stress Conditioning Hypothesis on a broad clinical basis.

Exercise, extracellular chaperones and inflammation

Luisa DiPietro (University of Illinois—Chicago) brought another topic of common interest to the meeting with a description of several experiments using exercise to modulate inflammation in mice. Mice were exercised on treadmills prior to surgery, a protocol which may have been stress conditioning depending on the timing. Interestingly, exercise reduced the levels of inflammatory mediators in healing wounds and improved wound closure. Dr. DiPietro pointed out that reduced inflammation is a characteristic of rapidly healing wounds and that there exists a critical balance created by up- and down-regulation of inflammation for optimal wound healing. Interest in exercise and the cellular stress response has increased dramatically following a report that exercise increases human serum levels of Hsp70 (Walsh et al. 2001). The immunomodulatory activity of extracellular Hsp70 was the subject of a recent review article (Johnson and Fleshner 2006). Another stress response protein with immunomodulatory activity is Hsp60. Injection of Hsp60 into normal and atopic canine skin induces cytokines of a regulatory and Th1 phenotype in healthy skin and partially in skin from dogs with atopic dermatitis. The authors suggested that heat shock protein interventions should be combined with anti-inflammatory compounds to neutralize the effects of pro-inflammatory cytokines (Jassies-van der Lee et al. 2008).

Sabine Eming (University of Cologne) continued the theme of the importance of a critical balance of inflammation. An influx of inflammatory cells across vascular endothelium stimulates the healing response, although later the inflammatory response must be turned down. In the stress response literature, there is evidence that blood vessels in injured tissue of stress-conditioned rats do not allow extravasation of monocytes, effectively blocking recruitment of additional inflammatory cells and putting the brakes on inflammation (House et al. 2001). Using an in vitro system of fibroblastic human synoviocytes, Luo et al. showed recently that extracellular Hsp70 can induce anti-inflammatory cytokine IL-10 production in these cells (Luo et al. 2008). IL-10 limits both innate and adaptive immune responses. Dr. Eming described studies with IL-10 deficient mice in which wounds actually healed faster. This was due primarily to increased epithelialization and enhanced wound tissue contraction. However, bursting strength of the partially repaired wounds in IL-10 deficient mice was reduced compared to wild-type mice, and later, thick bundles of collagen formed which increased scarring.

Vascular endothelial growth factor (VEGF) and inflammation

M.L. Petreaca (University of California—Riverside) discussed the role of VEGF in wound healing. This protein induces macrophage apoptosis. Wound healing begins with inflammation, followed by granulation tissue formation and finally tissue remodeling. Inflammatory cells are apoptosed resulting in the resolution of inflammation in normal wound healing. Chronic wounds have a longer inflammatory phase which does not result in proper resolution. When VEGF or its receptors were inhibited, an increase in macrophages occurred in wounds. VEGF may possibly be responsible for the loss of macrophages. In vitro work showed VEGF increased caspase 3 activation and stimulated apoptosis in primary macrophages. The role of Tumor Necrosis Factor Superfamily Member 14 (TNFSF14/LIGHT) was also studied in macrophages. An increase in VEGF resulted in an increase in LIGHT, which also lead to an increase in macrophage apoptosis. Lack of resolution of inflammation appears to be a factor in chronic wounds.

What is the link between VEGF and heat shock proteins? Masson-Gadais et al. (2003) showed that cross talk is needed between VEGFR2 and integrin $a_v b_3$ in vascular endothelial cells activated by VEGF. This cross talk stimulates transduction of VEGF signals to SAPK2/p38 and FAK, allowing Hsp90-dependent phosphorylation of focal adhesion kinase. The resulting increase in focal adhesions is instrumental in endothelial cell migration, an essential process in angiogenesis. Also, lipopolysaccharides induce apoptosis in primary cultures of porcine aortic endothelial cells and stimulate Hsp70 and Hsp32 production as well as release of VEGF extracellularly. This observation raises the possibility that the appearance of these molecules is a coordinated response to an inflammatory mediator and may indicate that acquisition of cytoprotection was also triggered as a brake on inflammation in vascular endothelium.

Wound healing molecular chaperones

Several molecular chaperones with particular roles in wound healing were noted in talks during the conference.

Calreticulin (CRT) is located in the lumen of the endoplasmic reticulum (ER) where it serves as a calcium-binding protein and as a molecular chaperone for molecules traveling through the ER with many destined for the cell surface and extracellular matrix. As Leslie I. Gold (NYU Medical Center) pointed out, extracellular functions are also emerging for CRT. Some of these functions outside of cells were reviewed by Michalak and colleagues (Johnson et al. 2001). Studies using CRT knockout mice have revealed an important role of CRT in organogenesis, especially in heart development. An N-terminal fragment of CRT, known as vasostatin, inhibits endothelial cell proliferation, angiogenesis and reduces tumor growth. Dr. Gold focused on the ability of CRT to enhance wound healing by stimulating production of matrix proteins and integrins by keratinocytes and fibroblasts. She described an interesting experiment in which CRT was applied topically to mouse skin. Wound healing was enhanced with increased granulation tissue and re-epithelialization. Not surprisingly, CRT-treated wounds were highly cellular and rich in extracellular matrix, suggesting roles in cell proliferation, migration and matrix formation. Evidence of roles of extracellular CRT in cell migration was reinforced by the outcome of chamber and scratch plate migration assays. The activity of CRT in focal adhesion disassembly may be involved here.

In a session on deep tissue injuries, Laura Edsberg (Daemen College) described an in vitro model for studying the effect on dermal tissue of pressure that produces compression ulcers. This can be viewed as a tissue-level stress response. In the model, human foreskin fibroblasts produced an elastin-rich tissue, and compression in vitro caused reorganization and thickening of elastin bundles. Collagen was realigned as well and deformation of the cells caused damage and cell death. While the morphological changes are most apparent, there is likely to be a biochemical component as well. At least one molecular chaperone, Hsp47, is a dedicated collagen chaperone (Marutani et al. 2004), and a good candidate to be involved in tissue remodeling in this response. In this regard, Jian-Fei Wang (University of Alberta) hypothesized that hypertrophic scar fibroblasts are derived from deep dermal fibroblasts. He showed that Hsp47 mRNA up-regulation in these deep cells coincided with increases in collagen and TGF-β.

Psychosocial stress

Stressful conditions to which humans are commonly exposed such as exam taking (Marucha et al. 1998) or marital discord (Kiecolt-Glaser et al. 2005) can contribute to delayed wound healing. The mechanisms involved are currently under study and may involve dysregulation of acute inflammatory responses. This session began to address the complex interplay between chronic diseases like diabetes mellitus and psychosocial stress. A potentially vicious, self-perpetuating cycle may become established in which chronic disease leads to chronic psychosocial stress which leads to significant psychosocial dysfunction (including psychiatric disease) which further impairs the patient's ability to heal. It is very significant that one of the most frequent wound-healing problems is that of the diabetic foot ulcer. The non-healing foot ulcer my be initiated by compromised blood flow, but patients may face months or even years of dealing with the wound problem in addition to the diabetic disease itself. The latter convergence of problems frequently overwhelms the patient, family and care-givers, leaving the patient with a feeling of hopelessness and depression. It is clear the cellular aspects of the stress response and wound healing are influenced by the psychosocial health of the patient. This underscores the need to treat the entire patient and not simply the wound. Dr. Schneiderman (University of Miami) reviewed the important role social contacts play in the systemic response to stress. Wound healing in rodents was reduced in animals that were isolated from littermates compared to wounded animals permitted to have baseline social contacts. This impaired wound healing could be reversed by oxytocin administration. Atherosclerotic rabbits were found to have exacerbation of vascular disease when exposed to the social stress of a new cage mate every 2 weeks (McCabe et al. 2002). It is clear that chronic disease is stressful and that chronic stress can worsen disease states. Dr. Polonsky (University of California—San Diego) very elegantly reviewed the interplay between diabetes mellitus and psychiatric illness. Approximately 30–40% of diabetic patients suffer from depression. Diabetes and depression are a "toxic combination" as diabetic patients who are depressed have significantly poorer blood sugar control and worse overall cardiac and ocular health than their non-depressed cohorts. A better understanding of the biochemical pathways by which psychosocial stress and depression impair inflammation and wound healing should provide potential therapeutic targets in the future.

Concluding remarks

The great experimental endocrinologist, Hans Selye defined stress as "any stimulus to which the system is not adapted." Dr. Selye cautioned that with any experimental model there will be the introduction willingly or unwillingly of stresses into the model and these additional stresses will influence the outcome being measured. Therefore, he recommended in all studies involving stress that two types of controls be

included in the design: (1) unstressed control which is completely unmanipulated and (2) Sham Stress Control in which the animals were manipulated except for the stressor being studied. Thus, the difference in response between control 1 and 2 would reflect the unmeasured, often unwanted and underappreciated stress to which control # 2 was exposed. Simply removing a rodent from its cage or housing facility will induce a stress response that will influence the inflammatory and cytoprotective responses in that animal. Given new knowledge about the vital role played by the stress proteins as molecular chaperones, and the ability of the cellular stress response to down-regulate cellular and tissue functions, Dr. Selye's wisdom should be heeded by all (Selye 1950).

It may be useful to consider wound healing in the context of a tissue-level stress response. The triggering stress would be tissue trauma, causing a break in homeostasis followed by invasion of the injured tissue by microbial cells. The initial response of the resident cells may be alterations in patterns of gene expression, release of stored chemokines and cytokines including stress proteins with the establishment of a pro-inflammatory state. Molecular chaperones may be among the extracellular mediators of inflammation and they may also accumulate within some of the cells at the site of tissue injury, contributing to a cytoprotected state. Cytoprotected cells in nearby blood vessels, particularly vascular endothelial cells, and cytoprotected cells at the site of injury would help put the brake on inflammation and down-regulate this response. How this succession of events might fit into the well-described phases of wound healing needs to be explored. Systems biology approaches, a panel discussion topic at this meeting, is applicable here, since this is indeed a problem encompassing biological complexity: Does the progression of events in the stress response fit into the multiple phases of wound healing and how might these sequences of events correspond? A combined genomic and proteomic approach to determine patterns of gene expression during the phases of wound healing and whether or not stress gene expression is part of those patterns would be very useful. Similarly, do chronic non-healing wounds represent different patterns of gene expression than patterns in normal wound healing or are these non-healing wounds "stuck" in an intermediate phase of normal wound healing? Much basic work remains to be done.

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