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ALTERED GENE EXPRESSION OF IL-1 ALPHA, IL-1 BETA, AND KGF-1 IN EARLY CUTANEOUS WOUNDS OF RESTRAINED MICE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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The Ohio State University 2001

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Chronic stress can considerably impair wound healing. Previous studies in humans showed that chronic stress caused significant delays in cutaneous and mucosal wound healing. In a murine model, cutaneous wounds of restrained-stressed (RST) mice healed significantly slower than controls. Stress was also related to significant decreases in proinflammatory cytokine Interleukin-1β (IL-1β) mRNA responses. These studies suggested that the suppressive effects of stress on inflammation could compromise subsequent proliferative events that lead to wound closure. Expression of proinflammatory cytokines, such as IL-1β and IL-1α, may effect expression of mediators of epithelial proliferation, such as keratinocyte growth factor-1 (KGF-1). To investigate the mechanisms by which stress influences molecular processes of inflammation and proliferation, cutaneous gene expression of IL-1α, IL-1β, and KGF-1 was studied in the murine model of wound healing. Female SKH-1 mice were RST for 3 days before and for 5 days after wounding. RT-PCR demonstrated that expression of IL-1α and IL-1β mRNA in control mice peaked at day 1, declined at day 3, and returned to constitutive levels by day 5. In contrast, day 1 wounds of RST mice had significantly low levels of IL-1β mRNA, while at day 5 there were significantly high levels of IL-1α and IL-1β mRNA. Treatment of RST mice with glucocorticoid receptor antagonist RU486 restored
IL-1β expression at day 1, suggesting that stress-induced disturbances in serum corticosterone were involved in early suppression of IL-1β. *In situ* hybridization revealed a low frequency of IL-1β mRNA-expressing fibroblasts at day 1. At day 3, control mice had thicker proliferating zones of epithelium than RST mice. This could be related to low frequency of KGF-1 mRNA-expressing fibroblasts in wounds of RST mice. There was evidence of leukocytic foci and reduced granulation tissue formation in day 5 wounds of RST mice. Our results demonstrate that stress induces alterations in the kinetics of cutaneous IL-1α, IL-1β, and KGF-1 gene expression, associated with delayed resolution of the inflammatory response, decreased epithelial proliferation, and reduced granulation tissue formation. Therefore, stress-induced alterations in proinflammatory cytokine and growth factor expression may account for significant changes in the quality of the healing tissues.
Dedicated to my husband, Henry,

*For your loving trust and encouragement:*

And to my baby son, Gabriel,

*For your unconditional smile.*
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I wish to thank my adviser, Dr. Phil Marucha, for trusting in my potential as a researcher ever since I started in his laboratory. Under his guidance throughout the Mentored Clinical Scientist program, I have developed my abilities as a scientist and have also been able to pursue my goals as a clinician.

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<td>AD</td>
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<tr>
<td>AMV-RT</td>
<td>Avian Myeloblastosis Virus-Reverse Transcriptase</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>bp</td>
<td>Basepair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CINC</td>
<td>Cytokine-induced Neutrophil Chemoattractant</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
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<td>DEX</td>
<td>Dexamethasone</td>
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<td>ELAM-1</td>
<td>Endothelial Cell Adhesion Molecule-1</td>
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<td>FGF-7</td>
<td>Fibroblast Growth Factor-7</td>
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<td>FWD</td>
<td>Food and Water-Deprived</td>
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<td>G3PDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GCs</td>
<td>Glucocorticoid hormones</td>
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<tr>
<td>GRO-α</td>
<td>Growth-related Oncogene-α</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic Pituitary Adrenal</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
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<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
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<td>IL-1β</td>
<td>Interleukin-1 beta</td>
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<tr>
<td>KC</td>
<td>Keratinocyte</td>
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<tr>
<td>kDa</td>
<td>KiloDaltons</td>
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<td>KGF-1</td>
<td>Keratinocyte Growth Factor -1</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<tr>
<td>LO</td>
<td>Lypooxygenase</td>
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<td>LPS</td>
<td>Lypopolysaccharide</td>
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<td>LTB₄</td>
<td>Leukotriene B4</td>
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<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>MCP-1</td>
<td>Macrophage Chemotactic Protein-1</td>
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<td>Macrophage Inflammatory Protein-1α</td>
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<td>MIP-2</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>NAP-2</td>
<td>Neutrophil-activating Peptide-2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>PGE₂</td>
<td>Prostaglandin E2</td>
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<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear Leukocyte</td>
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<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T-cell Expressed and Secreted</td>
</tr>
<tr>
<td>RST</td>
<td>Restraint, Restraint-stressed</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
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<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>SNS</td>
<td>Sympathetic Nervous System</td>
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<td>SSC</td>
<td>Saline Sodium Citrate</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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CHAPTER 1
INTRODUCTION

Overview

Wound healing is a complex process involving the progression of inflammatory, proliferative, and remodeling events ultimately aimed to restore the integrity of the injured tissues. After hemostasis is attained during the early inflammatory phase, circulating polymorphonuclear leukocytes (PMNs) infiltrate the wound in order to eradicate contaminating bacteria and debride the tissues. Monocytes infiltrate shortly thereafter and transform into macrophages, which phagocytose pathogenic organisms. As the PMN infiltrate resolves and macrophages continue to accumulate, a late inflammatory phase ensues. Macrophages promote the transition to the granulation tissue phase, when new connective tissue matrix is laid and neovascularization of the wound occurs. At the wound surface, re-epithelialization is taking place simultaneously in order to re-establish the protective barrier of the skin. The remodeling phase involves transition of the provisional extracellular matrix into mature collagenous tissue. These processes greatly overlap each other. Moreover, later stages like tissue proliferation and remodeling are strongly dependent upon earlier events such as inflammation (Hübner, Brauchle, Smola, Madlener, Fässler, & Werner, 1996).
Starting immediately after injury, recruited cells (PMNs and monocytes) as well as resident cells (keratinocytes, Langerhans cells, and fibroblasts) become activated. They establish communication pathways among each other via the release of diffusible polypeptides known as cytokines, essential mediators of inflammation. Extraneous conditions imposed upon the host, such as stress, may alter the neuroendocrine equilibrium that is necessary for a protective response to injury (Padgett, Marucha, & Sheridan, 1998). For instance, if stress provokes a change in host cytokine expression during wounding, this will compromise the function of inflammatory effector cells at the site of injury, altering subsequent reparative events and ultimately resulting in deregulation of wound healing (Hübner et al., 1996). Therefore, understanding the effects of stress at the molecular level, specifically on cytokine gene expression, may help to explain its impact on important biological processes such as wound healing.
The first cytokines released after injury are the primary proinflammatory cytokines Interleukin-1α and Interleukin-1β (IL-1α and IL-1β) (Fahey III, Sherry, Tracey, van Deventer, Jones II, Minei, Morgello, Shires, & Cerami, 1990; Hübner et al., 1996; Wood, Jackson, Elias, Grunfeld, & Feingold, 1992). Their presence within the wound stimulates the release of growth factors, such as Keratinocyte Growth Factor-1 (KGF-1), which is important for re-epithelialization of the wound surface (Brauchle, Angermeyer, Hübner, & Werner, 1994; Chedid, Rubin, Csaky, & Aaronson, 1994; Marchese, Chedid, Dirsch, Csaky, Santanelli, Latini, LaRochelle, Tossisi, & Aaronson, 1995; Werner, Peters, Longaker, Fuller-Pace, Banda, & Williams, 1992). Thus, IL-1α and IL-1β not only play a key role during the inflammatory response, but they also contribute to tissue repair by promoting re-epithelialization.

1.1 Expression of IL-1 in peripheral blood cells and skin cells

IL-1 is produced in two different bioactive forms, IL-1α and IL-1β (Dinarello, 1996). It is widely known that IL-1α and IL-1β are synthesized by peripheral blood cells such as monocytes and PMNs (Dinarello, 1996). Epidermal cells, such as keratinocytes and Langerhans cells, also produce IL-1. Keratinocytes (KCs), which account for more than 90% of epidermal cells, are responsible for creating a physical barrier to environmental agents (Salmon, Armstrong, & Ansel, 1994). Cultured normal human KCs express both IL-1α and -β mRNA, but IL-1α mRNA is the predominant form (Kupper, Ballard, Chua, McGuire, Flood, Horowitz, Langdon, Lightfoot, & Gubler, 1986; Lee, Morhenn, Ilnicka, Eugui, & Allison, 1991). Only IL-1α biological activity has been identified in KC
cultures (Salmon et al., 1994). Interestingly, the only biologically active cytokine detected in normal epidermis in the absence of disease is IL-1α (Blanton, Kupper, McDougall, & Dower, 1989; Kupper, 1989; Kupper, & et al., 1988). These findings suggest that KCs are the major source of IL-1 in normal skin. Langerhans cells (LCs) are dendritic cells derived from the monocytic lineage that intercalate between KCs in the epidermis (Salmon et al., 1994). In contrast to KCs, unstimulated LCs predominantly express IL-1β mRNA over IL-1α mRNA in vitro (Matsue, Cruz, Bergstresser, & Takashima, 1992). Matsue et al. reported that LCs are the major source of IL-1β mRNA among unstimulated mouse epidermal cells (Matsue et al., 1992). There is IL-1 bioactivity accumulated in culture supernatants of human epidermal cells that were highly enriched for LCs (Sauder, Dinarello, & Morhenn, 1984).

As described above, IL-1 is expressed in normal epidermis at low levels and is produced mainly by resting KCs and LCs. Thus, a constitutive pool of proinflammatory cytokines forms part of the immunoprotective barrier of the skin. Upon tissue injury, resident cells become alert. Studies of epidermal barrier perturbation in human skin have suggested that KCs become directly activated by exogenous environmental stimuli (Barker, Mitra, Griffiths, Dixit, & Nickoloff, 1991; Nickoloff, & Naidu, 1994). After repeated tape stripping of human skin, there is increased IL-1β mRNA in the epidermis accompanied by KC intercellular adhesion molecule-1 (ICAM-1) expression and the appearance of endothelial cell adhesion molecules (ELAM-1). Wood et al. (Wood et al., 1992) demonstrated in a variety of murine models of barrier disruption that epidermal mRNA levels of IL-1α and IL-1β are increased after injury. Levels of epidermal TNF
protein were also increased. Fahey et al. (Fahey III et al., 1990) used subcutaneously implanted wound chambers in mice to study cytokine mRNA appearance. IL-1β mRNA was expressed in inflammatory cells of wound fluid. IL-1 protein was also detected in wound fluid samples. Hübner et al. (Hübner et al., 1996) detected higher levels of IL-1α and IL-1β mRNA in wounds versus normal skin of mice, which correlated with increased protein levels of these cytokines. Therefore, induction of proinflammatory cytokine expression in resident epidermal cells contributes to the inflammatory reaction to tissue injury.

1.2 Effects of IL-1 at the wound site

The main action of IL-1 at the wound site is proinflammatory; that is, to promote inflammation. Localized inflammation involves a “wheal and flare” tissue reaction and the recruitment of peripheral leukocytes to the site of injury. Once the inflammatory reaction has started, IL-1 serves to enhance leukocyte antibacterial mechanisms. IL-1 is also involved in the promotion of wound re-epithelialization. Each one of these actions is considered separately.
Induction of the cutaneous wheal and flare reaction

Within seconds after blunt injury to the skin, a red area of local erythema develops at the site. In the next several minutes, a wave of arteriolar vasodilation—the "flare"—spreads to adjacent skin. In the area of initial erythema, increased venular permeability leads to plasma extravasation and edema—the "wheal". Hyperalgesia, the increased sensitivity to innocuous stimuli, occurs in the area of the flare. These characteristic tissue responses to skin injury are mediated by IL-1. Upon epidermal disruption, IL-1 is released immediately from resident KCs and from neutrophils that have extravasated into the clot after severance of dermal blood vessels.

IL-1 participates in the formation of eicosanoids, metabolites with potent proinflammatory characteristics. The eicosanoids, which include the prostanoids and the leukotrienes, are oxygenated products of arachidonic acid metabolism. IL-1 induces the expression of phospholipase A2, which catalyzes the release of arachidonic acid from the cell membrane. Arachidonic acid is subsequently metabolized via two pathways, the cyclooxygenase (COX) pathway and the lipoxygenase (LO) pathway. IL-1 induces the enzyme cyclooxygenase-2, which metabolizes arachidonic acid into Prostaglandin E2 (PGE2). IL-1 also increases the release of Leukotriene B4 (LTB4), a product of the LO pathway. PGE2 and LTB4 are mediators of the "wheal and flare" response to injury. Intradermal injections of PGE2 into human skin cause erythema (Flower, Harvey, & Kingston, 1976) and edema (Crunkhorn, & Willis, 1971). PGE2 can amplify the vascular permeability changes induced by other mediators (Basran, Morley, Paul, & Turners-Warsick, 1982; Crunkhorn et al., 1971) via a direct effect on blood vessels. The presence
of PGE\textsubscript{2} and IL-1 at the peripheral ends of nociceptive neurons can lead to hyperalgesia (Dray, Urban, & Dickerson, 1994). \textit{In vitro}, LTB\textsubscript{4} is a potent, although nonspecific, chemoattractant (Ford-Hutchinson, Bray, Doig, Shipley, & Smith, 1980). In neutrophils, LTB\textsubscript{4} causes degranulation (Showell, Naccache, Borgeat, Picard, Valerand, Becker, & Sha'afi, 1982), cation fluxes (Molski, Naccache, Borgeat, & Sha'afi, 1981) and enhanced binding to endothelial cells (Bray, Ford-Hutchinson, & Smith, 1981). Intraepidermal injection of LTB\textsubscript{4} elicits a transient wheal and flare reaction with accumulation of neutrophils perivascularly (Soter, Lewis, Corey, & Austen, 1987).

\textit{Induction of leukocyte infiltration}

Emigration of leukocytes from the circulation into the injured tissues constitutes the hallmark of the inflammatory response. After acute injury, extravasated neutrophils and activated KCs release IL-1. IL-1 promotes leukocyte infiltration via two events: up-regulation of vascular adhesion molecules and induction of chemotactic cytokines (chemokines). Upon interaction with receptors on endothelial cells, IL-1 induces Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1, or E-selectin) (Groves, Ross, Barker, Ross, Camp, & MacDonald, 1992). ELAM-1, expressed on the surface of endothelial cells, binds to carbohydrate ligands on the surface of circulating neutrophils and monocytes. This low affinity binding leads to leukocyte rolling along the vessel walls (Borregaard, Kjeldsen, Sengelov, Diamon, Springer, Anderson, Kishimoto, & Bainton, 1994; Carlos, & Harlan, 1994). Autocrine and juxtacrine effects of IL-1 on KCs lead to production of chemokines. In addition, IL-1 induces expression of Intercellular Adhesion Molecule-1 (ICAM-1) on endothelial cells (Dustin, Rothlein, & Bhan, 1986).
Chemokines stimulate adherent leukocytes to express integrins that bind with high affinity to endothelial ICAM-1. High affinity binding leads to firm adhesion, or sticking. This is followed by transendothelial migration. The chemotactic gradient established between the tissue and the circulation is the driving force for transendothelial migration and for migration of neutrophils and monocytes toward the site of injury.

There are other agents in the wound that participate in cell recruitment. For example, bacterial products like LPS, components of the complement cascade like C5a, and products of arachidonic acid metabolism like LTB4 all exhibit chemoattractant properties to leukocytes and support their rapid trafficking. However, these factors do not act in a leukocyte subtype-specific manner. Thus, the actions of these factors may not explain the recruitment of different types of leukocytes at different time points during inflammation. Sequential expression of chemokines at the wound site allows for such timely and differentially regulated leukocyte recruitment. Initial extravasation and migration of neutrophils occurs via platelet-derived Neutrophil-activating Peptide-2 (NAP-2) and endothelial cell-derived Growth-related Oncogene α (GRO-α). Infiltrated neutrophils and monocytes in the provisional matrix of the wound produce IL-8 and GRO-α, both chemoattractants for neutrophils (Gillitzer, & Goebeler, 2001). Activated KCs also produce IL-8 and GRO-α (Venner, Sauder, Feliciani, & McKenzie, 1995). This serves to stimulate further accumulation of neutrophils, which are the predominant cells at day 1. In mice, Cytokine-induced Neutrophil Chemoattractant (CINC) and Macrophage Inflammatory Protein-2 (MIP-2) are homologs of GRO. At later days, Monocyte Chemoattractant Protein-1 (MCP-1) is expressed by proliferating KCs at the
wound edge, endothelial cells, and macrophages (Barker, Jones, Swenson, Sarma, Mitra, Ward, Johnson, Fantone, Dixit, & Nickoloff, 1991; Gillitzer et al., 2001; Gillitzer, Wolff, Tong, Muller, Yoshimura, Hartmann, Stingl, & Berger, 1993). MCP-1 is a potent chemoattractant for monocytes, which become the predominant cells after day 2. In murine cutaneous wound healing, other macrophage-specific chemokines include Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) and Macrophage Inflammatory Protein-1α (MIP-1α) (DiPietro, Burdick, Low, Kunkel, & Strieter, 1998; Frank, Kämpfe, Weltzer, Stallmeyer, & Pfeilschifter, 2000; Jackman, Yoak, Keerthy, & Beaver, 2000; Weltzer, Kämpfe, Stallmeyer, Pfeilschifter, & Frank, 2000).

Enhancement of antibacterial mechanisms

The first step in the inflammatory response to invading pathogenic organisms is accomplished by IL-1-mediated recruitment of leukocytes into the wound site. However, infiltrated leukocytes must become activated in order to eradicate infection. IL-1 is involved in the activation of neutrophils and macrophages for increased bacterial killing.

Neutrophils are the "first line of defense" against infectious agents or "nonself" substances that penetrate the epidermal barrier. As neutrophils phagocytose opsonized microorganisms, microbicidal mechanisms are activated. Neutrophils have two microbicidal mechanisms: (1) the oxidative burst, which results in production of cytotoxic reactive oxygen species, and (2) degranulation, which results in release of proteolytic enzymes and antimicrobial polypeptides from the granules into the phagosome. While activation of neutrophils triggers the immediate expression of
microbicidal activity, priming stimuli amplify the magnitude of the response. IL-1, released by monocytes and also released by neutrophils in an autocrine manner, contributes to the microbicidal activity of neutrophils by priming the release of various enzymes involved in the oxidative burst, like NADPH oxidase and myeloperoxidase (MPO) (Dularay, Elson, Clements-Jewery, Damais, & Lando, 1990). LTB₄, which is also induced by IL-1 (Dinarello, 1996), causes degranulation of neutrophils (Showell et al., 1982). These actions have physiological implications because priming of neutrophils by nontoxic doses of IL-1 appears to be responsible for the increased resistance of mice to bacterial infection (Cross, Sadoff, Kelly, Bernton, & Gemski, 1989).

When monocytes are recruited to the site of injury, they differentiate into macrophages, which then need to become activated into reparative, or inflammatory, macrophages. Macrophages phagocytose and digest pathogenic organisms and effete neutrophils. Activated macrophages also release cytokines and growth factors necessary for initiation and propagation of new tissue formation. IL-1 released by KCs, neutrophils, and by injured endothelial cells within early wounds is capable of activating macrophages (Ford, Hoffman, Wing, Magee, McIntyre, & Simmons, 1989). In addition, activated macrophages within the early wound produce IL-1 that acts in a paracrine fashion to activate incoming monocytes (Ford et al., 1989).

**Promotion of wound re-epithelialization**

Re-epithelialization of wounds begins within hours after injury and occurs simultaneously with inflammation. It involves migration of KCs at the wound margins, proliferation of KCs behind the actively migrating cells, and degradation of the
extracellular matrix. IL-1 released during the inflammatory phase may play a role in re-epithelialization. It has been demonstrated that IL-1 is mitogenic for KCs (Kupper et al., 1986), and it induces KC migration on collagen (Chen, Lapiere, Sauder, Peavy, & Woodley, 1995). IL-1 produced by KCs has an autocrine effect (Kupper et al., 1986), stimulating further production of IL-1 and other cytokines. IL-1 may promote KC proliferation via the release of LTB₄ from extravasated neutrophils. It has been shown that topical application of LTB₄ leads to epidermal hyperproliferation (Bauer, Van de Kerhof, & De Grood, 1986), probably by a direct mitogenic effect observed in cultured human KCs (Kragballe, Desharlais, & Voorhees, 1985). In addition, IL-1 and TNF-α induce the production of Matrix Metalloproteinases (MMPs), such as collagenase-1 and stromelysin (Madlener, Mauch, Conca, Brauchle, Parks, & Werner, 1996; Tracey, Vlassara, & Cerami, 1989). MMPs cleave proteins of the extracellular matrix, thereby releasing the migrating KCs at the wound edge from their attachment to the basal lamina and dermal substratum. IL-1 may also contribute to re-epithelialization via induction of KGF-1 in dermal fibroblasts (Brauchle et al., 1994; Chedid et al., 1994; Mass-Szabowski, Shimotoyodome, & Fusenig, 1999). The role of KGF-1 is described in the following section.
1.3 Role of KGF-1 in re-epithelialization

While all the inflammatory events described above are proceeding deep within the wound, re-epithelialization is taking place at the wound surface. There is proliferation and migration of epithelial cells from the wound edge across the defect. Re-epithelialization is stimulated by local release of fibroblast growth factors, particularly KGF-1. KGF-1 is a potent chemoattractant and mitogen for KCs (Rubin, Osada, Finch, Taylor, Rudikoff, & Aaronson, 1989), and it is constitutively expressed in normal human skin (Finch, Rubin, Miki, Ron, & Aaronson, 1989). Furthermore, exogenous stimuli to the skin can induce KGF-1 production. During wound healing in mice, expression of KGF-1 mRNA is considerably increased within 12 hours and up to 7 days after injury, being the most predominant fibroblast growth factor induced (Werner et al., 1992). During re-epithelialization of normal human skin, KGF-1 transcript levels are increased during the early postwounding period and remain elevated until the 8th day after biopsy. This increase is accompanied by an elevation in protein expression in the same tissues (Marchese et al., 1995). These findings strongly suggest that the rapid induction of KGF-1 gene expression and its persistance several days after injury underlie the migration and proliferation of epithelial cells during re-epithelialization. Other studies found that exogenous KGF-1 promoted re-epithelialization of partial and full thickness wounds (Pierce, Yanagihara, Klopchin, Danilenko, Hsu, Kenney, & Morris, 1994; Staiano-Coico, Kruger, Rubin, D'Limi, Vallar, Valentino, Fahey III, Hawes, Kingston, Maidden, Mathwich, Gottlieb, & Aaronson, 1993). Further in vivo studies underscored the importance of KGF-1 in wound repair. Werner et al. (Werner, Smola, Liao, Longaker,
Krieg, Hofschneider, & Williams, 1994) assessed wound healing in transgenic mice expressing a dominant negative receptor for KGF-1 in KCs only. Wound re-epithelialization in transgenic mice was significantly delayed due to a reduced proliferation rate of epidermal KCs at the wound edge. Therefore, KGF-1 is a critical stimulatory signal in the prompt re-establishment of tissue integrity.

Several mediators of KGF-1 expression have been proposed. *In vitro* studies by Brauchle *et al.* found that KGF-1 gene expression in murine and human fibroblasts is strongly induced by IL-1β, TNF-α, and IL-6 (Brauchle *et al.*, 1994). Chedid *et al.* found increased KGF-1 gene transcription by IL-1 and a corresponding increase in mitogenically active KGF-1 protein in conditioned medium obtained from IL-1-treated fibroblasts (Chedid *et al.*, 1994). These studies suggest that production of proinflammatory cytokines within the early wound is an important signal not only for decontamination and debridement of the area, but also for wound re-epithelialization via KGF-1 induction. As previously described, IL-1 also contributes to re-epithelialization via the induction of MMPs, which detach the KCs from the basal lamina and thereby promote their migration beyond the wound margins.

1.4 The architecture of the wound favors the process of inflammation and repair

There are multiple tissue compartments in a wound, each with effector cells that play critical roles during wound healing. At the epidermal margins, KCs become activated, alert cells from the nearby blood vessels to infiltrate the area, and start migrating and proliferating in order to bridge the defect. At the wound clot, neutrophils and
macrophages destroy invading microorganisms and debris. At the dermal margins, macrophages eradicate penetrated pathogens and remove damaged tissue. Macrophages also activate fibroblasts and promote granulation tissue formation. Within the connective tissue, fibroblasts secrete growth factors that promote surface re-epithelialization and synthesize proteins for the new extracellular matrix. The architecture of the wound, with close apposition of the different tissue compartments, facilitates interactions among effector cells that start immediately upon skin injury. These interactions are established by cytokines like IL-1, which is released very early after injury. In the wound, IL-1 mediates inflammation, proliferation, and eventual remodeling. IL-1 is produced by infiltrating leukocytes as well as by resident tissue cells in the epidermis and dermis. This suggests that IL-1 serves as the "connecting signal" among effector cells at different tissue compartments of the wound, taking advantage of the specialized architecture of the wound to exert its decisive role in tissue repair. Figure 1.1 illustrates a model for the multiple interactions established by IL-1 within the architecture of the wound. Each interaction is explained as follows:

(1) IL-1 released from activated KCs diffuses from the epidermis into the dermis to effect changes in blood vessels. Anatomically, these events occur at the tip of the dermal papillae where the space between KCs and capillary loops is very small. IL-1 acts on endothelial cells to increase surface expression of adhesion molecules, which will favor the rolling and sticking of leukocytes to the vessel wall.
(2, 3) IL-1 also induces the expression of adhesion molecules on the surface of monocytes and neutrophils which will stimulate their migration across the vessel wall.

(4) Autocrine stimulation of KCs by IL-1 leads to the induction of chemokine synthesis and release from KCs. Thus, KCs contribute to establish a chemokine gradient that will favor the transendothelial migration of leukocytes from the blood vessel toward the surface of the wound. Autocrine stimulation by IL-1 also induces proliferation and migration of KCs, thus promoting re-epithelialization.

(5) IL-1 released by activated KCs primes neutrophils infiltrated in the dermis for enhanced phagocytosis and killing of bacteria at the wound margins.

(6) IL-1 released by neutrophils at the blood clot stimulates nearby KCs for migration, proliferation, and synthesis of chemokines. In turn, IL-1 released by KCs primes neutrophils for increased bacterial killing.

(7) High amounts of IL-1 produced by extravasated neutrophils activate incoming monocytes into macrophages. IL-1 also induces the release of PGE$_2$ and LTB$_4$, which increase vascular permeability and vasodilation. This results in local edema, erythema, and hyperalgesia.

(8) IL-1 released by macrophages at the blood clot stimulates nearby KCs for migration, proliferation and synthesis of chemokines. IL-1 released by KCs activates macrophages for increased phagocytosis and release of cytokines and growth factors.

(9) IL-1 released by macrophages may help in the recruitment of monocytes from blood vessels and also may activate them into macrophages.
Macrophages in the dermis release IL-1 to stimulate KC proliferation and migration. IL-1 released by KCs activates dermal macrophages for phagocytosis and for the release of cytokines and growth factors.

IL-1 is also produced by fibroblasts within the connective tissue. Here, it serves to activate macrophages within the dermal margins to eradicate any remaining pathogens that may have penetrated the tissues beyond the initial scab. Activated macrophages in the dermal margins release collagenases that help remodel the injured tissues. IL-1 also stimulates macrophages to produce growth factors important in fibroplasia and angiogenesis. IL-1 produced by macrophages in the dermis stimulates the fibroblasts to synthesize collagen, fibronectin, and proteoglycans that will make up the new extracellular matrix. Macrophage-derived IL-1 also induces fibroblasts to release KGF-1.

KCs and fibroblasts interact via a double paracrine mechanism. Activated KCs release IL-1, which diffuses into the dermis (black arrow). IL-1 induces the release of KGF-1 by fibroblasts (white arrowhead). KGF-1 stimulates KC proliferation and migration, thus promoting re-epithelialization.

As demonstrated in the above model, the effects of IL-1 at the wound site are multiple and occur at different compartments. This suggests that any molecular study that aims to characterize the expression of IL-1 during wound healing should evaluate not only the total amount of IL-1 mRNA expressed from whole excised tissues, but also the expression of IL-1 from cells at different tissue compartments during the course of healing.
1.5 Stress, inflammation, and wound healing

After discussing the essential role of inflammatory mediators within the early wound, it would be reasonable to suspect that conditions affecting the host inflammatory response to injury could in turn compromise wound repair. An example of such a condition is stress. Two major neuroendocrine systems, the sympathetic nervous system (SNS), and the hypothalamic-pituitary-adrenal (HPA) axis mediate many of the somatic effects of stress. Activation of the SNS results in the production of plasma catecholamines. Catecholamines are known for their contribution to local tissue edema, increasing endothelial cell wall adhesion (Koopman, 1995). Catecholamines have also been shown to have an inhibitory effect on epidermal cell migration (Donaldson, & Mahan, 1984). When used in concentrations comparable to the levels found in the sera of postsurgical stress patients, catecholamines inhibit fibroblast proliferation (Saito, Tazawa, Yokoyama, & Saito, 1997). This suggests that increased levels of catecholamines at the wound site, either arising from the peripheral circulation or released from local sympathetic nerve endings, could alter wound healing processes by increasing edema, retarding re-epithelialization, and decreasing fibroplasia. On the other hand, pathophysiological activation of the HPA axis results in production of glucocorticoid hormones (GCs) (Sundar, Cierpial, Kilts, Ritchie, & Weiss, 1990). GCs have been more extensively studied due to their anti-inflammatory as well as immunosuppressive actions. Incidentally, it is the potent anti-inflammatory effect of GCs that accounts for their profound inhibitory effect on wound healing. After administration of exogenous GCs, neutrophil (Clark, Gallin, & Fauci, 1979) and monocyte (Norris,
Capin, & Weston, 1982) recruitment is decreased, and macrophage phagocytosis and bacterial killing are suppressed (Fauci, Dale, & Balow, 1976). This results in delayed wound debridement, including foreign body and bacteria elimination. In the epidermis, KC proliferation is inhibited (Edwards, & Dunphy, 1958), producing a thinned, abnormal epidermis. Wound strength, epithelialization, and closure of open wounds are impaired. As described previously, most of these processes are primarily mediated by proinflammatory cytokines like IL-1 and by growth factors like KGF-1. Indeed, one of the proposed mechanisms of action for GCs is inhibition of cytokine gene expression and/or protein production.

1.6 Effect of glucocorticoids on IL-1 gene expression

GCs are known to suppress expression of IL-1. This was originally reported as a reduced production of IL-1 by PMA-stimulated murine peritoneal macrophages under hydrocortisone (Snyder, & Unanue, 1982). Recent studies by Amano et al. (Amano, Lee, & Allison, 1993) demonstrated that cells of the human pro-monocytic line U937 activated by PMA and LPS had lower IL-1α and -β mRNA levels under dexamethasone (DEX), an effect that was abrogated by preincubation with the steroid receptor antagonist RU486. Similarly, LPS-stimulated human monocytes treated with DEX had decreased expression of both IL-1α and IL-1β mRNA, decreased cell-associated levels of IL-1α and IL-1β, and decreased amounts of secreted IL-1β in culture supernatants. Interestingly, DEX significantly inhibited IL-1-induced IL-6 mRNA expression in synoviocytes of patients with rheumatoid arthritis (Amano et al., 1993). This suggests
that down-regulated IL-1 gene expression caused by GCs will result in reduced expression of other cytokine genes known to be induced by IL-1, thereby suppressing the cascade of mediators elicited by IL-1 during inflammation. In addition to circulating mononuclear cells, resident skin cells can also be a target for GC-mediated cytokine regulation. Lee et al. found that hydrocortisone decreases the expression of IL-1α mRNA in human KCs (Lee et al., 1991). Although no studies have specifically addressed the effect of GCs on IL-1β expression in dermal fibroblasts, it has been demonstrated that in lung fibroblasts GCs inhibit IL-1- or TNF-induced expression of IL-6 and IL-8 (Monick, Aksamit, Geist, & Hunninghake, 1994; Zitnik, Whiting, & Elias, 1994). GCs induce degradation of AU-rich sequences in 3' untranslated regions of IL-6 and IL-8 (Peppel, Vinci, & Baglioni, 1991), resulting in mRNA instability. This motif is also present in IL-1β transcripts, which are thereby destabilized by GCs (Amano et al., 1993; Lee, Tsou, Chan, Thomas, Petrie, Eugui, & Allison, 1988). This suggests that GCs could also down-regulate IL-1β gene expression via post-transcriptional degradation. Therefore, GCs can down-regulate gene expression of IL-1 in peripheral and local cell compartments, both of which participate and interact decisively during wound healing.

1.7 Effect of glucocorticoids on KGF-1 expression and re-epithelialization

Recent studies attempted to elucidate both in vivo and in vitro the relationship among exogenously-administered GCs, cytokine gene expression, and impaired wound healing. Presumably, since GCs decrease the expression of proinflammatory cytokines important in the early phases of wound healing, it is possible that expression of growth factors
important in re-epithelialization is also downregulated by GCs either directly or indirectly. Brauchle et al. found a dramatically delayed re-epithelialization in histological wound sections of DEX-treated mice (Brauchle, Fässler, & Werner, 1995). They also found that the typical induction of epidermal KGF-1 mRNA after injury is reduced in DEX-treated mice. Cultured murine fibroblasts treated with DEX showed an inhibition of KGF-1 mRNA levels. Chedid et al. showed that addition of DEX significantly reduces the level of constitutively produced KGF-1 mRNA, protein and bioactivity in conditioned medium from human dermal fibroblasts (Chedid, Hoyle, Csaky, & Rubin, 1996). These results suggest that down-regulation of KGF-1 expression by GCs may be due to a direct effect on mesenchymal cells. However, it may also be partially explained by an inhibition of KGF-inducing factors in the wound, such as IL-1β, which is known to be down-regulated by GCs. Indeed, it was found that addition of IL-1β to DEX-treated fibroblasts reversed the inhibitory effect of GCs on KGF-1 expression. Furthermore, later studies by Hübner et al. (Hübner et al., 1996) demonstrated that induction of IL-1α and IL-1β mRNA and protein after injury was significantly reduced in wounds of GC-treated mice, which also presented reduced infiltration of inflammatory cells, delayed wound re-epithelialization, and impaired granulation tissue formation. Therefore, elevated levels of exogenous GCs can dysregulate the profile of proinflammatory cytokines and growth factors that interact locally for a rapid and effective restoration of skin integrity. However, the effect of endogenously elevated GC levels (such as those elicited by stress) upon proinflammatory cytokine gene expression and wound healing has not been investigated.
1.8 Human models for the study of stress and wound healing

Several models have been used to study the effects of stress on wound healing in humans. The first model involved female caregivers of Alzheimer’s disease (AD) patients, compared to age and socio-economically matched noncaregiver controls (Kiecolt-Glaser, Marucha, Malarkey, Mercado, & Glaser, 1995). It was found that caregivers are more psychologically stressed than controls. Caregivers took 9 days longer to heal a full-thickness skin wound, with the largest differences in wound size between groups appearing in the first 2 weeks after wounding. These results suggest that stress has more impact on the earlier phases of wound repair, specifically the inflammatory phase, resulting in an overall delayed re-epithelialization. Further support for this theory was provided by Northern blot analyses of whole blood, in which IL-1β mRNA responses to LPS stimulation were 56% lower in caregivers as compared to controls. Moreover, caregivers that showed the lowest IL-1β mRNA responses to LPS stimulation took the longest time to heal than the rest of the study subjects.
In another model, Marucha et al. (1998) placed wounds on the hard palate of dental students during summer vacation and again 3 days before the first major examination of the term. Thus, each student served as her or his own control. It was found that dental students experiencing examination stress had a 40% delay in mucosal wound healing compared to healing in the same students during vacation. Whole blood IL-1β responses to LPS declined by 68% during examinations. Moreover, no student healed as rapidly or produced as much IL-1β mRNA during examinations as during vacation. Therefore, these studies in humans provide evidence of a strong interaction among stress, decreased IL-1β mRNA responses, and delayed wound healing.

Further investigation is necessary to elucidate the precise molecular mechanisms among stress, inflammation, and wound healing. Human models are not ideally suitable for this purpose due to a number of limitations. For example, sample sizes are usually small since it is necessary to match stressed and non-stressed individuals according to a variety of factors that may affect perception of stress and/or physiological responses. Subject numbers are typically reduced along the course of the study due to subjects’ noncompliance. In human studies, the number of wounds and the methods for wound sampling are limited, hindering time-course studies and the variety of assays performed. Furthermore, in vivo manipulation of cellular and molecular processes with the use of pharmacological agents is limited due to ethical concerns. This is of outmost importance for any controlled mechanistic study. Animal models greatly overcome such limitations.
1.9 Murine model of stress for the study of immune responses

Sheridan and co-workers have developed a murine model of stress based on physical restraint (RST). They have used this model extensively for the investigation of stress-induced modulatory mechanisms in viral pathogenesis and immunity. The ability of mice to roam in their cages is restricted when they are placed inside conical tubes for 12-hour cycles before and during the course of infection. RST mice infected with virus have a significantly suppressed lymphadenopathy in the draining lymph nodes and a reduced mononuclear cell infiltration in target tissues (Bonneau, Sheridan, Feng, & Glaser, 1991; Dobbs, Vasquez, Glaser, & Sheridan, 1993; Feng, Pagniano, Tovar, Bonneau, Glaser, & Sheridan, 1991; Sheridan, Feng, Bonneau, Allen, Huneycutt, & Glaser, 1991). These findings coincided with elevated levels of plasma corticosterone in RST mice (Dobbs et al., 1993; Sheridan et al., 1991), suggesting a possible role for endogenous GCs in inflammatory cell trafficking. Indeed, treatment of infected, RST mice with a GC receptor antagonist (RU486) restored cellularity to the draining lymph nodes and enhanced accumulation of mononuclear cells in target tissues (Dobbs et al., 1993; Hermann, Beck, & Sheridan, 1995). Based on such findings, it could be speculated that the stress-induced increase in plasma corticosterone also compromises cell trafficking and infiltration to other sites of inflammation, such as a skin wound. If the tissue inflammatory reaction to injury were depressed, this would jeopardize efficient wound healing. In addition, RST-induced elevation in both GC and catecholamine levels are responsible for suppression of cytotoxic T cell activation in RST mice (Dobbs et al., 1993). This suggests that elevation in catecholamines as a result of stress can also affect
immune processes in this murine model that could be important locally and systemically after an injurious stimulus. As previously mentioned, catecholamines have been shown to inhibit migration of epidermal cells (Donaldson et al., 1984), which is important in re-epithelialization. Thus, the murine model of RST stress is characterized by predictable neuroendocrine responses, which should be evaluated for their potential modulatory role on important biological events such as wound healing.

1.10 Cutaneous wound healing in the murine model of stress

Padgett and co-workers (1998) used the murine model of RST stress to study cutaneous wound healing (Padgett et al., 1998). They showed that RST mice take an average of 3 days longer to heal and have reduced wound cellularity than control animals. Confirming previous reports on this model (Dobbs et al., 1993; Sheridan et al., 1991), plasma corticosterone levels of RST mice were elevated over a sustained period of time. Moreover, treatment of RST mice with GC receptor antagonist RU40555 restored wound healing kinetics and wound cellularity. These results suggest that elevated plasma corticosterone may decrease leukocyte trafficking and recruitment to wounds in RST mice. Consistent with this idea, previous studies demonstrated that treatment of virally infected, RST mice with GC receptor antagonist restored cellularity to the draining lymph nodes and enhanced accumulation of mononuclear cells in target tissues (Dobbs et al., 1993; Hermann et al., 1995). If the inflammatory response to cutaneous injury is depressed, overlapping events such as re-epithelialization and granulation tissue formation could be compromised. Furthermore, Rojas et al. (2001) demonstrated that
RST caused a 2 to 5-log increase in opportunistic bacteria in the wound as compared to control mice (Rojas, Padgett, Sheridan, & Marucha, 2000). Treatment of RST mice with GC receptor antagonist RU486 resulted in a nearly one-log reduction in opportunistic bacteria as compared to RST mice treated with vehicle. Therefore, another consequence of a depressed inflammatory response in RST mice is impairment of bacterial clearance during wound healing. Molecular studies are needed to investigate if the effects of RST on wound healing are associated with changes in cytokine gene expression similar to those found in the human studies (Kiecolt-Glaser et al., 1995; Marucha, Kiecolt-Glaser, & Favagehi, 1998).
There are multiple advantages for studying cutaneous wound healing in the murine model. Mice are relatively inexpensive to purchase and can be easily and economically maintained. The SKH-1 mouse, used by Padgett et al. (1998) and Rojas et al. (2000), is immunologically intact although hairless, which facilitates wound healing evaluation and tissue handling for a variety of assays. Infliction of a wound allows for measurement of wound size across time (determining rate of healing). The period of healing of a standardized 3.5mm punch biopsy is from 9 to 16 days, which is a relatively short period as compared to that of humans (in our caregiver study, from 24 to 68 days). In addition, it has been demonstrated that the RST stress paradigm can be uniformly applied to animals, with predictable neuroendocrinological changes (Bonneau et al., 1991; Dobbs et al., 1993; Feng et al., 1991; Sheridan et al., 1991). Pharmacological agents (i.e., GC receptor antagonists) can be administered to study hormone effects on wound healing. Moreover, wounds and surrounding tissues can be excised at given time points for molecular biology studies, such as characterization of gene expression.

1.11 A proposed mechanism for neuroendocrine influences on wound healing

In summary, several pieces of information can be gathered into a hypothesis concerning the neuroendocrine mechanisms of wound healing. First, stress activates the HPA axis, resulting in elevation of serum GC levels. Upon the action of GCs, infiltrating as well as resident cells show a lessened inflammatory response to tissue injury, characterized by down-regulated gene expression of proinflammatory cytokines IL-1α and IL-1-β within the wound. Reduced levels of these important mediators of
inflammation will result in decreased recruitment and activation of leukocytes necessary for debridement of the early wound. An attenuated inflammatory response will delay the transition to other phases such as granulation tissue formation. Gene expression of proliferation mediators such as KGF-1 may also be down-regulated. As a result, there is slower re-epithelialization of the wound surface. The above explanation is only hypothetical, since it is yet uncertain if elevated levels of GCs in restrained mice could be associated with down-regulated gene expression at the wound site, and if this could in turn contribute to the observed delay in wound healing.

1.12 Purpose of the present studies

The present studies were intended to investigate gene expression of proinflammatory cytokines and growth factors in full thickness excisional wounds of control versus RST mice during the inflammatory and proliferative stages of healing. To this end, the following specific aims were formulated:

♦ Characterize the kinetics of IL-1α, IL-1β, and KGF-1 gene expression in homogenized whole wounds from control versus RST mice at days 1, 3, and 5 of healing.

♦ Determine the effect of GC receptor blockade on IL-1α, IL-1β, and KGF-1 gene expression in early wounds from control versus RST mice.

♦ Characterize the distribution and cellular localization of IL-1α, IL-1β, and KGF-1 mRNA in wounds from control versus RST mice at days 1, 3, and 5 of healing.
Characterize histological changes in the quality of the healing tissues that could be associated with stress-induced alterations in proinflammatory cytokine and growth factor gene expression.
Figure 1.1. Model for the multiple interactions established by IL-1 within the architecture of the wound. Adapted from Gillitzer et al., (2001).
CHAPTER 2

ALTERED KINETICS OF IL-1α, IL-1β, AND KGF-1 GENE EXPRESSION
IN EARLY WOUNDS OF RESTRAINED MICE

2.1 Introduction

A skin wound elicits an inflammatory response upon which later regenerative processes such as tissue proliferation and remodeling will depend. Extraneous conditions that are known to alter the inflammatory response to injury, such as stress, could in turn compromise wound repair. Indeed, our previous studies suggested that there are interactions among stress, inflammation and wound healing. Chronically stressed individuals (caregivers of dementia patients) took 24% longer to heal a cutaneous wound as compared to matched controls (Kiecolt-Glaser et al., 1995). Marucha et al. (Marucha et al., 1998) demonstrated that dental students experiencing examination stress had a 40% delay in mucosal wound healing as compared to healing during vacation. In both studies stress was related to significant decreases in whole blood Interleukin-1β (IL-1β) responses to lipopolysaccharide (LPS).
Proinflammatory cytokines IL-1α and IL-1β modulate the expression of chemokines and adhesion molecules necessary for continued recruitment of inflammatory cells (Bickel, Nöthen, Freiburghaus, & Shire, 1996). They may also contribute to simultaneous repair of the wound surface by inducing expression of keratinocyte growth factor-1 (KGF-1 or FGF-7), a mitogen for keratinocytes which is critical during re-epithelialization (Pierce et al., 1994; Staiano-Coico et al., 1993; Werner et al., 1994). Therefore, dysregulation of IL-1 and KGF expression may be an important molecular mechanism for the delaying effect of stress on wound healing.

Stress disturbs the neuroendocrine equilibrium of the host by activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in elevation of circulating glucocorticoid hormones (GCs). GCs are known to suppress in vitro expression of IL-1α and IL-1β (Amano et al., 1993; Kern, Lamb, Reed, Daniele, & Nowell, 1988; Knudsen, Dinarello, & Strom, 1987; Lee et al., 1991; Lee et al., 1988; Lew, Oppenheim, & Matsushima, 1988; Snyder et al., 1982). Moreover, several studies have demonstrated that the typical induction of IL-1α, IL-1β, and KGF-1 mRNA is reduced in wounds of dexamethasone-treated mice, which also present delayed wound re-epithelialization (Brauchle et al., 1995; Hübner et al., 1996). Therefore, exogenous GCs can dysregulate the profile of proinflammatory cytokines and growth factors that interact locally for a timely restoration of skin integrity.

Recent studies in a murine model of wound healing have suggested that stress-induced disruption of neuroendocrine homeostasis may have a modulatory effect on wound inflammation and re-epithelialization. Padgett et al. (Padgett et al., 1998) showed
that repeated cycles of restraint stress (RST) resulted in elevated levels of plasma corticosterone. RST mice had reduced wound cellularity and delayed wound closure as compared to controls. Glucocorticoid receptor blockade restored wound cellularity and wound healing kinetics in RST mice. However, the effect of a stress-induced elevation of endogenous GCs upon IL-1 and KGF-1 gene expression has not been fully investigated in the context of wound healing.

The present study was intended to characterize neuroendocrine regulation of proinflammatory cytokine and growth factor gene expression in the murine model of wound healing. It was hypothesized that stress-induced GCs down-regulate the inflammatory response to injury, which could in turn impair important signals involved in re-epithelialization. To test this hypothesis, the kinetics of IL-1α, IL-1β, and KGF-1 mRNA expression were investigated in full thickness excisional wounds of RST and control mice. In addition, RU486 was administered to block the action of stress-induced GC at the receptor level and to investigate the effect of endogenous GCs on cutaneous cytokine and growth factor mRNA expression.
2.2 Materials and Methods

Animals

Virus-antibody-free SKH-1 female mice, 6-8 weeks of age, were obtained from Charles River, Inc. (Wilmington, MA) and housed in a female-only room. Prior to the start of the experiment, mice were allowed to acclimate to their surroundings for 7-10 days. Mice were housed five per cage and provided free access to food and water. All animal experiments were carried out in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). This facility is maintained on a 12-h light/dark cycle (lights on at 6:00 AM).

Restraint Stress Paradigm

Mice were placed in well-ventilated, loose-fitting 50 ml conical centrifuge tubes for 3 cycles prior to wounding and for 5 subsequent cycles. Restraint started at 6:00PM (lights out at 6:00PM) and ended at 9:00AM (lights on at 6:00AM). While in the tubes, mice did not have access to food or water. Therefore, non-restrained mice were food and water deprived (FWD) during the same time period, although they were free to roam in their cages (Padgett et al., 1998).

Measurement of plasma corticosterone

Blood samples were obtained via intranasal bleeding prior to and after RST or FWD. All samples were collected at 10:00 am to guard against circadian rhythm fluctuations in serum corticosterone levels. Approximately 50-100μl of whole blood was collected from
each mouse at baseline, at day 0, and at day 5. Serum corticosterone levels were determined by radioimmunoassay (ICN, Costa Mesa, CA) using a standard curve and expressed in ng/ml.

**Treatment with RU486**

Beginning 1 day prior to the initiation of RST, mice were injected subcutaneously with 25 mg/kg of the type II GC receptor antagonist RU486 (Sigma, St. Louis, MO) dissolved in polyethylene glycol 400 (PEG 400, Sigma, St. Louis, MO) or control vehicle. All injections were done each day of the RST paradigm approximately 2h prior to the beginning of each cycle.

**Measurement of wound size**

Wounds from individual mice were photographed at day 1, 3, and 5 after wounding. Photographs of the wound site were taken with a standard-size dot placed beside the wound. Photographic slides were digitized by a scanner and analyzed by photoplanimetry (Marucha et al., 1998). An investigator blinded to treatment group and day of photograph measured wound size. Wound size was expressed as the percentage of the wound area (each day after wounding) compared to the initial area of the wound.
**Wounding and harvesting**

Mice were anesthetized with a 0.3 ml intraperitoneal injection of 0.44 mg/ml Rompum (Haver-Lockhart, Shawnee, KS) and 7.8 mg/ml Ketaset (Aveco, Fort Dodge, IA). After cleansing the area, a sterile 3.5 mm punch (Premier Medical Products, King of Prussia, PA) was used to create two full-thickness wounds on the dorsal side of each mouse, just below the shoulder blades. Wounds placed at this location cannot be reached by the animals for self-licking. After days 1, 3, and 5, mice were euthanized with an overdose of anesthesia and wounds were excised with a 6 mm punch. Control, nonwounded skin was excised in the same fashion. Harvested tissues were immediately submerged in TRIzol reagent (Life Technologies, Rockville, MD) and frozen at -80°C.

**Extraction of total RNA and mRNA selection**

Tissue samples (two wounds per animal) were homogenized with a ground glass mortar and pestle in TRIzol reagent and total RNA was extracted according to manufacturer’s protocol. RNA spectrophotometry readings were done at absorbances of 260 and 280nm, and the 260/280 ratios were determined for nucleic acid purity. Selection of mRNA from 20 μg of total RNA samples was done using magnetic oligo (dT)25 beads and a magnetic particle concentrator (Dynal A.S., Oslo, Norway), according to manufacturer’s protocol.
Reverse Transcription (RT)

Poly(A)-tailed RNA was added to a solution containing oligo-dT primer, dNTP mix, ribonuclease inhibitor Rnasin (Promega, Madison, WI) and reverse transcribed with 15 U of AMV reverse transcriptase (Promega, Madison, WI). The reaction buffer contained 250 mM Tris-HCl, pH 8.3; 250 mM KCl; 50 mM MgCl₂; 50 mM DTT; and 2.5 mM spermidine. The reaction was incubated at 42°C for 60 min. After the reaction, AMV-RT was inactivated by heating to 90°C for 5 min. The resulting single-stranded cDNA was cooled to 4°C.

Competitive Polymerase Chain Reaction (PCR)

Internal standard DNA sequences (competitors) which compete with target cDNA for the same primers during PCR were designed for each gene and constructed according to the PCR MIMIC construction kit (Clontech, Palo Alto, CA). PCR products generated with the competitors differed from the target products in length and were distinguished based on molecular size. Molecular weight correction factors were calculated for each target/competitor pair and used to adjust densitometric ratios between co-amplified competitor and target cDNA.

Table 2.1 shows primer sequences used to amplify each mouse target/competitor gene and the sizes of their respective PCR products: IL-1α (Lomedico, Gubler, Hellman, Dukovich, Giri, Pan, Collier, Semionow, Chua, & Mizel, 1984), IL-1β (Gray, Glaister, Chen, Goeddel, & Pennica, 1986), KGF-1 (Mason, Fuller-Pace, Smith, & Dickson, 1994), and the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (G3PDH)
PCR for each gene was conducted separately in reaction volumes of 25 μl. Reactions contained 10 mM Tris-HCl, 50 mM KCl, 3 mM MgCl₂, 0.2 mM of each dNTP, 0.1-1.4 μM of 5' and 3' primers of each gene and 1 unit of Platinum Taq DNA polymerase (Gibco BRL, Gaithersburg, MD). cDNA was amplified for 35 cycles (Perkin Elmer Gene-Amp PCR 960). Each cycle consisted of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and primer extension at 72°C for 2 min. Before amplification, reactions were incubated at 94°C for 1 min, and after amplification extension was performed at 72°C for 7 min. Samples were electrophoresed in a 1.8% agarose gel stained with ethidium bromide and visualized in an UV transilluminator. Densitometry was done with NIH Image 1.61 (Bethesda, MD). Figure 2.1 depicts an example of a gel for competitive RT-PCR of G3PDH.

Standard cDNA samples containing high amounts of each gene were quantitated using serial 2-fold dilutions of competitor in the presence of a single dilution of the standard cDNA. The concentration of competitor that resulted in a target cDNA/competitor ratio of 1.0 was determined. At this ratio, the concentration of competitor was equivalent to the concentration of the specific cDNA being amplified. By amplifying serial 2-fold dilutions of the standard cDNA with a fixed amount of competitor, a standard curve was generated for each gene. Experimental cDNA samples were also amplified with this fixed competitor concentration. The cDNA/competitor ratios of standard and experimental samples were determined and adjusted by the gene's molecular weight correction factor. The amount of specific cDNA in each experimental sample was determined by polynomial regression analysis using the standard curve.
(Statview 5.0, SAS Institute Inc., Cary, NC). Figure 2.2 depicts an example of a polynomial regression plot for G3PDH standard curve. Attomoles of cytokine or growth factor mRNA for each sample were divided by the corresponding attomoles of G3PDH mRNA and represented as ratios.

**Statistical methods**

Differences in gene expression, wound size, and serum corticosterone levels between groups were analyzed by Student’s *t* test and ANOVA. Differences were considered statistically significant when *p*<0.05. Data were analyzed using statistical program JMP-IN 3.2.1 (SAS Institute Inc., Cary, NC).

**2.3 Results**

**Effects of Restraint Stress on Wound Healing Kinetics**

To evaluate the effects of RST on wound healing kinetics, wounds were photographed at days 1, 3, and 5 and their sizes were determined by photoplanimetry. As shown in Figure 2.3, RST mice had significant delay in wound closure at day 1, day 3, and day 5 of healing as compared to FWD mice (*p*<.001 at day 1, *p*<.0001 at day 3, and *p*<.01 at day 5, *t*-test). The biggest difference in wound size between groups was detected at day 5 (average wound size in the RST group was 1.5-fold larger than the average wound size in the FWD group).
Effects of Restraint Stress on the Kinetics of Cutaneous IL-1α and IL-1β Gene Expression

To assess the effects of RST on mRNA expression of proinflammatory cytokines IL-1α and IL-1β, RNA was isolated from control (non-wounded) skin or from excisional wounds at days 1, 3, and 5 after wounding and competitive RT-PCR was performed. Attomoles of cytokine mRNA were divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. In control skin of FWD and RST mice, IL-1α mRNA was expressed at low levels (Figure 2.4), while IL-1β mRNA was barely detectable (Figure 2.5). Expression of both cytokines was dramatically induced after wounding, particularly for IL-1β mRNA. At day 1, there was no difference in IL-1α mRNA expression between FWD and RST mice. However, IL-1β mRNA expression was significantly lower in RST as compared to FWD mice (p<0.01, t-test). At day 3, mRNA levels of both cytokines decreased with no difference between groups. At day 5, levels of IL-1α and IL-1β mRNA in FWD animals returned to near control levels, while RST mice had significantly elevated mRNA expression compared to FWD (p<0.01 for IL-1α, p<0.001 for IL-1β, t-test). In preliminary studies, we also looked at TNF-α, another proinflammatory cytokine induced early after injury. However, the levels of cutaneous TNF-α gene expression detected by our competitive PCR methods were extremely low and variable. Therefore, analysis of TNF-α was not included in subsequent experiments.
Effects of Restraint Stress on the Kinetics of Cutaneous KGF-1 Gene Expression

To assess the effects of RST on mRNA expression of KGF-1, RNA was isolated from control (non-wounded) skin or from excisional wounds at days 1, 3, and 5 after wounding and competitive RT-PCR was performed. Attomoles of KGF-1 mRNA were divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Low, constitutive levels of KGF-1 mRNA were detected in control skin of FWD and RST mice (Figure 2.6). Strong KGF-1 induction was found at day 1, with significantly lower expression in RST as compared to FWD mice (p<0.05, t-test). At day 3, KGF-1 mRNA expression in FWD mice started to decline, while expression in RST mice increased to its peak. At day 5, KGF-1 mRNA expression decreased in both FWD and RST animals, but it was still significantly higher than in control skin (p<0.001 for FWD, p<0.01 for RST, t-test).

Effect of Restraint Stress on Serum Corticosterone Levels

To evaluate the effects of RST on serum corticosterone levels, blood samples were obtained each morning at baseline, day 0, and day 5. Prior to the beginning of the experiments, basal levels between FWD and RST groups were comparable (146.2 ± 46 ng/ml in FWD, 139 ± 40 ng/ml in RST) (Figure 2.7). Basal levels for both groups were higher than those reported by Padgett et al. (1998), who demonstrated that basal levels for FWD and RST animals were in the range of 35 to 39 ng/ml. At day 0, there was a significant increase in the serum corticosterone levels of RST (184.9 ± 45 ng/ml) as compared to FWD mice (45.6 ± 4.5 ng/ml) (p<02, t-test). At day 5, serum corticosterone
levels were elevated in FWD mice, resulting in no significant difference between FWD and RST mice (136.2 ± 29 ng/ml in FWD, 209.2 ± 79 ng/ml in RST). It should be noted that, at the day of baseline blood collection and at day 5, the cages where the animals were housed had been changed for clean cages prior to blood sample collection, without previous knowledge by the investigators. This extra manipulation of the animals could have induced undesired stress in mice and thus resulted in elevated corticosterone levels at baseline in both groups and at day 5 in FWD animals.
Effects of Glucocorticoid Receptor Blockade on IL-1α, IL-1β, and KGF-1 Gene Expression in RST mice

As described previously in this murine model of wound healing (Padgett et al., 1998), repeated cycles of RST resulted in significantly higher levels of serum GCs as compared to FWD. In addition, RST mice had a delay in the onset of closure during the early proinflammatory phase of healing. The present studies showed that RST had a major effect on IL-1β gene expression at day 1, while the magnitude of the difference found at day 5 was fairly small. Taken together, these results suggest that stress-induced GCs had a stronger suppressive effect on the early inflammatory response to injury, which could compromise later events in wound healing such as proliferation and remodeling. Therefore, experiments with a GC receptor antagonist were focused on day 1 after wounding, an early time point when IL-1β is strongly induced. To evaluate the effect of RST-induced GCs on mRNA expression of IL-1α, IL-1β, and KGF-1 mRNA, mice were treated with the GC receptor antagonist RU486. RNA was isolated from excisional wounds at day 1 after wounding and competitive RT-PCR was performed.
In Figure 2.8, the ratios of cytokine or growth factor to G3PDH gene expression are represented as percent of the average ratios of the same gene expressed in FWD mice. Neither RST (alone or with vehicle) nor RST with RU486 had an effect on IL-1α mRNA expression at day 1. For IL-1β, RST alone or with vehicle resulted in significantly lower mRNA levels as compared to FWD ($p<0.05$, ANOVA), while treatment of RST mice with RU486 restored IL-1β mRNA expression to levels comparable with FWD mice. For KGF-1, RST alone or with vehicle resulted in significantly lower mRNA levels as compared to FWD ($p<0.05$, ANOVA). However, treatment of RST mice with RU486 did not restore KGF-1 mRNA expression.

2.4 Discussion

Several studies have suggested that the suppressive effects of GCs on the inflammatory response to injury may ultimately have a deleterious impact on wound repair (Hübner et al., 1996; Padgett et al., 1998). However, the effect of stress-induced elevation of GCs on cutaneous expression of proinflammatory cytokines IL-1α and IL-1β has not been investigated. The present study demonstrated a significant down-regulation of IL-1β mRNA expression in day 1 wounds of RST as compared to FWD mice, while IL-1α mRNA expression was not altered. The suppression of IL-1β expression is likely mediated by stress-induced GCs, since treatment of RST mice with RU486 restored IL-1β mRNA responses to the levels expressed in FWD mice. Since it was anticipated that the early restoration of IL-1β gene expression at day 1 would in turn result in normalized cytokine gene expression at later time points, the present study did not evaluate the effect
of RU486 at days 3 or 5. One explanation for the effect of stress-induced GCs on IL-1β expression is a reduced responsiveness of leukocytes to inflammatory stimuli, as shown by numerous in vitro studies using synthetic GCs (Bendrups, Hilton, Meager, & Hamilton, 1993; Kern et al., 1988; Knudsen et al., 1987; Lee et al., 1988; Lew et al., 1988; Russo-Marie, 1992; Snyder et al., 1982). This was also suggested by our previous human studies, in which stress was related to lower whole blood IL-1β mRNA responses to LPS (Kiecolt-Glaser et al., 1995; Marucha et al., 1998). Lowered IL-1β expression from circulating and infiltrating cells will compromise the continued recruitment and activation of inflammatory cells to the site of injury. Moreover, a GC-mediated reduction in the number of infiltrating leukocytes (Leibovich, & Ross, 1975) may also account for lower IL-1β mRNA levels in early wounds of RST mice. Indeed, Hübner et al. (1996) showed reduced infiltration of inflammatory cells in wounds of dexamethasone-treated mice. In addition, previous studies in this model demonstrated that one-day old wounds from RST mice had reduced cellularity as compared to FWD mice (Padgett et al., 1998).
The apparent lack of a RST effect on IL-1α mRNA expression during the first three days after wounding suggests that a stress-induced rise in serum GCs did not alter the molecular responsiveness of keratinocytes, which are the major producers of IL-1α (Kupper et al., 1986; Lee et al., 1991; Salmon et al., 1994). However, studies by Hübner et al. (1996) demonstrated a down-regulation in IL-1α mRNA in wounds of mice treated with dexamethasone. It is possible that pharmacological doses of this synthetic form of GC, which has a higher affinity for GC receptors than corticosterone (Svec, 1985), exert a more profound suppressive effect on keratinocyte responsiveness and consequently on IL-1α gene expression.

At day 5, IL-1α and IL-1β mRNA expression in FWD animals decreased nearly to the levels expressed in nonwounded skin, suggesting that the inflammatory phase was approaching resolution. However, RST mice expressed significantly high mRNA levels of both IL-1α and IL-1β as compared to FWD mice and nonwounded skin. This shift in the kinetics of proinflammatory cytokine gene expression at the wound site may be a major contributory factor to delayed wound closure in RST mice. First, a delay in the kinetics of the inflammatory response may alter the transition into the granulation phase, which is important for fibroblast proliferation, collagen synthesis, and neovascularization. Moreover, a prolonged inflammatory response to injury may result in destruction of viable tissue due to the persistence of neutrophils and their continued release of oxygen radicals and proteases (Martin, 1997; Ravage, Gomez, Czermak, Watkins, & Till, 1998). Although Padgett et al. (1998) found no difference in cellular infiltrate at day 5 in wounds of FWD vs. RST mice, the method employed for assessment of wound cellularity
could not differentiate which types of leukocytes were present, i.e., neutrophils or macrophages. Thus, it is possible that day 5 wounds of RST mice had more areas of neutrophilic rather than monocytic infiltration in comparison to FWD mice, while maintaining similar overall leukocyte numbers between groups. Since macrophages are critical for the clearance of neutrophils and the development of granulation tissue, this could have a deleterious impact on wound repair in mice under restraint stress. Future immunocytochemical studies should aim to evaluate the quality of the cellular infiltrate and identify which leukocyte cell types are predominantly expressed in wounds of FWD vs. RST mice.

While the inflammatory phase proceeds, re-epithelialization of the wound surface is taking place simultaneously via regulation by growth factors such as KGF-1, which is produced by dermal fibroblasts (Brauchle et al., 1994; Chedid et al., 1994). The present study demonstrated that one-day-old wounds of RST mice had significantly lower KGF-1 mRNA expression as compared to FWD mice. This correlated with in vivo studies which found that the typical induction of KGF-1 mRNA after injury was reduced in dexamethasone-treated mice (Brauchle et al., 1995). Thus, the depression in KGF-1 mRNA in RST mice could be mediated by stress-induced GCs, by either a direct or an indirect effect. First, cultured fibroblasts treated with dexamethasone have reduced KGF-1 mRNA expression (Brauchle et al., 1995; Chedid et al., 1996), which suggests that there may be a direct GC effect on mesenchymal cells. Alternatively, the early down-regulation of IL-1β, a positive regulator of KGF-1 (Brauchle et al., 1994; Chedid et al., 1994), in wounds of RST mice may be responsible for low expression of KGF-1.
Nevertheless, the present study showed that treatment of RST mice with RU486 did not restore KGF-1 mRNA expression at day 1, even when the IL-1β response had recovered. Other hormones that are elevated during stress, such as catecholamines, may be involved in down-regulation of KGF-1 expression. For example, it has been shown that catecholamines inhibit fibroblast proliferation when used in concentrations comparable to the levels found in the sera of post-surgical stress patients (Saito et al., 1997).

Using the same murine model of wound healing, Padgett et al. (1998) demonstrated that treatment of RST mice with GC receptor antagonists restored normal kinetics of wound closure, suggesting that the signals for keratinocyte proliferation and migration across the wound edges, i.e., re-epithelialization, had been recovered. However, although wound closure was restored, the present study showed that treatment with RU486 did not restore KGF-1 gene expression in wounds of RST mice. It should be noted that KGF-1 is only one of the multiple mediators that play an interactive role during tissue repair. For example, expression of chemokines such as MCP-1 is necessary for recruitment of macrophages, which phagocytose effete neutrophils and continue tissue debridement (Engelhardt, Toksoy, Goebeler, Debus, Brocker, & Gillitzer, 1998). Growth factors released by macrophages and epidermal cells (e.g., PDGF, TGF-β, bFGF, and VEGF) stimulate fibroblast proliferation (Heldin, & Westermark, 1996; Roberts, & Sporn, 1996), angiogenesis (Nissen, Polverini, Koch, Volin, Gamelli, & DiPietro, 1998), synthesis of a provisional extracellular matrix (Clark, Nielsen, Welch, & McPherson, 1995; Welch, Odland, & Clark, 1990), and its eventual remodeling into mature collagen (Clark et al., 1995; Welch et al., 1990). Release of matrix metalloproteinases, such as collagenase-1
by epidermal cells, is necessary for degradation of the existing extracellular matrix, thereby allowing migration of keratinocytes (Pilcher, Dumin, Sudbeck, Krane, Welgus, & Parks, 1997). KGF-2 (FGF-10), a newly identified member of the fibroblast growth factor family, is mitogenic for keratinocytes and also contributes to re-epithelialization (Beer, Florence, Dammeier, McGuire, Werner, & Duan, 1997a; Tagashira, Harada, Katsumata, Itoh, & Nakatsuka, 1997; Yamasaki, Miyake, Tagashira, & Itoh, 1996b). Reduction of the wound size and eventual closure of the defect is partially achieved by contraction, which requires stimulation by TGF-β and PDGF (Clark, Folkvord, Hart, Murray, & McPherson, 1989; Montesano, & Orci, 1988), adherence of contractile myofibroblasts to one another and to the collagen matrix, and collagen cross-linking (Clark et al., 1989; Montesano et al., 1988; Schiro, Chan, & Roswit, 1991; Woodley, Yamauchi, Wynn, Mechanic, & Briggaman, 1991). Any of these mediators of proliferation and remodeling could also be a direct target of neuroendocrine regulation under conditions of stress, as well as through the regulation of proinflammatory cytokines such as IL-1.

In conclusion, restraint stress induced an alteration in the kinetics of IL-1α, IL-1β, and KGF-1 gene expression during the first five days after wounding. Early suppression of IL-1β may reduce the responsiveness of leukocytes and their recruitment to the site of injury, thereby compromising tissue decontamination. On the other hand, a late up-regulation of IL-1α and IL-1β may prolong inflammatory events at a time when proliferative processes such as granulation should prevail. Moreover, suppression of KGF-1 may reduce the number of epidermal cells available for re-epithelialization of the
defect. Therefore, the present study suggests that disruption of neuroendocrine homeostasis by stress not only has an overall delaying effect on the kinetics of wound closure, but it may also impair the quality of the healing tissues by altering molecular signals of inflammation and proliferation.
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<th>Primer</th>
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Table 2.1. Primer sequences used for competitive RT-PCR
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<td>mimic</td>
<td>blank</td>
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**Figure 2.1.** Example of gel for competitive RT-PCR of G3PDH.

![Graph](https://via.placeholder.com/150)

\[ Y = 0.02 - 0.127X + 0.607X^2 \]

- \( R^2 = 1 \), \( p < 0.0001 \)

**Figure 2.2.** Polynomial regression plot for G3PDH standard curve.
Figure 2.3. Effect of restraint stress on wound healing kinetics. Mice were subjected to three cycles of RST or FWD beginning 3 days prior to wounding. Wound size was determined by photoplanimetry from the day of wounding until day 5. Data represent means ± SEM. n=23 animals per group at day 1, 15 animals per group at day 3, and 4 animals per group at day 5. *p<.01 as compared to FWD mice. **p<.001 as compared to FWD mice. ***p<.0001 as compared to FWD mice.
Figure 2.4. Effect of restraint stress on the kinetics of cutaneous IL-1α gene expression. Mice were euthanized at days 1, 3, and 5 after wounding. Wounds and control (non-wounded) skin were excised and homogenized. Poly(A)-tailed RNA was subjected to competitive RT-PCR. Attomoles of cytokine mRNA were divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Data represent mean ratios ±SEM of three pooled experiments. At each time point, n=8-10 mice per group, per experiment.

*p<0.01 as compared to FWD mice.
Figure 2.5. Effect of restraint stress on the kinetics of cutaneous IL-1β gene expression. Mice were euthanized at days 1, 3, and 5 after wounding. Wounds and control (non-wounded) skin were excised and homogenized. Poly(A)-tailed RNA was subjected to competitive RT-PCR. Attomoles of cytokine mRNA were divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Data represent mean ratios ±SEM of three pooled experiments. At each time point, n=8-10 mice per group, per experiment.

*p<0.01 as compared to FWD mice. **p<0.001 as compared to FWD mice.
Figure 2.6. Effect of restraint stress on the kinetics of cutaneous KGF-1 gene expression. Mice were euthanized at days 1, 3, and 5 after wounding. Wounds and control (non-wounded) skin were excised and homogenized. Poly(A)-tailed RNA was subjected to competitive RT-PCR. Attomoles of growth factor mRNA were divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. Data represent mean ratios ± SEM of three pooled experiments. At each time point, n=8-10 mice per group, per experiment.
*p<0.05 as compared to FWD mice.
Figure 2.7. Effect of restraint stress on serum corticosterone levels. From each group, blood was collected from 7-8 mice for corticosterone determination by RIA. Baseline (BL) and experimental samples were obtained at 10:00AM. Stressed animals were restrained for 12h each night beginning on the evening 3 days prior to wounding. Wounds were made in the morning of day 0 after blood collection. Restraint stress was continued for 5 days after wounding. Data represent means ±SEM. *p< .02 as compared to FWD mice at day 0.
Figure 2.8. Effect of glucocorticoid receptor blocker on IL-1α, IL-1β, and KGF-1 gene expression in day 1 wounds. Beginning one day prior to the initiation of restraint and 4 days prior to wounding, mice were injected subcutaneously with 25 mg/kg of glucocorticoid receptor antagonist RU486 or vehicle (VEH) each day of the restraint paradigm 2h prior to the beginning of restraint. Mice were euthanized at day 1 after wounding. Wounds were excised and homogenized, and poly(A)-tailed RNA was subjected to competitive RT-PCR. Gene expression is defined as attomoles of cytokine or growth factor mRNA divided by the corresponding attomoles of G3PDH mRNA expressed in each sample. The ratio of gene expression was then divided by the average ratio of the same gene expressed in the FWD group and multiplied by 100. Data represent means ± SEM of three pooled experiments. At each time point, n=10-12 mice per group, per experiment.

*p<0.05 as compared to FWD and RST/RU486.

**p<0.05 as compared to RST, RST/VEH, and RST/RU486.
CHAPTER 3

RESTRAINT STRESS ALTERS THE EXPRESSION OF INTERLEUKIN-1
AND KERATINOCYTE GROWTH FACTOR AT THE WOUND SITE:
AN IN SITU HYBRIDIZATION STUDY

3.1 Introduction

Chronic stress can significantly impair wound healing. Caregivers of Alzheimer’s disease (AD) patients, who were found to be chronically stressed, took 24% longer to heal a cutaneous wound than control subjects (Kiecolt-Glaser et al., 1995). In dental students, examination stress caused a 40% delay in mucosal wound healing compared to healing in the same students during vacation (Marucha et al., 1998). The mechanisms by which stress influences the cellular processes involved in wound healing are not well understood. It has been postulated that activation of the hypothalamic-pituitary-adrenal (HPA) axis, with its consequent release of glucocorticoid hormones (GCs), may be the main cause of stress-related impairments in wound healing. Indeed, Padgett et al. (1998) showed that elevated levels of plasma corticosterone induced by repeated cycles of restraint stress (RST) were associated with delayed wound closure in mice (Padgett et al., 1998).
The profound inhibitory effect of GCs on wound healing has been widely studied. Exogenous GCs cause dehiscence of surgical wounds, increased wound infection, and delayed wound closure (Anstead, 1998). GCs inhibit proliferation of keratinocytes (KCs) at the wound site, resulting in a thin, abnormal epithelium (Edwards et al., 1958). The effect of GCs on wound repair may be related to their anti-inflammatory action. In vitro studies have demonstrated that GCs inhibit gene expression of proinflammatory cytokines Interleukin-1α (IL-1α) and Interleukin-1β (IL-1β) (Amano et al., 1993; Kern et al., 1988; Knudsen et al., 1987; Lee et al., 1991; Lee et al., 1988; Lew et al., 1988; Snyder et al., 1982). IL-1α and IL-1β modulate the expression of chemokines and adhesion molecules necessary for recruitment of inflammatory cells to the site of injury (Bickel et al., 1996; Larsen, Anderson, Oppenheim, & Matsushima, 1989). IL-1 stimulates proliferation of KCs and induces KC migration on collagen (Chen et al., 1995; Kupper et al., 1986). A suppression of IL-1 by GCs may compromise wound debridement, decontamination, and re-epithelialization. Consistent with this idea, Rojas et al. (2001) found that high levels of GCs were associated with increased incidence of opportunistic bacterial infections at the wound site and delayed wound healing in mice (Rojas et al., 2000). IL-1 further contributes to wound re-epithelialization via the induction of fibroblast-derived keratinocyte growth factor-1 (KGF-1, or FGF-7), a mitogen for KCs (Pierce et al., 1994; Rubin et al., 1989; Staiano-Coico et al., 1993; Werner et al., 1994). KGF-1 gene expression is also suppressed by GCs (Brauchle et al., 1995; Chedid et al., 1996; Tang, & Gilchrest, 1996). In wounds of mice treated with dexamethasone (DEX), the typical induction of IL-1α, IL-1β, and KGF-1 mRNA is
reduced, with delayed wound re-epithelialization and reduced granulation tissue formation (Brauchle et al., 1995; Hübner et al., 1996). Therefore, GC-mediated suppression of IL-1 and KGF-1 expression may compromise early signals of inflammation and proliferation at the wound site.

Our previous studies showed that wounds of RST mice had an alteration in the kinetics of IL-1α, IL-1β, and KGF-1 mRNA expression during the first 5 days of healing, as evaluated by competitive RT-PCR (Mercado, Padgett, Sheridan, & Marucha, 2001). These findings suggested that stress can dysregulate proinflammatory cytokine and growth factor gene expression at the wound site, which may account for its negative effect on bacterial clearance and wound closure. The present study extends initial molecular investigations into the cellular level, evaluating the effects of stress on IL-1 and KGF-1 gene expression by specific cell types at the wound site. To this end, in situ hybridization immunohistochemistry for IL-1α, IL-1β, and KGF-1 was done in full-thickness excisional wounds of RST and control mice. In addition, histological changes in the quality of the healing tissues that could be associated with stress-induced alterations in proinflammatory cytokine and growth factor gene expression were characterized.
3.2 Materials and Methods

Animals

Same as described previously in Section 2.2.

Restraint Stress Paradigm

Same as described previously in Section 2.2.

Wounding and harvesting

Mice were anesthetized with a 0.3 ml intraperitoneal injection of 0.44 mg/ml Rompum (Haver-Lockhart, Shawnee, KS) and 7.8 mg/ml Ketaset (Aveco, Fort Dodge, IA). After cleansing the area, a sterile 3.5 mm punch (Premier Medical Products, King of Prussia, PA) was used to create two full-thickness wounds on the dorsal side of each mouse, just below the shoulder blades. Wounds placed at this location cannot be reached by the animals for self-licking. After days 1, 3, and 5, mice were euthanized with an overdose of anesthesia and wounds were excised with an 8 mm punch. Harvested tissues were fixed in formalin, processed and embedded in paraffin. Microtome sections 5 μm thick were cut and adhered to slides. Figure 3.1 illustrates the preparation of wounds for histology.
Preparation of Digoxigenin-labeled RNA probes

A 490 bp DNA sequence corresponding to nucleotides 241 to 731 of the murine IL-1α mRNA (accession X01450), and a 439 bp DNA sequence corresponding to nucleotides 474 to 913 of the human FGF-7 mRNA (accession NM 002009) were purified from PCR amplification of mouse cDNA using primers flanking these sequences. A 579 bp DNA sequence corresponding to nucleotides 514 to 1093 of the murine IL-1β mRNA (accession M15131) was prepared from commercially available insert #963357 in vector pT7T3D-pac cloned in host Escherichia coli (ATCC, Monassas, VA). In vitro transcription of antisense RNAs for IL-1α, IL-1β, and KGF-1 was done with T7 RNA polymerase (Boehringer Mannheim GmbH, Germany) and labeled with digoxigenin-conjugated UTP. Probes were treated with DNase I (Boehringer Mannheim GmbH, Germany), precipitated with lithium chloride, washed in ethanol, and dissolved in DEPC-treated distilled water. Aliquots of the probes were stored at -80°C.

In situ hybridization procedure

Paraffin sections were de-waxed in xylene and ethanol, washed in phosphate-buffered saline (PBS) twice for 5 min, and digested with pepsin (Sigma, St. Louis, MO) for 30 min at 37°C. Slides were immersed in 4% paraformaldehyde for 5 min at room temperature and in triethanolamine/acetic anhydride for 10 min at room temperature. Slides were then placed in hybridization buffer (composed of formamide, bovine serum albumin, sodium citrate-SSC, polyvinylpyrrolidone, Ficoll, dextran, yeast tRNA, and salmon sperm) with digoxigenin-labeled probe, formamide, sodium dodecyl sulfate (SDS), and
sodium thiosulfate. Glass coverslips were applied and hybridization was carried out at 55°C overnight. Slides were washed in 2x SSC and incubated in 1x SSC/50% formamide for 1 hour at 55°C. They were treated with RNase A buffer for 15 min at 37°C. After washing, slides were incubated in blocking buffer (composed of BMB blocking reagent, maleic acid, and sodium chloride) for 30 min at 37°C. Slides were then placed in a solution containing mouse antidogoxigenin (Boehringer Mannheim GmbH, Germany) and blocking buffer for 2 hours at room temperature. They were washed twice for 5 min in PBS and incubated with biotinylated antimouse IgG secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After washing in PBS, Streptavidin horseradish peroxidase (ABC kit, Vector Laboratories, Burlingame, CA) was applied to sections and allowed to incubate for 30 min at room temperature. After washing again in PBS, slides were incubated with 50 mM Tris-HCl for 5 min at room temperature. The immunoperoxidase complex was revealed with diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in an alkaline buffer for 3-4 min at room temperature, then washed in distilled water twice. Finally, slides were counterstained with hematoxylin and mounted in aqueous mounting medium. In addition, serial tissue sections were stained with hematoxylin and eosin and mounted.

**Analysis of mRNA-expressing cells**

A Zeiss microscope (Carl Zeiss, Oberkochen, Germany) was used for evaluation of slides under bright-field illumination. Images were captured by a digital color camera (DAGE-MTI, Michigan City, IN) with a plug-in to Adobe PhotoShop 5.0 (San Jose, CA).
The three major cell-types within a healing wound, e.g., leukocytes, KCs, and fibroblasts, were identified and distinguished based on location and morphology. Cells that were positive for the expression of specific mRNA species showed a characteristic reddish-brown staining of their cytoplasms. Sections were examined without knowledge of their corresponding treatment group. Positive cells were counted at 100X magnification within fields of a fixed size (1350 X 950 pixels). Counting fields were located at the wound edges and at the wound bed (2-3 counting fields per section). Figure 3.1 illustrates the location of the counting fields. The percentage of mRNA-expressing (or labeled) cells per counting field was determined and averaged for each section. One section per wound, per animal was examined. Data represent the means ± SEM from 3-4 animals per group, per time point.

Statistical methods

Differences in the mean percentages of mRNA-expressing cells were analyzed by Student’s t test. Differences were considered statistically significant when \( p < .05 \). Data were analyzed using statistical program JMP-IN 3.2.1 (SAS Institute Inc., Cary, NC).

3.3 Results

Histological analysis of wounds

To describe histological features of full-thickness wounds from FWD and RST animals, wounds excised at days 1, 3, and 5 were processed, stained with hematoxylin/eosin, and visualized under bright field illumination. Representative
photomicrographs are shown in Figures 3.2 to 3.4. At day 1 (Figure 3.2A, B), a clot rich in fibrin and inflammatory cells filled the area of the defect. Dermal margins of the wounds presented extensive infiltration of leukocytes. Epithelial margins were composed of elongated, flattened KCs that had apparently started migrating over the dermis along the wound bed. These characteristics were consistent within groups (observations are representative of 4 animals per group), with no marked qualitative difference between FWD and RST animals.

At day 3 (Figure 3.3A, B), differences were observed in the thickness of the epithelial margins. In wounds of FWD mice, the proliferating zones of epithelium were thickened by several layers of KCs. The leading edges of epithelium had migrated a short distance underneath the eschar. In contrast, the proliferating zones of epithelium in wounds of RST mice were thin with fewer layers of KCs. The leading edges of epithelium had apparently migrated a longer distance underneath the eschar. These observations are representative of 4 animals per group.

At day 5 (Figure 3.4A, B), wounds of FWD mice presented extensive areas of granulation tissue formation with no evidence of acute inflammation under a thick, continuous layer of remodeling epithelium. In wounds of RST mice, there were foci of leukocytic infiltration within the connective tissue with reduced granulation tissue formation under thin margins of epithelium. These observations are representative of 3 animals per group.
Expression of IL-1α mRNA at the wound site

To study the cellular distribution of IL-1α mRNA, wounds were excised at days 1, 3, and 5 and subjected to *in situ* hybridization immunohistochemistry. Representative photomicrographs are shown in Figures 3.5 and 3.6. Cell-specific signals for IL-1α mRNA were detected predominantly in leukocytes, fibroblasts, and, to a lesser frequency, KCs. At day 1, wounds of FWD and RST mice presented no difference in the mean percentages of IL-1α-labeled leukocytes or fibroblasts (Figure 3.5A, B). At day 3, no group differences were observed in the mean percentages of IL-1α-labeled leukocytes, KCs, or fibroblasts. At day 5, no group differences were observed in the mean percentages of IL-1α-labeled KCs or fibroblasts. Likewise, the mean percentages of leukocytes positive for IL-1α mRNA were similar between groups, although wounds of RST animals had more areas of leukocytic infiltration and less granulation tissue formation than wounds of FWD mice (Figure 3.6A, B). Quantitative data for IL-1α are summarized in Table 3.1.

Expression of IL-1β mRNA at the wound site

To evaluate the cellular distribution of IL-1β mRNA, wounds were excised at days 1, 3, and 5 and subjected to *in situ* hybridization immunohistochemistry. Representative photomicrographs are shown in Figures 3.7 to 3.10. Cell-specific signals for IL-1β mRNA were predominantly expressed by leukocytes, fibroblasts, and, to a lesser frequency, KCs. At day 1 (Figure 3.7A, B), wounds of RST animals had a significant 31% reduction in the mean percentage of IL-1β-labeled fibroblasts as compared to
wounds of FWD mice ($p < .05$, $t$ test), with no group differences in the mean percentages of labeled leukocytes or KCs. At day 3, there were no group differences in the mean percentages of IL-1β-labeled KCs or fibroblasts. When compared to FWD mice, day 3 wounds of RST mice had a significant 40% reduction in the mean percentage of labeled leukocytes ($p < .01$, $t$ test) (Figure 3.8A, B). At day 5, no group differences were observed in the mean percentage of IL-1β-labeled fibroblasts. RST animals had a significant 150% increase in the mean percentage of KCs labeled for IL-1β mRNA as compared to FWD mice ($p < .05$, $t$ test) (Figure 3.9A, B). The mean percentages of leukocytes positive for IL-1β mRNA were similar between groups, although wounds of RST animals had more areas of leukocytic infiltration and less granulation tissue formation than wounds of FWD mice (Figure 3.10A, B). Quantitative data for IL-1β are summarized in Table 3.2.
Expression of KGF-1 mRNA at the wound site

To study the cellular distribution of KGF-1 gene expression, wounds were excised at days 1, 3, and 5 and subjected to in situ hybridization immunohistochemistry. Representative photomicrographs are shown in Figures 3.11, 3.12, and 3.13. At day 1, cell-specific signals for KGF-1 mRNA were detected in dermal fibroblasts near the wound margins with no difference between groups (Figure 3.11A, B). At day 3, wounds of RST mice showed a tendency towards a decrease in the mean percentage of labeled fibroblasts when compared to wounds of FWD mice (close to statistical significance at \( p < .08, t \) test) (Figure 3.12A, B). At day 5, mean percentages of KGF-1 mRNA positive cells were highly variable with no difference between groups (Figure 3.13A, B).

Quantitative data for KGF-1 are summarized in Table 3.3.

3.4 Discussion

Previous studies in the murine model of cutaneous wound healing have suggested that the delaying effect of stress on wound closure results from alterations in the kinetics of IL-1 and KGF-1 gene expression at the site of injury (Mercado et al., 2001). However, the mechanisms by which stress-induced changes in molecular signals may impact cellular processes of wound healing have not been investigated. The present in situ hybridization study demonstrated that stress exerts a differential regulation of gene expression targeted at leukocytes, keratinocytes, and fibroblasts within the wound, and that this may ultimately impair the quality of the tissues undergoing repair.
Leukocytes are the major producers of IL-1β during the inflammatory phase of wound healing (Hübner et al., 1996). RT-PCR analysis by Mercado et al. (2001) showed a 50% down-regulation of IL-1β mRNA in day 1 wounds of RST as compared to FWD mice. It could be speculated that a stress-induced down-regulation of IL-1β gene expression occurred primarily at the leukocyte, resulting from either a decrease in the number of infiltrated leukocytes or a decrease in leukocyte responsiveness. The results of the present study showed that there were no significant group differences in the frequency of IL-1β mRNA-labeled leukocytes at day 1. It should be noted that non-isotopic in situ hybridization was used to identify the presence of specific mRNA-expressing cells at various tissue compartments. This method is not suitable for measurement of cellular responsiveness, i.e., the amount of mRNA expressed per cell. It is possible that the responsiveness of leukocytes, rather than their number, was affected by stress. Padgett et al. (1998) demonstrated that RST mice had elevated levels of plasma corticosterone. Several in vitro studies have shown that GCs suppress expression of IL-1β in leukocytes (Amano et al., 1993; Kern et al., 1988; Knudsen et al., 1987; Lee et al., 1988; Lew et al., 1988; Snyder et al., 1982). This suggests that stress-induced elevations in GCs may decrease the responsiveness of leukocytes for proinflammatory cytokine expression. Our previous studies in chronically stressed AD caregivers (Kiecolt-Glaser et al., 1995) and dental students under examination stress (Marucha et al., 1998) showed 56% and 68% decreases, respectively, in whole blood IL-1β mRNA responses to lipopolysaccharide. This demonstrated that the responsiveness of peripheral blood cells is attenuated in stressed individuals without changes in cell numbers. RST
may have also altered other cells, like fibroblasts. Interestingly, day 1 wounds of RST mice had a significant decrease in the percentage of dermal fibroblasts expressing IL-1β mRNA. Stress-induced elevations in GCs may account for the decrease in IL-1β-expressing fibroblasts. GCs are known to inhibit cytokine expression in fibroblasts (Amano et al., 1993; Lee et al., 1988; Peppel et al., 1991). Taken together, these findings suggest that the down-regulation of IL-1β gene expression in day 1 wounds of RST mice as previously shown by RT-PCR (Mercado et al., 2001) may be the result of lower responsiveness of leukocytes and decreased numbers of dermal fibroblasts expressing IL-1β mRNA at the wound site.

At day 3, the percentage of IL-1β mRNA-expressing leukocytes was significantly decreased in wounds of RST mice. At day 5, no group differences were detected in the percentages of IL-1α mRNA-expressing cells or in the percentages of leukocytes and fibroblasts labeled for IL-1β mRNA. These results on the frequencies of IL-1α and IL-1β mRNA-expressing cells in days 3 and 5 wounds can not directly explain previous findings in this model. RT-PCR studies showed that there were no group differences in overall IL-1β mRNA expression in day 3 wounds (Mercado et al., 2001). In day 5 wounds, RT-PCR demonstrated that IL-1α and IL-1β mRNA returned to constitutive levels in FWD mice while expression in RST mice was still significantly elevated (Mercado et al., 2001). Stress may have altered the responsiveness of cells more than their frequency, possibly by influencing the stimuli encountered at the site of injury. Rojas et al. (2000) demonstrated that day 3 and day 5 wounds of RST mice had significantly higher counts of opportunistic bacteria as compared to wounds of FWD.
mice. This suggests that, in an effort to eradicate the undermining infection, wound cells in RST mice continued to express high levels of proinflammatory cytokines as compared to wound cells in FWD mice, which had returned to constitutive expression of proinflammatory cytokines. Alternatively, higher RT-PCR levels of proinflammatory cytokines at day 5 may be due to considerably larger wounded areas (Padgett et al., 1998; Rojas et al., 2000), resulting in more cells per sample expressing IL-1α and IL-1β mRNA in RST as compared to FWD mice. The presence of inflammatory foci in day 5 wounds of RST mice confirmed that leukocytes persisted in the tissues of stressed animals. Persistent leukocytes were probably in an activated state of proinflammatory cytokine expression in response to increased bacterial load in day 5 wounds of RST mice. Therefore, variations in wound size alone can not account for elevated IL-1α and IL-1β gene expression at this time. On the other hand, group differences in wound closure could be responsible for the observed increase in the frequency of IL-1β mRNA-expressing KCs in day 5 wounds of RST mice. Histological analysis revealed that most wounds of FWD mice were completely re-epithelialized at day 5. This suggests that in FWD mice IL-1β expression by KCs had nearly returned to constitutive levels. In contrast, most wounds of RST mice were not completely re-epithelialized at day 5. This suggests that in RST mice KCs were still proliferating and migrating. During these processes, KCs express high levels of IL-1β. IL-1 produced by KCs has an autocrine effect, stimulating further production of IL-1 and enhancing KC growth (Kupper et al., 1986). It is possible that the abundance of IL-1β-labeled KCs in day 5 wounds of RST mice served to stimulate re-epithelialization of the defect.
The major group difference in KGF-1 mRNA expression was found at day 3, when there was a tendency toward fewer labeled fibroblasts in wounds of RST as compared to FWD mice ($p < .08$, t test). Stress may have exerted a stronger effect on the responsiveness than on the frequency of KGF-1 mRNA-expressing fibroblasts. It has been demonstrated that DEX decreases KGF-1 mRNA expression in cultured dermal fibroblasts and also in wounds (Brauchle et al., 1995). This suggests that elevated levels of GCs in RST mice could mediate a suppressive effect of stress on the responsiveness of fibroblasts for KGF-1 gene expression at day 3.

Histological analysis revealed marked differences between wounds of FWD and RST mice. At day 3, wounds of FWD mice had thicker proliferating zones than wounds of RST mice. A decrease in the frequency or responsiveness of fibroblasts for KGF-1 gene expression at this time could result in attenuated signals for KC proliferation in day 3 wounds of RST mice. At day 5, there was persistence of leukocytic foci in wounds of RST mice, showing that there was a delay in resolution of the inflammatory phase. In addition, day 5 wounds of RST mice had reduced granulation tissue formation. These two findings may be interrelated, with the common effector cell being the macrophage. Macrophages play a key role in the transition from the inflammatory to the granulation phase. They infiltrate the site of injury shortly after the neutrophils, aiding in tissue decontamination and destroying effete neutrophils. Macrophages produce the anti-inflammatory cytokine IL-10, which inhibits synthesis of proinflammatory cytokines including IL-1α and IL-1β (DeVries, 1995). Thus, macrophages overlap and contain the early inflammatory response. It is possible that the activation of macrophages in day 5
wounds was lower in RST as compared to FWD mice. It has been demonstrated that stress decreases phagocytic activity of macrophages (Brown, Sheridan, Pearl, & Zwilling, 1993; Zhang, Kishihara, Wang, Mizobe, Kubo, & Nomoto, 1998). Down-regulation of IL-1β expression at day 1 may result in decreased macrophage activation in wounds of RST mice. If macrophage activation is low, they are unable to control the inflammatory response initiated by neutrophils. Neutrophils could then become the predominant cells in leukocytic infiltrates of RST mice at day 5, persistently expressing proinflammatory cytokines in response to increased bacterial invasion. Macrophages also secrete growth factors that are necessary to promote angiogenesis and fibroplasia, key components of granulation tissue formation.
Decreased macrophage activation may also result in decreased production of macrophage-derived growth factors (TGF-β, PDGF) involved in granulation tissue formation. Stress-induced elevations in GCs may be related to decreased expression of macrophage-derived growth factors in RST mice. Cutaneous wounds of mice treated with DEX have decreased gene expression of TGF-β1, TGF-β2 (Frank, Madlener, & Werner, 1996), and PDGF (Beer, Longaker, & Werner, 1997b). Moreover, Brauchle et al. (1995) demonstrated that day 5 wounds of DEX-treated mice had decreased granulation tissue formation as compared to wounds of control mice (Brauchle et al., 1995). Therefore, it is postulated that the protracted inflammatory response and impaired granulation tissue formation in day 5 wounds of RST mice could be the result of decreased macrophage activity mediated by stress-induced GCs. Future immunocytochemical studies are required to identify which leukocyte cell types, i.e., neutrophils or macrophages, populate the wounds of FWD versus RST mice.

In summary, our previous studies in humans provided evidence that, in addition to its delaying effect on wound healing, chronic stress is related to decreased molecular responsiveness of peripheral blood cells to inflammatory stimuli (Kiecolt-Glaser et al., 1995; Marucha et al., 1998). In the murine model of cutaneous wound healing, we have shown that stress-induced disruption of neuroendocrine homeostasis results in delayed wound closure (Padgett et al., 1998), increased susceptibility to wound infection (Rojas et al., 2000), and alterations in the kinetics of proinflammatory and growth factor gene expression directly at the wound (Mercado et al., 2001). The present study demonstrated that stress-induced alterations in IL-1α, IL-1β, and KGF-1 gene expression result from
differential regulation of specific cells, *i.e.*, leukocytes, keratinocytes and fibroblasts, at the wound site. Furthermore, changes in molecular signals of inflammation and proliferation may account for impairments in the histological quality of healing tissues from stressed individuals.
Wound excised with 8mm punch and flattened over dry ice block.

Wound bisected and fixed in formalin.

Wound placed on its side, embedded in paraffin.

Sections were cut 5μm thick, mounted, and stained with H&E.

[Image: Representative location of counting fields.]

Figure 3.1. Preparation of wounds for histology.
Figure 3.2. Histological appearance of wound margins at day 1 after wounding. Photomicrographs taken from Day 1 wounds of FWD (A) and RST (B) mice. Wounds were bisected, fixed in formalin, paraffin-embedded, and sectioned. Sections were stained with hematoxylin/eosin and visualized under bright field illumination at 25X magnification. Wounds were oriented to the right side of each figure. Hair follicle (hf), dermis (d), epithelium (e), clot (c), sebaceous gland (sg).
Figure 3.3. Histological appearance of wound margins at day 3 after wounding. Photomicrographs taken from Day 3 wounds of FWD (A) and RST (B) mice. Wounds were bisected, fixed in formalin, paraffin-embedded, and sectioned. Sections were stained with hematoxylin/eosin and visualized under bright field illumination at 25X magnification. Wounds were oriented to the right side of each figure. Proliferating zone (pz), leading edge (le).
Figure 3.4. Histological appearance of wound margins at day 5 after wounding. Photomicrographs taken from Day 5 wounds of FWD (A) and RST (B) mice. Wounds were bisected, fixed in formalin, paraffin-embedded, and sectioned. Sections were stained with hematoxylin/eosin and visualized under bright field illumination at 25X magnification. Wounds were oriented to the right side of each figure. Granulation tissue (gt), leukocytic infiltration (LI).
Figure 3.5. Expression of IL-1α mRNA at day 1 after wounding. Photomicrographs taken from Day 1 wounds of FWD (A) and RST (B) mice. *In situ* hybridization was carried out with a digoxigenin-labeled antisense probe for mouse IL-1α. FWD and RST mice had comparable frequency of labeled dermal fibroblasts (black arrows), epidermal keratinocytes (arrowheads), and leukocytes (white arrows). All slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.6. Expression of IL-1α mRNA at day 5 after wounding. Photomicrographs taken from Day 5 wounds of FWD (A) and RST (B) mice. *In situ* hybridization was carried out with a digoxigenin-labeled antisense probe for mouse IL-1α. No group differences in the frequency of labeled cells were detected, although wounds of RST mice had more leukocytic infiltration (white arrows). All slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.7. Expression of IL-1β mRNA at day 1 after wounding. Photomicrographs taken from Day 1 wounds of FWD (A) and RST (B) mice. In situ hybridization was carried out with a digoxigenin-labeled antisense probe for mouse IL-1β. At day 1, FWD had positive leukocytes (white arrows), keratinocytes (arrowheads), and fibroblasts (black arrows), while RST mice had fewer labeled fibroblasts. Slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.8. Expression of IL-1β mRNA at day 3 after wounding. Photomicrographs taken from Day 3 wounds of FWD (A) and RST (B) mice. In situ hybridization was carried out with a digoxigenin-labeled antisense probe for mouse IL-1β. At day 3, wounds of RST mice had lesser frequency of labeled leukocytes (arrows) as compared to wounds of FWD mice. Slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.9. Expression of IL-1β mRNA at day 5 after wounding. Photomicrographs taken from Day 5 wounds of FWD (A) and RST (B) mice. *In situ* hybridization was carried out with a digoxigenin-labeled antisense probe for mouse IL-1β. At day 5, wounds of RST mice had more labeled keratinocytes (arrows) than wounds of FWD mice. Slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.10. Expression of IL-1β mRNA in the connective tissue of day 5 wounds. Photomicrographs taken from Day 5 wounds of FWD (A) and RST (B) mice. In situ hybridization was carried out with a digoxigenin-labeled antisense probe for IL-1β. No group differences in the frequency of labeled cells were detected, although wounds of RST mice had more infiltrated leukocytes (arrows). Slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.11. Expression of KGF-1 mRNA at day 1 after wounding. Photomicrographs taken from Day 1 wounds of FWD (A) and RST (B) mice. *In situ* hybridization was carried out with a digoxigenin-labeled antisense probe for KGF-1. FWD and RST mice had comparable frequencies of labeled fibroblasts (arrows). Slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.12. Expression of KGF-1 mRNA at day 3 after wounding.
Photomicrographs taken from Day 3 wounds of FWD (A) and RST (B) mice. In situ hybridization was carried out with a digoxigenin-labeled antisense probe for KGF-1. Wounds of RST mice had fewer labeled fibroblasts than wounds of FWD mice (arrows). Slides were counterstained with hematoxylin, shown at 100X magnification.
Figure 3.13. Expression of KGF-1 mRNA at day 5 after wounding. Photomicrographs taken from Day 5 wounds of FWD (A) and RST (B) mice. In situ hybridization was carried out with a digoxigenin-labeled antisense probe for KGF-1. Wounds of FWD and RST mice had comparable frequency of labeled fibroblasts (arrows). Slides were counterstained with hematoxylin, shown at 100X magnification.
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Table 3.1. Quantification of IL-1α mRNA-expressing cells in wounds of FWD vs. RST mice. Values represent mean percentage of IL-1β mRNA-expressing cells per counting field (± SEM) of 3-4 mice per group (one lesion section per animal for each time point). No significant group differences were found.
### Table 3.2. Quantification of IL-1β mRNA-expressing cells in wounds of FWD vs. RST mice.

Values represent mean percentage of IL-1β mRNA-expressing cells per counting field (± SEM) of 3-4 mice per group (one lesion section per animal for each time point).

* $p < .05$

** $p < .01$

<table>
<thead>
<tr>
<th>Days after wounding</th>
<th>Treatment group</th>
<th>Leukocytes</th>
<th>Keratinocytes</th>
<th>Fibroblasts</th>
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<td>70 ± 11</td>
<td>24 ± 6</td>
<td>74 ± 5 *</td>
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<tr>
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<td>RST</td>
<td>61 ± 9</td>
<td>27 ± 1</td>
<td>51 ± 6 *</td>
</tr>
<tr>
<td>3</td>
<td>FWD</td>
<td>88 ± 6 **</td>
<td>27 ± 7</td>
<td>57 ± 12</td>
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<td></td>
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<td>17 ± 7</td>
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<td>FWD</td>
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<td>16 ± 3 *</td>
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<td>40 ± 2 *</td>
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<tr>
<td>Days after wounding</td>
<td>Treatment group</td>
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Table 3.3. Quantification of KGF-1 mRNA-expressing fibroblasts in wounds of FWD vs. RST mice. Values represent mean percentage of KGF-1 mRNA-expressing cells per counting field (± SEM) of 3-4 mice per group (one lesion section per animal for each time point). No significant group differences were found.
CHAPTER 4

DISCUSSION

Previous studies in the murine model of cutaneous wound healing have suggested that stress-induced disruption of neuroendocrine homeostasis has a modulatory effect on local inflammation and rate of wound closure (Padgett et al., 1998). However, the impact of stress on cellular and molecular mechanisms of wound healing has not been investigated. The present studies demonstrated that stress alters the kinetics of IL-1α, IL-1β, and KGF-1 gene expression at the wound site, resulting from differential regulation of multiple mRNA-expressing cells within the architecture of the wound. Alterations at the molecular and cellular levels helped to explain morphological changes in the quality of the tissues undergoing repair. Therefore, stress results not only in an overall delay in wound closure kinetics, but it may also jeopardize the restoration of a healthy, functional skin barrier.

RT-PCR analysis showed constitutive mRNA expression of IL-1α and IL-1β mRNA in control, non-wounded skin of FWD and RST mice. IL-1α mRNA was expressed at low levels, while IL-1β mRNA was barely detectable. This may be explained by the fact that resting KCs, which account for more than 90% of epidermal cells (Salmon et al., 1994), express more IL-1α than IL-1β mRNA (Kupper et al., 1986; Lee et al., 1991).
Therefore, IL-1α is the predominant form of IL-1 in non-inflamed skin, expressed constitutively by resting KCs. Non-wounded skin from FWD and RST mice had comparable levels of IL-1α or IL-1β mRNA, indicating that RST had no effect on constitutive expression of cutaneous proinflammatory cytokines. These findings correlate with those of Hübner et al. (1996), who demonstrated that non-wounded skin of mice had low levels of IL-1α mRNA compared to hardly detectable levels of IL-1β mRNA. Wounds of DEX-treated mice expressed equally low levels of IL-1α and IL-1β mRNA than wounds of control mice.

At day 1 after wounding, RT-PCR showed a strong induction of both IL-1α and IL-1β gene expression. Induction of IL-1α mRNA in FWD mice was almost 12-fold the level in control skin. Induction of IL-1β mRNA was almost 380-fold the level in control skin. This indicated that the up-regulation of IL-1β mRNA is more robust than the up-regulation of IL-1α mRNA, which correlated with previous in vivo findings by Hübner et al. (1996). They demonstrated that early after injury, the predominant proinflammatory cytokine expressed is IL-1β, localized in PMNs at the dermal edges and particularly at the clot. This suggests that early expression of IL-1β mRNA originates mostly from numerous inflammatory cells infiltrated at the clot and wound edges, while expression of IL-1α mRNA originates mostly from activated KCs that line the wound edges. Interestingly, the present studies showed that the levels of mRNA expression for IL-1α and IL-1β are considerably different. The mean ratio of IL-1α mRNA expression (attomoles of IL-1α mRNA adjusted by the corresponding attomoles of G3PDH mRNA) in day 1 wounds of FWD mice was 5.83 ± 2. The mean ratio of IL-1β mRNA expression....
(attomoles of IL-1β mRNA adjusted by the corresponding attomoles of G3PDH mRNA) in day 1 wounds of FWD mice was 108 ± 26. This finding may be explained by the difference in the scope of actions of these two cytokines. Several studies have demonstrated that IL-1α plays an essential role locally in the skin, regulating normal KC cellular differentiation (Hauser, Saurat, Schmitt, Jaunin, & Dayer, 1986; Maier, Voulalas, Roeder, & Maciag, 1990). Accordingly, low expression of IL-1α at the wound site suggests that this cytokine is a primary regulator of intracellular events and mediator of local inflammation. On the other hand, IL-1β is a key mediator of immune-to-brain communication. IL-1β can signal the brain to create “sickness responses” including fever, decreases in food and water intake, increased sleep, and hyperalgesia (Kent, Bluthe, Kelley, & Dantzer, 1992; Maier, Goehler, Fleshner, & Watkins, 1998; Watkins, Maier, & Goehler, 1995). It can also regulate hormones of the HPA axis (Maier, & Watkins, 1998). Although high expression of IL-1β may act on the CNS to induce fever, and behavioral changes, this was unlikely the case in the present model since no signs of fever or illness behavior were observed in wounded mice.

There was a significant down-regulation of IL-1β mRNA expression in day 1 wounds of RST as compared to FWD mice. The suppression of IL-1β expression is likely mediated by stress-induced GCs, since treatment of RST mice with the GC receptor antagonist RU486 restored IL-1β mRNA responses to the levels expressed in FWD mice. Leukocytes are the major producers of IL-1β during the inflammatory phase of wound healing (Hübner et al., 1996). It could be speculated that the effect of stress-induced GCs on IL-1β gene expression occurred primarily at the leukocyte, resulting from a decrease
in the number of infiltrated leukocytes at the wound site. GCs are known to reduce the
number of infiltrating leukocytes (Leibovich et al., 1975). Hübner et al. (1996) showed
reduced infiltration of inflammatory cells in wounds of DEX-treated mice. However, in
situ hybridization analysis showed no significant group differences in the frequency of
IL-1β mRNA-labeled leukocytes at day 1. This correlates with data from Rojas et al.
(2001) which showed that at day 1 there were no differences in the numbers of
neutrophils or macrophages in wounds of FWD and RST mice (Rojas, Sheridan,
Mercado, Quan, Padgett, & Marucha, 2001).
Another explanation for the effect of stress-induced GCs on IL-1β expression could be a reduced responsiveness of leukocytes to inflammatory stimuli. It should be noted that non-isotopic in situ hybridization was used in the present studies to identify the presence of specific mRNA-expressing cells at various tissue compartments. This method is not suitable for measurement of cellular responsiveness, i.e., the amount of mRNA expressed per cell. Numerous in vitro studies have demonstrated the inhibitory effect of GCs on IL-1β gene expression (Amano et al., 1993; Bendrups et al., 1993; Kern et al., 1988; Knudsen et al., 1987; Lee et al., 1988; Lew et al., 1988; Russo-Marie, 1992; Snyder et al., 1982). Furthermore, our previous studies in chronically stressed AD caregivers (Kiecolt-Glaser et al., 1995) and dental students under examination stress (Marucha et al., 1998) showed 56% and 68% decreases, respectively, in whole blood IL-1β mRNA responses to LPS. This demonstrated that the responsiveness of peripheral blood cells is attenuated in stressed individuals without changes in cell numbers. Future studies should investigate the responsiveness of infiltrated wound leukocytes for the expression of IL-1β mRNA during the early inflammatory response.
RST may have also altered other cells at the wound, like the dermal fibroblasts, for the expression of IL-1β mRNA. Interestingly, day 1 wounds of RST mice had a significant decrease in the percentage of dermal fibroblasts expressing IL-1β mRNA. Stress-induced elevations in GCs may account for the decrease in IL-1β-expressing fibroblasts. GCs are known to inhibit cytokine expression in fibroblasts (Monick et al., 1994; Zitnik et al., 1994). Therefore, a decrease in the number of IL-1β mRNA-expressing fibroblasts and possibly a decrease in the responsiveness of fibroblasts for the expression of IL-1β mRNA may contribute to explain the down-regulation of IL-1β mRNA levels in day 1 wounds of RST mice as shown by RT-PCR.

RT-PCR of day 1 and day 3 wounds revealed no significant differences in IL-1α mRNA expression in FWD as compared to RST mice. Likewise, in situ hybridization detected no group differences in the percentages of leukocytes, KCs, or fibroblasts labeled for IL-1α mRNA in day 1 and day 3 wounds. KCs are the major source of IL-1α in the skin (Kupper et al., 1986; Lee et al., 1991; Salmon et al., 1994), suggesting that any effects of RST on IL-1α gene expression at the wound site would primarily be directed at the KC population. The apparent lack of a RST effect on IL-1α mRNA levels and on the frequency of IL-1α mRNA-labeled cells during the first three days after wounding suggests that a stress-induced rise in serum GCs did not alter the number or molecular responsiveness of KCs upon injury. However, studies by Hübner et al. (1996) demonstrated a down-regulation in IL-1α mRNA in wounds of mice treated with DEX. It is possible that pharmacological doses of this synthetic form of GC, which has a higher affinity for GC receptors than corticosterone (Svec, 1985), exert a profound suppressive
effect on KC responsiveness and consequently on IL-1α gene expression. Stress-induced elevations in endogenous GCs may not be sufficient to alter gene expression of local IL-1α, which plays an essential role in normal KC cellular differentiation (Hauser et al., 1986; Maier et al., 1990) and thus may be more resistant to neuroendocrine regulation.

The initial induction of IL-1β mRNA levels observed in day 1 wounds of FWD mice decreased sharply by day 3 (81% reduction from the expression at day 1), whereas it decreased modestly in wounds of RST mice (56% reduction from the expression at day 1). As a result, IL-1β mRNA levels in wounds of FWD and RST mice were comparable at day 3. In day 5 wounds, RT-PCR showed that IL-1α and IL-1β mRNA returned to constitutive levels in FWD mice while expression in RST mice was still significantly elevated. These results regarding the levels of IL-1α and IL-1β mRNA in days 3 and 5 wounds can not be directly explained by in situ hybridization findings on the frequency of labeled cells at the wound site. At day 3, the percentage of IL-1β mRNA-expressing leukocytes was significantly decreased in wounds of RST mice. At day 5, no group differences were detected in the percentages of IL-1α mRNA-expressing cells or in the percentages of leukocytes and fibroblasts labeled for IL-1β mRNA. This suggests that stress may have altered the responsiveness of wound cells for expression of proinflammatory cytokines more than their frequency, possibly by influencing the stimuli encountered at the site of injury. For example, Rojas et al. (2000) demonstrated that day 3 and day 5 wounds of RST mice had significantly higher counts of opportunistic bacteria as compared to wounds of FWD mice (Rojas et al., 2000). This suggests that, in response to high numbers of contaminating bacteria, cells in day 3 and day 5 wounds of
RST mice continued to express high levels of proinflammatory cytokines as compared to those in FWD mice, which by day 5 were expressing proinflammatory cytokines at constitutive levels. Therefore, at day 5, wounds of FWD mice had nearly resolved the initial inflammation, while wounds of RST mice had an extended inflammatory response.
Group differences in the levels of proinflammatory cytokine mRNA detected by RT-PCR at day 5 could be related to the marked differences in wound size previously demonstrated at this time point in the murine model of cutaneous wound healing (Padgett et al., 1998; Rojas et al., 2000). Wounds of RST mice had significantly greater areas than wounds of FWD mice. It is possible that wounds of RST mice contained more leukocytes, KCs and fibroblasts than wounds of FWD mice due to their larger size. Accordingly, higher RT-PCR levels of proinflammatory cytokines at day 5 may be due to significantly larger wounded areas, resulting in more cells per sample expressing IL-1α and IL-1β mRNA in RST as compared to FWD mice. It should be noted that the photoplanimetry analysis for assessment of wound size yields an estimation of a wound’s circular area, which is a two-dimensional parameter defined as the square of the radius multiplied by \( \pi \). In reality, a wound is a three-dimensional object in the shape of a cylinder with a volume defined as the square of its radius, multiplied by \( \pi \), and multiplied by its height. Calculation of the wound area can not accurately describe its full dimensions since it does not take into account the depth of the wound. Therefore, changes detected by RT-PCR, which required homogenization of three-dimensional whole wounds, can not be correlated to changes in wound size, which are only two-dimensional. The development of wound scanning methods and computerized graphic reconstruction of wounds would allow for three-dimensional, more comprehensive analysis of changes in wound size and volume during the healing process.
In addition, even after a wound has clinically attained closure as determined by photoplanimetry, there could be multiple active inflammatory, proliferative, and remodeling processes still taking place underneath the surface. Indeed, histological analysis revealed the presence of inflammatory foci in day 5 wounds of RST mice, confirming that leukocytes persisted in the tissues of stressed animals. Persistent leukocytes, major producers of proinflammatory cytokines, were probably in an activated state of IL-1α and IL-1β expression in response to increased bacterial load in day 5 wounds of RST mice. On the other hand, day 5 wounds of FWD mice had no evidence of acute leukocytic infiltration. Instead, they had extensive areas of granulation, typically composed of macrophages, proliferating fibroblasts, and newly formed blood vessels under a continuous layer of new epithelium. During granulation tissue formation, growth factors for fibroblast and endothelial cell proliferation are abundantly produced and expression of IL-1 is down-regulated by anti-inflammatory cytokines. This suggests that the quality of the tissues and the types of cells present, rather than the size of the wound, is responsible for the levels of cytokines expressed at a particular time point. Therefore, variations in wound size alone can not account for elevated IL-1α and IL-1β gene expression at day 5.

Histological analysis of day 5 wounds from FWD and RST mice revealed marked qualitative changes that correlated with the differences detected in proinflammatory cytokine expression. First, there was presence of leukocytic foci in wounds of RST mice with no evidence of acute inflammation in wounds of FWD mice. This confirmed that RST mice had a delay in resolution of the inflammatory phase at the wound site. In
addition, day 5 wounds of RST mice had reduced granulation tissue formation. These findings may be interrelated, and point to the macrophage as the common effector cell. Macrophages play a key role in the transition from the inflammatory to the granulation phase. They infiltrate the site of injury shortly after the neutrophils, aiding in tissue decontamination and destroying effete neutrophils. Macrophages produce anti-inflammatory cytokines such as IL-10, which inhibits synthesis of proinflammatory cytokines including IL-1α and IL-1β (DeVries, 1995). Thus, macrophages overlap and contain the early inflammatory response. Stress could be related to changes in the number of macrophages infiltrated at the wound site. Immunocytochemical studies by Rojas et al. (2001) showed no differences in the numbers of macrophages from day 5 wounds of FWD and RST mice. This points to possible impairments in the function of macrophages in RST mice. Impaired macrophage function may involve a depressed ability to recognize effete neutrophils, decreased phagocytosis, decreased bacterial killing, or decreased tissue debridement mechanisms. It has been demonstrated that stress decreases phagocytic activity of macrophages (Brown et al., 1993; Zhang et al., 1998). Down-regulation of IL-1β expression at day 1, shown in the present studies by RT-PCR, may result in decreased macrophage function in wounds of RST mice. In addition, Rojas et al. (2001) showed that gene expression of MCP-1, a chemokine that stimulates macrophage function, is suppressed in day 1 wounds of RST mice. Recent work by Low et al. (2001) demonstrated that wounds of MCP-1-deficient mice had altered healing of cutaneous wounds, yet they had normal levels of macrophages as compared to wounds from wild-type control mice (Low, Drugea, Duffner, Quinn, Cook,
Rollins, Kovacs, & DiPietro, 2001). This suggested that it is not only the presence but also the activity of macrophages that is important for wound repair. Therefore, it is speculated that RST results in decreased macrophage function via early suppression of IL-1β and MCP-1 expression at the wound site.

Stress-induced impairments in phagocytic function may compromise the macrophages' ability to control the acute inflammatory response initiated by neutrophils. Neutrophils could then become the predominant cells in leukocytic infiltrates of RST mice at day 5. Indeed, it has been demonstrated that day 5 wounds of RST mice have a seven-fold increase in myeloperoxidase, a marker of neutrophil infiltration, as compared to FWD mice (Rojas et al., 2001). Increased infiltration of neutrophils in day 5 wounds of RST mice may occur in response to significant increases in opportunistic bacterial load as compared to wounds from FWD mice (Rojas et al., 2000). Recent studies in a mouse model of pneumococcal pneumonia demonstrated that pathogen-derived chemoattractant factors play an important role in neutrophil and macrophage recruitment during infection (Fillion, Ouellet, Simard, Bergeron, Sato, & Bergeron, 2001). This suggests that chemotactic factors derived from bacteria, such as formyl peptides, may contribute to neutrophil recruitment in stressed mice. In addition, day 5 wounds of RST mice had significant up-regulation of Macrophage Inflammatory Protein-2 (MIP-2) and Cytokine-Induced Neutrophil Chemoattractant (CINC) gene expression (Rojas et al., 2001), chemokines involved in the recruitment of neutrophils (Kernacki, Barret, Hobden, & Hazlett, 2000; Kielian, Barry, & Hickey, 2001). Taken together, these studies suggest that decreased macrophage function, increased bacterial load, and up-regulated
expression of neutrophil-attracting chemokines may all contribute to high numbers of neutrophils in day 5 wounds of RST mice. Prolonged expression of IL-1α and IL-1β mRNA detected by RT-PCR in day 5 wounds of RST mice probably originates from persistent neutrophils at the site. Continued release of oxygen radicals and proteases from persistent neutrophils may also result in destruction of viable tissue (Martin, 1997; Ravage et al., 1998). Therefore, the persistence of neutrophils later in the inflammatory response may lead to tissue damage, thereby contributing to delayed wound closure in RST mice.

A decrease in macrophage function may also help to explain the reduced granulation tissue areas observed in day 5 wounds of RST mice. There could be down-regulation of macrophage-derived growth factors involved in granulation tissue formation. For example, TGF-β1 stimulates fibroblast proliferation (Roberts et al., 1996) and synthesis of a provisional extracellular matrix (Clark et al., 1995; Welch et al., 1990). VEGF is mitogenic for endothelial cells, thus stimulating neovascularization of (Nissen et al., 1998). Preliminary studies in our laboratory (Horan et al., 2001, unpublished observations) have demonstrated significant down-regulation of TGF-β1 and VEGF gene expression in wounds of RST as compared to FWD mice. Stress-induced elevations in GCs may be related to decreased expression of macrophage-derived growth factors in RST mice. It has been shown that cutaneous wounds of mice treated with DEX have decreased gene expression of TGF-β1 (Frank et al., 1996). Moreover, Brauchle et al. (1995) demonstrated that day 5 wounds of DEX-treated mice had decreased granulation tissue formation as compared to wounds of control mice (Brauchle et al., 1995).
Therefore, it is postulated that the protracted inflammatory response and impaired granulation tissue formation in day 5 wounds of RST mice is the result of decreased macrophage function mediated by stress-induced GCs. Future studies should evaluate the effect of stress-induced GCs on the activation and function of infiltrated macrophages during wound healing.
In situ hybridization revealed that day 5 wounds of RST mice had higher percentage of IL-1β mRNA-expressing KCs than wounds of FWD mice. This finding could be related to group differences in epithelial healing. Histological analysis revealed that most wounds of FWD mice were completely re-epithelialized at day 5. This suggests that in FWD mice IL-1β expression by KCs had nearly returned to constitutive levels. In contrast, most wounds of RST mice were not completely re-epithelialized at day 5. This suggests that in RST mice KCs were still proliferating and migrating. During these processes, KCs express high levels of IL-1β. IL-1 produced by KCs has an autocrine effect, stimulating further production of IL-1 and enhancing KC growth (Kupper et al., 1986). IL-1 also induces KC migration on collagen (Chen et al., 1995). It is possible that the abundance of IL-1β-labeled KCs in day 5 wounds of RST mice served to stimulate re-epithelialization of the defect. In addition, environmental challenges such as infection may activate epidermal KCs for increased expression of proinflammatory cytokines (Nickoloff, & Turka, 1993). This suggests that the presence of high counts of opportunistic bacteria in day 5 wounds of RST mice (Rojas et al., 2000) may have stimulated KCs for increased expression of IL-1β mRNA. A high frequency of IL-1β mRNA-expressing KCs may partially explain high levels of IL-1β mRNA detected by RT-PCR in day 5 wounds of RST mice.

As described above, IL-1 released during the inflammatory phase promotes re-epithelialization of the wound surface. IL-1 may further contribute to re-epithelialization by inducing expression of keratinocyte growth factor (KGF-1 or FGF-7) (Brauchle et al., 1994; Chedid et al., 1994). Released by dermal fibroblasts, KGF-1 stimulates
proliferation of KCs from the wound margins and remaining skin appendages (Rubin et al., 1989). There is strong up-regulation of KGF-1 during cutaneous wound repair (Marchese et al., 1995; Werner et al., 1992). Transgenic mice expressing a dominant-negative KGF-1 receptor in the epidermis had defects in re-epithelialization of full-thickness excisional wounds (Werner et al., 1994), suggesting that KGF-1 plays a critical role during wound healing. In the present studies, RT-PCR analysis demonstrated low, constitutive levels of KGF-1 mRNA expression in control skin, as shown previously by Finch et al. (1989). Restraint stress had no effect on KGF-1 gene expression in non-wounded skin. In wounds of FWD mice, strong KGF-1 induction was found at day 1, which started to decline at day 3 but was still significantly higher than in control skin at day 5. This kinetic pattern is consistent with previous in vivo studies which demonstrated that peak KGF-1 gene expression occurred within 24 hr after wounding and continued significantly elevated in day 7 wounds (Brauchle et al., 1995; Marchese et al., 1995; Werner et al., 1992). In the present studies, the major group difference was detected at day 1, when wounds of RST mice had significantly lower KGF-1 mRNA levels as compared to FWD mice.

*In situ* hybridization demonstrated that the major group difference in KGF-1 mRNA occurred at day 3, when labeled fibroblasts had a tendency to be fewer in wounds of RST as compared to FWD mice ($p < .08, t$ test). This is in contrast with RT-PCR findings, which demonstrated that KGF-1 mRNA down-regulation in RST animals occurred at day 1. It should be noted that in repeated experiments, stress-induced down-regulation in KGF-1 mRNA could be detected either at day 1 or at day 3. After pooling the data from
three replicate experiments for analysis and report of RT-PCR results, the main group difference in KGF-1 expression occurred at day one with no difference at day 3. The data from the *in situ* hybridization study corresponds to an experiment in which previous RT-PCR analysis had shown a significant group difference at day 3. In this regard, results from the two studies are in agreement and confirm that there is an effect of stress on fibroblast expression of KGF-1 mRNA. It should not be excluded that the differences detected by RT-PCR at day 1 may be due to decreased responsiveness of fibroblasts for expression of KGF-1 mRNA, with no significant changes in their frequency as demonstrated by *in situ* hybridization. Histological analysis of wounds from FWD and RST mice revealed marked differences in the epithelium that could be related to changes in KGF-1 expression. At day 3, wounds of FWD mice had thick proliferating zones composed of several layers of KCs. In contrast, wounds of RST mice had thin proliferating zones composed of fewer layers of KCs. A decrease in the frequency or responsiveness of fibroblasts for KGF-1 gene expression could compromise molecular signals for KC proliferation and consequently result in thin proliferating zones in day 3 wounds of RST mice.

The down-regulation of KGF-1 gene expression in wounds of RST mice correlated with results from *in vivo* studies by Brauchle *et al.* (1995). They found that the typical induction of KGF-1 mRNA after injury was reduced in DEX-treated mice, which also presented a dramatically delayed wound re-epithelialization. Thus, the depression in KGF-1 mRNA observed in RST mice could be mediated by stress-induced GCs, either by a direct or an indirect effect. First, cultured fibroblasts treated with DEX have reduced
KGF-1 mRNA expression (Brauchle et al., 1995; Chedid et al., 1996), which suggests that there may be a direct effect of GCs on mesenchymal cells. Alternatively, the early down-regulation of IL-1β, a positive regulator of KGF-1 (Brauchle et al., 1994; Chedid et al., 1994), in wounds of RST mice may be responsible for low expression of KGF-1. We could speculate that blockade of GC receptors indirectly restores KGF-1 mRNA expression by restoring IL-1β mRNA levels in wounds of RST mice. Indeed, Brauchle et al. (1995) demonstrated that the inhibitory effect of DEX on cultured murine fibroblasts was compensated for by addition of positive regulators of the KGF-1 gene, such as IL-1β. Nevertheless, the present study showed that treatment of RST mice with RU486 did not restore KGF-1 mRNA expression at day 1, even when the IL-1β response had recovered. Other hormones that are elevated during stress, such as catecholamines, may be involved in down-regulation of KGF-1 expression. However, no studies have addressed the effect of catecholamines on KGF-1 gene expression.

Catecholamine hormones are the major products of sympathetic nervous system (SNS) activation (Elenkov, Wilder, Chrousos, & Vizi, 2000). Several studies have suggested that catecholamines may play a role during wound repair. Catecholamines inhibit migration of epidermal cells via adrenergic receptors on the cell surface (Donaldson et al., 1984). In addition, catecholamines inhibit the growth of cultured fibroblasts when used in concentrations comparable to the levels found in the sera of post-surgical stress patients (Saito et al., 1997). These studies suggest that, by inhibiting migration of KCs and proliferation of fibroblasts, catecholamines may delay the rate of wound re-epithelialization and decrease the formation of granulation tissue. It is possible
that the molecular mechanisms behind these inhibitory actions of catecholamines involve alterations in KGF-1 gene expression. Future studies in the present model should investigate the influence of stress-induced catecholamines on KGF-1 gene expression and wound healing using adrenergic receptor blockers such as nadolol. Vascular responses modulated by cathecholamines such as edema (Koopman, 1995) and peripheral vasoconstriction (Baraniuk, 1997) may potentially impact wound repair processes and thus merit further investigation.

Using the same murine model of wound healing, Padgett et al. (1998) demonstrated that treatment of RST mice with the GC receptor antagonist RU486 restored normal kinetics of wound closure. This suggested that the signals for KC proliferation and migration across the wound edges, i.e., re-epithelialization, had been recovered. However, although wound closure was restored, the present studies showed that treatment with RU486 did not restore KGF-1 gene expression in wounds of RST mice. It should be noted that in full-thickness wounds KGF-1 has multiple other functions besides the promotion of re-epithelialization. These functions can only be evaluated with histological analysis of specially stained sections and with electron microscopy. Using such techniques, Staiano-Coico et al. (1993) demonstrated that KGF-1-treated wounds have increased number of serrated basal cells associated with increased deposition of collagen fibers in the superficial dermis. In addition, KGF-1-treated wounds had better developed hemidesmosomes associated with thicker bundles of tonofilaments in basal cells, suggesting that KGF-1 strengthens the basement membrane junction (Staiano-Coico et al., 1993). Therefore, low expression of KGF-1 mRNA in wounds of RST mice
treated with RU486 may have caused subclinical alterations in collagen deposition and basement membrane strength, thus compromising the structural and functional properties of the healing tissues despite clinical restoration of wound closure kinetics.

Recent studies have suggested that KGF-1 is not essential for wound repair. Guo et al. (1996) demonstrated that the healing process of incisional wounds appeared normal in KGF-1 knockout mice (Guo, Degenstein, & Fuchs, 1996). Although incisional wounds do not depend as extensively on re-epithelialization as full-thickness wounds do, the KGF-1 knockout study showed that at least incisional wounds could heal in the absence of KGF-1. This suggests that there could be other fibroblast-derived factors that compensate for KGF-1 in promoting KC proliferation and migration. Recently, a new member of the fibroblast growth factor family has been identified as KGF-2 or FGF-10 (Duan, Jimenez, Gruber, Liu, Feng, Florence, Blunt, Huddleston, Teliska, Alfonso, Coleman, Ornitz, & Dillon, 1997; Yamasaki, Miyake, Tagashira, & Itoh, 1996a). KGF-2 shares homology with KGF-1/FGF-7. Like KGF-1, KGF-2 acts in a paracrine manner: it is produced by dermal fibroblasts and exerts its action on epidermal KCs (Beer et al., 1997a). In mouse full-thickness excisional wounds, KGF-2 mRNA was highly induced 1 day after injury and decreased rapidly by day 3 (Tagashira et al., 1997). These results suggest that KGF-2 could compensate for the lack of KGF-1 during wound healing in KGF-1 null mice. KGF-2 binds to the same receptor that binds KGF-1 (Beer et al., 1997a). Thus, the defect in re-epithelialization observed in wounds of transgenic mice expressing a truncated receptor (Werner et al., 1994) could be explained by the receptor’s failure to respond to KGF-2 binding, rather than by an inability to respond to KGF-1
binding as previously thought. Other studies by Jimenez et al. (1997) demonstrated that KGF-2 promoted wound closure in excisional wounds of methylprednisolone-treated rats, which also had a significant increase in re-epithelialization, reduction in wound gap, increased granulation tissue with collagen deposition, abundant fibroblasts, and neovascularization (Jimenez, Teliska, Liu, Judis, Rampy, & Antonaccio, 1997). These studies show that KGF-2/FGF-10 is a novel mediator of re-epithelialization that merits more extensive investigation especially in models of impaired wound healing. Therefore, further attempts to elucidate the molecular mechanisms of delayed wound healing under conditions of stress should include the analysis of KGF-2/FGF-10 gene expression at the wound site.

The present studies have focused on the effects of stress on expression of proinflammatory cytokines and growth factors at the mRNA level. It is believed that the most important steps in gene regulation occur early, from control of gene transcription within the cell nucleus to control of mRNA degradation in the cell cytoplasm. GCs are widely known for their profound suppressive effects on mediators of inflammation and cell growth, involving GC interference with transcription factors needed to turn on transcription, induction of transcription inhibitors, and stimulating mRNA destabilization. Thus, it became evident that an investigation of the molecular effects of stress-induced GCs on wound healing had to focus on the mRNA level of cytokine and growth factor expression. Previous studies on cytokine and growth factor expression in skin during wound healing have looked at both the mRNA and the protein levels. Hübner et al. (1996) demonstrated that IL-1α transcript levels in full-thickness wounds of mice
correlate with tissue levels of IL-1α protein. On the other hand, mature IL-1β proteins (17 kDa) were hardly detectable and the precursor form of IL-1β protein (33 kDa) was only found at low levels in comparison to the dramatic induction observed in IL-1β mRNA levels. This suggested that translation of the IL-1β mRNA and maturation of the precursor protein was not efficient during wound repair. However, the amounts of mature protein detected may be sufficient to induce biological effects since IL-1β protein is biologically active at low concentrations (Hübner et al., 1996). For KGF-1, Marchese et al. (1995) demonstrated that transcript levels in full-thickness wounds of humans correlate with tissue levels of the growth factor. Therefore, it is anticipated that in the present murine model of cutaneous wound healing the induction and kinetics of IL-1α and KGF-1 protein synthesis will follow the pattern observed at the mRNA level. For IL-1β, however, changes at the protein level may not correlate with the changes observed at the mRNA level. Future studies should investigate the amounts of IL-1α, IL-1β, and KGF-1 protein present in lysates from wounded skin by immunoprecipitation and also the tissue distribution of protein-secreting cells by immunocytochemistry.

Cellular processes that occur during the first days after injury are overlapped by other processes that occur over weeks. For example, reduction of the wound size is initially achieved by re-epithelialization. Eventual closure of the defect is achieved by contraction, which continues even after the wound is healed. Wound contraction is mediated by TGF-β1, which is produced by platelets, activated macrophages, neutrophils, fibroblasts, and KCs (Roberts et al., 1996). TGF-β1 promotes fibroblast proliferation, migration, and differentiation. Fibroblasts differentiate into specialized contractile cells
known as myofibroblasts, which contain α-smooth muscle actin. TGF-β1 induces the expression of α-smooth muscle actin in granulation tissue fibroblasts (Desmouliere, Geinoz, Gabbiani, & Gabbiani, 1993). Myofibroblasts adhere to one another and to the wound margins (Montandon, D'Andiran, & Gabbiani, 1977). As a result, the entire granulation bed contracts, drawing the edges together. At the same time, collagen is synthesized, deposited, and cross-linked to form a rigid scaffold holding the wound in place (Montesano et al., 1988; Woodley et al., 1991). A decrease in the signals for fibroblast proliferation and differentiation may compromise contraction, and in turn wound closure. Preliminary studies in our laboratory (Horan et al., 2001, unpublished observations) showed that TGF-β1 gene expression was down-regulated in day 3 wounds of RST mice. Expression of α-smooth muscle actin mRNA was also down-regulated in wounds of RST mice, suggesting that there could be a decrease in fibroblast differentiation or lower numbers of myofibroblasts. Moreover, there was a significant decrease in wound contraction and closure in RST mice during the first five days after wounding. These results suggest that stress may suppress molecular signals important for wound contraction. Impairments in wound contraction may be another contributory factor to delayed wound closure in RST mice. Future studies should investigate if stress-induced GCs regulate wound contraction and expression of TGF-β in wounds of FWD and RST mice.

In conclusion, the present studies have demonstrated that the effects of stress on wound healing start during the early inflammatory response and take place at different tissue compartments of the wound. Based on these findings, Figure 4.1 illustrates the
proposed mechanism for the effect of RST on inflammation and wound healing. First, stress causes a depression in the early inflammatory response, which occurs within one day after wounding. The following events take place at this time point within the architecture of the wound:

(1) There is decreased frequency of IL-1β mRNA-expressing fibroblasts in the dermis. It is also possible that fibroblasts have a decreased responsiveness for expression of IL-1β mRNA. This will compromise signals for activation and priming of infiltrated leukocytes.

(2) Although no differences were detected in the frequency of IL-1β mRNA-expressing leukocytes, it is proposed that these cells have a decreased early responsiveness for expression of IL-1β mRNA. As a result, chemotactic cytokines (such as MCP-1) that are important for stimulating the phagocytic function of leukocytes may also be down-regulated. This will compromise the antimicrobial capabilities of infiltrated leukocytes.

(3) Decreased responsiveness of leukocytes for IL-1β mRNA expression may attenuate signals necessary for KC proliferation.

(4) There is decreased expression of KGF-1 mRNA from dermal fibroblasts, which will also compromise KC proliferation.

Therefore, a depressed early inflammatory response to injury fails to prepare the infiltrated neutrophils and macrophages to efficiently eradicate invading bacteria at the wound clot and edges, thus allowing an opportunistic infectious process to develop. In addition, there is considerable less cell growth at the epithelial wound margins. The
consequences of the early suppressive effect of stress on inflammation and KC proliferation will extend into the proliferative phase of healing, which occurs at day 5 after wounding. These events are explained as follows:

(5) Although KCs seem to have migrated across the wound edges and have partially covered the defect, there has been considerably less KC proliferation.

(6) There are high counts of opportunistic bacteria due to decreased phagocytic function of leukocytes in the wound. Increased bacterial accumulation leads to the release of bacterial products and induction of chemotactic cytokines that will recruit numerous neutrophils into the area. Infiltrated neutrophils predominate in the wound and are inefficiently cleared by dysfunctional macrophages. Release of oxygen radicals and proteases from persistent neutrophils will result in destruction of viable tissue. Neutrophils and activated KCs are responsible for high levels of IL-1α and IL-1β mRNA at a time when proinflammatory cytokine gene expression should have returned to constitutive levels.

(7) There are multiple foci of dense leukocytic infiltration, which is indicative of a protracted inflammatory process in response to increased bacterial invasion.

(8) In the dermis, there is reduced granulation tissue formation. This may be due to a decreased responsiveness of macrophages for the synthesis and release of growth factors necessary to promote fibroplasia, neovascularization, and collagen deposition. Ultimately, the aforementioned effects of stress on wound healing will result in an overall delay in the kinetics of wound closure. More important clinically, however, are the profound impairments in the quality of the healing tissues under conditions of stress.
First, there is increased incidence in opportunistic bacterial infections, resulting in development of cutaneous microabscesses. There is reduced granulation tissue formation, thus compromising oxygenation of the tissues, proliferation of fibroblasts, and synthesis of a new extracellular matrix. This will jeopardize the structural reconstitution of tissues, resulting in low functional strength and poor cosmetic quality of the healed skin. There is a thin epithelial layer covering the defect, probably with weak attachment of basal KCs to the basement membrane. This will compromise the barrier function of the epidermis and render it highly vulnerable to frictional trauma. Therefore, chronic stress imposes significant changes on local molecular and cellular processes that render the host inadequately prepared to respond to tissue trauma and consequently result in dysregulated and even debilitating mechanisms of self-repair.

Understanding the mechanisms by which stress impacts wound healing helps to anticipate and explain healing impairments in surgical patients and in individuals with immunocompromising conditions such as diabetes. Based on these mechanisms, therapeutic strategies for the improvement of cutaneous repair may be designed and implemented. First, psychological intervention in pre-surgical patients can be attempted to reduce their anticipatory anxiety levels. Post-surgically, psychological interventions should continue to ameliorate pain stress and depression. These interventions will assist in reducing circulating levels of stress hormones and therefore may enhance the physiological ability for healing of the surgery site. Pharmacological modulation of soft tissue repair is currently under clinical testing. A variety of cytokines and growth factors are considered candidate therapeutic agents because of their ability to stimulate cells
required for tissue repair and because they have shown to be deficient in a variety of models of chronic nonhealing wounds. For example, IL-1β has been used to enhance healing of infected open wounds (Kucukcelebi, Hui, Sahara, & al, 1992). A clinical trial demonstrated that IL-1β is effective in extending the ability of chronic pressure ulcer fibroblasts to replicate and that it had a major effect on the development of the extracellular matrix (Vande Berg, Robson, & Mikhail, 1995). More recent studies showed that sequential therapy consisting of priming of tissue with an inflammatory cytokine followed by application of a proliferative cytokine at the time of incision closure nearly doubled the breaking strength of an acute wound in a rat dermal model (Smith, Kuhn, Franz, Wachtel, Wright, & Robson, 2000). Clinical trials are currently in progress to evaluate the safety and efficacy of KGF-1 as a cytoprotective agent against radiation- and/or chemotherapy-induced oral and gastrointestinal mucositis (Danilenko, 1999). Thus, preparing the host to mount a robust early inflammatory response with proinflammatory cytokines and manipulating the proliferative phases of wound healing with growth factors may enhance wound repair. These pharmacological modalities may also hold promise for the restoration of prompt and efficient wound repair in individuals undergoing chronic stress.
Figure 4.1. Proposed mechanism of stress, inflammation, and wound healing.

Day 1: Early inflammatory phase
- Keratinocytes
- Wound clot
- Decreased IL-1β
- Decreased KGF-1

Day 5: Proliferative phase
- Capillary loop at the dermal papilla
- Leukocytic infiltrate
- Up-regulation of IL-1α and IL-1β expression due to increased bacterial invasion

STRESS

Stress reduces the responsiveness of cells for the expression of IL-1β and KGF-1.

Impaired quality of healing tissues
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