Effects of High Estrogen Levels on Monocyte Chemoattractant Protein-1 and Wound Healing

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Objective: Herein, we tested the effects of high levels of supplemental estrogen treatment on cutaneous wound healing.

Approach: Female mice were implanted with a 17 β -estradiol (E₂) secreting pellet or placebo before receiving a full-thickness dermal excisional wound. Mice receiving the E₂ pellet attained hormone levels that are comparable to those achieved during pregnancy. At 1, 3, and 5 days after injury, the dermal excision wound was examined for their histologic appearance, rate of closure, and chemokine levels.

Results: Wound closure, assessed by percent reepithelialization, was slower in E₂-treated mice relative to placebo (42.6% $\pm$ 6.6% vs. 70.0% $\pm$ 5.3%, respectively, 3 days after injury). In addition, there was a marked reduction in the subepithelial inflammatory infiltrate and granulation tissue in E₂-treated mice relative to placebo. Wound levels of monocyte chemoattractant protein-1 (MCP-1) were increased by 3 days after injury and continued to rise at 5 days after injury in placebo-treated mice ($p < 0.01$). By contrast, MCP-1 levels were significantly reduced at 3 and 5 days after injury in E₂-treated mice relative to placebo-treated controls ($p < 0.01$). This attenuation could be reversed by treatment with an estrogen receptor antagonist.

Innovation: High levels of estrogen are able to suppress normal wound closure.

Conclusion: Dermal wound healing can be altered by manipulating the gonadal steroid hormone levels. In particular, high levels of estrogen can be utilized to slow down the rate of wound healing through a reduction in the inflammatory response.

INTRODUCTION

THE GONADAL STEROID hormone estrogen not only controls the sexual dimorphism of the immune response,¹ but also influences wound healing through a variety of noninflammatory mechanisms.^{2–4} This is supported by the observation that autoimmune connective tissue disorders, such as scleroderma and lupus erythematosus, are more prevalent in women of reproductive age than in postmenopausal women or in men.⁵ Furthermore, high levels of estrogen

may be therapeutic in these and other disease states, as symptoms are often suppressed during pregnancy when circulating levels of estrogen reach 100 to 1,000 times that of cycling females.⁶ In addition, because of the ameliorating effects of high physiologic levels of estrogen in rheumatoid arthritis, there are anecdotal reports of deliberate use of pregnancy as a therapeutic intervention.⁷ Animal models designed to evaluate the role of estrogen in connective tissue formation reveal

that high levels of estrogen suppress peritoneal adhesion formation,² whereas lower proestrus levels of estrogen enhance connective tissue deposition.^{8,9} Taken together, these observations suggest that it is the concentration of estrogen, not only the presence of the hormone, which dictates outcomes.

The critical role of macrophages in the repair process after tissue injury has been known for nearly half a century,^{10,11} however, the mechanisms by which circulating monocytes and tissue macrophages migrate to sites of injury are only partially understood. Chemotactic cytokines, including monocyte chemoattractant protein-1 (MCP-1), are produced by macrophages and fibroblasts, as well as numerous other cell types.¹² Local production of these mediators leads to recruitment and activation of cells at the site of injury and initiates the cascade of events leading to wound healing.^{13,14}

The mechanisms by which estrogen modulates inflammation and wound healing have not been fully elucidated. Interestingly, estrogen receptors (ERs) have been found not only in classical reproductive tissues but also in immune cell populations (including lymphocytes, monocytes, and macrophages),¹ suggesting that the hormone could directly affect cellular function. Studies from several different groups have shown that estrogen has a biphasic effect on the production of macrophage-derived cytokines commonly involved in immune and inflammatory processes, including tumor necrosis factor α , interleukin-1 β , interleukin-6, and MCP-1.^{1,5-19} Thus, the effects of estrogen on inflammation and wound healing could be mediated by controlling the recruitment and activation of key regulatory cells. While the effect of E₂ at lower concentrations are more well understood,^{3,4} its effect at pregnancy levels are not.

CLINICAL PROBLEM ADDRESSED

It is well accepted that pregnancy is associated with a dampening of inflammatory responses and that this is largely mediated by the effects of estrogen on the immune system.²⁰ Clinically, these high levels of estrogen can be used to promote the healing of chronic wounds that have failed to close secondary to a persistent inflammatory state.²¹ However, a separate and distinct issue is that of wound healing for an acute wound that is not associated with an inflammatory state. This study seeks to understand how high levels of estrogen can influence wound healing to better understand wound healing in the pregnant patient.

MATERIALS AND METHODS

Animals

Female adult C57Bl/6 mice (25–30 g) (Harlan Laboratories, Indianapolis, IN) were maintained on a 12-h light/12-h dark cycle (7 am lights on) and provided with food and water ad libitum. All experimental procedures were performed in accordance with the Loyola University Animal Research Facility guidelines. Mice were randomly assigned to different treatment groups. Out of concern for potential effects of the circadian rhythm on corticosteroids and how hormone fluctuations might influence inflammation and wound healing, all animal procedures were carried out between 8 and 10 in the morning.

Hormone treatment

Under methoxyflurane (Pitman-Moore, Inc., Mundelein, IL) anesthesia, intact mice were given subcutaneous implants of 21-day slow diffusion polymer pellets containing E₂ (0.5 mg), 17 α -estradiol (5.0 mg), or placebo (Innovative Research of America, Tampa, FL), as previously described.¹⁹ The E₂ pellet, the biologically active form of estrogen, delivers a constant circulating hormone level that has previously been shown to be equivalent to the high levels found during pregnancy.²² A 10-fold excess of the antagonist, 17 α -estradiol, was used to ensure complete blockage of the agonist. Mice were allowed to recover from the implantation procedure for a minimum of 5 days to allow for equilibrium of hormone levels.

Generation of full-thickness excisional wounds

While the mice were under methoxyflurane anesthesia, full-thickness excisional wounds were produced, as previously described.²³ Briefly, six full-thickness excisional wounds were made with a standard 3-mm biopsy punch (Acuderm, Inc., Ft. Lauderdale, FL) on the shaved dorsal surface of the mice. On day 1, 3, and 5 after injury, groups of animals were euthanized. Peripheral blood was collected and the wounds were removed with the surrounding tissue. Serum was frozen at -80°C before measurement of circulating estradiol levels.

Wound tissue and adjacent skin were collected. To ensure that equal amounts of tissue were obtained for analysis, skin was removed by surgical excision that extended to the skeletal muscle of the back, such that all epidermal, dermal, and panniculus carnosus components were preserved. This was accomplished by spreading the pelt on a rubber block and cutting a uniform size wound specimen with a 5-mm biopsy punch, as previously

described.²⁴ The wounds were either fixed in formalin for histology or flash-frozen in liquid nitrogen for cytokine analysis.

Analysis of inflammation, collagen deposition, and reepithelialization

For histologic analysis, tissue samples were placed in 10% buffered formalin and fixed overnight, as previously described.² Following fixation, samples were paraffin embedded and sectioned in 6 μ m increments throughout the entire wound. Every 5th section was saved so that the midpoint (widest portion of the wound) could be measured. Hematoxylin and eosin (H&E)-stained sections were used to assess the inflammatory infiltrate based upon cell morphology. The collagen content was evaluated in adjacent sections stained with Masson's trichrome.

Skin samples from wounded mice were examined by light microscopy for evaluation of reepithelialization, as previously described.²³ To measure the degree of reepithelialization, the central (widest) portion of the wound was viewed under 100X or 400X power. The distance within the width of the wound and the distance that the epithelium had traversed were measured. The percent reepithelialization was calculated with the following formula: % reepithelialization = (distance covered by epithelium/width of wound bed) $\times$ 100.

In addition to evaluating dorsal skin from wounded mice, skin from unmanipulated mice was also taken for comparative purposes. Unmanipulated mice were not administered methoxyflurane nor were they subjected to injury or hormone manipulation.

Determination of dermal MCP-1 content

For cytokine measurements, tissue samples were obtained at days 1, 3, and 5 after injury, as described above. The tissue samples were homogenized in a protease inhibitor cocktail (Sigma, St Louis, MO), as previously described.²⁵ Aliquots of the filtered homogenates were frozen at -80°C before assay. MCP-1 levels were assayed by enzyme-linked immunosorbent assay according to the manufacturer's protocol (Biosource International, Camarillo, CA). The total protein concentration of homogenates was measured in mg using the BioRad Protein Assay (BioRad, Hercules, CA), and the ratio of protein:biopsy was found not to differ throughout the time course. Data are expressed as the amount of chemokine in pg per 5 mm biopsy. The minimum detectable levels of MCP-1 in the immunoassays were less than 2.0 pg/mL. In addition to evaluating normal unwounded skin from animals of each treatment group, skin from

unmanipulated mice was also assessed for MCP-1 levels. For those samples, a 5 mm biopsy was taken from the animal. A 3 mm punch was taken out of the center of the biopsy and discarded. This was done to more closely replicate the skin biopsy samples of the manipulated groups. Data are expressed as percent control using the following formula: % control = (MCP-1 of manipulated group/average MCP-1 of uninjured animals) $\times$ 100.

Determination of serum estradiol level

Whole blood was obtained by cardiac puncture and allowed to coagulate at room temperature for 2 h, as previously described.²² The serum concentration of E_2 was determined by RIA (Diagnostic Products Corp., Los Angeles, CA).

Statistical analysis

Data are expressed as treatment group mean $\pm$ the standard error of the mean. Data are compared using a two-way ANOVA followed by Tukey's honestly significant difference, where appropriate, with InStat II software. A p -value of less than 0.05 was considered significant.

RESULTS

Assessment of dermal inflammation in the absence of estrogen treatment

H&E staining of uninjured skin demonstrated a complete absence of inflammatory processes throughout the dermal and hypodermal regions (Fig. 1A). In contrast, at 1 day after injury, wounded skin revealed considerable widespread inflammatory cell infiltration (Fig. 1B). High-power microscopic evaluation of the inflammatory cells in the dermis and hypodermis showed that the majority of the inflammatory cells present were neutrophils at this time point. In addition, numerous neutrophils were visualized adhering to the endothelium of vessel walls throughout the hypodermis. In contrast, at 3 days after injury, the inflammatory infiltrate contained a mixture of neutrophils and mononuclear cells, which were located throughout the underlying regions (Fig. 1C). By 5 days after injury, the cellularity was composed of mononuclear cells and fibroblasts, with few neutrophils (Fig. 1D). The infiltration of inflammatory cells was consistent among numerous histologic preparations that were examined.

Effects of high levels of E_2 on inflammation

Examination of H&E-stained slides of wounded tissue revealed that the influx of inflammatory cells was different in mice given high levels of E_2 and placebo treatments. In E_2 -treated mice, there

Figure 1. Time course of dermal repair after full-thickness excisional wounds. Representative hematoxylin and eosin (H&E)-stained sections of skin from female mice are shown. Control skin from an unmanipulated mouse (A) Skin obtained 1 day (B) 3 days (C) and 5 days (D) after administration of a 3 mm full-thickness wound. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

was a larger percentage of neutrophils on days 3 and 5 than following placebo treatment. At 3 days after injury, in placebo-treated mice, the inflammatory infiltrate contained both neutrophils and mononuclear cells (Fig. 2A), whereas in E_2 -treated animals, the infiltrate included more neutrophils (Fig. 2B). In addition, by 5 days after injury, when few, if any, neutrophils were present in the dermis and hypodermis of the placebo-treated mice, this cell type was abundant in the skin of E_2 -treated animals (data not shown). At this time point, the dermis of the placebo-treated animals contained primarily mononuclear cells and fibroblasts, and a greater amount of granulation tissue was present relative to E_2 -treated animals. This delay in the

progression of the inflammatory response and the presence of granulation tissue is consistent with a delay in wound healing.

Effects of E_2 on reepithelialization

There were no differences between the inflammatory infiltrate or rate of wound closure in placebo-treated and untreated mice subjected to dorsal wounding (data not shown). In contrast, both parameters were markedly affected by E_2 treatment (Fig. 2). The degree of reepithelialization was significantly diminished in E_2 -treated female mice relative to placebo treatment ($p < 0.01$). At 3 days after injury, wound closure in female mice given high levels of E_2 was approximately 60% that

Figure 2. Effect of estrogen on dermal wound healing. Representative trichrome-stained slides are shown. Female mice were given E_2 (or placebo) in the form of slow releasing polymer pellets designed to achieve pregnancy levels of the hormone. A comparison of placebo-treated (A) and E_2 -treated (B) wounds are shown at 3 days after injury. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

of placebo treatment (Fig. 3). Although there was a significant delay in wound reepithelialization, by 10 days after injury wound closure was complete, demonstrating that the delay was brief.

Since administration of high levels of E_2 slowed wound closure in female mice, further studies focused on the effects of hormone manipulation on wound healing in female mice. To confirm the role of high levels of E_2 in delaying wound closure, additional studies examined the degree of reepithelialization at 3 days after wounding in females given the estrogen antagonist 17α -estradiol alone or in combination with E_2 (Fig. 3). These studies showed that treatment with 17α -estradiol did not alter the degree of reepithelialization. Treatment with both E_2 and 17α -estradiol restored reepithelialization to the levels seen in placebo-treated mice.

Production of MCP-1 at the wound site

Since it appeared that wound healing was delayed in E_2 -treated mice and estrogen was shown to inhibit MCP-1 mRNA expression in other animal models [2], we tested whether E_2 -mediated effects on dermal wound healing correlated with local production of MCP-1. To accomplish this, MCP-1 levels were measured in the wound tissue at 1, 3, and 5 days after injury. These studies revealed that at 1 day after wounding, the level of MCP-1 in skin of E_2 -treated female mice was threefold above that of skin from unmanipulated animals. The levels of MCP-1 in dermal wounds from placebo-treated female mice continued to increase at 5 days to approximately twofold higher than E_2 -treated female mice, after which it gradually diminished (Fig. 4).

Figure 3. Effects of estradiol on the degree of wound closure in female mice. After hormone equilibration, mice were subjected to full-thickness excisional dermal wounds. Wounds were examined for the degree of reepithelialization on day 3 after wounding. Data are shown as mean $\pm$ SEM. $n=12$ for placebo and E_2 groups, $n=6$ for 17α -estradiol and E_2 + 17α -estradiol. * $p<0.05$ from all other groups, as determined by ANOVA.

Figure 4. Effects of E_2 on dermal monocyte chemoattractant protein-1 (MCP-1) levels during the course of wound healing. Female mice were given E_2 or placebo before administration of excisional wounds. The wound and adjacent dermal tissue were collected on days 1, 3, and 5 after injury. Tissue samples were homogenized in a protease inhibitor cocktail, as described in the Materials and Methods. MCP-1 levels were determined and are expressed as a mean $\pm$ SEM relative to uninjured mice. $n=4$ on day 1 after wounding and $n=6$ on days 3 and 5 after wounding. * $p<0.001$ from placebo day 3, # $p=0.05$ from all other groups, + $p<0.01$ from placebo day 5, as determined by ANOVA.

For E_2 -treated female mice, at both 3 and 5 days after wounding, the levels of MCP-1 in the skin was less than half that of placebo-treated animals ($p<0.05$). Treatment with the ER antagonist 17α -estradiol alone had no effect on MCP-1 levels, and the combined treatment with E_2 and 17α -estradiol resulted in MCP-1 levels similar to that of placebo-treated female mice 3 days after wounding (Fig. 5).

Effect of hormone replacement on circulating estradiol levels

To determine whether hormone treatment resulted in similar levels of E_2 , serum obtained from

Figure 5. Effects of an estrogen antagonist on MCP-1 levels in excisional wounds. Female mice were given E_2 , 17α -estradiol, or a combination of the two before excisional wounding. Three days after injury, the wounds were excised and assessed for MCP-1 levels. Data are expressed as a mean $\pm$ SEM relative to biopsies from uninjured mice. $n=12$ for E_2 and placebo groups, $n=6$ for 17α -estradiol and E_2 + 17α -estradiol groups. * $p<0.01$ from all other groups, as determined by ANOVA.

female mice was tested for hormone content. Circulating levels of E_2 were 43 ± 33 pg/mL in placebo-treated female mice, which are consistent with the range of hormone concentration over the estrus cycle of intact nonsynchronized female mice. The hormone level in E_2 -treated female mice was 184 ± 46 pg/mL ($p < 0.05$ relative to placebo-treated males or females), which is consistent with hormone levels seen during pregnancy in mice.

DISCUSSION

Previous work from this laboratory demonstrated that ovariectomized mice with high pregnancy levels of E_2 had a marked attenuation of peritoneal adhesion formation relative to placebo treatment.² This suppression in connective tissue deposition was concomitant with a reduction in local expression of MCP-1 mRNA. Furthermore, the culture of macrophages and fibroblasts with pregnancy levels of E_2 significantly suppressed MCP-1 mRNA and protein production, suggesting that the hormone could have direct effects on the cell types, which play a central role in the deposition of connective tissue.^{26,27} Herein, we report that treatment of intact female mice with comparable levels of E_2 resulted in delayed reepithelialization and macrophage recruitment at the site of injury and that this was associated with significant suppression in dermal levels of the chemoattractant cytokine MCP-1. In addition, the effects of pregnancy levels of E_2 in females could be reversed by treatment with an ER antagonist, confirming that they are specific to that hormone.

We are yet to determine the mechanism(s) by which estrogen attenuates connective tissue deposition in either the dermal wound healing or peritoneal adhesion model, but it is likely that mononuclear phagocytes play a key role in the process. The involvement of monocytes/macrophages in the tissue response to injury was demonstrated by the studies of Lebovich and Ross, in which wound healing was delayed in monocytopenic animals.¹⁰ Further studies suggest that MCP-1 is critically involved in dermal wound healing as reepithelialization, leukocyte infiltration, angiogenesis, and collagen deposition are delayed in MCP-1-deficient mice.¹⁵

A likely mechanism for the suppressive effects of E_2 on connective tissue deposition would involve attenuating local MCP-1 levels and resulting in less recruitment of mononuclear phagocytes to the wound bed. This, in turn, would yield less production of various fibrogenic mediators, such as transforming growth factor β (TGF β), a response that is

heavily mediated by macrophages.¹⁵ This process is supported by the observation that MCP-1^{-/-} mice are known to produce less TGF β following wounding.²³ The suppressed levels of TGF β hinder fibroblast proliferation and delay wound closure.

Based on these observations, it suggests that treatment with pregnancy levels of estrogen would be expected to slow wound healing. While this has not been examined directly, there is anecdotal evidence that patients who undergo cesarean sections are less likely to develop peritoneal adhesions than those who undergo other types of abdominal surgeries.²⁸ Along with other differences in the surgical procedures, cesarean section patients have higher circulating levels of estrogen at the time of the procedure. Of additional clinical relevance is the potential for an estrogen-induced delay in wound healing, increasing the rates of infections and/or incisional hernia formation. Not surprisingly, others have demonstrated that cesarean sections make up a disproportionate percentage of index surgeries among women undergoing incisional hernia repairs.²⁹ While the authors concluded that pregnancy was a predisposing risk factor for incisional hernia formation, the results of this study might suggest that the risk is not due to pregnancy *per se*, but rather due to the effects of high levels of estrogen. Taken together, these studies suggest a need to better understand what effect high levels of estrogen may have on wound healing.

The results in the present study differ from most studies examining the effects of estrogen on wound healing. Estrogen has classically been thought to enhance cutaneous wound healing.^{3,4,30-32} These stimulatory effects on wound healing have been shown to be related to ER β on keratinocytes and not from anti-inflammatory effects of macrophages or other cell types.^{31,33} However, these studies have focused on much lower levels of estrogen administration and/or supplementation that are often 1/10th of the dose being used in the present experiments. While these studies have shown a beneficial effect to low-dose estrogen supplementation in promoting wound healing, the effects of high levels of estrogen have remained uncertain before the present study. The present results should not be viewed as contradictory to these prior findings, but rather should be viewed as somewhat complementary as they highlight that the dose effect of E_2 on wound healing follows a bell-shaped curve.

Multiple cell types in the healing wound have been shown to produce MCP-1.^{23,26,34} While the present study does not determine which of these cell populations were producing the chemokine nor the genetic mechanism responsible for the effect of

estrogen on the production of the chemokine, evidence from others suggests that there is a direct action of the hormone on MCP-1 expression in keratinocytes and fibroblasts.^{35,36} In addition, estrogen has a direct effect, through ER α , on impairing macrophage recruitment and function in other wound healing and inflammatory models.³⁷ These models have shown that the anti-inflammatory effects of estrogen are dose dependent with immunosuppression of monocytes not occurring until higher doses of E₂.³⁸ This decrease in inflammation may be mediated, in part, though decreased recruitment, as high levels of estrogen have been shown to decrease MCP-1 and mRNA production, and lower levels had increased its production.^{26,36} This relationship may explain the divergent effects of E₂ on wound healing with lower doses having a stimulatory effect on the keratinocytes and minimal effect on the macrophages, whereas with higher doses, the anti-inflammatory effects may be so great that any benefit from keratinocyte activation is never realized. Further studies are needed to confirm this clinical suspicion.

INNOVATION

These data show that pregnancy levels of estrogen inhibit the rate of dermal wound healing and local production of MCP-1. Furthermore, antagonism of endogenous estrogen does not alter these wound healing parameters. Overall, these studies have enabled us to achieve a better understanding of the effects of estrogen on inflammatory and fibrotic responses and may lead to the development of therapeutic regimens to treat or lessen the severity of disorders resulting from excessive connective tissue production.

KEY FINDINGS

- Pregnancy levels of E₂ inhibit local production of MCP-1
- Pregnancy levels of E₂ inhibit neutrophil migration into the wound
- Pregnancy levels of E₂ decrease wound reepithelialization
- Pregnancy levels of E₂ delay wound healing

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Abbreviations and Acronyms

E₂ = 17 β -estradiol
 ER = estrogen receptor
 H&E = hematoxylin and eosin
 MCP-1 = monocyte chemoattractant protein-1
 TGF β = transforming growth factor β