Effects of Steroids and Retinoids on Wound Healing

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Hypothesis: Anti-inflammatory corticosteroids significantly impair wound healing. Retinoids partially, but significantly, reverse this effect. Little is known about the mechanism of steroid retardation or retinoid reversal. We hypothesized that corticosteroids lower transforming growth factor-β (TGF-β) and insulin-like growth factor-I (IGF-I) levels and tissue deposition in wounds and that retinoids stimulate corticosteroid-impaired TGF-β and IGF-I release and collagen production.

Design: Randomized controlled trial.

Setting: Wound healing research laboratory.

Participants: Animal study.

Interventions: Four wire mesh wound cylinders were implanted subcutaneously into the backs of 72 male Sprague-Dawley rats. Wound healing was impaired by a single subcutaneous injection of 6 mg of methylprednisolone acetate (Depo-Medrol). Two preparations of retinoids were used in separate experiments: all-trans-retinoic acid and 9-cis-retinoic acid that were fed orally.

Main Outcome Measures: Hydroxyproline content was measured in the healing tissue and TGF-β and IGF-I levels were analyzed in the wound fluid.

Results: Methylprednisolone treatment significantly decreased TGF-β and IGF-I levels in the wound fluid and hydroxyproline content in the tissue (P < .05). Oral all-trans- and 9-cis-retinoic acid partially reversed the TGF-β and IGF-I decrease and significantly increased hydroxyproline content toward normal levels (P < .05). Oral all-trans-retinoic acid enhanced collagen deposition, TGF-β and IGF-I levels over normal chow fed control animals (P < .05).

Conclusions: Steroids and retinoids have antagonistic effects on growth factors and collagen deposition in wound healing. These effects can be relevant for treatment options in a clinical setting.

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Anti-inflammatory glucocorticosteroids markedly affect most aspects of wound healing. When administered sufficiently early after injury, high corticosteroid levels delay the appearance of inflammatory cells, fibroblasts, the deposition of ground substance, collagen, regenerating capillaries, contraction, and epithelial migration. Retinoids have the unique ability to reverse these inhibitory effects, except for wound contraction. Impairment of the inflammatory response, tensile strength, and collagen accumulation in cutaneous wounds after steroid treatment are partially, but significantly, reversed by retinoid administration (retinyl ester, retinol, and/or retinoic acid). Similar observations were made studying flexor tendon repair, healing of rat femoral fractures, vessel repair, and healing of intestinal anastomoses. Although steroid retardation of healing is a significant clinical problem and a commonly used laboratory model in the study of repair processes, little is known about the mechanisms of either steroid retardation or reversal by retinoids.

Retinoids are global regulators of the growth and differentiation of many cell types. It is now appreciated that all actions of retinoids are mediated through 2 families of nuclear receptors belonging to the steroid hormone receptor superfamily. These receptors, termed retinoic acid receptors (RARs) and retinoid X receptors (RXRs), bind and transactivate distinct response elements of numerous genes required to maintain differentiation and proliferation of epithelial tissues. The ligand for these receptors is retinoic acid. Other, more
MATERIALS AND METHODS

Four wire mesh wound cylinders were implanted under the dorsal skin of 72 male Sprague-Dawley rats (weighing 350 g). Wire mesh cylinders were chosen to obtain wound fluid and wound tissue simultaneously and to assess wound fluid at different times. Wound healing was impaired by a single subcutaneous injection of 6 mg of methylprednisolone acetate at the time of surgery. This time point was chosen since the inhibitory effect of glucocorticosteroids on wound healing is most pronounced when administration of the hormone begins just prior to the onset of inflammation in the healing process.26

The following preparations of retinoids were used:

1. all-trans-retinoic acid (120 mg of all-trans-retinoic acid per kilogram of diet); 20 g of diet fed daily (approximately 2.4 mg of all-trans-retinoic acid per rat per day); and

2. 9-cis-retinoic acid (120 mg of 9-cis-retinoic acid per kilogram of diet); 20 g of diet fed daily (approximately 2.4 mg of 9-cis-retinoic acid per rat per day).

The animals were assigned to 4 treatment arms:

1. corticosteroids alone,

2. either of the 2 retinoids alone,

3. corticosteroids and either of the 2 retinoids, and

4. control animals. The controls were fed regular rat chow (Purina Ground Laboratory Chow; Purina, Ralston Purina, Atlanta, Ga, containing 17.6 IU—approximately 5 µg—of vitamin A per gram). All animals drank water ad libitum. The study protocol was approved by the University of California, San Francisco Committee of Animal Research.

Wound fluid was harvested by sterile aspiration with a syringe at different times in the various experiments. Transforming growth factor β levels were assayed on day 3, 7, 11, 15, and/or 17 using a nonradioactive bioassay based on its ability to induce expression of plasminogen activator inhibitor-1 in mink lung epithelial cells stably expressing a portion of the plasminogen activator inhibitor-1 promoter linked to luciferase as described.27

Wound fluid samples were diluted 1:25 in serum-free medium to a final protein concentration of 50 to 100 µg/mL and heat activated (80°C for 8 minutes). Samples were then chilled on ice and 200 µg of bovine serum albumin was added as carrier. Each sample was assayed individually in a 6-point dilution curve in 24-well plates and its activity compared with a standard curve of recombinant human TGF-β1 run in the same assay to determine the concentration of active TGF-β in the wound fluid. Each sample was assayed in 2 or 3 replicate assays and the results averaged. The wound fluid from each wound cylinder was assayed individually for TGF-β content. Insulin-like growth factor-I levels were measured at day 17 by radioimmunoassay. Insulin-like growth factor-I was separated from its binding proteins prior to radioimmunoassay by the formic acid–acetone extraction method.28 For assessment of IGF-I levels only, the wound fluid from all 4 wound chambers was pooled for each animal. The animals were killed on day 17 and the cylinders were explanted. All accessible wound tissue was removed mechanically from the implants. Hydroxyproline content of the tissue within the wound cylinders was assessed by high-performance liquid chromatography with a modified method after Lindblad and Diegelmann.29

Immunohistochemistry was performed on full-thickness skin sections obtained from the back of the animals from an unwounded area. The sections were stored in 10% formalin solution and stained with the 3 anti–TGF-β antibodies (anti–TGF-β1, anti–TGF-β2, anti–TGF-β3). Rabbit polyclonal antibodies to the TGF-βs were used as follows: TGF-β1, anti-P (1-30) LC at 7 µg/mL; TGF-β2, anti-P (50-75) at 1.5 µg/mL; TGF-β3, anti-P (50-60) at 2.4 µg/mL.30 The results were statistically analyzed by SuperANOVA (Department of Biostatistics, University of California, San Francisco).

Reduced metabolites function to control the storage, trafficking, and conversion to the metabolically unstable active form, retinoic acid.

Certain actions of the retinoids on cells are now known to be mediated through regulation of the levels of expression of secreted growth factors and/or their receptors.9-11 Two of the peptides known to be regulated by retinoic acid include the transforming growth factor β (TGF-β) isoforms, TGF-β1, 2, and 3, and insulin-like growth factor I (IGF-I).7,12 Each of these growth factors has been shown to regulate important phases of wound healing on its release from platelet α granules.13

The TGF-βs and IGF-I are synthesized and secreted by cells constituting the newly formed granulation tissue, they can each act as local mediators of cellular response and they can regulate interactions between cells based on the ubiquitous nature of their receptors.14 Platelets release 2 distinct forms of latent TGF-β: the “small” form containing TGF-β and the latency associated protein is directly released into the supernatant of clotted blood, whereas the “large” form containing mature TGF-β, latency associated protein, and latent TGF-β–binding protein, which binds latency associated protein covalently, remains associated with the clot and is made available only slowly as the clot is proteolyzed.15 Transforming growth factor β affects all phases of the healing process, including the inflammatory response, angiogenesis, and matrix deposition.16 Transforming growth factor β is mitogenic for fibroblasts and stimulates the production of fibronectin and collagen. Its effects on the extracellular matrix are more complex and more profound than that of any other growth factor. In animals, TGF-β enhances strength in incisional wounds.17 It also corrects the wound healing defect created by age or corticosterone administration.18 No mechanism beyond the fact that inflammation is restored has been offered. Topical and systemic TGF-β administration improves healing in dermal as well as nondermal sites such as bone19 and intestine.20

Insulin-like growth factor I is a major regulator of growth and development and of wound healing.21 Wounds deprived of 90% of their IGFs by hypophysectomy show a marked impairment in cell replication and deposition of collagen and a decrease of wound macrophages.22,23 Insulin-like growth factor-I has direct actions on fibroblasts, endothelial, and epithelial cells.

The mechanisms whereby the retinoids regulate the activity of the TGF-βs and IGF-I are myriad and include...
both transcriptional and posttranscriptional mechanisms as well as regulation of the activity of the mature peptides. For example, it has been demonstrated that retinoids control not only the stability of messenger RNAs encoding the TGF-β isoforms, but also regulate activation of TGF-β from its latent forms. Similarly, retinoids have been shown to regulate not only synthesis and secretion of the IGFs, but, in certain cases, synthesis of IGF-binding protein as well.

Based on the known antagonistic effects of steroids and retinoids on wound healing, and on the well-documented effects of retinoids in regulating the activity of peptide growth factors, we proposed that steroid treatment would lower basal levels of TGF-β and IGF-I and would suppress tissue deposition in wounds. Furthermore, we proposed that retinoids would antagonize these suppressive effects by their ability to stimulate corticosteroid-impaired TGF-β and IGF-I release and thereby stimulate tissue deposition. To test this hypothesis, we have examined the effects of methylprednisolone acetate Depo-Medrol treatment on the levels of TGF-β and IGF-I in wound fluid and on tissue deposition in wounds, and have assessed the ability of various retinoids to antagonize this effect.

**RESULTS**

**TGF-β LEVELS IN WOUND FLUID**

Transforming growth factor-β levels in wound fluid were significantly reduced after 7, 11, 15, and 17 days in rats treated with steroids when compared with controls (Figure 1 and Figure 2). Oral all-trans-retinoic acid reversed the steroid-suppressed TGF-β levels toward normal levels to within approximately 80% of control levels (Figure 2A). Oral all-trans-retinoic acid given alone significantly increased TGF-β levels beyond normal when compared with controls (Figure 2A). Oral 9-cis-retinoic acid also reversed the steroid-suppressed TGF-β levels toward normal to within approximately 80% of control levels (Figure 2B).

**IGF-I LEVELS IN WOUND FLUID**

Methylprednisolone treatment significantly decreased IGF-I levels in wound fluid (Figure 3). Oral all-trans-retinoic acid significantly reversed the steroid-induced IGF-I decrease toward control levels (Figure 3A). Oral all-trans-retinoic acid given alone significantly increased IGF-I levels beyond control levels (Figure 3A). Oral 9-cis-retinoic acid also significantly reversed the steroid-induced IGF-I decrease (Figure 3B).

**COLLAGEN DEPOSITION IN HEALING TISSUE**

Methylprednisolone treatment significantly decreased hydroxyproline content in the wound cylinders by approximately 50% compared with controls at day 17 (Figure 4). Feeding the steroid-treated animals with all-trans-retinoic acid increased hydroxyproline content toward normal levels to within approximately 80% of controls at day 17 (Figure 4A). Oral all-trans-retinoic acid alone significantly increased hydroxyproline content beyond normal levels when compared with animals fed the control diet at day 17 (Figure 4A). Feeding the steroid-treated animals with 9-cis-retinoic acid significantly increased hydroxyproline content toward normal levels.

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**Figure 1.** Transforming growth factor-β (TGF-β) levels in wound fluid over the course of 15 days. Asterisks indicate P < .05, n=48 cylinders per group.

**Figure 2.** Transforming growth factor-β (TGF-β) levels in wound fluid at day 17 in all-trans-isomer (A) and 9-cis-isomer (B). Asterisks indicate P < .05, n=48 cylinders per group. See “Materials and Methods” section for an explanation of the 4 treatment arms of this study.
to within approximately 80% of controls at day 17 (Figure 4B).

IMMUNOHISTOCHEMISTRY

The histological features and immunohistochemical staining patterns reflected the biochemical results and showed the systemic effects of steroid and/or retinoid treatment on TGF-β expression. At day 17 control sections of the skin stained with the TGF-β1 antibody showed maximal staining of hair follicles and muscles. Control sections of the skin stained with the TGF-β2 antibody showed similarly distributed staining of epidermis, dermis, and muscle. The muscle staining was less pronounced than in the control sections stained with TGF-β1 antibody. Control sections of the skin stained with the TGF-β3 antibody showed the maximum intensity of staining in the epidermis and a granular distribution of the growth factor. Skin sections from animals treated with steroids showed thinning of the epidermal layer, fewer hair follicles, and less staining of all 3 TGF-β isoforms. Sections of dermal wounds from animals that received supplemental retinoids showed thicker epidermis, more hair follicles per microscopic field, and intensified staining for all 3 TGF-β isoforms when compared with the steroid-treated group (Figure 5). As previously shown, systemic retinoic acid alone induced expression of all TGF-β isoforms in the epidermis in these experiments.10

COMMENT

Corticosteroid hormones are widely used clinically to treat a variety of diseases by suppressing inflammation and immune functions. Soon after the discovery of the therapeutic potential of corticosteroids, their adverse effects on wound healing became evident.1 Subsequently, impaired healing during glucocorticoid therapy has become a serious clinical problem. This is well recognized and steroid-retarded repair has been used frequently as a test for the effect of various agents including TGF-β and IGF-I.18

The depressive effect of steroids on wound healing has long been presumed to depend on the postponement of the inflammatory reaction, without which the healing sequence cannot proceed. Ehrlich et al2 serendipitously noted that glucocorticoid-mediated reductions in inflammatory cell infiltration, fibroplasia, and deposition of collagen fibers are prevented by concurrent vitamin A therapy. The proposed mechanism is an antagonistic effect of corticosteroids and vitamin A on the inflammatory response.

Figure 3. Insulin-like growth factor-I (IGF-I) levels in wound fluid at day 17 in all-trans-isomer (A) and 9-cis-isomer (B). Asterisks indicates P<.05, n=9 animals per group. See “Materials and Methods” section for an explanation of the 4 treatment arms of this study.

Figure 4. Hydroxyproline (HOPRO) content in the wound cylinders at day 17 in all-trans-isomer (A) and 9-cis-isomer (B). Asterisks indicate P<.05, n=48 cylinders per group. See “Materials and Methods” section for an explanation of the 4 treatment arms of this study.
multiple inflammatory components. However, no other mechanistic detail has been added since then.

Glucocorticosteroids decrease collagen synthesis also in unwounded connective tissues and fibroblast cell culture. The decrease of type I collagen synthesis caused by steroids has been attributed to a decrease of the steady-state level of total cellular type I procollagen messenger RNAs. Both glucocorticoids and retinoids regulate $\alpha_2$-type I procollagen promoter activity.\textsuperscript{31}

Retinoids regulate the expression of secreted growth factors. They increase the levels of secreted TGF-$\beta$ isoforms by as much as 50-fold in certain cells such as keratinocytes.\textsuperscript{7} Nuclear run-on experiments have confirmed that this effect is not transcriptional, a result consistent with the lack of identifiable retinoid response elements in the promoters for the TGF-$\beta$ isoforms. Rather, the increased expression seems to derive from an increased half-life of the TGF-$\beta$ messenger RNAs and from increased translational efficiency.\textsuperscript{32} Treatment of cells with retinoids also increases the proportion of TGF-$\beta$ that is secreted in the active form. At least for certain cells this can be correlated with the ability of retinoids to increase the expression of transglutaminase, one of the components in the activation mechanism.\textsuperscript{33}

The goal of this study was to identify whether the above data could be used to explain the effects of corticosteroids and retinoids during wound healing. The results show the predicted suppressive effects of steroids and the stimulatory effects of retinoids on the expression of TGF-$\beta$ and IGF-1. Suppression and/or stimulation of TGF-$\beta$ and IGF-1 expression is paralleled by a decrease and/or an increase in collagen deposition.

All-trans-retinoic acid significantly increased hydroxyproline levels beyond control levels, an effect that could not be shown with the 9-cis-isomer. This information might be valuable for further studies and could be significant for therapy of healing deficits. It is tempting to speculate that all-trans-retinoic acid might actually enhance normal healing.

Differences in the pharmacokinetic properties of orally administered all-trans-retinoic acid and 9-cis-retinoic acid have been described.\textsuperscript{34} In rat breast cancer prevention the 9-cis-isomer is clearly superior to the all-trans-isomer.\textsuperscript{35}

The 9-cis-isomer has high affinity for both the RAR and RXR families of retinoid receptors, whereas all-trans-RA binds only to the RAR receptors. Potentially, broader receptor activation may contribute to the greater activity of the 9-cis-isomer. Additionally, 9-cis-retinoic acid does not induce its own oxidative inactivation to the same extent as all-trans-retinoic acid. Further studies on the comparative pharmacokinetics of 9-cis-retinoic acid and all-trans-retinoic acid as well as their activation of target genes might be useful for enhancing the control that surgeons have over wound healing.

We attempted but could not repeat prior data showing that parental vitamin A palmitate has the same effects as seen for orally fed all-trans- and 9-cis-retinoic acid.\textsuperscript{2} Parenteral (subcutaneous) vitamin A did not increase the suppressed TGF-$\beta$ or IGF-1 levels in the wound fluid over 17 days (data not shown). In higher concentrations parenteral vitamin A led to toxic effects including weight loss. According to the manufacturer, the parenteral retinoid preparations used in earlier experiments, which are now unavailable, were different mixtures of the various retinoid metabolites and isomers. The palmitate ester is metabolized differently than other forms, and this difference rather than the route of administration, in our opinion, best explains the failure of the parenteral preparation to share the vulnerability results of the orally given forms.

The immunohistochemical studies were performed for 2 reasons. First, it was necessary to demonstrate that the animals had developed significant systemic effects of the steroids despite the short treatment duration. Second, we wished to test a long-held hypothesis that systemic retinoids given in treatment of steroid-suppressed repair might diminish the therapeutic potential for which the steroids are being given. Clearly, the animals were significantly affected, and there is potential for diminishing the desired effects of steroid therapy. These observations raise several caveats. First, treatment of wound failure in this circumstance should be cautious when interruption of steroid effect might become problematic. Local retinoid therapy to wounds is also clinically effective. Second, systemic use should be short-
term recognizing that excess vitamin A is stored in the liver. It is unknown whether systemic retinoids might be used to forestall wound failure when interruption of the steroid effect is not critical as for patients with low-grade rheumatoid arthritis who are being prepared for operations. However, these data also raise the idea that retinoids might be used to ameliorate signs and symptoms of Cushing’s disease or even hasten recovery from Cushing’s syndrome after the need for steroid therapy is ended.

CONCLUSIONS

The results of this study indicate that steroids reduce TGF-β and IGF-I production in wounds and that collagen deposition suffers by that mechanism. Conversely, retinoids enhance steroid-retarded healing toward normal levels by restoring TGF-β and IGF-I levels and thereby reinvigorate collagen production. These effects might well be shared by other growth factors and substances that are relevant for the healing process. This study implies that glucocorticosteroids, retinoids, and the TGF-β family share similar pathways in controlling cell differentiation and proliferation.

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