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The Effect of Bacterial Endotoxin on the Early Tensile Strength of Healing Surgical Wounds

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Wound healing in the oral cavity occurs in a bacteria-rich environment, which may affect its outcome. Furthermore, it takes place where forces are frequently applied to the healing tissue. The effect of bacterial endotoxin on the development of tensile strength in healing wounds was studied using surgical skin wounds in rats as a model. Collagen membranes soaked with 0.01 μg of bacterial endotoxin were inserted into surgical skin wounds, and their effect was studied on days 6 and 10. Membranes with no endotoxin served as controls. Endotoxin inhibited the early development of tensile strength in 6 days, healing wounds by 38%, whereas the collagen membrane alone had no effect. Dexamethasone (0.5 mg/kg every 72 h) had a suppressive effect on the development of tensile strength in healing noncontaminated wounds, but not in those containing bacterial endotoxin. These results suggest that bacterial endotoxin may interfere with the early healing of wounds. Understanding the mechanisms of this inhibition may result in treatments that will allow this response to be faster and more reproducible.

The healing of surgical wounds is a complex sequence of events in which cells, growth factor, and matrix constituents interact to result in reepithelialization, as well as reestablishing the strength and resilience of the underlying connective tissue (1, 2). At the stage of suture removal, reepithelialization has usually been completed, however, the full strength of the dermis is still far from being achieved (3). A potential interference of bacterial contamination with the early development of wound strength and its delay may lead to jeopardizing its integrity at the stage after suture removal.

The blood clot formed in the wound provides an initial temporary seal for the severed tissue. Nevertheless, the fibrin clot has yet another major function: it serves as a temporary matrix in which cells migrate and in which angiogenesis takes place. The platelet-derived growth factor (PDGF) in the clot will serve as both a potent chemoattractant and an essential growth factor. The healing wound is initially invaded by large numbers of polymorphonuclear leukocytes (PMNs), which will be replaced in 2 to 3 days by macrophages. Both cells function as phagocytes but are also the source of a plethora of cytokines, chemoattractants, and growth factors, which will eventually result in fibroblast proliferation and migration into the fibrin clot. Activation of the fibroblasts to form a new extracellular matrix will lead to formation of the early new connective tissue, which will be gradually remodeled and replaced by the scar.

Wound strength gradually develops during this process with only a fraction of its final strength reached by day 6, when sutures are commonly removed (3). During the initial stages, type III collagen is mainly formed, and collagen bundles bridging the tissue gap are first encountered only on day 5. From days 5 through 14, the fibroblastic stage of wound healing is hallmarked by intensive production of collagen and other matrix constituents, as well as by increasing wound strength (3). From day 7, type I collagen is mainly produced, and type III collagen is gradually replaced by the former.

Active macrophages are considered a key element in the initial fibroplasia in the healing wound (1, 4). If they are eliminated or inhibited, wound repair will not progress and wound strength will lag, as well (4). Nevertheless, a higher state of activation of macrophages, such as that achieved by their exposure to bacterial endotoxin (LPS), may lead to inhibition of fibroblast proliferation, migration, and growth (5, 6). It has recently been demonstrated that down-regulation of this state of activation by glucocorticoids may free the fibroblasts from this suppressive effect and restore their function (6).

The present study was designed to study the potential of LPS contamination of healing wounds to inhibit their strength development and to explore the potential of dexamethasone to reverse this inhibition.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 170 ($\pm$ 20) g were used in this study. They were allowed to adapt to the animal facility for 7 days before starting the experiments.

Bacterial endotoxin-soaked collagen membranes were used to introduce the endotoxin into surgical wounds. Collagen membranes were prepared from rat tail type I collagen by the method described by Pitaru et al. (7). The membranes were cut into strips

of 2.5 × 15 mm that weighed 5 (± 1) mg. The membranes were soaked with 15 μl of LPS solution (0.67 μg/ml in sterile water, phenol extract from *Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO) and allowed to dry. This volume of fluid was previously established as one that is easily absorbed by the membrane, leaving no surplus. The LPS concentration was adapted so that each membrane contained 0.01 μg of LPS. Membranes which were soaked with the same amount of sterile water and dried served as controls.

The animals were anesthetized and their backs shaved and disinfected using Polydine surgical scrub. The surgical wound consisted of a full-thickness 15-mm cut through the skin on the back of the animal. The wound lips were closed with three separate 000 silk sutures. When collagen membranes were used, the membrane was placed on the subcutaneous tissue under the center of the wound, and the wound was sutured as described above. Because the dry collagen membrane adhered tightly to the underlying tissues on which they were placed, there was no need for any further stabilization. The loose subcutaneous connective tissue allowed the lips of the wound to relate similarly to each other, whether a membrane was present or not. The animals were caged separately after surgery.

Dexamethasone (Dexacort, Teva, Israel) was injected intramuscularly at a dose of 0.5 mg/kg. The first administration was 48 h before surgery, and it was repeated every 3 days throughout the experiment.

The effect of bacterial endotoxin on wound healing was studied using a wound strength assay (described below). Two experimental groups were used: the first consisted of 14 animals, each with a wound containing a LPS-soaked collagen membrane; the second was composed of 14 animals that were surgically treated, as in the first, with the addition of dexamethasone administration. Control groups included (group A) 14 animals with two wounds, one of which contained a collagen membrane with no LPS and the other with no membrane; (group B) 14 animals with two wounds similar to group A, with the addition of dexamethasone administration. Group A was designed to evaluate the effect of the membrane alone on the wound strength, whereas group B was designed to evaluate the effect of dexamethasone on the strength of healing wounds, with and without a membrane. In half of the animals in each group, skin with healing wounds was harvested on day 6 (as described below), whereas in the other half, harvesting was done on day 10. A third control group consisted of animals that were not wounded and served to evaluate the strength of normal skin and that of intact skin of animals treated with dexamethasone.

Six or 10 days after surgery the animals were killed, and a full-thickness skin strip measuring 1 × 8 cm was harvested from the wounded area. Each strip contained the healing wound at its midpoint with two 1 × 4-cm skin strips on each side. The loose connective tissue allowed the harvesting of the skin strips without applying forces to the skin specimen. The specimens were kept for 24 h in phosphate buffered saline with antibiotics at 4°C. This was done to minimize the differences in handling time between the first and last sample, both in harvesting process and in strength testing.

The sutures were carefully removed with no tension applied to the specimens. Each end of the skin strip was adapted with a strip made of two layers of an overhead projector film that served as a connector to the testing apparatus. The two layers of strip were attached to the skin on both sides (dermal and epidermal), using cyanoacrylate cement (Renfert, Hilzingen, Germany). It was established in pilot experiments that the bond strength between the film strips and the skin was greater than the force required to tear a normal skin strip of similar dimensions. Each strip was mounted in a loading machine (Instron 1065, High Wycombe, England) using the film strips as attachment sites; tension was applied at 5 mm/min, using a 5 kg load cell to separate the wound lips from each other. The maximal force the specimen sustained during each test was recorded and referred to as the breaking strength of the wound (3); dividing this value by the area of the cross-section of the strip in the wound site resulted in the tensile strength (3).

For each group a mean tensile strength of the wound was calculated (± SEM). An analysis of variance with Bonferroni correction was used to compare the results. When samples from the same animals were compared (control groups A and B), analysis of variance with repeated measures was used.

RESULTS

Early Wound Strength Compared with Normal Skin

By day 6 the mean tensile strength of wounds with no membrane, endotoxin, or dexamethasone was 31.0 (± 4.4) g/mm^2, which was 13% of that of intact skin of normal animals, which was 240.2 (± 45.0) g/mm^2. By day 10 the strength increased to 70.1 (± 14.9) g/mm^2, which was 29% of that of intact skin.

Effect of Collagen Membrane on Early Wound Strength

The collagen membrane inserted under the healing wound had no significant effect on early wound strength; by day 6 the mean tensile strength was 37.1(± 11.8), compared with 31.0 (± 4.4) g/mm^2 for wounds with and without collagen membranes, respectively. On the other hand, by day 10 the membrane inhibited the development of wound strength: 44.0 (± 12.1) with collagen, compared with 70.1 (± 14.9) g/mm^2 ($p = 0.001$) without collagen.

Effect of Bacterial Endotoxin on Wound Strength

Endotoxin present in the healing wound inhibited the early development of its tensile strength, which by day 6 reached a mean of 23.3 (± 5.5) g/mm^2, compared with a strength of 37.1 (± 11.8) g/mm^2 in wounds containing a membrane with no LPS ($p = 0.0013$). This difference could not be observed on day 10, when a significant difference was no longer found between wounds containing LPS and those with only a membrane.

Effect of Dexamethasone on Wound Strength

When no endotoxin was present, dexamethasone inhibited the development of the tensile strength of the wound. By day 6, tensile strength of wounds containing collagen membranes was reduced by 22% from a mean of 37.1 (± 11.8) to 29.1 (± 7.1) g/mm^2 ($p = 0.051$). The slight decrease in strength that was observed on day 10 was not significant. In wounds containing no membrane, the tensile strength on day 6 was reduced by 19% from 31.0 (± 4.4) to 25 (± 6.0) g/mm^2, but the difference was not significant. Dexamethasone did not affect the strength of normal intact skin (data not presented).

Effect of Dexamethasone on LPS Inhibition of Wound Strength

Dexamethasone increased the tensile strength of wounds containing LPS from an average of 23.3 (± 5.5) to 28.5 (± 7.3) g/mm^2 on day 6 and from 46.7 (± 15.3) to 51.9 (± 13.8) g/mm^2 on day 10, but these differences were not significant.

DISCUSSION

Tensile strength of healing surgical wounds, which gradually develops during the fibroblastic and remodeling stages, takes several weeks to reach its peak (3). It is in its early development that the wound is most sensitive to mechanical disruption. Because day 6 is a common clinical time of suture removal, wound strength was measured in the present study on day 6 and a few days later on day 10 of healing. Even with this short interval, the strength doubled from 31.0 (± 4.4) to 70.1 (± 14.9) g/mm^2.

Bacterial contamination has previously been shown to interfere with the normal progress of wound healing (8). In the present study, we chose to study the effect of a nonliving constituent of Gram-negative bacteria, bacterial endotoxin LPS, which has been shown to persist in infected sites such as the periodontal pocket long after the bacteria had been eliminated because of its attachment to the cementum (9). To form a reproducible reservoir of LPS next to the healing wounds, we used a collagen membrane soaked with this agent. It has previously been shown that LPS binds to collagen and is active in this form (7, 10). The introduction of a LPS containing membrane next to the healing wound resulted in reduction of its early (6-day) strength by 38%. By day 10 the wound strength was no longer effected by the LPS that was initially present. The inhibition of early strength may be of importance, because the common schedule of suture removal at day 5 to day 6 assumes that the minimal tensile strength that is required to maintain the integrity of the wound already exists. This may not be true in LPS-contaminated healing wounds.

The inhibition observed might be the result of either the direct effect of LPS on the fibroblasts or an indirect one by its activation of macrophages in the wound. Direct effects of LPS on fibroblasts have previously been demonstrated, however, they usually require a relatively high concentration of this agent (11, 12). It has recently been shown that macrophage activation, which occurs at much lower LPS concentrations (such as the one used here), may result in a substantial inhibition of fibroblast proliferation and migration in vitro (5, 6). The LPS was used in the present study in a quantity that if fully released from the membrane into the subcutaneous space adjacent to the healing wound would result in a calculated concentration of 1.0 μg/ml. The actual concentration available in the site was most probably much lower than that, because the LPS binds to the collagen in the membrane. Additional small amounts of the material may be made available at the site while the collagen of the membrane is gradually resorbed by metaloproteases. It is, therefore, more likely that the suppressive effect of LPS was via the activation of macrophages, which readily occurs at the low concentrations of LPS that may be present in the site.

Macrophage stimulation is an essential step in wound healing (13). These cells are a main source of growth factors, such as PDGF and transforming growth factor, which are essential for the activation of fibroblasts to proliferate and migrate into the wound (1, 2). Even though the blood clot is an initial rich source of PDGF, these cells perpetuate the supply of this and other growth factors, without which the healing will not properly proceed (1). Nevertheless, high levels of activation may turn the macrophage into a suppressor cell that may inhibit essential fibroblast functions, such as proliferation and migration (5, 6). It is most probable that such activation contributed to the inhibitory effect observed in the present study.

The glucocorticoid dexamethasone was used in an attempt to reverse the suppressive effect. Glucocorticoids have been shown to inhibit the expression of macrophage activation both in vivo and in vitro. In vivo tumoricidal activity of activated macrophages could be inhibited by administration of hydrocortisone (14), and many effector functions of these cells such as inflammatory cytokine production were prevented by in vitro exposure to dexamethasone (15). The inhibitory effect of the steroid was at the gene transcriptional level and resulted in a reduction of mRNA related to the genes encoding for the proteins associated with the enhanced functions of the activated macrophages (15). Furthermore, it has recently been demonstrated that when fibroblast colonies were profoundly suppressed by LPS-activated macrophages, hydrocortisone totally reversed the suppression (6).

We therefore used a glucocorticoid in an attempt to reverse the suppressive effect. Dexamethasone was chosen because of its prolonged half life, which permits its clinical administration every second or third day. The dose chosen was an equivalent to the high, short-term dose that is clinically used in humans. Even though the dexamethasone had some of the expected effect, the difference between the treated and nontreated groups was not significant. On the other hand, the steroid alone had a suppressive effect on the wound strength of the control wounds that did not contain LPS. One would expect that if the drug alone had a suppressive effect, as had the LPS, they might have an additive suppression. Nevertheless, such additive effect did not occur.

The lack of reversal by the steroid of the LPS-induced suppression may be explained in two ways: (a) the drug does not have in vivo the effects observed in vitro by Metzger et al. (6); and (b) the lack of effect is due to a difference between local and systemic administration of the glucocorticoid. When used in vitro, the steroid had only a local effect on the activated macrophages, with no direct effect on the fibroblasts. In contrast, when injected in vivo the steroid had most probably both local and systemic effects. Systemic administration of steroids has been shown to inhibit normal wound healing (1, 4). This effect was attributed to systemic inhibition of monocyte/macrophages that resulted in a lack of adequate numbers of macrophages in the healing wound, which could not proceed normally (4).

In the present study, the systemic administration of dexamethasone resulted in inhibition of early strength development in wounds that did not contain LPS. It is possible that this systemic effect masked and did not allow the expression of the local effect of the steroid on the interaction between the LPS-activated macrophages and the fibroblasts. A similar discrepancy between an in vivo local effect of dexamethasone and a lack of its effect when the steroid was systemically administered were recently reported by Sae-Lim et al. (16). Further studies, with local administration of the dexamethasone in LPS contaminated wounds, will be required to explore this possibility.

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