Impaired wound healing in an acute diabetic pig model and the effects of local hyperglycemia

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ABSTRACT

Diabetic wounds result in significant morbidity, prolonged hospitalization, and enormous health-care expenses. Pigs have been shown to have wound healing resembling that in humans. The aim of this study was to develop a large-animal model for diabetic wound healing. Diabetes was induced by streptozotocin injection in Yorkshire pigs. Full-thickness wounds were created and dressed with a sealed chamber. Nondiabetic pigs with or without high glucose wound fluid concentration served as controls. Glucose concentration in serum and wound fluid was measured and collected. Wound contraction was monitored, and biopsies were obtained for measurement of reepithelialization. Wound fluid was analyzed for insulin-like growth factor-1 (IGF-1), platelet-derived growth factor, and transforming growth factor. Glucose concentration in wound fluid initially followed serum levels and then decreased to undetectable on day 9. Reepithelialization was significantly delayed in diabetic pigs. In nondiabetic pigs, wounds treated in a local hyperglycemic environment, and thus excluding the effects of systemic hyperglycemia, showed no difference in wound closure compared with controls. This suggests that delayed wound healing in diabetes is not induced by local high-glucose concentration itself. Analysis of growth factor expression showed a marked reduction in IGF-1 in the diabetic wounds. Diabetic pigs have impaired healing that is accompanied by a reduction of IGF-1 in the healing wound and is not due to the local hyperglycemia condition itself.

Approximately 5 million patients in the United States suffer from chronic wounds.[1] With the increased longevity, obesity, and diabetes, the problem of chronic wounds has increased, resulting in significant morbidity, lost time from work, and enormous health-care expenses. According to the American Diabetes Association, 25% of people with diabetes will suffer from a wound problem during their lifetime, and approximately 82,000 limb amputations for nontraumatic wounds were performed in people with diabetes in 2002.[2] The Agency for Health Care Policy and Research reports that wound care for pressure ulcers uses $200 billion a year for hospitalization, durable medical goods, nursing home care, physicians, and transportation.[3] Surgical treatment of diabetic wounds remains difficult and often insufficient, leading to high morbidity among those patients.[4] We need better ways to treat diabetic wounds and relevant preclinical models are needed to develop new therapeutic strategies.

Numerous diabetic wound healing models have been described.[5,6] Small mammals, such as rats, rabbits, guinea pigs, and mice, are frequently used in wound healing studies because of cost and ease of handling. However, the anatomy and physiology of small mammals differ from those of humans in many ways.[7] Pig physiology and wound healing has been found to be significantly more similar to humans.[8,9] In wound healing models investigating basic fibroblast growth factor (bFGF), wounds in genetically diabetic db/db mice treated with bFGF healed significantly faster than wounds in control mice.[10] However, studies evaluating the use of bFGF in porcine partial-thickness wound models did not support these findings,[11] and a small randomized, double-blind study of 11 human patients found no effect of bFGF on reepithelialization of split skin graft donor sites as compared with vehicle-treated control wounds.[12]

Furthermore, the overall physiology of pigs is close to that of humans, with the anatomy and function of most key organ systems being similar. The many similarities between humans and pigs have led to the conclusion that the pig is an excellent animal model for wound-healing studies.

Sullivan et al.[7] evaluated 25 different wound therapies and showed that, in studies that could be compared with human studies, the results in porcine models agreed with those of human studies 78% of the time, whereas results of small-mammal models showed only 53 and 57% for in vitro studies.

These and other findings show that the pig is more suitable than small-animal models for simulation of human wound healing.

Although streptozotocin-induced diabetic pigs have been well established for the study of diabetes[13]

(especially islet cell transplantation), no diabetic wound healing model in large animals is available to date.

Our study aim was to develop a large-animal model of wound healing for diabetes-impaired wound healing and to investigate pathopysiologic mechanisms of diabetic wound healing.

Several growth factors and cytokines are considered to be important in mediating, coordinating, and controlling cellular interactions that occur during normal wound healing.[14] They influence cell proliferation and cellular activity, induce migration of inflammatory cells into the wound environment, and stimulate protein synthesis or down-regulate certain cellular functions as healing progresses.[15,16] Therefore, changes in the levels and timing of their expression in diabetes could alter the wound-healing process. Little is known about the influence of different disease states such as diabetes on the levels or actions of growth factors in the wound environment.[17] Growth factors and cytokines such as insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β), have been found to influence wound healing in a variety of small animal models and are diminished in diabetic wounds in humans.[18–24] To confirm that this new wound healing model is comparable to the situation in humans, we analyzed these growth factors in our preclinical wound healing model.

To study wound healing, we used a previously developed external polyurethane chamber that can be sealed around the edges of the wound.[25] The chambers protect the wound like a dressing but allow the wound environment to be standardized and monitored and provide access for delivery of growth medium and potential therapeutic agents. It functions as an in vivo incubator, providing a bridge between in vitro and in vivo experimentation. The wounds were analyzed for glucose concentration, reepithelialization, contraction, and concentrations of different growth factors during healing.

METHODS

Animals

All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals. All procedures conformed to the regulations related to animal use and other federal statutes. Female Yorkshire pigs (Parson's Farm, MA) weighing 60 kg at arrival were allowed to acclimatize for 1 week before initiation of the experiment. They were kept in smooth-walled stainless-steel cages to minimize wound trauma and disruption of applied wound chambers. $N=10$ animals were used in this study ($n=3$ served as diabetic models, $n=4$ as nondiabetic controls, and $n=3$ for high-glucose wound concentration study).

Induction of diabetes

Pigs were fasted for 12 hours before diabetes was induced to prevent possible asphyxia and nausea during and after the procedure. At the day of operation, the animals received induction anesthesia with ketamine (Hospira, Lake Forest, IL)/xylazine (Xyla-Ject, Phoenix, St. Joseph, MO) via intramuscular injection and were weighed. While animals were under general anesthesia with isoflurane (Novaplus, Hospira), a 21-gauge intravenous catheter (Becton Dickinson, Franklin Lakes, NJ) was inserted into an ear vein. Streptozotocin (Zanosar, Pharmacia, Pfizer, NY) was prepared at a dose of 150 mg/kg body weight diluted in 9.5 mL/mg sterile saline (0.9% NaCl injection USP, Baxter, Deerfield, IL) and sterilized by filtration. The solution was administered through the catheter over 1 minute. Intramuscular injections of buprenorphine (0.001 mg/kg; Bedford Laboratories, Bedford, OH) and metoclopramide (0.15 mg/kg; GensiaSicox, Irvine, CA) were used as postoperative analgesia and anti-emetic, respectively. Buprenorphine and metoclopramide were administered intramuscularly every 12 hours for the first 2 days. Serum glucose concentrations were measured on an hourly basis for the first 36 hours and twice daily for the remainder of the experiment. The pigs were treated with a subcutaneous injection of short-acting insulin (Normulin, Novo Nordisk, Princeton, NJ) and long-acting insulin zinc suspension (Humulin, Eli Lilly, Indianapolis, IN) to keep the blood glucose concentration between 350 and 550 mg/dL.

Wounding and chamber treatment

Fourteen days after induction of diabetes, pigs received anesthesia as mentioned above and were transferred to a panepinto sling (Fulton, Mosinee, WI). The pig's dorsum was waxed thoroughly (Nair, Church & Dwight, Princeton, NJ), shaved, and thoroughly disinfected. Eighteen to 21 squares measuring 1.5×1.5 cm were outlined on the dorsum with a template and a skin marker. The edges of these squares were retraced with a tattoo machine (Special Electric Tattoo Marker, Spaulding Enterprises, Voorheesville, NY) to allow measurement of wound contraction. After the skin was prepared with povidone iodine, the skin within the squares was excised with a no. −11 blade to create 18–21 full-thickness wounds measuring 1.5×1.5×0.8 cm. Adhesive polyurethane chambers (Corium International, Grand Rapids, MI) were applied over each wound, and the chambers were then injected with 2 mL of sterile saline containing penicillin 100 U/mL (Gibco, Invitrogen, Carlsbad, CA) and streptomycin 100 μg/mL, (Gibco, Invitrogen). For nondiabetic high-glucose wound concentration study, the 2 mL sterile saline solution was adjusted to 1,800 mg/dL (100 mM) glucose concentration. The wound fluid collected in the chambers was completely aspirated at 24-hour intervals and chilled on ice before being stored at −80 °C for later analysis. Each chamber was reinjected with 2 mL of sterile saline (containing penicillin/streptomycin; Gibco, Invitrogen). For analgesia, buprenorphin was administered intramuscularly every 12 hours for the first 2 days. Before the wounds were retrieved, the pigs were killed by intravenous injection of Euthasol (Virbac AH, Fort Worth, TX).

Wound contraction

Wound contraction was determined by digitized planimetry of the tattooed margins. The area of the wounds at specific days was measured using Scion image software (Scion, Frederick, MD) and the percentage of contraction was calculated by the formula (area at biopsy day)/(area on wounding day) ×100.

Histology

Cross-sectional wound biopsies 2 mm wide were taken from the middle of the wounds; the sample included unwounded skin at the sides and subcutaneous tissue at the bottom. Biopsies were collected on days 8, 12, 16, and 18 after wounding. Samples were fixed in 4% buffered paraformaldehyde and processed for routine hematoxylin–eosin staining.

Reepithelialization was calculated by scanning the slides (Epson Perfection 3600, Epson, Long Beach, CA) and measuring the epithelial tongues from the computerized image with Paintshop Pro 7.0 (Jasc Software, Corel, Ottawa, Canada).

Measurements of growth factor and protein

Levels of PDGF-BB, TGF-β, and IGF-1 in the wound environment were measured in wound fluid by a specific enzyme-linked immunosorbent assay and a protein assay (R&D Systems, Minneapolis, MN). Results were calculated by using a Vmax kinetic Microplate Reader and Soft Max Pro software (Molecular Devices, Sunnyvale, CA).

Statistics

Values are presented as means ± SE. Groups were compared with a nonparametrical test, and statistical calculations were performed with GraphPad Instat software (GraphPad Software, San Diego, CA). A p-value < 0.05 was considered statistically significant.

RESULTS

Serum glucose levels

All the animals that received streptozotocin survived in good general condition for the duration of the experiment. Following the injection of streptozotocin there was an initial triphasic glucose response during the first 30 hours with an initial hyperglycemia lasting from hours 1–8, followed by a marked hypoglycemia that lasts from hours 10–20. A permanent hyperglycemic state (blood glucose above 350 mg/dL) established at 22 hours postinduction.

Glucose levels in wound fluid and urine

Wound fluid was collected on a daily basis, and the glucose concentration was measured. The glucose levels in wound fluid closely followed the levels in serum until day 5, at which time the glucose levels in wound fluid progressively decreased until they reached undetectable levels at day 9 (Figure 1). Nondiabetic pigs showed no detectable glucose concentrations in the wound fluid throughout the experiment, whereas wounds with local hyperglycemia in nondiabetic pigs showed a mean glucose concentration of 201.2 mg/dL.

Urine glucose was 100.25 ± 7.1 mg/dL in the fasting nondiabetic pig and > 500 mg/dL in the diabetic pig.

Reepithelialization

In all pigs, cross-sectional biopsies were done on days 8, 12, 14, 16, and 18 after wounding. Epidermal healing in diabetic pigs was significantly delayed as compared with healing in nondiabetic pigs: On day 8, the wounds in diabetic pigs were 12% reepithelialized, whereas those in nondiabetic pigs were 44% reepithelialized. On day 12, the wounds of diabetic pigs were 42% reepithelialized and those in nondiabetic pigs were 96% epithelialized. Full reepithelialization was delayed in diabetic pigs (day 18) as compared with nondiabetic animals (days 12–14) (Figures 2 and 3). Local hyperglycemic wounds on nondiabetic animals showed no statistical difference compared with control wounds on day 12 (83 vs. 88%; Figure 4)

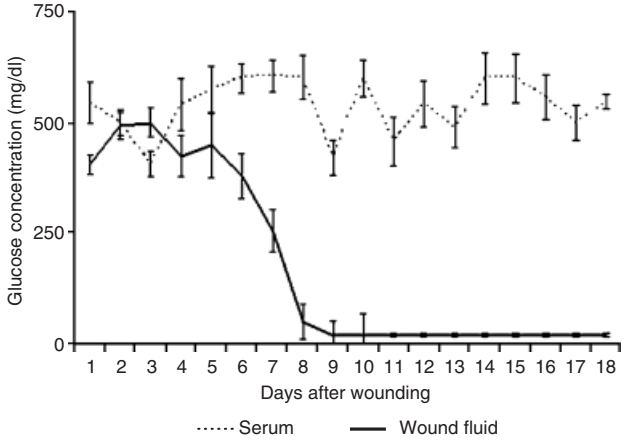

Figure 1. Relation between the glucose concentration in wound fluid and in serum in the diabetic pig after the creation of wounds (n=52 in three pigs). Glucose concentrations in wound fluid followed that in serum closely until day 5, when it decreased, becoming undetectable on day 9.

Wound contraction

Diabetic wounds contracted 24% on day 8 and 34% on day 12. Wounds in nondiabetic pigs contracted 35% on day 8 and 42% on day 12. Diabetic wounds contracted less

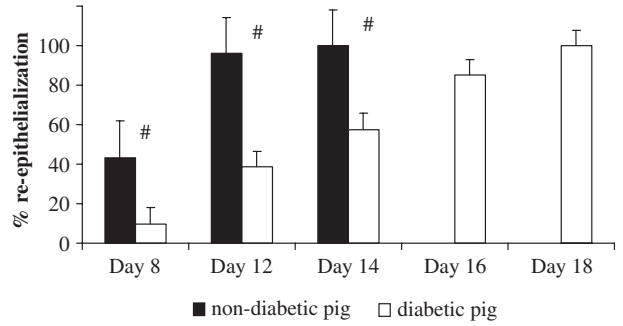

Figure 2. Level of reepithelialization of full-thickness wounds in nondiabetic and diabetic pigs. Wounds measuring 1.5× 1.5 cm were created on the backs of the pigs. Biopsy samples were obtained on days 8 (n=4), 12 (n=12), 14 (n=12), 16 (n=12), and 18 (n=12) for the diabetic pigs and on days 8 (n=12), 12 (n=12), and 14 (n=10) for the healthy pigs. #=diabetic vs. nondiabetic group: $p < 0.001$.

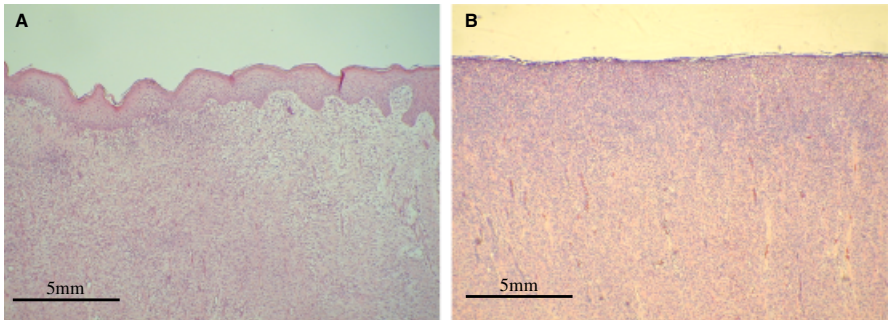

Figure 3. Histology of wounds on day 12 after wounding (H&E staining). Pictures show the center of the wounds at ×10 magnification. (A) Nondiabetic pig at day 12; (B) Diabetic pig at day 12.

at both time points, but the difference between the two groups was not statistically significant.

Cytokine and growth factors

Expression of IGF-1 in diabetic wounds was 15-fold lower that expression in nondiabetic wounds at day 3, 10-fold lower at day 5, and fourfold lower at day 7 (Figure 5A). The concentration of TGF-β was lower in the wound fluid of diabetic pig than in the wound fluid of nondiabetic pigs (Figure 5B) with a significant lower expression of TGF-β at day 7 in diabetic pigs as compared with nondiabetic pigs. The difference in expression of PDGF-BB in the wound fluid of diabetic and nondiabetic pigs was not statistically significant.

DISCUSSION

In this study, streptozotocin injection was well tolerated by the animals and a stable long-term diabetic state was achieved in the Yorkshire pigs. Multiple studies have shown a zero rate of mortality and little morbidity, such as polydypsia and polyuria.[13,26,27]

Insulin therapy helped maintain blood glucose on a high level throughout the experiment. The glucose concentration in wound fluid followed the serum concentration closely until day 5, when the wound fluid concentration decreased. The glucose level in the wound fluid seems to be associated with the state of healing in the diabetic wound. This may be due to decreasing vessel permeability in the healing wound.[14]

We could show that wound healing in diabetic pigs was significantly delayed as reepithelialization was decreased as compared with nondiabetic pigs. A diabetic state for 4 months before wounding did not further delay wound healing significantly (data not shown).

A state of systemic hyperglycemia, as seen in diabetes, may influence wound closure in numerous ways. Several hypotheses have been described in the literature such as the formation of glycation end products,[28] hyperosmolarity,[29] and altered insulin signaling in various ways.[30] However, molecular mechanisms whereby diabetes alters skin and wound physiology have not been elucidated to date.[31] To evaluate the sole effect of local hyperglycemia in wounds, the other potential effects of systemic hyperglycemia need to be excluded. To achieve this, we studied the effects of a high-glucose wound concentration on the healing of full-thickness wounds in nondiabetic pigs. Wounds that were subjected to a local hyperglycemic environment healed slightly but not significantly faster than control wounds. These findings suggest

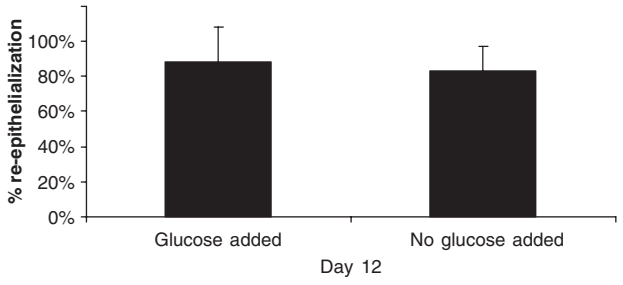

Figure 4. Reepithelialization of nondiabetic full-thickness wounds under local high-glucose condition and normoglycemic controls on day 12.

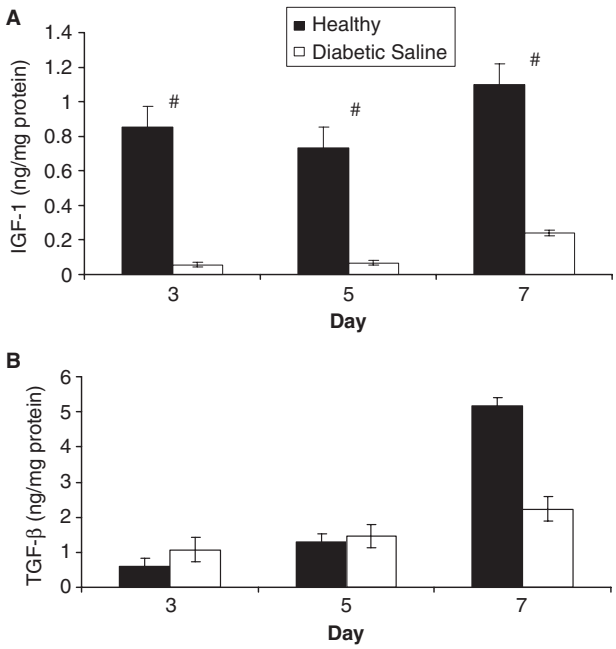

Figure 5. Level of expression of growth factor from the wound fluid of nondiabetic and diabetic pigs. Each bar represents the mean ± standard deviation for three diabetic pigs or four healthy pigs. All samples were assayed in duplicate. (A) IGF-1: # diabetic vs. nondiabetic pigs; $p < 0.05$. TGF-β: # diabetic vs. nondiabetic pigs; $p < 0.05$.

that wound-healing impairment in diabetes is not induced by local hyperglycemia within the wound environment itself and that local hyperosmolarity does not play a key role in our study.

Neuropathy, vascular damage, and malnutrition are described as further reasons for complicated wound healing.[32] In our model, a diabetic metabolic state was induced only 2 weeks before the experiment, which suggests that no long-term side effects such as neuropathy are responsible for impaired wound healing. This highlights a limitation of our model, since it omits long-term effects such as neuropathy and vasculopathy.

PDGF-BB growth factor has become the only growth factor licensed for topical application to stimulate the healing of chronic full-thickness diabetic foot ulcers.[33] Doxey et al.[34] reported significantly lower concentrations of PDGF-BB protein in a diabetic rat wound model. Their findings suggest that the absence of an initial increase in PDGF may play a role in the poor wound healing observed in diabetic rats.

In clinical studies, the response rate to PDGF-BB application varies widely.[35,36] PDGF has not been detected in chronic nonhealing wounds in humans. Pierce et al. detected only a minimal expression of PDGF isoforms in normal skin and in nonhealing dermal ulcers.[37] In accord with this finding, we found only low levels of PDGF-BB in the wound fluid (0.9–1.2 ng/mg protein in the wound fluid) and no statistically significant differences in the expression of diabetic and nondiabetic animals.

Ferguson et al.[38] reported an increase in acute inflammatory cells and an absence of cellular growth and migration of the epidermis over the wound in diabetic humans. The healing wound is a site of intense metabolism, involving a cascade of complex molecular interactions. Many alterations at the molecular level have been found in patients with diabetes that together could severely compromise healing, potentially leading to ulcer formation.

In other animal studies, topical application of TGF-β improved acute wound healing.[39] TGF-β has important functions in the normal healing process, including as a chemoattractant for monocytes, leukocytes, macrophages, lymphocytes, neutrophils, keratinocytes, and fibroblasts, and the induction of these cells to release growth factors.[39,40] In the present experiment in diabetic pigs, the expression of TGF-β in the wound environment decreased significantly on day 7 as compared with expression in nondiabetic pigs.

In this study, the most significant decrease of the concentrations of growth factors in the wound fluid was in the expression of IGF-1. The level of expression during the first 7 days after wounding decreased up to 15-fold.

Systemic concentrations of IGF-1 detected on day 14 after induction of diabetic metabolic state was 0.18 ng/mL compared with 1.33 ng/mL before streptozotocin induction, which is 7.4-fold lower. This observation indicates that the lower levels of IGF-1 detected in the diabetic wounds is in part due to the reduced IGF-1 expression in circulation.

In other studies, IGF-1 was shown to induce chemotactic activity in endothelial cell lines, as well as to stimulate keratinocyte and fibroblast proliferation and reepithelialization and to increase wound strength.[41] Human studies have been limited. Blakytny et al.[22] showed that IGF-1 is reduced within the basal layer of the epidermis in human diabetic skin, in fibroblasts from diabetic patients, and in their ulcer margins. We could show that the expression levels of the growth factors analyzed in our porcine model followed the findings in humans.

From the review of different animal models of wound healing, it becomes clear that skin wounds are difficult to manipulate and analyze in a standardized fashion. Therefore, we developed a polyurethane chamber that fits tightly around a wound and is simple to use for healing of porcine wounds in a liquid environment.[25] Previous reports have documented the beneficial effect of a wet over a dry wound environment, in both partial- and full-thickness wounds in nondiabetic pigs.[42,43] We confirmed, as documented earlier by Gabel et al.,[27] that a stable long-term diabetic state can be achieved in the Yorkshire pig. We also observed a high concentration of glucose in the wound microenvironment and showed a significant delay in epidermal healing, with full reepithelialization occurring 50% later in the diabetic than in the nondiabetic pig.

In this study, we developed the first model of diabetic impaired wound healing for large-animal studies that can be directed toward the discovery of pathophysiologic mechanisms in impaired wound healing and investigation of the efficacy of new approaches to the treatment of wounds.

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