Effects of insulin on wound healing in diabetic mice

Elora J. Weringer, John M. Kelso, Irene Y. Tamai
and Edward R. Arquilla

Department of Pathology, University of California Irvine, Irvine USA

Abstract. In the present study wound healing was examined in normal C57B1/6 male mice, diabetic mice, non-treated; and in diabetic mice treated with insulin. Small dermal wounds were made in the ears of the mice 40 h after the initial injection of insulin or vehicle alone. All animals were biopsied 8 h later. The wounds were examined by light and electron microscopy and wound components (capillaries, fibroblasts, PMN’s, oedema, collagen) were quantitated by lineal point analysis. The non-treated, diabetic mice demonstrated an inability to heal wounds when compared to controls; whereas, diabetic mice given a multidose insulin regimen demonstrated a response similar to controls. Insulin treatment of diabetic mice reduced the mean level of hyperglycaemia when compared to the non-treated diabetic mice. There was no detectable difference in the healing response with duration of diabetes in either the insulin-treated or non-treated diabetic mice. Although there was a mild reduction in hyperglycaemia, these data support the hypotheses that insulin is a necessary component of an adequate wound healing response.

Hyperglycaemia is a cardinal sign for recognition and evaluation of the diabetic state (Bondy & Felig 1971), and insulin therapy is initially aimed at correcting the high blood glucose. Rigid control of blood glucose, however, does not prevent the onset of vascular lesions, although it may have an effect on the ultimate course of the pathology (Raskin 1978). Thus, other factors may be operative in the insidious onset of diabetic complications. Decreased availability of insulin to the tissues most certainly contributes to the ensuing vascular pathology. Studies have shown that insulin, supplied by frequent injections or transplanted islets, not only normalized blood glucose, but also delayed or reversed diabetic kidney lesions in rats (Siperstein et al. 1977) and diabetic retinopathy in dogs (Engerman et al. 1977).

Insulin is intimately involved in the composite of metabolism (Czech 1977; Steiner 1966), and of particular interest, is the role of insulin as a regulatory hormone for cell growth (Steiner et al. 1978). It is the action of insulin in this capacity which may contribute to the initiation and promotion of cell growth observed in wound healing.

The relationship between wound healing, diabetes, insulin and hyperglycaemia is a complex issue. This study was designed to examine the acute effects of exogenous insulin on healing wounds in diabetic mice.

Methods

A) Induction of chronic diabetes with alloxan

Male C57 B1/6 mice weighing 25–30 g were divided into age and weight-matched groups. Chronic diabetes was induced by a single iv injection of 100 mg/kg alloxan.
solution in phosphate buffered saline (PBS, pH 7.4). Control mice received PBS alone at a dose of 0.1 ml/g body weight. Both groups of mice were weighed and tested for urine glucose (Diasstix, Ames Co.) weekly for the duration of the experimental period. Blood glucose levels were determined twice a month on samples from the periocular sinus. Blood samples (0.025 ml) were diluted 1:5 and read on a glucose analyzer (Yellow Springs, YSI-Model 23A). The alloxan diabetic mice (D) were considered diabetic according to the following criteria: 1) weight loss, 23% below control (C) (C, 30 g ± 5; D, 23 g ± 2, \( P < 0.01 \)) 2) hyperglycaemia, 27.5% above control (C, 120 mg/dl ± 2; D, 454 mg/dl ± 8, \( P < 0.01 \)) 3) glucosuria (C, 0 g/dl; D, > 4 g/dl in urine samples, and 4) low serum insulin, 56% below control (C, 25 mU/ml; D, 11.1 mU/ml). All chronic diabetic mice used in these experiments were determined to be diabetic by the above criteria for at least four weeks prior to use.

The effect of duration of diabetes on wound healing was examined in 4 separate paired groups of experimental animals which were diabetic for 4, 11, 22 or 33 weeks. All animals were monitored and evaluated for diabetes by the above methods.

B) Insulin injections
Alloxan diabetic mice were randomly selected for a series of insulin injections. Lente insulin (Hlentum, U-100, Lilly) was diluted in PBS to 160 mU/0.2 ml, and injected ip at 0.5, 1.5, 4, 5.5, 9, 10.5, 15, 16.5, 22, 23.5, 30, 31.5, 38, 39.5, 46 and 47.5 h. Blood glucose was measured 30 min before and 60 min after each injection as above.

In parallel, alloxan diabetic mice and control, non-diabetic mice from the same age-matched group were injected ip with 0.2 ml PBS according to the above schedule and served as the diabetic, non-treated and normal control groups, respectively.

C) Wounding, biopsy, tissue preparation
The ears of all the animals were wounded 40 h after the first injection and biopsied 8 h later. The mice received two circular perforating wounds in each ear with a 1.0 mm dermal trephine; the ears were biopsied by removing the entire ear and trimming to final size in the fixative. The biopsies were then processed for light and electron microscopy.

Tissues for light microscopy were fixed in neutral-buffered formalin (pH 7.4) and embedded in paraffin. Four \( \mu \)m sections were cut and stained with haematoxylin-eosin (H and E), haematoxylin-phloxine-safran (HPS) or Masson’s trichrome connective tissue stain. For electron microscopy the tissues were placed in a combined glutaraldehyde-paraformaldehyde fixative (Karnovsky 1965). The wounds were post-fixed in 1.33% osmium tetroxide buffered with 0.2 M S-collidine (pH 7.4) for 2 h at room temperature. The tissues were dehydrated in a graded series of alcohols and 100% propylene oxide and embedded in Araldite 502 in flat blocks. For orientation and quantitation 0.5 \( \mu \)m sections were cut and stained with methylene blue-azure II. Thin sections of the wound edge were cut on a MT2-B ultramicrotome in an identical manner for all biopsies examined. The sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. The electron microscope observations were from a sample of 10 tissue blocks taken from each experimental group (see Table 2).

D) Morphometry
The relative volumes occupied by the major cellular and extracellular components present at the 8 h wound edge were quantitated. A 10 mm square reticle with 1.0 mm line intervals was inserted into the eyepiece of a Leitz-Dialux microscope (Leitz, Wetzlar, Germany) equipped with a Wild Photomat MPS-55 (Wild, Heerbrugg, Switzerland). Morphometry was performed on 0.5 \( \mu \)m sections stained with methylene blue-azure II under oil at a magnification of 1400x. The area quantitated was determined according to a standard protocol for each wound examined in every experimental group. A calibrated reticle was superimposed over the tissue section beginning at the wound edge. The area of the reticle at 1400x was calculated to 85 \( \mu \)m\(^2\). The per cent volume occupied by each component was determined by lineal point analysis according to the method of Weibel et al (1968). The structures lying under the points of intersection in the reticle were counted. The components quantitated were capillaries, polymorphonuclear leukocytes (PMN-s), fibroblasts, oedema and collagen. Oedema was defined as that area devoid of visible tissue components but was within the defined zone. For each group, control \( (n = 45) \), diabetic, non-treated \( (n = 70) \) and diabetic, insulin-treated \( (n = 74) \), two blocks were randomly chosen per animal and one slide per block was quantitated in a double-blind design. The mean ± standard error was determined and the significance was determined by Student’s \( t \)-test.

Results
In preliminary studies, doses of 80 or 160 mU lente insulin were given in 2 injections 8 h apart. In these experiments 80 mU of insulin did not reduce hyperglycaemia in the mice, but insulin at 160 mU produced a significant depression in blood glucose up to 6 h after injection. The blood glucose level dropped from 550 mg/dl to 145 mg/dl. A dose of 160 mU lente insulin proved effective in reducing hyperglycaemia with no adverse effects on the mice and provided a 6 h interval of depressed blood glucose after each injection. These parameters
Mean (± SE) plasma glucose profiles of PBS-treated (---) and insulin-treated (—) diabetic mice and control, non-diabetic mice (baseline) for the 48 h treatment period. Insulin injections were given at intervals shown; the mice were wounded at 40 h and biopsied at 48 h. Values are mean ± SEM.

**Fig. 1.**

Low power electron micrograph of wound edge from an insulin-treated diabetic mouse taken 8 h after injury. The dense collagen stroma (Co) is infiltrated with PMN's ( ▪ ▪ ▪ ▪), fibroblasts (F) and capillaries (C). (× 5000).

**Fig. 2.**
provided the basis for the choice of insulin dose and multiple injection regimen for the reported experiments.

The blood glucose curve in Fig. 1 represents the combination of 4 experimental groups of mice that were diabetic for 4, 11, 22 and 33 weeks, respectively. ip Injections of insulin given at progressive intervals of 4, 5, 6, 7 and 8 h, produced regular depressions in blood glucose, beginning 1 h after injection (lowest point).

Fig. 3.
Low power electron micrograph of wound edge from an untreated diabetic mouse taken 8 h after injury. The collagen stroma (Co) is separated by large areas of dense material consistent with oedema (E). Cellular infiltrate is minimal. A large immature fibroblast-like cell (F) and dilated capillary are observed in the field. (× 8000)
**Morphology**

All biopsies were taken 8 h after injury. At this time, wound biopsies from control mice had a dense collagen stroma which was infiltrated with capillaries, fibroblasts and PMN’s. Wounds from diabetic mice contained few cells, reduced collagen stroma and large areas of interstitial oedema. In contrast to diabetic mice, wounds from insulin-treated diabetic mice produced a response comparable to control mice.

By electron microscopy, 8 h wounds from insulin-treated diabetic mice demonstrated a vascular and cellular infiltrate comprised of capillaries, fibroblasts and PMN’s within a compact collagen stroma (Fig. 2). In the untreated diabetic mice, a sparse cellular infiltrate was disposed around small isolated bundles of collagen; the remainder of the tissue volume was occupied by large areas of interstitial oedema (Fig. 3).

---

**Morphometry**

The results from the morphometric analysis are shown in Tables 1 and 2. The wound areas examined were calculated according to a standard method for all groups examined, and results are expressed as the per cent wound volume occupied by each component (Figs. 4 and 5).

In Table 1, the healing response is evaluated with regard to duration of diabetes. Untreated diabetes for 4, 11, 22 or 33 weeks produced a significant cellular and extracellular drop in healing components, accompanied by a substantial rise in wound oedema. This pattern is consistent and does not vary with the duration of diabetes. Similarly, the response observed after insulin treatment was significantly different from the diabetic, non-treated for every component, except for PMN’s at 4 weeks, and consistent for all stages of duration of diabetes. Insulin treatment appeared to induce a

---

<table>
<thead>
<tr>
<th>Table 1. Evaluation of healing response in control, diabetic and diabetic mice after insulin treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
</tr>
<tr>
<td>Control (10)</td>
</tr>
<tr>
<td>Diabetic In-Tx&lt;sup&gt;a&lt;/sup&gt; (10)</td>
</tr>
<tr>
<td>Diabetic N-Tx&lt;sup&gt;b&lt;/sup&gt; (10)</td>
</tr>
</tbody>
</table>

11 weeks | Control (10) | 5.38 ± 0.73 | 9.50 ± 0.82 | 12.90 ± 0.93 | 9.00 ± 0.98 | 58.10 ± 1.20 |
| Diabetic In-Tx (24) | 5.20 ± 0.48 | 7.12 ± 0.35 | 10.05 ± 0.78 | 14.20 ± 1.34 | 56.95 ± 1.47 |
| Diabetic N-Tx (24) | 1.75 ± 0.26 | 3.26 ± 0.36 | 2.98 ± 0.57 | 47.29 ± 1.64 | 34.52 ± 1.99 |

22 weeks | Control (15) | 4.25 ± 0.38 | 7.27 ± 0.61 | 9.64 ± 1.18 | 11.79 ± 1.94 | 63.75 ± 1.36 |
| Diabetic In-Tx (19) | 3.50 ± 0.39 | 6.00 ± 0.29 | 7.11 ± 0.92 | 14.61 ± 1.73 | 56.72 ± 1.54 |
| Diabetic N-Tx (19) | 1.65 ± 0.30 | 2.31 ± 0.34 | 1.92 ± 0.44 | 41.81 ± 1.93 | 43.48 ± 1.75 |

33 weeks | Control (10) | 4.88 ± 0.61 | 7.86 ± 0.70 | 11.15 ± 1.50 | 8.60 ± 1.51 | 59.09 ± 1.72 |
| Diabetic In-Tx (21) | 4.17 ± 0.30 | 7.36 ± 0.54 | 11.01 ± 0.92 | 11.09 ± 0.81 | 61.05 ± 1.23 |
| Diabetic N-Tx (22) | 1.59 ± 0.28 | 2.90 ± 0.32 | 1.84 ± 0.35 | 50.10 ± 1.65 | 37.99 ± 1.35 |

---

a: In-Tx, insulin-treated; b: N-Tx, non-treated; c: no significant difference between control vs. diabetic, insulin-treated for components quantitated, except for PMN’s (P < 0.05). The level of significance between control vs. diabetic, non-treated and diabetic, insulin-treated vs. diabetic, non-treated was P < 0.001 unless otherwise indicated (d: P < 0.05; e: not significant).

The values are expressed as per cent wound volume occupied by each component and are the mean ± SEM. Numbers of animals shown in parentheses.
Table 2.
Total per cent wound volume for wound healing components.

<table>
<thead>
<tr>
<th></th>
<th>Capillaries</th>
<th>Fibroblasts</th>
<th>Polymorphonuclear leukocytes</th>
<th>Oedema</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 45)</td>
<td>4.50 ± 0.33</td>
<td>7.50 ± 0.45</td>
<td>10.24 ± 0.92</td>
<td>10.52 ± 1.33</td>
<td>61.88 ± 1.14</td>
</tr>
<tr>
<td>Diabetic, insulin-treated (n = 74)</td>
<td>4.51 ± 0.23</td>
<td>6.83 ± 0.24</td>
<td>9.44 ± 0.65</td>
<td>13.51 ± 0.70</td>
<td>58.14 ± 0.75</td>
</tr>
<tr>
<td>Diabetic, non-treated (n = 70)</td>
<td>1.71a ± 0.15</td>
<td>2.87a ± 0.20</td>
<td>2.50a ± 0.27</td>
<td>44.03a ± 1.21</td>
<td>37.20a ± 1.03</td>
</tr>
</tbody>
</table>

The values are expressed as per cent wound occupied by each component and are the mean ± SEM. Numbers in parentheses indicate numbers of animals.

a: Level of significance between control vs. diabetic, non-treated and diabetic, insulin-treated vs. diabetic, non-treated was P < 0.001.

The healing response very similar to that in the control mice, regardless of the length of the diabetic state. In Table 2, the total per cent wound volume is calculated to include all of the time periods studied.

In Fig. 4, the response of capillaries, fibroblasts and polymorphonuclear leukocytes is seen. Both the cellular and vascular elements were significantly greater in wounds from insulin-treated diabetic mice when compared to non-treated diabetic mice. Furthermore, the response to insulin treatment was not significantly different from that observed in control mice. In every instance the insulin treatment appeared to stimulate the cellular components of the healing response as measured by the methods in this study.

The per cent volume occupied by oedema, as shown in Fig. 5, in wounds from insulin-treated diabetic mice was significantly reduced after insulin treatment in fact, similar to the non-diabetic control levels. The space occupied by oedema in the...
untreated diabetic mice remained elevated in the presence of decreased cellular, vascular and fiber volume 8 h after injury.

Collagen also remained depressed in the untreated diabetic mice, in contrast to the response observed after insulin treatment. The increased oedema in the tissues around the wound margin plus a decrease in fibroblasts probably contribute to the decreased wound volume occupied by collagen.

Discussion

The effect of insulin on the cellular and vascular events in healing wounds is of great interest. A few reports have stated that more rapid and efficient healing was achieved by topical administration of insulin directly into the wound (Van Ort 1976). This claim, however, had not been substantiated in laboratory studies with animals (Rosenthal & Enquist 1968). More recently, studies in which diabetic animals were given an ip insulin dose after wounding revealed that the infiltration of granulation tissue into wound cylinder implants was normal in amount (Goodson & Hunt 1978).

The results of these experiments suggest that the administration of exogenous insulin given over a time interval before wounding restored the impaired healing response in diabetic mice. Examination of wound components (capillaries, fibroblasts, PMN’s collagen, oedema) from control, diabetic, untreated and diabetic mice treated with insulin demonstrated that the response to injury was impaired in diabetic mice when compared to control; and, further, that this impairment was improved after insulin treatment.

Healing in the insulin-treated mice progressed despite periods of hyperglycaemia, indicating that the exogenous insulin did contribute to the improved response. Diabetic vascular complications are frequently attributed to the metabolic alterations induced by hyperglycaemia (Bondy & Felig 1971). Recent studies, however, suggest that hyperglycaemia alone may not significantly contribute to the poor healing response in diabetic mice (Weringer et al. 1981). Animals with high blood glucose and low insulin levels had an impaired healing response, while those with high blood glucose and normal insulin levels had a healing response comparable to controls. In addition, a third group, with low blood glucose and low insulin also showed an impaired healing response. These data support the hypothesis that insulin is an important and necessary component for wound healing.

Insulin is known to possess growth stimulating properties at concentrations lower than that required for control of hyperglycaemia (Steiner et al. 1978). Most of the biological events that characterize wound healing, initiation of DNA and protein synthesis, also define growth. In wound healing these events are observed as capillary proliferation, fibroblast activation and collagen synthesis. Promotion of growth is modulated by a variety of agents which share many biological properties with insulin (Rechner & Nissley 1977). As a group these polypeptide hormones have similar physiological actions (Baxter et al. 1979). This is consistent with the hypothesis that insulin is a cellular growth factor in many peripheral tissues (Czech 1977).

In addition to insulin, serum contains many growth promoting factors in high concentrations (Gospodarowicz & Moran 1976). Serum from diabetic patients promotes growth of aortic smooth muscle cells by in vitro analysis. In other tissues, notably cornea and retina, normal serum and/or oedema fluid (plasma filtrate) initiates a pronounced neovascular response (Ben Ezra 1978). Collectively, these data suggest that cellular stimulation is mediated by diffusible factor(s) found in serum. Cells in vivo are normally exposed to filtered plasma where growth factor levels are low; but at the wound margin, cells are exposed to whole serum and serum fractions and high levels of the growth factors (Gospodarowicz & Moran 1976).

Yet, in the wound tissue of diabetic mice, the increased oedema failed to induce any cellular or vascular response to injury. The cells appeared to be non-responsive in the presence of growth factors, perhaps due to a lack of receptors for these factors or as a result of a prolonged insulin insufficiency in the diabetic animals. However, when insulin was administered in large doses prior to wounding the cells were primed and responded to injury. The concentration of insulin in the wound area may be a critical constituent for improved healing observed in these studies.

The reaction of fibroblasts to injury was also impaired in diabetic, non-treated mice. In diabetes, fibroblasts and probably other cell types have a decreased capacity to initiate DNA or protein synthesis. In vitro studies of fibroblasts from diabetic patients demonstrate that the cells are compromised and unable to respond to injury (Goldstein et
al. 1969). They no longer synthesize collagen and have a shorter life span (Rosenbloom 1978). This may be, in part, due to the lack of insulin. Other studies show that fibroblasts, when deprived of nutrients, can be stimulated to DNA synthesis by the addition of insulin (Fujimoto & Williams 1974; Kamely & Rudland 1976). Diabetic mice treated with insulin exhibited many of the anabolic effects of the hormone as evidenced by an increase in fibroblasts, capillaries, PMN's and collagen in the wounds at 8 h. The growth and proliferation observed may be attributed to an action of insulin on the cells and a similar mechanism of response by the cells involved.

In conclusion, a significant impairment in the wound healing response of diabetic mice has been reported. This was characterized by an inability of the cellular and vascular elements in the wounds to undergo proliferation and growth. After insulin treatment there was a marked improvement in the healing response of diabetic mice. This restoration of the healing response supports the hypothesis that insulin is a necessary component for wound healing.

Acknowledgments

The authors wish to express their appreciation to Ms. Brenda McDougall for her invaluable technical assistance. This investigation was supported by a research grant from the Juvenile Diabetes Foundation and a research grant from the Kroc Foundation.

References


Received on December 12th, 1980.