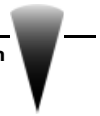# PAPER 1

Aminated β-1,3-D-glucan improves wound healing in diabetic *db/db* mice

Margrete Berdal, MD[1]; Hege I. Appelbom, BS[1]; Jorunn H. Eikrem, BS[1]; Åse Lund, BS[1]; Svetlana Zykova, MD, PhD[2]; Lill-Tove Busund, MD, PhD[2,3]; Rolf Seljelid, MD, PhD[2]; Trond Jenssen, MD, PhD[1,4]

1. Institute of Clinical Medicine,
2. Institute of Medical Biology, University of Tromsø, Tromsø, Norway,
3. Department of Pathology, University Hospital of Northern Norway, Tromsø, Norway, and
4. Department of Medicine, Rikshospitalet University Hospital, Oslo, Norway

Reprint requests:
Margrete Berdal, MD, Institute of Clinical Medicine, Faculty of Medicine, University of Tromsø, N-9037 Tromsø, Norway.
Tel: +47 776 69019
Fax: +47 776 44650;
Email: mberdal@fagmed.uit.no

ABSTRACT

Delayed wound healing in diabetes is caused by neuropathy, vascular changes, and impaired cellular response to the injury. Macrophages are crucial in normal wound healing, and impaired functions of these cells have been shown in diabetes. β-1,3-D-glucans stimulate macrophage function. This open-label study was performed to see if aminated β-1,3-D-glucan (AG) stimulates wound healing in diabetes. Four groups (1–4) of diabetic *db/db* mice and one nondiabetic control group, *db/+*(5) were studied: group 1 ($n=11$): topical AG; group 2 ($n=10$): topical AG and subcutaneous insulin; group 3 ($n=14$): topical placebo and subcutaneous insulin; group 4 ($n=10$): diabetic control (placebo); group 5 ($n=12$): normal control (placebo). At the end of the experiments fasting blood glucose and A1C were (mean ± SE) as follows: Group 1: 30.5 ± 1.9 mmol/L and $11.3 \pm 0.6\%$; group 2: 12.0 ± 1.7 mmol/L and $8.0 \pm 0.6\%$; group 3: 15.4 ± 2.4 mmol/L and $7.4 \pm 0.3\%$; group 4: 32.6 ± 2.6 mmol/L and $12.3 \pm 0.6\%$; group 5: 7.2 ± 0.4 mmol/L and $3.9 \pm 0.04\%$, respectively. The closed wound area was the same in group 1 (AG alone) and group 2 (AG plus insulin) after 17 days, 57.3 ± 4.7 vs. $50.1 \pm 4.9\%$ ($p=0.7$). The results of these two groups were superior to group 3 (insulin treatment alone, $32.0 \pm 4.3\%$, $p < 0.001$) and diabetic controls ($38.2 \pm 5.1\%$, $p=0.001$). The macrophage-stimulant AG improves wound healing in *db/db* mice.

Certain diabetic complications, such as increased susceptibility to infection,[1] frequent occurrence of ulcers,[2] delayed wound healing,[3] and aggravated arteriosclerosis[4] implicate an aberrant course of inflammation.

It has indeed been reported that blood monocytes from diabetic patients display phenotypic abnormalities, including aberrant chemotactic responses as well as decreased phagocytosis and cytokine release.[5–7] The data from these studies are difficult to interpret with respect to the *in vivo* situation because functional tissue macrophages, crucial to wound healing,[8] are a different subset from free-floating monocytes in the blood.

Data from studies on human, diabetic tissue macrophages are not available—a fact that motivates studies on relevant animal models. Studies on streptozotocin-treated diabetic rats and alloxan-treated diabetic mice, both type 1-like conditions, have revealed decreased production of cytokines and growth factors from macrophages *in vitro*.[9,10]

Recently, Zykova and coworkers reported altered macrophage morphology and their decreased release of cytokines and vascular endothelial growth factor (VEGF) in a model with type 2 diabetes-like animals, i.e., *db/db* mice.[11]

β-Glucans stimulate cells from the monocyte–macrophage system in animals and humans.[12–16] They also exert antitumor activity *in vitro* and *in vivo*,[13,17,18] and protect against life-threatening infections,[12,16,19] qualities possibly caused by induction of cytokine expression from the monocyte–macrophage system.[14,18,20]

The present study was undertaken to see if topical applications of the macrophage-stimulant, aminated β-1,3-D-glucan (AG), could improve wound healing in an animal model with diabetes mellitus (*db/db* mice).

MATERIALS AND METHODS

Animals

Diabetic C57Bl/KsBom-*db/db* mice, and the nondiabetic strain C57Bl/KsBom-*db/+* were studied. All animals were purchased from M & B A/S, Ry, Denmark. The *db/db* strain has an autosomal recessive mutation in the db-gene on chromosome 4, with a deficient leptin receptor,[21] and they develop hyperphagia, obesity, insulin resistance, and transient hyperinsulinemia at the age of 2–3 months. After that the pancreatic insulin production decompensates, and serious hyperglycemia and polyuria occur as the pancreatic β-cells atrophy.[22]

Three to five animals were housed per cage under controlled conditions: Room temperature $21 \pm 1\,^{\circ}$C, relative

AG	aminated β-1,3-D-glucan
A1C	glycosylated hemoglobin A1c
IL-1β	interleukin-1β
TNF-α	tumor necrosis factor-α

humidity $55 \pm 10\%$ and a 12:12 light:darkness cycle (light 08:00–20:00 hours). The animals were offered rodent food, SDS RM 1 (E), (Special Diets Services, Essex, UK) and water *ad libitum*. The Norwegian Ethics Committee for Research on Animals approved the experimental protocols.

The experiments started when the diabetic animals ($n=45$) were 3–4 months of age. Only *db/db* mice with blood glucose ≥ 16 mmol/L were included in the diabetic groups. In addition, (*db/+*) animals ($n=12$) were studied as a nondiabetic control group.

Anesthesia and blood sampling

General anesthesia was introduced after 4 hours of fasting (but still with water *ad libitum*) using a mixture of fentanyl/fluanisone and midazolam (final concentrations 0.079 mg/mL fentanyl, 2.50 mg/mL fluanisone, and 1.25 mg/mL midazolam; dose: 0.0075 mL/g body wt administered subcutaneously). Blood samples were drawn from the large saphenous vein on anesthetized animals for measurements of blood glucose, lactate, and A1C.

Wounding

The wound model is a modification of the procedure described by Greenhalgh et al.[3] With the animals in anesthesia the midpart of their back was shaved, chemically depilated using Nair® cream (Carter-Wallace Ltd., Folkestone, Kent, UK), and washed with tap water.

A template was then used to mark a 1.5×1.5 cm^2 area on the skin. The depilated area was disinfected with chlorhexidine 5 mg/mL prepared at the Hospital pharmacy and washed with sterile water. The back of the animals was then covered with a sterile utility drape, Klinidrape® (Mölnlycke Health Care AB, Gothenburg, Sweden), leaving the wound area open. A full-thickness skin wound was made on the back of the animals by excising the skin and panniculus carnosus. The wound was thereafter covered with a semipermeable, transparent polyurethane dressing, Opsite Flexigrid® (Smith & Nephew Medical Ltd., Hull, UK), which was fixed with the tissue adhesive, enbucrilate (Histoacryl®, B. Braun Melsungen AG, Melsungen, Germany), and 12 interrupted 5–0 Monosof™ sutures (Auto Suture Company, Norwalk, CT). The wound margins were finally traced onto glass microscope slides (=area day 0) before buprenorphine was given subcutaneously as analgesia (final concentration 0.030 mg/mL buprenorphine; dose: 0.0033–0.010 mL/g body wt). Another dose of buprenorphine was given 12 hours after the surgical procedure. An isotonic electrolyte solution, Ringer Acetate® (Fresenius Kabi Norge AS, Halden, Norway) (dose: 0.030–0.050 mL/g body wt), was given subcutaneously 0 and 2 hours after wounding. The following groups were studied:

1. *Db/db* mice ($n=11$) receiving local wound application of the macrophage stimulant AG (final concentration 11.10 mg/mL in 9 mg/mL NaCl; Fresenius Kabi Norge AS, Halden, Norway).
2. *Db/db* mice ($n=10$) receiving both AG and a subcutaneous insulin implant, Linplant® (Linshin Canada Inc., Scarborough, ON, Canada).

3. *Db/db* mice ($n=14$) receiving placebo wound treatment with NaCl 9 mg/mL and insulin Linplant®.
4. *Db/db* mice ($n=10$) receiving placebo wound treatment with NaCl 9 mg/mL only.
5. *Db/+* mice ($n=12$) receiving placebo wound treatment with NaCl 9 mg/mL.

During the observation period the animals behaved in an unstressed manner with unaffected food and water intake. The animals were anesthetized at the end of the experimental period, as described above. Blood samples were drawn from the retro-orbital plexus for measurement of blood glucose, lactate, and A1C. The animals were thereafter sacrificed by cervical dislocation.

Insertion of insulin implants

Insulin treatment was given with the bovine insulin implant, Linplant®, with a release rate of ~ 2 U/24 hours/implant for > 40 days.[23]

Implantation was performed with local anesthesia with a lidocaine–prilocaine plaster (Emla®, AstraZeneca, Södertälje, Sweden) on depilated skin in the neck region: The skin and the implant were disinfected with 2% povidone-iodine, PVP-iodine Doubldone (Marcus Research Laboratory Inc., St. Louis, MO). The implant was then inserted by a sterile, 12-gauge hypodermic needle.

Preparation and application of AG

AG, a water-soluble derivative of Curdlan, was prepared as previously described. Curdlan is a linear homopolymer composed of β-1,3-linked ᴅ-glucose subunits.[13]

All batches of AG were tested for lipopolysaccharide (LPS)-activity by the endotoxin-specific assay, Endospecy (Seikagaku Kogyo, Tokyo, Japan). They were found to have an activity below 60 ng/mL. Application was performed through a 3–4 mm wide opening made by carefully loosening the Opsite® dressing. Fifty to 100 μL of the agent were injected onto the wound, completely covering the wound bed (defined as the area surrounded by unwounded skin, fascia, regenerated epidermis, and scab). The dressing was finally fixed with enbucrilate.

The application frequency was based on the study on growth factor induced wound healing as reported by Greenhalgh and coworkers.[3] The agent was applied as intermittent treatment from the day of surgery (once daily for 5 days, followed by 4 days without treatment) to avoid skin maceration. A minimum of 15 doses was applied in all animals.

Wound closure measurement

The percentage wound closure was calculated at seven different time points over 17 days. The wound margins were traced onto glass microscope slides every second to fourth day during the experimental period. The wound area was measured by two different methods:

1. *Manual method (all animals, $n=57$)*: The wound perimeter was traced onto transparent film (3M®, St. Paul, MN), the traced area cut out and weighed on a micro balance, Mettler AT 250 (Mettler Instrumente AG, Greifensee, Switzerland). A pre-made standard curve

of area vs. weight was used to determine the wound areas.

2. *Digital method (n=50)*: The wound perimeter was scanned using a color image scanner, Canoscan™ N656U (Canon Inc., Tokyo, Japan), the software Scan-Gear™ Toolbox CS, version 1.01 (Canon Inc.) and Arc-Soft Photo Studio 2000 (ArcSoft Inc., Fremont, CA). The image analysis program Kontron Elektronik Imaging System KS 100, version 2.02 (Kontron Elektronik GmbH, Munich, Germany) was used to calculate the wound area.

Percentage wound closure for dayX was calculated using the following formula, where day0 is the day of surgery:

$$\frac{(\text{Area}_{day0} - \text{Area}_{day\,X}) \times 100}{\text{Area}_{day0}}\%$$

Comparison of the methods

Bland–Altman plots (Figure 1) were used to evaluate the agreement between the two methods:[24] The average of the areas (manual, digital; cm^2) vs. the difference between the areas (manual minus digital; cm^2) and the percentage difference between the areas (manual minus digital; %) were compared. The analysis included 600 observations from 50 mice (12 per animal) for each method of wound area measurement (cm^2). The Bland–Altman plot showed highly correlated average and difference values. The coefficient of correlation, adjusted for repeated observations, was 0.92 (95% CI 0.86–0.97; $p < 0.001$). Adjustment of the coefficient was performed by a linear mixed model analysis[25] with Z-score for "average" as covariate, and "time" as well as Z-score for "difference" as fixed factors. The covariances associated with the repeated measurements declined exponentially over time and were modeled by the first order autoregressive structure.

The coefficients of variation were 2.2 and 0.71% for the manual and the digital method, respectively. There was good agreement between the two methods with highly correlated average and difference values as well as low coefficients of variation. As the manual method was performed in all animals ($n=57$) and digital measurements were performed in only 50 animals, our calculations were performed on the results from the manual measurements.

Metabolic parameters

Glucose and lactate were measured by the YSI Glucose and L-Lactate Analyzer Model 2300-GL STAT (Yellow Springs Instrument Co., Yellow Springs, OH). A1C was analysed with the DCA 2000®+Analyzer Model 5031 C (Bayer Corporation, Elkhart, IN).

Histologic evaluation

In another 19 experimental animals ($n=6\;db/+$, $n=13\;db/db$) skin samples from the wound area were collected on day 13 and fixed in 4% para-formaldehyde with 0.2 M sucrose and embedded in paraffin. Six of the db/db mice had been treated with placebo and seven with AG. All animals were anesthetized before the procedure and thereafter sacrificed. Five-micrometer thick sections of the samples were hematoxylin–eosin (H/E) stained for light microscopy. The wound samples were blinded and histologically scored according to Greenhalgh et al.[3] The scores ranged from 1 to 12: 1 corresponded to no healing and 12 corresponded to a completely reepithelialized wound.[3] The scoring was based on the degree of cellular density, granulation tissue formation, vascularity, and reepithelialization. Two of the authors (L.T.B. and M.B.) scored the slides independently. In 16 out of 19 cases (84%), scoring was similar, and the remaining samples were reviewed until final agreement was achieved.

Bacteriological examination and fungus cultivation

Samples were harvested from wound bed abradant on anesthetized animals at the end of the study period. Signs of a probable wound infection were green-yellowish secretion and stagnation of wound closure over time. Animals with signs of an infection and growth of wound pathogens (e.g., *Staphylococcus aureus*) were excluded from the study.

In vitro release of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)

Peritoneal macrophages were harvested at the end of the study with the animals in general anaesthesia. Peritoneal lavage was performed with 8 mL ice cold RPMI-1640 with synthetic serum replacement medium (SSR-II; Medicult, Copenhagen, Denmark). The cells were seeded at a density of 1.0×10^6 cells per 0.8–1 mL per well into Falcon® 24-
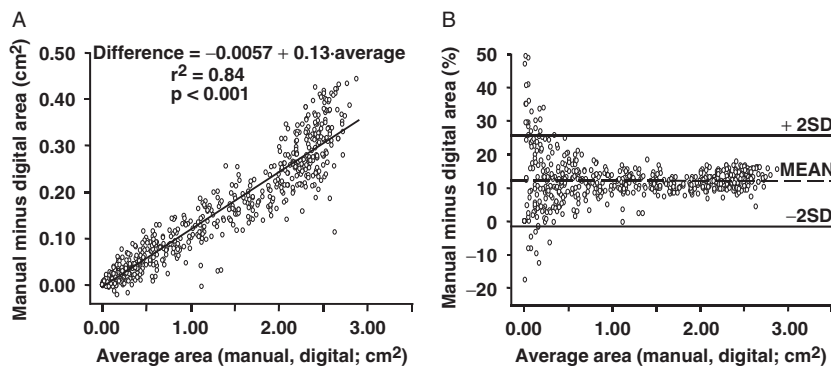

Figure 1. Bland–Altman plots of the two methods of wound area measurement (cm^2) showing the relationship between average area measurements (cm^2) and differences between measurements (manual minus digital area), in (A) absolute (cm^2) and (B) relative (%) values. The mean of the difference areas was 12% (standard deviation 6.8).

well plates (Becton Dickinson Labware, Franklin Lakes, NJ), and incubated at 37 °C in a humidified atmosphere at 5% CO_2 for 2 hours. Nonadherent cells were subsequently washed off, and adherent cells were cultivated over night in the same SSR-II medium. A final wash was performed before adding fresh medium and *E. coli* LPS (*Escherichia coli:* O26:B6 LPS; Difco Laboratories, Detroit, MI) at a final concentration of 0.5 μg/mL for macrophage stimulation over 24 hours.

The supernatants were thereafter used for measurement of TNF-α and IL-1β concentrations. The concentrations were adjusted for cell numbers.[26] The analyses were performed by commercial enzyme-linked immunosorbent assay kits purchased from R&D Systems Europe (Abingdon, UK). Unstimulated cultures served as controls.

Statistical analysis

All data, except for cytokine data (TNF-α, IL-1β) and histologic scores, were normally distributed and are presented as mean ± standard errors of the mean (SE). Statistical significance between groups was tested by independent samples *t*-test and one-way analysis of variance (ANOVA). The data on percentage wound closure between day 0 and day 17 were analyzed by repeated measurements ANOVA, and Bonferroni correction was applied for multiple comparisons. Analysis of the skewed data for the cytokines and of the histologic scores was made by nonparametric tests, Kruskal–Wallis and Mann–Whitney test. Analysis was performed by the statistical package SPSS 13.0 for Windows. $p < 0.05$ (two-tailed) was considered as statistically significant.

RESULTS

Baseline characteristics

At the start of the experiments the *db/db* mice showed gross appearance and metabolic characteristics consistent with diabetes (Table 1). They had polyuria and were obese.

At baseline the four diabetic groups were similar with respect to age, blood glucose, A1C, blood lactate, and body weight.

Wound closure rates

Diabetic vs. nondiabetic mice

As compared with the nondiabetic mice, wound closure in the diabetic control mice was delayed (mean difference $-40.7 \pm 2.7\%$, $p < 0.001$).

Wound contraction appeared not to contribute significantly to diminish wound area in the diabetic animals, irrespective of intervention group. This, however, was the case in the nondiabetic animals.

It took about 15 days to close 30% of the wound area in the diabetic control animals compared to 7 days in the nondiabetic control animals.

Effects of AG and insulin

Percentage wound closure in the insulin-treated group (group 3) was not different from that in the diabetic control animals (group 4) 17 days after surgery, 32.0 ± 3.8 vs. $38.2 \pm 6.3\%$ (NS). Both the AG-treated groups, with (group 2) or without (group 1) addition of insulin treatment, closed more completely at day 17: 50.1 ± 5.8 and $57.3 \pm 3.8\%$, respectively (Figure 2).

As the insulin-treated group (group 3) and the control group (group 4) appeared similar, and the same was the case with groups 1 and 2, with a treatment effect in favor of AG, AG− (groups 1+2) and non-AG (groups 3+4) treatments were also analyzed together. Treatment with AG showed a significantly higher percentage wound closure over 17 days than non-AG treatments, the mean difference in the 0–17 days period was $13.7 \pm 2.4\%$, $p < 0.001$. All wounds, regardless of treatment, were healed within 45 days.

Table 1. Characteristics of the experimental animals at the start and the end of the experiments

Intervention group (*n*)	AG (11)		AG+subcutaneous insulin (10)		Placebo+ subcutaneous insulin (14*)		Diabetic placebo (10)		Nondiabetic placebo (12[†])	
	Start	End	Start	End	Start	End	Start	End	Start	End
Age (months)	3–4	4–5	3–4	4–5	3–4	4–5	3–4	4–5	2.5–3.5	3.5–4.5
BG (mmol/L)	25.3 ± 1.5	30.5 ± 1.9	25.2 ± 2.0	12.0 ± 1.7	26.3 ± 1.1	15.4 ± 2.4	26.3 ± 2.3	32.6 ± 2.6	8.7 ± 0.3	7.2 ± 0.4
A1C (%)	10.3 ± 0.5	11.3 ± 0.6	9.9 ± 0.4	8.0 ± 0.6	10.8 ± 0.5	7.4 ± 0.3	9.7 ± 0.5	12.3 ± 0.6	3.8 ± 0.04	3.9 ± 0.04
Lactate (mmol/L)	1.4 ± 0.1	2.1 ± 0.1	1.8 ± 0.2	2.4 ± 0.2	1.5 ± 0.1	2.6 ± 0.3	1.7 ± 0.1	2.2 ± 0.3	0.8 ± 0.04	1.3 ± 0.2
Body weight (g)	40.4 ± 2.7	35.1 ± 3.2	46.9 ± 1.0	45.3 ± 0.8	39.9 ± 1.7	41.4 ± 1.5	46.4 ± 1.2	40.9 ± 1.9	24.8 ± 0.8	26.3 ± 0.9

Data are mean ± SE. Blood samples were obtained after 4 hours of fasting; however, there was no fasting in insulin-treated animals because of hypoglycemia risk.

*Blood sampling was unsuccessful in follow-up in two animals.

[†]*n*=7 for the A1C measurements.

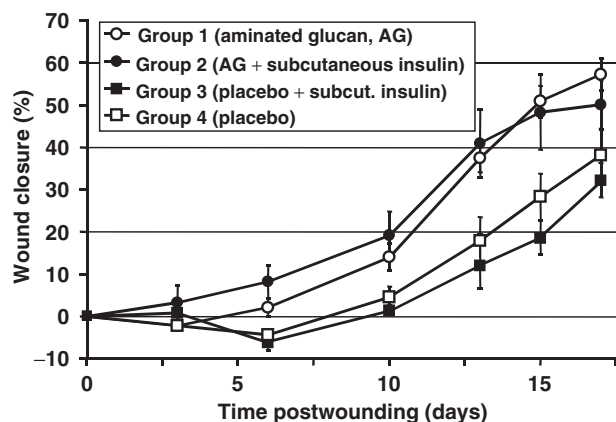AG, aminated glucan; BG, blood glucose.

Figure 2. Time course of the wound closure, i.e., percentage reduction of the original wound area, in the four intervention groups of diabetic (*db/db*) mice. Data are expressed as mean ± SE. The two AG-groups, groups 1 and 2, had significantly higher closure rate compared with the non-AG groups, groups 3 and 4 (repeated measurements ANOVA, $p < 0.001$).

Impact of wound infection

When we analyzed 11 animals from group 2 that had infectious wounds, the closure rate was only $40.4 \pm 5.6\%$ after 30 days compared with $89.2 \pm 5.0\%$ in the noninfected animals ($n=10$; $p < 0.001$). In groups 1, 3, and 4 the numbers of infected wounds were too small (≤ 4) to draw conclusions about the course of wound closure.

Histologic results

Histologic scoring of sections from the wound area at day 13 postwounding revealed significantly higher scores in the AG-treated group vs. the placebo group (Table 2), indicating accelerated granulation tissue formation associated with AG-treatment.

Representative sections are shown in Figure 3.

Sections from nondiabetic mice (group 5)

There was significantly thickened epithelium consisting of 6–8 cell layers in the periphery of the wound area. A granulation tissue rich in capillaries, fibroblasts, and inflammatory cells was observed beneath the wound bed.

Sections from placebo-treated diabetic mice (group 4)

Compared with the nondiabetic case, the granulation tissue appeared less extensive and less vascularized in the periphery of the wound bed, with extravasations of erythrocytes, a dense cellular infiltrate, and sparse bundles of collagen, indicating a relatively immature tissue.

Sections from AG-treated diabetic mice (group 1)

Compared with the diabetic control case, there was an epithelial thickening in a more extensive area in the periphery of the wound bed, and a highly vascularized, cell-rich granulation tissue appearing more vascularized and more extensive, that could be seen.

In vitro TNF-α and IL-1β release from peritoneal macrophages

TNF-α and IL-1β release were not significantly different between the four diabetic intervention groups ($n=44$), but significantly lower in the diabetic placebo-treated animals ($n=16$) vs. the nondiabetic animals ($n=6$) (Table 3).

DISCUSSION

Our study shows that diabetic (*db/db*) mice given topical applications of AG (groups 1 and 2) obtained significantly higher wound closure rates compared with the placebo-treated mice (group 4).

Histologic examinations of wound specimens (day 13) from the AG-treated group (group 1) revealed a more cell-rich and vascularized granulation tissue and increased re-epithelialization compared with the placebo-treated group (group 4).

Histologic scoring of these sections revealed significantly higher scores in the AG-treated group vs. the placebo group (Table 2), indicating accelerated granulation tissue formation.

We suggest that an inflammatory response with increased wound cellularity promoted the wound healing process, as is also reported with effective growth factor applications to diabetic wounds.[3] This may indicate a higher degree of inflammatory response preceding granulation tissue formation.

Cytokines and growth factors are important components of inflammation, and *in vitro* studies, including the present study (Table 3), have shown reduced expression of cytokines and growth factors in diabetic rodents.[9-11] In our experiments we chose to study cytokine release from peritoneal macrophages. These cells are easier to access

Table 2. Histologic scores and associated percentage wound closure at day 13 after surgery in three groups

Intervention group (*n*)	Aminated glucan (7)	Diabetic placebo (6)	Nondiabetic placebo (6)
Histologic score	8.0 (6–9)*,†	6.0 (5–8)	11.5 (10–12)
Wound closure (%)	25.7 ± 2.0	17.9 ± 2.8	90.0 ± 3.5

Values for histologic scores are expressed as median and associated range and were significantly different between the groups (Kruskal–Wallis test, $p < 0.001$).
*$p < 0.05$ vs. diabetic placebo.
†$p=0.001$ vs. nondiabetic placebo (Mann–Whitney tests). Data for wound closure (%) are mean ± SE.
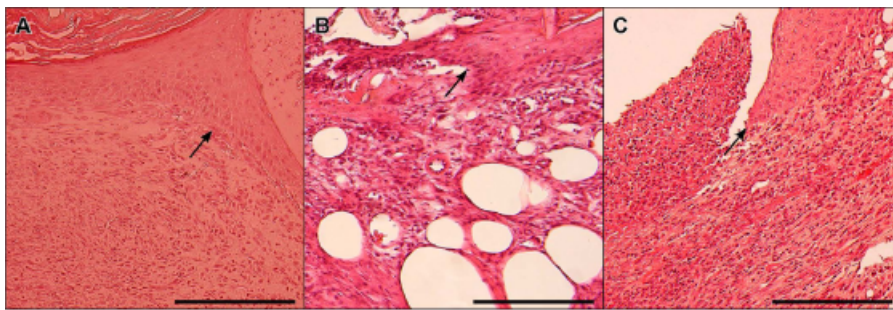
Figure 3. Histologic appearance of hematoxylin–eosin stained sections of skin from the wound area 13 days after surgery in a nondiabetic, *db/+* (A), placebo-treated diabetic, *db/db* (B), and aminated glucan (AG)-treated diabetic (*db/db*) mouse, (C). The magnification is ×200. A: The wound bed appears with a crust-like surface (upper right corner), and a significantly thickened epidermis in its periphery (the arrow indicates the basal cell layer) covered by squamous material (upper left corner). There is a cell-rich granulation tissue in the dermis below dominated by fibroblasts and inflammatory cells. (B) The advancing edge of epidermal cells (indicated by the arrow) is seen covering the dermis with sparse granulation tissue including fibroblasts, inflammatory cells, extravasations of erythrocytes and adipose tissue. (C) The thickened epidermis and its edge (indicated by the arrow) are seen surrounded by abundant cell-rich granulation tissue dominated by rounded cells, presumably inflammatory cells. Scale bars=200 μm.

compared with wound macrophages and could be obtained in culture by the simple method of peritoneal lavage.

TNF-α and IL-1β were studied because they are considered the more important pro-inflammatory cytokines. Furthermore, the macrophages that secrete these cytokines are essential cells both in innate and adaptive immunity. Unfortunately, we do not have a method of studying the TNF-α and IL-1β-release from on-site wound macrophages, which could have explained further the mechanisms behind the AG-effects in our study.

In wound models of diabetes mellitus reduced growth factor expression has been reported,[27] and local application of growth factors to wounds has shown a significant effect on healing rates compared with controls.[3,28]

To our knowledge, there are not many experimental studies with glucan in diabetes. One study reported an increase in collagen accumulation in streptozotocin-induced diabetic mice, although the increase was apparently not significant.[29] In that study, another glucan was used, and relatively few animals were studied under more moderate hyperglycemia than in our study.

The AG used in our study, is a water-soluble derivative of Curdlan that has shown strong immunomodulatory effects.[18,19] Furthermore, for the first time we here report effect of locally applied AG on wound healing in diabetic *db/db* mice, but the mechanisms that promote wound healing are not well understood. Among the AG-effects of direct relevance to wound healing in diabetes mellitus is the ability to stimulate production and release of inflammatory mediators, such as cytokines and arachidonic acid metabolites, as well as stimulation of antibacterial activi-

ties.[14,15] In this study we found no significant differences between the diabetic intervention groups regarding cytokine release from peritoneal macrophages (Table 3). This may indicate that the AG worked through a topical effect, which is nontoxic, as compared with other immunomodulators, e.g., LPS.

Macrophages are important sources of growth factors at the wound site, and *in vitro* studies have shown altered morphological appearance of these cells.[11] Application of β-1,3-glucan to nondiabetic wounds is reported to give a higher number of macrophages in the early inflammatory stage of repair compared with controls.[30] Furthermore, reepithelialization and the onset of fibroblast proliferation commences at an earlier stage in glucan-treated wounds.[30] This is consistent with our findings in diabetic mice.

In yet another study on nondiabetic animals, glucan increased the tensile strength of the wound tissue in the early phase of wound healing, probably due to increased cross-linking of collagen.[31]

The molecular mechanisms behind the effects of β-glucans are poorly understood. However, *in vitro* experiments have recently indicated that fibroblasts express β-glucan receptors, and that glucan may stimulate collagen synthesis in these cells.[32]

Also leukocytes, including macrophages, express receptors for β-glucans.[33] These receptors, as well as the toll-like receptors, function as pattern recognition receptors, crucial for innate immunity toward pathogenic microorganisms. The β-glucan receptor can recognize the molecular pattern of β-1,3-glucans, which is the structure of AG used in the present study. Previous studies have shown that AG

Table 3. *In vitro* TNF-α and IL-1β release from peritoneal macrophages

Intervention group (*n*)	AG (4)	AG+subcutaneous insulin (12)	Placebo+subcutaneous insulin (12)	Diabetic placebo (16)	Nondiabetic placebo (6)
TNF-α (pg/mL)	105 (83–127)	170 (51–439)	94 (32–255)	110 (70–357)	493 (423–767)
IL-1β (pg/mL)	51 (32–70)	42 (20–57)	29 (11–96)	56 (12–136)	151 (142–178)

Data are medians and associated range. TNF-α and IL-1β release were not significantly different between the four diabetic intervention groups, $p=0.6$ and $p=0.3$, respectively (Kruskal–Wallis test), but significantly lower in the diabetic placebo group vs. the nondiabetic placebo group, $p < 0.005$ and $p=0.001$, respectively (Mann–Whitney test).
AG, aminated glucan; TNF, tumor necrosis factor; IL, interleukin.

is bound and internalized via β-glucan receptors on mouse peritoneal macrophages.[34] Therefore, we could reasonably assume that the presence of β-glucan receptors on wound macrophages may be implicated into the healing process of AG-treated animals, e.g., by induction of cytokine expression.[15,18]

There is evidence of nitric oxide (NO) deficiency at the wound site in diabetic rats, and wounds heal more readily with NO supplementation, which indicates a crucial role for NO in wound repair.[35] Administration of β-glucans increases the NO production of cells in the peritoneal exudate,[17] and macrophages secrete NO,[11] a mechanism that also could have contributed to the wound healing effect in our model. Also glucan has been shown to scavenge free radicals, which could have improved wound healing.[36] However, neither NO nor free radicals were measured in this study.

As far as we know, there have only been a few reports on wound healing effects by structurally different glucans in diabetes.[29,37,38] In a study on 10 diabetic patients with sodium carboxyl-methyl-cellulose dressings wound healing improved significantly compared with controls treated with saline-moistened gauze.[37] In another study, which was a randomized, prospective, controlled multi-center trial with 276 patients treated either with a collagen/oxidized-regenerated cellulose dressing or moistened gauze, a clinical improvement in wound healing could be seen over 12 weeks.[38]

In the present study the addition of insulin (group 2) did surprisingly not improve the rate of wound closure further compared with the animals that were given AG alone (group 1), although insulin treatment was associated with a significant improvement in metabolic control, as measured by blood sugar and A1C, and also improvement in caloric balance with prevention of weight loss (Table 1). However, in group 2 a higher frequency of wound infections affecting wound closure was observed. This could possibly be explained by a larger volume of insulin implants in the proximity of the wound.

Insulin treatment alone (group 3) was also associated with a metabolic improvement, but not with a higher rate of wound closure compared with the placebo-treated group (group 4).

One may speculate that there is a threshold level of metabolic control (A1C) that has to be reached before the wound closure rate is improved in this animal model.

Although the insulin-treated animals were observed by weekly blood sugar measurements, and the insulin doses were titrated accordingly, we were not able to completely normalize the blood sugars in these extremely insulin resistant animals.[22] This could have blurred a beneficial effect of insulin treatment and normoglycemia on wound healing. Alternatively, one could speculate that the lack of a positive effect of insulin on the closure rates may rely on a species difference.

A1C-levels, which reflect long-term (2–3 months) glycation in humans with diabetes, were not normalized in our study. The life span of mouse-erythrocytes, of approximately 50 days, is shorter compared with human erythrocytes, and the synthesis of A1C is linear for at least the first 50 days of the mouse-erythrocyte's life.[39] Therefore, A1C-measurements performed at the end of our experiments, reflected glycemia during the whole period of insulin treatment, and in addition 10–15 days of hyperglycemia before the experiments started. A short-term glycation marker, e.g., serum fructosamine, could appear as a better choice.[40]

However, in rodents, fructosamine measurements only reflect a few days of nonenzymatic glycation as the half-life of circulating albumin is only 2 days in these animals. Multiple measurements during the study period would therefore have been necessary.

In conclusion, the structurally well-defined macrophage stimulant, AG, has a significant wound healing effect in a model of diabetic *db/db* mice. This indicates that the intrinsic impairment of growth factor and cytokine production in diabetic macrophages[11] can, at least partly, be overcome by local application of macrophage-stimulants.

The effect of AG on wound healing has so far not been explored in humans. However, our findings suggest that AG, or other possibly more effective derivatives of the same family of substances, should be considered as candidates for clinical application to wounds in diabetic patients.

REFERENCES

1. Kontras SB, Bodenbender JG. Studies of the inflammatory cycle in juvenile diabetes. *Am J Dis Child* 1968; 116: 130–4.
2. Abbott CA, Garrow AP, Carrington AL, Morris J, Van Ross ER, Boulton AJ. Foot ulcer risk is lower in South-Asian and African-Caribbean compared with European diabetic patients in the UK The North-West Diabetes Foot Care Study. *Diabetes Care* 2005; 28: 1869–75.
3. Greenhalgh DG, Sprugel KH, Murray MJ, Ross R. PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am J Pathol* 1990; 136: 1235–46.
4. Orlidge A, Hollis TM. Aortic endothelial and smooth-muscle histamine-metabolism in experimental diabetes. *Arteriosclerosis* 1982; 2: 142–50.
5. Hill HR, Augustine NH, Rallison ML, Santos JI. Defective monocyte chemotactic responses in diabetes mellitus. *J Clin Immunol* 1983; 3: 70–77.
6. Katz S, Klein B, Elian I, Fishman P, Djaldetti M. Phagocytotic activity of monocytes from diabetic patients. *Diabetes Care* 1983; 6: 479–82.
7. Luger A, Schernthaner G, Urbanski A, Luger TA. Cytokine production in patients with newly diagnosed insulin-dependent (type I) diabetes mellitus. *Eur J Clin Invest* 1988; 18: 233–6.
8. Leibovich SJ, Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol* 1975; 78: 71–100.

9. Ptak W, Klimek M, Bryniarski K, Ptak M, Majcher P. Macrophage function in alloxan diabetic mice: expression of adhesion molecules, generation of monokines and oxygen and NO radicals. *Clin Exp Immunol* 1998; 114: 13–18.

10. Doxey DL, Nares S, Park B, Trieu C, Cutler CW, Iacopino AM. Diabetes-induced impairment of macrophage cytokine release in a rat model: potential role of serum lipids. *Life Sci* 1998; 63: 1127–36.

11. Zykova SN, Jenssen TG, Berdal M, Olsen R, Myklebust R, Seljelid R. Altered cytokine and nitric oxide secretion in vitro by macrophages from diabetic type II-like db/db mice. *Diabetes* 2000; 49: 1451–8.

12. Kokoshis PL, Williams DL, Cook JA, Diluzio NR. Increased resistance to *Staphylococcus-aureus* infection and enhancement in serum lysozyme activity by glucan. *Science* 1978; 199: 1340–2.

13. Bogwald J, Seljelid R. Coupling of polysaccharides activated by means of chloroacetaldehyde dimethyl acetal to amines or proteins by reductive amination. *Carbohydrate Res* 1986; 148: 101–7.

14. Doita M, Rasmussen LT, Seljelid R, Lipsky PE. Effect of soluble aminated beta-1,3-D-polyglucose on human monocytes: stimulation of cytokine and prostaglandin E2 production but not antigen-presenting function. *J Leukoc Biol* 1991; 49: 342–51.

15. Rasmussen Busund LT, Konopski Z, Oian P, Seljelid R. Killing of *Escherichia coli* by mononuclear phagocytes and neutrophils stimulated in vitro with beta-1,3-D-polyglucose derivatives. *Microbiol Immunol* 1992; 36: 1173–88.

16. Browder W, Williams D, Pretus H, Olivero G, Enrichens F, Mao P, Franchello A. Beneficial effect of enhanced macrophage function in the trauma patient. *Ann Surg* 1990; 211: 605–12.

17. Suzuki T, Ohno N, Chiba N, Miura NN, Adachi Y, Yadomae T. Immunopharmacological activity of the purified insoluble glucan, zymocel, in mice. *J Pharm Pharmacol* 1996; 48: 1243–8.

18. Seljelid R, Figenschau Y, Bogwald J, Rasmussen LT, Austgulen R. Evidence that tumor necrosis induced by aminated beta 1-3D polyglucose is mediated by a concerted action of local and systemic cytokines. *Scand J Immunol* 1989; 30: 687–94.

19. Almdahl SM, Bogwald J, Hoffman J, Giercksky KE, Seljelid R. Protection by aminated glucan in experimental endogenous peritonitis. *Eur Surg Res* 1987; 19: 78–85.

20. Abel G, Czop JK. Stimulation of human monocyte beta-glucan receptors by glucan particles induces production of Tnf-Alpha and Il-1-Beta. *Int J Immunopharmacol* 1992; 14: 1363–73.

21. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996; 379: 632–5.

22. Coleman DL. Diabetes–obesity syndromes in mice. *Diabetes* 1982; 31 (Suppl. 1, Part 2): 1–6.

23. Wang PY. Palmitic acid as an excipient in implants for sustained release of insulin. *Biomaterials* 1991; 12: 57–62.

24. Bland JM, Altman DG. Comparing methods of measurement: why plotting difference against standard method is misleading. *Lancet* 1995; 346: 1085–7.

25. Littell RC, Pendergast J, Natarajan R. Modelling covariance structure in the analysis of repeated measures data. *Stat Med* 2000; 19: 1793–819.

26. Gillies RJ, Didier N, Denton M. Determination of cell number in monolayer-cultures. *Anal Biochem* 1986; 159: 109–13.

27. Doxey DL, Ng MC, Dill RE, Iacopino AM. Platelet-derived growth factor levels in wounds of diabetic rats. *Life Sci* 1995; 57: 1111–23.

28. Wieman TJ, Smiell JM, Su Y. Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (becaplermin) in patients with chronic neuropathic diabetic ulcers. A phase III randomized placebo-controlled double-blind study. *Diabetes Care* 1998; 21: 822–7.

29. Bhide MV, Dunphy MJ, Mirkopulos N, Smith DJ. Promotion of wound collagen formation in normal and diabetic mice by quadrol. *Immunopharmacol Immunotoxicol* 1988; 10: 513–22.

30. Leibovich SJ, Danon D. Promotion of wound repair in mice by application of glucan. *J Reticuloendothel Soc* 1980; 27: 1–11.

31. Browder W, Williams D, Lucore P, Pretus H, Jones E, McNamee R. Effect of enhanced macrophage function on early wound healing. *Surgery* 1988; 104: 224–30.

32. Wei D, Zhang L, Williams DL, Browder IW. Glucan stimulates human dermal fibroblast collagen biosynthesis through a nuclear factor-1 dependent mechanism. *Wound Repair Regen* 2002; 10: 161–8.

33. Willment JA, Gordon S, Brown GD. Characterization of the human beta-glucan receptor and its alternatively spliced isoforms. *J Biol Chem* 2001; 276: 43818–23.

34. Konopski Z, Smedsrod B, Seljelid R, Eskeland T. A novel immunomodulator soluble aminated beta-1,3-D-glucan: binding characteristics to mouse peritoneal macrophages. *Biochim Biophys Acta* 1994; 1221: 61–65.

35. Witte MB, Kiyama T, Barbul A. Nitric oxide enhances experimental wound healing in diabetes. *Br J Surg* 2002; 89: 1594–601.

36. Patchen ML, Dalesandro MM, Brook I, Blakely WF, Macvittie TJ. Glucan—mechanisms involved in its radioprotective effect. *J Leuk Biol* 1987; 42: 95–105.

37. Piaggesi A, Baccetti F, Rizzo L, Romanelli M, Navalesi R, Benzi L. Sodium carboxyl-methyl-cellulose dressings in the management of deep ulcerations of diabetic foot. *Diabet Med* 2001; 18: 320–4.

38. Veves A, Sheehan P, Pham HT. A randomized, controlled trial of Promogran (a collagen/oxidized regenerated cellulose dressing) vs standard treatment in the management of diabetic foot ulcers. *Arch Surg* 2002; 137: 822–7.

39. Koenig RJ, Cerami A. Synthesis of hemoglobin AIc in normal and diabetic mice: potential model of basement membrane thickening. *Proc Natl Acad Sci USA* 1975; 72: 3687–91.

40. Day JF, Ingebretsen CG, Ingebretsen WR Jr., Baynes JW, Thorpe SR. Nonenzymatic glucosylation of serum proteins and hemoglobin: response to changes in blood glucose levels in diabetic rats. *Diabetes* 1980; 29: 524–7.