

PAPER 2



Aminated β -1,3-D-glucan has a dose-dependent effect on wound healing in diabetic *db/db* mice

Margrete Berdal, MD¹; Hege I. Appelbom, BS¹; Jorunn H. Eikrem, BS¹; Åse Lund, BS¹; Lill-Tove Busund, MD, PhD^{2,3}; Robert Hanes, BSc²; Rolf Seljelid, MD, PhD²; Trond Jenssen, MD, PhD^{1,4}

1 Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway,

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 Organ Transplantation, Oslo University Hospital-Rikshospitalet, Oslo, Norway

Reprint requests:

Dr. Margrete Berdal, Institute of Clinical Medicine, Faculty of Health Sciences, University of Tromsø, N-9037 Tromsø, Norway.

Tel: +47 776 69019;

Fax: +47 776 45300;

Email: margrete.berdal@uit.no

Manuscript received: March 27, 2009

Accepted: June 15, 2011

DOI:10.1111/j.1524-475X.2011.00715.x

ABSTRACT

Inflammatory responses are common in diabetes and are operative in angiopathy, neuropathy, and wound healing. There are indications of incomplete macrophage activation in diabetes and reduced expression of growth factors. We have previously found that up to 15 topical applications of the macrophage-stimulant, aminated β -1,3-D-glucan (AG), improved wound healing in *db/db* mice. The present open-label study was undertaken to examine dose-dependent effects of AG over 40 days in *db/db* mice. AG was given as a single dose (group 1), one dose every 10th day (group 2), five initial doses on consecutive days (group 3), and ≥ 15 doses (group 4). Controls were *db/db* mice receiving platelet-derived growth factor + insulin-like growth factor-1 (group 5), topical placebo (NaCl 9 mg/mL) and insulin (group 6), placebo (group 7), and a nondiabetic group receiving placebo (group 8). Seven to 14 animals were allocated to each group. Percentage wound closure 17 days after surgery in groups 1 and 2 were (mean \pm standard error of the mean) 25.5 ± 5.3 and 32.2 ± 6.3 , respectively. Corresponding closure in groups 3, 4, and 5 was 55.7 ± 5.0 , 57.3 ± 5.0 , and 55.6 ± 4.8 , respectively ($p < 0.05$ vs. groups 1 and 2). Groups 6, 7, and 8 closed 32.0 ± 4.5 , 38.2 ± 5.3 , and $98.5 \pm 0.4\%$, respectively. Significant association between the number of AG-dosages and wound closure indicates dose-related effects in *db/db* mice.

The modulation of inflammatory pathways is implicated in diabetic complications, such as nephropathy, neuropathy, cardiovascular disease, and impaired wound healing.¹⁻⁴ The monocyte-macrophage system is an important contributor in inflammation. Studies on human blood monocytes in diabetes and diabetes-related models have shown altered function, such as defective chemotaxis, decreased release of cytokines, and altered expression of growth factors, as compared to the nondiabetic condition.⁵⁻⁸

Hyperlipidemia in diabetes is associated with reduced tumor necrosis factor- α (TNF- α) mRNA production, and cultured monocytes from diabetic humans release less cytokines (interleukin-1 β [IL-1 β], TNF- α) and growth factors (platelet-derived growth factor [PDGF], TGF- β 1) when exposed to low-density lipoproteins and triglycerides compared to control cells.^{8,9}

Studies on wounds in diabetic mice, however, demonstrate higher expression of TNF- α mRNA and protein compared to their nondiabetic littermates.¹⁰ Furthermore, diabetic wounds express less growth factor protein and mRNA, both in animals and humans, and topical applications of growth factors, such as PDGF, basic fibroblast growth factor, and/or IGF-1, have improved wound healing in several species including humans.^{4,11-15} Zykova and coworkers found decreased cytokine and growth factor release from peritoneal macrophages in an animal model with type 2-like *db/db* mice.¹⁶

Beta glucans are macrophage stimulants associated with antibacterial and antitumor effects.¹⁷⁻²² Previously, we

reported an effect of improved wound healing by multiple doses of a beta glucan, namely aminated β -1,3-D-glucan (AG).²³ These experiments were performed in diabetic *db/db* mice with full-skin wounds treated topically with AG. However, dose-response effects of AG have so far not been assessed.

The present study was undertaken to determine if there was a dose-dependent effect of cumulative AG doses on wound healing. In control experiments, placebo, insulin, or topically applied growth factors were used.

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).

bFGF	Basic fibroblast growth factor
IGF-1	Insulin-like growth factor-1
IL-1 β	Interleukin-1 β
PDGF	Platelet-derived growth factor
TGF- β 1	Transforming growth factor- β 1
TNF- α	Tumor necrosis factor- α

The diabetic state in the *db/db* mice results from an autosomal recessive mutation in the *db* gene on chromosome four encoding the leptin receptor.²⁴ Based on our objective of adjustment for *db*-gene effects as far as possible, and our observation of a similar progress of wound healing in *db/+* compared to normal C57Bl/KsBom *+/+* mice (unpublished data), we chose to use *db/+* mice as controls in our studies.^{24,25}

The animals were housed under the same conditions as previously reported²³ and offered rodent food, SDS RM 1 (E) (Special Diets Services, Essex, England) and water ad libitum. The Norwegian Ethics Committee for Research on Animals approved the experimental protocols.

The experiments started when the diabetic animals ($n = 75$) were 2 to 4 months of age. Only *db/db* mice with plasma 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 weight administered subcutaneously). Blood samples were drawn from the large saphenous vein on anesthetized animals for measurements of plasma glucose, plasma lactate, and glycosylated hemoglobin A1C (A1C).

Wounding

We used the same wound model as previously reported, which is a modification of the procedure described by Greenhalgh et al.^{4,23}

In brief, the procedure was performed on anesthetized animals having the midpart of their back shaved, chemically depilated using Nair cream (Carter-Wallace Ltd., Folkestone, Kent, England), and washed with tap water. A template was then used to mark a 1.5×1.5 cm² area on the skin. The depilated area was disinfected with chlorhexidine 5 mg/mL prepared at the hospital pharmacy and washed with sterile water. A full-thickness skin wound was made on the back of the animals by excising the skin and panniculus carnosus under optimal clean conditions. The wound was thereafter covered with a semipermeable, transparent polyurethane dressing, Opsite Flexigrid (Smith & Nephew Medical Ltd., Hull, England), which was fixed with the tissue adhesive, eubucrilate (Histoacryl, B. Braun Melsungen AG, Melsungen, Germany), and 5-0 Monosof sutures (Auto Suture Company, Norwalk, CT).

The wound margins were finally traced onto glass microscope slides (= area day 0), and buprenorphine was given subcutaneously as analgesia (final concentration 0.030 mg/mL buprenorphine; dose according to recommendations from the Norwegian Institute of Public Health, National Lab Animal Center: 0.0033–0.010 mL/g body weight, an average of 0.007 mL/g body weight was injected). Another dose of buprenorphine was given 12 hours after the surgical procedure. An isotonic electrolyte solution, Ringer Acetate (Fresenius Kabi Norge AS, Halden, Norway), was given subcutaneously 0 and 2 hours after wounding.

The following eight groups were studied, of which four received local wound applications of the macrophage stimulant AG. All interventions started on the day of surgery, and the observation period was 45 days:

- (1) *Db/db* mice ($n = 10$) receiving one dose of AG (final concentration 11.10 mg/mL in NaCl 9 mg/mL [Fresenius Kabi Norge AS]).
- (2) *Db/db* mice ($n = 7$) receiving one dose of AG every 10th day.
- (3) *Db/db* mice ($n = 11$) receiving one daily dose of AG for 5 consecutive days.
- (4) *Db/db* mice ($n = 11$) receiving AG until complete closure of the wound, at least 15 doses.¹
- (5) *Db/db* mice ($n = 12$) receiving one daily topical application for 5 consecutive days of the mixture: 1 μ g recombinant human platelet-derived growth factor, isoform BB (rhPDGF-BB), and 1 μ g recombinant human insulin-like growth factor-1 (rhIGF-1) according to the study of Greenhalgh et al.⁴ Both substrates were purchased from R & D Systems (Abingdon, UK), and both were reconstituted in human serum albumin (Octapharma AG, Vienna, Austria; final concentration 5 mg/mL in NaCl 9 mg/mL).
- (6) *Db/db* mice ($n = 14$) receiving placebo wound treatment once daily for 5 consecutive days (NaCl 9 mg/mL) and insulin subcutaneously implanted, Linplant (Linshin Canada, Inc., Scarborough, Ontario, Canada).^{23,26}
- (7) *Db/db* mice ($n = 10$) receiving placebo wound treatment with NaCl 9 mg/mL once daily for 5 consecutive days.
- (8) *Db/+* mice ($n = 12$) receiving placebo wound treatment with NaCl 9 mg/mL once daily for 5 consecutive days.

The animals' conditions during the experiment and the procedures performed at the end of the study period were as previously reported.²³

Among several growth factors, the combination of PDGF-BB and IGF-1 was chosen because of their previously reported synergistic effects on wound healing, possibly implicating a PDGF-mediated increase in the level of the IGF-1 receptor.^{14,27}

As the subcutaneous administration of insulin does not require general anesthesia, and the duration of insulin delivery from the implants was supposed to be >40 days,²⁶ we chose this way of insulin treatment. If glucose control was not satisfactory, we added an extra $1/2-1$ implant during the observation period.

Parallel groups without AG treatment were compared in pilot studies (no applications [$n = 11$ animals] vs. 1 daily dose of NaCl 9 mg/mL for 5 consecutive days [$n = 12$ animals]). Wound closure was not significantly different with the presence or absence of carrier over time ($p = 0.4$, repeated measurements analysis of variance [ANOVA]), and we therefore chose to conduct all experiments with five doses of carrier (NaCl 9 mg/mL) as the placebo group.

All experiments were performed within a time frame of 24 months. Results from groups 4, 6, 7, and 8 have previously been presented.²³

¹Cycling 5 days with and 5 days without AG treatment to avoid maceration of the skin. All animals received at least three cycles.

Preparation and application of aminated β -1,3-D-glucan (AG)

AG, a water-soluble derivative of Curdlan, was prepared as previously described.²⁸ Curdlan is a linear polymer of β -1,3-linked D-glucose subunits. 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 as previously reported.²³

Preparation and application of PDGF and IGF-1

Lyophilized rhPDGF-BB and rhIGF-1 were reconstituted in human serum albumin and NaCl, as mentioned above, and the final concentration of both growth factors was 10 μ g/mL. A total volume of 100 μ l was injected onto the wound in the same manner as previously reported on AG.²³

Wound closure measurement

The measurements were performed at seven different time points over 17 days as previously reported.²³ Percentage wound closure for day X was calculated using the following formula, where day 0 is the day of surgery:

$$\frac{(Area_{day0} - Area_{dayX}) \times 100}{Area_{day0}} \%$$

In the previous study, a part of the measurements were performed using two different methods, manual and digital.²³ These methods were compared using 600 observations from 50 animals (12 per animal). As the comparison gave essentially the same results between these methods, we used the manual method for all animals included ($n = 87$) also in the present study.

Metabolic parameters

Plasma glucose and lactate measurements were performed with the YSI Glucose and L-Lactate Analyzer Model 2300-GL STAT (Yellow Springs Instrument Co., Yellow Springs, OH). A1C was analyzed with the DCA 2000 + Analyzer Model 5031 C (Bayer Corporation, Elkhart, IN).

Bacteriological examination and fungus cultivation

Samples were harvested from wound bed abraded on anesthetized animals at the end of the experimental period. Animals with signs of wound infection (green-yellowish secretion and decreased closure rate) and growth of wound pathogens (e.g., *Staphylococcus aureus*) were excluded from the study.

Histologic studies

Skin biopsies from wound areas of another 30 experimental animals (*db/db* mice) were collected, 24 of them on day 12 to 14 from: AG ($n = 14$), placebo (NaCl 9 mg/mL; $n = 7$), and PDGF + IGF-1-treated animals ($n = 3$). The remaining six

biopsies were collected from PDGF + IGF-1-treated animals on days 17 and 18. All animals were treated topically in the wound bed. The AG-treated mice were divided into three different dosage groups: one dose ($n = 4$), five doses ($n = 6$), and eight doses ($n = 4$). Results from seven of these animals treated with AG have previously been presented.²³ All animals were anesthetized prior to the procedure and thereafter sacrificed.

The specimens were fixed in 40 mg/mL paraformaldehyde with 0.2 mol/L sucrose and embedded in paraffin. Five-micrometer-thick sections of the samples were hematoxylin-eosin stained for light microscopy. The wound samples were blinded and histologically scored according to Greenhalgh et al.⁴ The scores ranged from 1 to 12 with 1 corresponding to no closure and 12 corresponding to a healed wound.⁴ Two of the authors (L.T.B. and M.B.) scored the slides independently and were blinded to any other information. The scoring was similar in 21 out of 30 cases (70%). The remaining samples were reviewed until a final agreement was achieved.

Sections from 21 biopsies (seven placebo-treated and 14 AG-treated), as previously mentioned, were also stained with Masson's trichrome (MT) for light microscopic evaluation of collagen deposition that was semi-quantitatively scored at 100 \times magnification according to a scale suggested by Abramov and colleagues²⁹: 0 = none, 1 = scant, 2 = moderate, and 3 = abundant. Scoring was performed independently and blinded for any other information by L.T.B. and M.B. Scoring was the same in 10 out of 21 cases (48%). The remaining differed by 1 score point and were reviewed until a final agreement.

Immunohistochemistry

Sections from 14 of the previously mentioned biopsies (six placebo-treated and eight AG-treated [five or eight doses]) were deparaffinized. To inactivate endogenous peroxidases, they were treated for 10 minutes with 3% hydrogen peroxide. Thereafter, the samples were rinsed and washed in phosphate buffered saline solution (PBS) followed by incubation with 100 μ L blocking buffer (1.5% goat serum in PBS) for 20 minutes. The tissue was then incubated with 100 μ L primary antibody (0.008 mg/mL) (PE anti-mouse F4/80 Ab, #123109, BioLegend, San Diego, CA) overnight at 4 $^{\circ}$ C, then rinsed and washed with PBS. Thereafter, incubation with two drops from Reagent 1, Rat Antibody enhancer from the Polink-2 HRP Plus Rat-NM DAB Detection System (D46-15, Golden Bridge International Inc., Mukilteo, WA) for 10 minutes followed by rinsing and washing with PBS. Then the slides were incubated with two drops from Reagent 2, Polymer HRP for Rat from the Polink-2 HRP Plus Rat-NM DAB Detection System for 10 minutes, rinsed, and washed with PBS. The samples were subsequently incubated with 100 μ L DAB+ reagent (two drops of DAB+ Chromogen blended with 1 mL of DAB+ Substrate Buffer from the DAKO EnVision+ System Kit; DAKO, K4011; DAKO Denmark A/S, Glostrup, Denmark) for 10 minutes, rinsed with distilled water, and washed in PBS. Furthermore, the sections were washed in water and counterstained with hematoxylin (HHS32-1L, Sigma-Aldrich Co., St. Louis, MO), dehydrated, and mounted.

For each section, the number of F4/80-positive cells (macrophages) was counted at 400 \times magnification in five visual

fields, including one at each wound edge and three in the wound bed. Then, an average was estimated.

Statistical analysis

The distributions of all variables were evaluated by visual inspection of frequency histograms. All data, except for the histologic scores, the respective number of AG-doses, and the numbers of (F4/80-positive) cells, were normally distributed and are presented as mean \pm standard error of the mean. Statistical significance between groups was tested by independent samples *t* test and one-way 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.

A mixed model analysis was applied to the repeated observations, from 49 mice, of the cumulative AG doses and their corresponding responses (percentage wound closure) to adjust the parameter estimates of the regression line for repeated measurements.³⁰ The percentage wound closure was the dependent variable, time was a fixed factor, and the number of AG doses was a continuous variable. The intercept of the regression equation was adjusted by the regression estimate of the 4th time point (day 10).

Furthermore, a mixed model analysis was used to adjust the coefficient of correlation for repeated measurements. In this analysis, the Z-score of the percentage wound closure was the dependent variable, time was a fixed factor, and Z-score for the number of AG-doses was a continuous variable. As the covariance associated with the repeated measurements declined exponentially over time, the residual covariance structure applied in both the mixed model analyses was the first-order autoregressive.

A chi-square test was used to test for differences in the rate of wound infection between intervention groups. Spearman's correlation coefficient was calculated for the skewed data of the AG doses and the respective histologic scores. Mann-Whitney test was performed for comparison of histologic scores and numbers of (F4/80-positive) cells between groups. Analysis was performed by the statistical package SPSS 14.0 for Windows (SPSS Inc. Chicago, IL). $p < 0.05$ (two-tailed) was considered as statistically significant.

RESULTS

Characteristics of the experimental animals

At the start of the experiments, the *db/db* mice had developed obesity and polyuria, both characteristics consistent with diabetes. The average body weight was 41.3 ± 0.7 g compared to 24.8 ± 0.8 g in the nondiabetic animals ($p < 0.001$). Analyses of blood from diabetic animals demonstrated increased levels of glucose, A1C, and lactate (Table 1).

At baseline, the seven diabetic groups were similar with respect to age, plasma glucose, A1C, plasma lactate, and body weight (one-way ANOVA, Table 1). Baseline and follow-up values for plasma glucose, A1C, and body weight were comparable in groups 1–5 and 7 (noninsulin groups), while follow-up plasma glucose and A1C were lower in the insulin group (Table 1). The A1C measurements were performed for the groups 3, 4, 6, and 7 only (Table 1).

Wound closure rates

Diabetic vs. nondiabetic mice

Wound closure in the diabetic animals was delayed compared to the nondiabetic control mice. Absence of wound contraction appeared to contribute significantly to this delay. After 17 days of observation, the nondiabetic wounds were 99% closed (data not shown).

Intervention in diabetic mice

Percentage wound closure 17 days after surgery in the two low dosage frequency AG groups, groups 1 and 2, were 25.5 ± 5.3 and 32.2 ± 6.3 , respectively, placebo plus insulin group (32.0 ± 4.5) and the diabetic placebo group (38.2 ± 5.3). These groups did not differ significantly from each other (Figure 1).

The corresponding wound closure of the higher dosage frequency AG groups (groups 3 and 4) and the growth factor group ($55.6 \pm 4.8\%$) was similar to each other, 55.7 ± 5.0 and 57.3 ± 5.0 , %, respectively (Figure 1). Time-dependent wound closure over the same period of time was significantly higher in groups 3–5 (high dose of AG or growth factors) compared to groups 1, 2, 6, and 7 (low-dose AG or placebo with or without insulin, Figure 1).

Furthermore, there was a significant association between the number of cumulative AG doses and the percentage wound closure during the course of the study. The association between the number of AG doses and wound closure is shown in Figure 2. All wounds, regardless of treatment, were healed within 45 days.

Wound infection

The occurrence of wounds with signs of infection was not significantly different between study groups, group 1: two of 12 (16.7%), group 2: one of eight (12.5%), group 3: one of 12

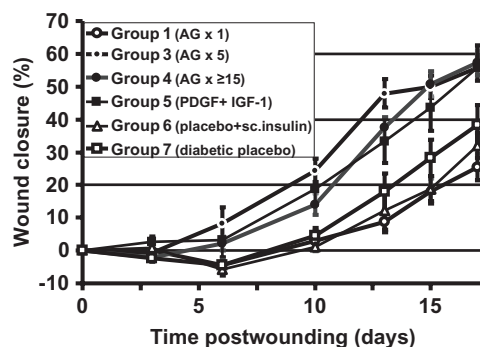


Figure 1. Time course of percentage wound closure in amniated β -1,3-D-glucan (AG)-treated diabetic *db/db* mice vs. three control groups (growth factors, placebo + subcutaneous (sc.) insulin, and placebo). The data are mean \pm SE. Time-dependent wound closure in either of groups 1, 2, 6, and 7 vs. either of groups 3–5 (all $p < 0.05$). Group 1 vs. group 7, $p = 1.0$; group 2 vs. group 7, $p = 1.0$; group 3 vs. group 4, $p = 1.0$ (repeated measurements ANOVA).

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

Group (n)	AG × 1 (10)		AG × 1/10th day (7)		AG × 5 (11)*		AG × ≥15 (11)		PDGF + IGF-1 (12)		Placebo + sc insulin (14)†		Diabetic placebo (10)		Nondiabetic placebo (12)‡	
	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
Age (months)	2-3	3-4	2.5-3	3.5-4	2.5-3.5	3.5-4.5	3-4	4-5	2.5-3	3.5-4	3-4	4-5	3-4	4-5	2.5-3.5	3.5-4.5
PG (mmol/L)	24.1 ± 1.2	31.2 ± 0.9	27.5 ± 3.1	29.2 ± 4.0	26.1 ± 1.2	33.1 ± 1.8	25.3 ± 1.5	30.5 ± 1.9	25.9 ± 2.3	32.2 ± 2.1	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 (%)	—	—	—	—	9.9 ± 0.6	12.4 ± 0.5	10.3 ± 0.5	11.3 ± 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)	1.3 ± 0.1	2.2 ± 0.1	1.7 ± 0.2	2.3 ± 0.3	1.7 ± 0.2	2.0 ± 0.2	1.4 ± 0.1	2.1 ± 0.1	1.6 ± 0.1	1.8 ± 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)	39.1 ± 1.1	35.5 ± 2.2	39.7 ± 1.5	30.6 ± 0.9	43.0 ± 1.8	34.8 ± 1.7	40.4 ± 2.7	35.1 ± 3.2	40.7 ± 1.6	33.6 ± 1.9	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 expressed as mean ± SE. Blood sampling was performed on 4-hour fasting animals, except for the insulin-treated mice at risk of hypoglycemia. All diabetic intervention groups were similar with respect to baseline characteristics (one-way analysis of variance and the Bonferroni correction for multiple comparisons).

*n = 7 for the A1C measurements.

†Blood sampling was unsuccessful in follow-up in 2 animals.

‡n = 7 for the A1C measurements.

AG, aminated β-1,3-D-glucan; PG, plasma glucose; A1C, glycosylated hemoglobin A1C; sc, subcutaneous; —, analysis not performed.

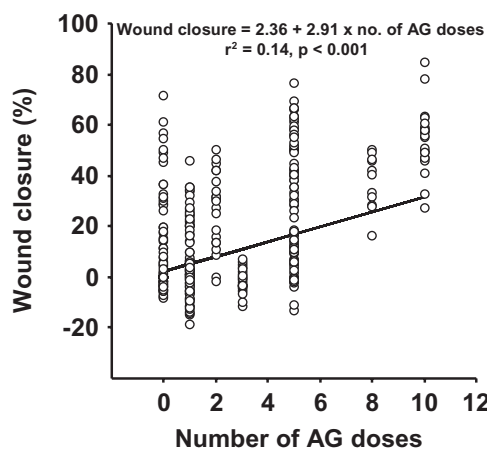


Figure 2. Aminated β-1,3-D-glucan (AG), dose vs. response. The association between the cumulative number of AG doses and the repeated observations of wound closure during 17 days after surgery, corrected for time-effect (see Methods, Statistics). Coefficient of correlation adjusted for repeated measurements 0.38 (95% CI 0.28–0.47; $p < 0.001$).

(8.3%), group 4: one of 12 (8.3%), group 7: three of 39 (7.7%). Specimens for bacterial growth were taken from four of these eight wounds, and wound pathogens were not detected in any of these samples. However, in three of them, Gram-negative rods (proteus species and coliform rods) were verified by bacterial culture, and in the fourth sample, sparse growth of *Staphylococcus epidermidis* was detected.

Histologic results

There was a significant association between the histologic scores of the wounds and the number of cumulative AG doses topically applied to the respective wounds during 12–14 days of experiments (Figure 3). Figure 4 shows photomicrographs of midsections from these wounds. Compared to the placebo-treated wounds, those treated with five or more AG doses showed a more mature, capillarized, and thicker granulation tissue, as did also the wounds treated with PDGF and IGF-1.

Scoring of MT-stained sections from AG-treated wounds (five or eight doses, $n = 10$) revealed significantly more collagen deposition compared to placebo-treated ($n = 7$) wounds (AG vs. placebo, $p = 0.02$). Furthermore, there was a significant association between the cumulative number of AG doses applied to the wounds, during 12–14 days of experiment, and the respective histologic scoring regarding collagen deposition ($r = 0.6$, $p = 0.01$, Spearman’s coefficient of correlation).

Cellular infiltration in the sparse granulation tissue in placebo-treated wounds (Figure 4E) appeared less extensive compared to AG-treated wounds (Figure 4F). Significantly lower numbers of F4/80-positive cells (macrophages) were also counted in these sections (Figure 5).

DISCUSSION

We have previously shown improved wound healing with the macrophage-stimulant AG in a model with full-thickness skin

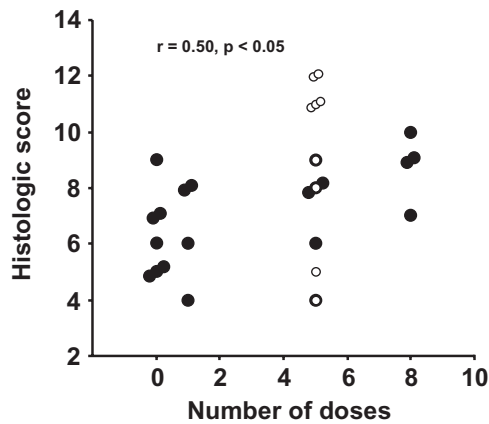


Figure 3. The relationship between the cumulative number of topical aminated β -1,3-D-glucan (AG) doses applied during the experiments and the respective histologic scores at 12–14 days (black circles) postwounding (21 cases, 12 overlaps; Spearman’s coefficient of correlation). The comparator was growth factor treatment (PDGF + IGF-1; open circles) after 13–18 days (nine cases, five overlaps).

wounds in diabetic *db/db* mice.²³ In the present study, we have explored the effect of cumulative AG-dosages, and as macrophage stimulation involves release of growth factors, we have also compared the findings to topical growth factor treatment.

The groups receiving five or more AG dosages (higher dosage frequency) showed a significant improvement in wound closure compared to low AG dosage frequency or placebo wound treatment. More than five AG applications were not associated with a further increase in wound closure compared to five applications only (Figure 1). Frequent AG applications induced a better wound closure which was comparable to topical growth factor treatment, involving a more extensive, vascularized, and mature granulation tissue in the wound bed (Figure 4).

In wound studies with growth factors, using similar wound models in *db/db* mice, one daily dose during the initial 5 days after wounding appeared to be the optimal dosage frequency both for wound closure and histologic scoring.^{4,31} More than five consecutive dosages of growth factors after the day of wounding have not been associated with an increased response of wound repair.⁴

The mechanisms of action of AG in wound healing in diabetes are not known, but cells from the monocyte-

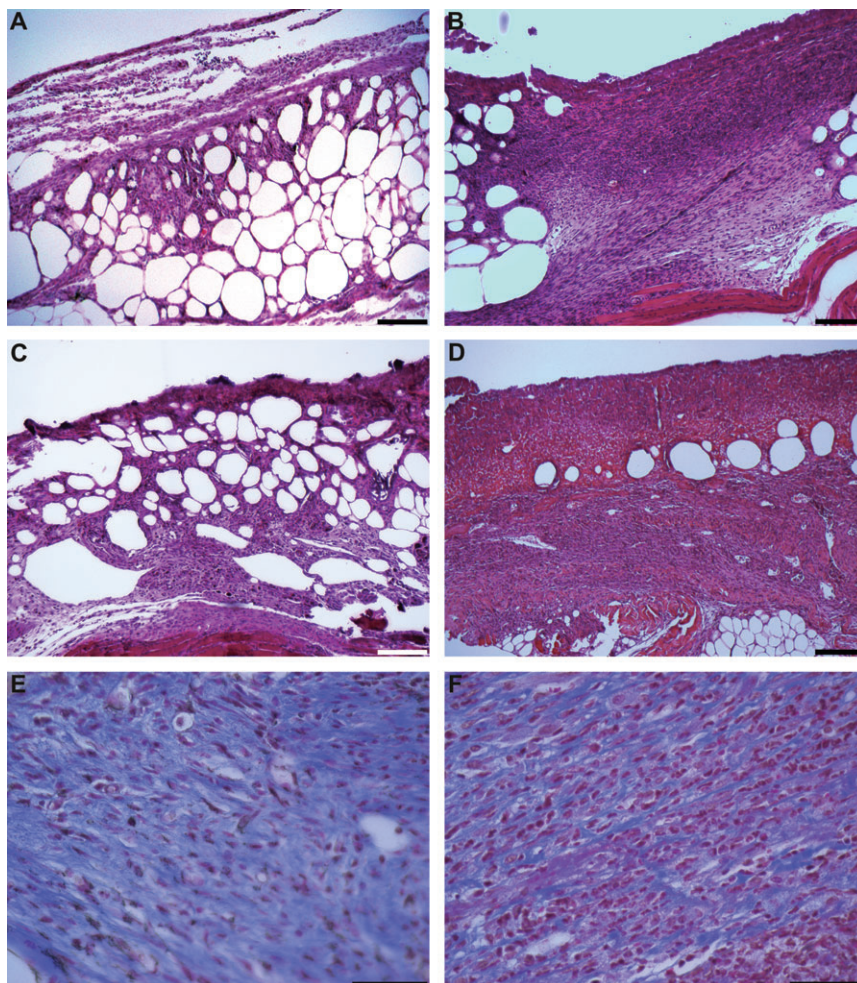


Figure 4. Histologic photomicrographs of representative hematoxylin–eosin (A–D, 100 \times magnification) and Masson’s trichrome (E, F, 600 \times magnification) stained midsections of 13-day postsurgery wounds from diabetic *db/db* mice topically treated with (A, E) placebo (NaCl 9 mg/mL), (B) PDGF and IGF-1, (C) one dose of aminated β -1,3-D-glucan (AG) given on day 0, and (D, F) five doses of AG given on days 0–4. (A) The sparse granulation tissue covers the subcutaneous fat with few capillaries and relatively little infiltration of inflammatory cells and fibroblasts. (B) A relatively cell-rich and well-vascularized granulation tissue. (C) A sparse, immature granulation tissue within the adipose tissue. (D) Granulation tissue with relatively higher cellular infiltration and more capillaries. (E) Collagen fibers (blue). (F) Collagen fibers and a relatively higher infiltration of inflammatory cells and fibroblasts. Scale bars = 200 μ m (A–D) and 50 μ m (E, F).

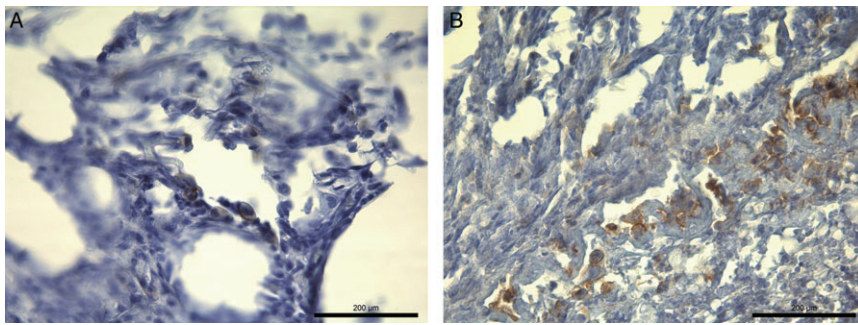


Figure 5. Immunostaining with F4/80 antibody of representative sections from (A) placebo-treated and (B) AG-treated wounds (see Methods, Immunohistochemistry). Magnification 400 \times . The number of F4/80-positive cells (macrophages) per visual field in sections from AG-treated wounds, 10 (3–24) was significantly higher vs. the placebo-treated, 2 (2–4) (median (range); $p < 0.003$). Scale bar = 200 μm . AG = aminated β -1,3-D-glucan.

macrophage system seem to be implicated. AG modulates the performance of the monocyte-macrophage system. First, at the molecular level, Konopski et al. performed *in vitro* experiments demonstrating binding of AG to β -glucan receptors in nondiabetic mouse macrophages.³² Later, the murine β -glucan receptor dectin-1 was identified on macrophages by Brown and Gordon.³³ The AG effects in our study, including a higher cellular infiltration compared to placebo, may be mediated through this receptor and, presumably, by subsequent cytokine production.^{20,21} Accordingly, previous experiments *in vivo* indicate insufficient macrophage recruitment and activation in diabetic, leptin receptor-deficient *db/db* murine wounds.³⁴ However, applications of IL-1 β -treated macrophages into these wounds accelerated granulation tissue formation and wound closure.³⁴ In the functional counterpart, the leptin-deficient *ob/ob* mouse, a decrease in cellular number together with systemic anti-TNF- α -treatment improved wound healing.¹⁰

Both *in vitro* and *in vivo*, the presence of AG was associated with macrophage-related activity such as induction of pro-inflammatory mediators (IL-1, TNF- α , prostaglandin E₂) and enhanced bactericidal effects.^{20,21}

Second, presence of β -glucan receptors on fibroblasts has also been reported, and there is *in vitro* evidence that glucan can stimulate collagen biosynthesis from these cells.³⁵ *In vivo* experiments with glucan in nondiabetic animals revealed increased wound breaking strength presumably because of increased collagen cross-linking.³⁶

Third, a reduced inflammatory reaction in diabetic compared to nondiabetic wounds is associated with reduced nitric oxide (NO) levels.³⁷ As AG is assumed partly to mediate its effects in wound healing through macrophage stimulation, and macrophages are crucial inflammatory cells secreting NO, this short-lived free radical may also play a role in the AG effects in the present model.¹⁶

Fourth, a possible mechanism behind impaired wound healing in diabetes is the increased production of reactive oxygen species leading to lipid peroxidation.³⁸ Agents inhibiting lipid peroxidation have been reported to improve wound healing in diabetes.³⁸ Indeed, there is evidence that β -glucans have antioxidant effects in nondiabetic animals with experimental wounds.³⁹

Furthermore, β -glucan-treated nondiabetic wounds have recruited a higher number of macrophages in the early inflammatory phase of repair compared to controls, which illustrates the macrophage-mobilizing abilities of glucan.⁴⁰ This is consistent with the histologic observations in our studies where AG treatment was associated with a more extensive, cell-rich

granulation tissue compared to placebo.²³ This could in part be attributed to a higher macrophage number, and our results from the immunostaining with the macrophage-specific F4/80 antibody indicate that (Figure 5).

In vivo experiments have revealed protection from life-threatening infections by AG and other glucans.^{17–19} This is relevant in diabetes as impaired infection defense is a known issue. However, in our experiments, we did not find a difference in the frequency of wound infection in the AG-treated groups compared to controls. It is therefore fair to say that this wound model in diabetic mice has a rather low rate of wound infection. The bacteria that were found in growth cultures could easily have been caused by colonization from the animals' feces. These findings may also be attributed to an insufficient number of animals to reveal a difference in such complications.

In wound studies with doses of 1 μg of PDGF in severely hyperglycemic *db/db* mice, Greenhalgh et al. showed a positive effect on histologic scores compared to controls, but no significant improvement in the degree of wound closure.⁴ In our experiments, we combined PDGF with IGF-1, and we observed an improvement in wound closure compared to placebo treatment. However, this can also be attributed to the fact that our animals suffered less severe hyperglycemia (average plasma glucose 32 mmol/L) than what was seen in the study of Greenhalgh et al. (average serum glucose 52 mmol/L).⁴ On the other hand, we did not observe any further improvement in wound healing by lowering plasma glucose to 15 mmol/L by insulin, but this does not exclude an effect of glycemia in our experimental model.

Age of the experimental animals could also have been an important confounder in our experiments. Greenhalgh and coworkers reported no significant synergism between 1 μg PDGF and 1 μg IGF-1 of wound treatment in 8- to 12-week-old *db/db* mice, while Kiritsy and colleagues found a significant synergistic effect by the same growth factors in more than 15-week-old *db/db* mice.^{14,31} We also used older animals (11 to 14 weeks).

In conclusion, a regime of one daily AG dosage for 5 days consecutively from the day of wounding appears to be superior to one or two dosages and not inferior to additional dosages beyond 5 days. This further indicates that AG exerts its primary effects in the early phase of wound healing and is coherent with an effect on macrophage functions in the wound. AG, or other derivatives of the same substance family, has so far not been tested in humans. Therefore, future studies including these substances could be considered in diabetic patients with chronic wounds.

ACKNOWLEDGMENTS

We appreciate the technical assistance by Anja I. Vepsä, Institute of Medical Biology, University of Tromsø. We also appreciate the statistical advice by Tom Wilsgaard, Institute of Community Medicine, University of Tromsø. We would also like to thank Peter McCourt, Institute of Medical Biology, University of Tromsø, who read the manuscript and gave linguistic advice. This work is supported by a grant from the Norwegian Diabetes Association and The Research Council of Norway.

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