Diabetes-induced fibrotic matrix inhibits intramembranous bone healing

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Abstract Diabetes diminishes bone healing and ossification. Reduced bone formation in intramembranous ossification is known, yet the mechanism(s) behind impaired intramembranous bone healing are unclear. Here we report the formation of a fibrotic matrix during healing of intramembranous calvarial bone defects that appears to exclude new bone growth. Our histological analyses of 7-day and 14-day calvaria bone healing tissue in chemically-induced diabetic mice and non-diabetic mice showed the accumulation of a non-mineralized fibrotic matrix, likely as a consequence of unresolved hematomas under diabetic conditions. Elevated mRNA and enzyme activity levels of lysyl oxidase on day 7 in diabetic bone healing tissues also supports that the formation of a fibrotic matrix occurs in these tissues. Based on these findings, we propose that elevated fibroblast proliferation and formation of a non-mineralized fibrotic extracellular matrix in diabetes contributes to deficient intramembranous bone healing in diabetes. A greater understanding of this process has relevance to managing dental procedures in diabetics in which successful outcomes depend on intramembranous bone formation.

Keywords Bone · Diabetes · Fibrosis · Lysyl oxidase · Wound healing

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Introduction

Diabetes is a chronic disease of impaired glucose metabolism and is associated with complications including osteopenia (Bouillon 1991). Diabetic bone complications can be divided into physiological bone remodeling complications, or impaired bone healing due to local factors (McCabe 2007) and stem from diabetes-induced cellular and extracellular matrix abnormalities.

Compromised physiological bone remodeling in diabetes is mainly attributed to diminished differentiation and functions of bone forming osteoblasts resulting in reduced bone formation (Verhaeghe et al. 1990). Additionally, hyperglycemia prompts non-enzymatic heterogeneous modifications known as Advanced Glycation End-products (AGEs). Collagens undergo advanced glycation in diabetes. These modifications interfere with its structure and functions leading to bone fragility in diabetes (Paul and Bailey 1996). In addition, activation of the receptor for advanced glycation end products (RAGE) activates pathways common to inflammation which contributes to diabetes complications including bone abnormalities and periodontal diseases (Lalla et al. 2000).

Bone formation occurs by either endochondral ossification, or intramembranous ossification (Zuscik and O’Keefe 2009). In endochondral ossification, bone matrix replaces a transient cartilaginous matrix and calcifies into mature bone. In healing diabetic endochondral bone fractures, reduced amounts of transient cartilage leads to defective bone healing (Ogasawara et al. 2008; Topping et al. 1994). By contrast, intramembranous ossification lacks the transient cartilage matrix, and migrated mesenchymal stromal cells directly differentiate into osteoblasts that synthesize and deposit bone matrix. A mechanism of impaired bone healing common to both endochondral and intramembranous bones is reduced bone formation due primarily to osteoblast dysfunction. Our laboratory previously reported that activation of AGE-RAGE axis

is a mechanism of osteoblast dysfunction and reduced intramembranous bone formation in diabetic mice (Santana et al. 2003). In vitro studies of osteoblast cells also suggested an impaired regulation of lysyl oxidase, a required enzyme in the maturation of collagen, under diabetic conditions, which could affect diabetic bone healing (Khosravi et al. 2014). In the present study, we sought to examine aspects of non-mineralized and mineralized extracellular matrix formation and development in intramembranous bone healing under diabetic conditions. Data suggest that fibroblast-derived lysyl oxidase levels are elevated in healing intramembranous bone, possibly leading to a non-mineralized fibrotic matrix which can limit the ability of osteoblasts to proliferate, differentiate and produce bone.

Materials and methods

Induction of type I diabetes Animal studies reported here were approved by Boston University Medical Center IACUC. Diabetes was induced in BALB/cByJ male mice by five consecutive multiple low dose intraperitoneal injections of streptozotocin (STZ) (40 mg/Kg/day) (Like and Rossini 1976; Rossini et al. 1977). STZ powder (EMD cat#572201) was dissolved in 0.1 M sodium citrate buffer solution pH 4.5 and the control non-diabetic mice were injected with the equivalent volume of citrate buffer (vehicle). Non-fasting blood glucose levels were measured from tail bleeds using an ACCU-CHEK Compact Plus kit (Roche Diagnostics) beginning on day 7 after the first STZ injection, and diabetes was declared in mice with blood glucose of ≥ 250 mg/dl (Santana et al. 2003).

Calvarial bone healing model A 1 mm diameter calvaria defect in the parietal bone and one in the frontal bone of each mouse was created 1 week after the onset of diabetes and in control non-diabetic mice (Fig. 1) (Santana et al. 2003). Defects were created in anesthetized mice with shaved heads and a midline incision with a carbide cylinder square bur (Meisinger cat#BA-2021-010) under normal saline irrigation. Flaps were then replaced and sutured with polytetrafluoroethylene non-absorbable suture (Cytoplast cat#CS0618PREM). Frontal and parietal lesions were studied separately to examine potential differences in lysyl oxidase regulation between neural crest-derived (frontal) and mesoderm-derived (parietal) derived tissues, since different osteoblast phenotypes occur in these two embryonic lineages (Behr et al. 2010; Quarto et al. 2010).

Histological analyses Diabetic and non-diabetic mice were sacrificed 7 and 14 days after the surgery. The skull tissues were fixed in 10 % formalin, decalcified for 4 weeks in Cal-Ex Decalcifier (Fisher cat#CS510). Decalcified samples were

dissected, mounted in paraffin and sectioned and stained with Masson's trichrome or hematoxylin and eosin and photographed with an inverted Zeiss microscope at 100 $\times$ magnification.

Histomorphometric analysis Image J software was used to measure the percentage surface area of hematomas or collagen-rich non-mineralized fibrotic tissues in hematoxylin and eosin stained samples of 7 day healing tissues. In addition, the percentage surface area of newly formed bone was measured in samples of 14 day healing tissues. Specifically, total surface area of healing tissue was measured for each sample. Next, surface area of hematomas, fibrotic tissues, or newly formed bone was measured in each sample, and divided by total surface area of the healing tissue to calculate the percentage hematomas, fibrotic tissues, or newly formed bone.

Isolation of total RNA from calvarial bone healing tissue Mice were sacrificed on day seven after defect creation and the skull tissue was immersed in RNA Later solution (Life Sciences cat#AM7021). Total RNA was extracted from pooled mouse tissues from three mice per sample, three samples per group. Thus, the total number of pooled samples was three per group ($n=3$), made from nine mice. RNA was isolated using the RNeasy Micro Kit (Qiagen cat# 74004). Specifically, calvarial bone healing tissues were isolated using a 1.5 mm diameter biopsy punch (Premier cat# 9033509). Biopsy samples were pooled in 150 μ l RLT buffer. Samples were homogenized with a PowerGen homogenizer for 1 min on ice, and total RNA was extracted according to the manufacturer's protocol.

Protein extraction from calvarial bone healing tissues Mice were sacrificed on day seven post-surgery and the skull tissues were dissected, placed in empty 1.5 ml microcentrifuge tubes, immediately snap frozen by dipping the sealed tubes in liquid nitrogen and stored at -80°C . Lesions were dissected from the skulls with a 1.5 mm diameter biopsy punch. Biopsy samples from four mice were pooled in 200 μ l of the extraction buffer (4 M urea, 0.150 M NaCl, 0.05 M borate, pH 8.0 supplemented with 0.25 mM phenylmethanesulfonyl fluoride and 1 μ l/ml Aprotinin). Tissue samples were then ground and homogenized using a 2 ml Konet mini tissue grinder (Fisher cat# K885470-0000). The tissue grinder was kept on dry ice for at least 15 min prior to use. Ground samples in buffer were kept on ice for 10 min, then transferred into cold centrifuge tubes, and spun down at 10,000 $\times g$ for 15 min at 4°C . The supernatant was collected and its total protein was measured using a BCA protein quantification kit.

Measurement of lysyl oxidase mRNA and enzyme activity levels Total RNA was extracted from the calvarial bone

Fig. 1 Flow chart for diabetes induction, calvaria defect creation and analyses. Diabetes was induced in 8-week old male BALB/cByJ mice by five consecutive multiple low dose intraperitoneal injections of streptozotocin (STZ) (40 mg/kg/day) (Like and Rossini 1976; Rossini et al. 1977)

tissues as described above. cDNA was synthesized from 1 μ g total RNA, using TaqMan Reverse Transcription Reagents (Applied Biosystems cat#N8080234). cDNAs were then subjected to real time PCR analysis (TaqMan gene expression) using Applied Biosystems® 7300 Real-Time PCR Systems to measure lysyl oxidase mRNA levels. The following TaqMan probes (Applied Biosystems) were mouse lysyl oxidase (cat#Mm00495386_m1) and 18S rRNA (cat#Hs03003631_g1). A previously established protocol was followed to measure lysyl oxidase enzyme activity using Amplex Ultra-Red Fluorescent dye (Palamakumbura and Trackman 2002). Equal amounts of extracted proteins from calvarial bone healing tissues were divided into two aliquots. β -aminopropionitrile (0.5 mM final) was added to one of the two aliquots for each sample to inhibit lysyl oxidase enzyme activity and to establish the baseline for experimental samples. Triplicate replicates of aliquots were then transferred followed by a master mix containing 1.2 M urea 50 mM sodium borate, pH 8.2, supplemented with 10 mM diaminopentane HCl, 10 μ M Amplex Red, and 1 unit/ml horseradish peroxidase (HRP). The tubes next were incubated for 30 min at 37 °C and fluorescence readings for experimental samples and for a standard curve of hydrogen peroxide set up at the same time were obtained on a Berthold TriStar LB 941 plate reader at excitation and emission wavelengths of 540 and 600 nm, respectively. For each experimental sample, the reading with BAPN was subtracted from that without BAPN, and converted to equivalent nM hydrogen peroxide according to the equation for the line of best fit derived from the standard curve. The lysyl oxidase enzyme activity levels are reported as nmoles $\text{H}_2\text{O}_2/23 \mu\text{g protein}/30 \text{ min incubation}$.

Statistical analysis Student's *t*-test with unequal variance was employed because the standard deviations varied for groups for which the null hypothesis was tested. Significance was declared at $p < 0.05$.

Results

Diabetes induction

The experimental design for diabetes induction by the multiple low dose streptozotocin procedure in 8-week old mice, calvaria defect generation, and analyses are outlined in Fig. 1. The weights of mice at 0, 7, 14, 21, and 28 days following the first STZ injection were determined. Figure 2a and b shows that STZ prompts weight loss in the first week after the first injection; however, diabetic mice regain this weight, consistent with our previous work (Santana et al. 2003). Non-fasting blood glucose data (Fig. 2c) indicate increased blood glucose as a function of time; and the green dotted-line identifies the threshold for diabetes (250 mg/dl), while data in Fig. 2d shows the blood glucose of all mice. Mice in the diabetic group that did not reach the threshold were excluded from the study before the surgical procedure. Diabetes was induced in 80 % of the STZ injected mice. Thirty mice per group were used in the current study.

Diabetes results in formation of a fibrotic matrix in bone healing tissues

In order to assess the structure of the extracellular matrix during calvarial defect healing, we performed histological analyses on day 7 and 14 of diabetic and non-diabetic lesions. Defects were created in diabetic and non-diabetic mice 21 days after the initiation of STZ injections, corresponding to 7 days after the onset of diabetes (Fig. 1). Mice were euthanized at 1 or 2 weeks after the surgery and skulls were fixed and decalcified as explained in Materials and methods. Decalcified samples were dissected, mounted in paraffin, sectioned and then stained with Masson's trichrome and hematoxylin and eosin. Representative histological sections showed inhibited bone formation in diabetic defects, as expected. Sections from day 7 healing defects in diabetic mice

Fig. 2 Weight and non-fasting blood glucose levels in the diabetic and non-diabetic mice. Data are pooled from all mice generated for the current report, 30 diabetic and 30 non-diabetic mice. **a** and **b** Animal weights; **c** and **d** blood glucose levels, all as a function of time after injection with vehicle or STZ. Only mice with glucose levels in excess of 250 mg/dl were classified as diabetic and utilized in the diabetic group. All non-diabetic mice used in the study were injected only with vehicle

contained hematomas (red arrow) in parietal defects, which was not seen in the controls at this time point (Fig. 3). Frontal defects contained primarily excess non-mineralizing extracellular matrix at this time point (Fig. 3, green arrow). Five out of seven diabetic mice (71 %) showed unresolved hematomas or fibrotic healing tissues in their cranial defects; while, one out of eight non-diabetic mice showed a fibrotic healing tissue (12 %). As we previously reported, diabetes interferes with bone formation in parietal bone. Purple arrows in Fig. 3 mark newly formed bone tissue on day 14 post-surgery in non-diabetic mice, which is absent in diabetic mice. These histological observations collectively suggest that hematomas, which form in the initial stage of bone healing, do not resolve in diabetic mice. Moreover, an accumulation of excess non-mineralized fibrotic extracellular matrix in diabetic mice seemed to accumulate in the interstitial bone healing tissues and likely interferes with the normal course of bone healing.

Histomorphometric analyses of images indicate that the area of apparent fibrotic tissue in both the diabetic frontal and parietal bones (Fig. 4) was clearly higher than in the non-diabetic bones, and that bone formation was inhibited in diabetes. In the non-diabetic frontal bones there appeared to be a greater degree of bone formation, suggesting that bone formation occurs more quickly in these tissues compared to the parietal bones.

Diabetes up-regulates lysyl oxidase mRNA and enzyme activity levels in calvarial defect healing on day 7

To independently examine the formation of a fibrotic matrix during intramembranous bone healing under diabetic conditions, lysyl oxidase enzyme activity and mRNA levels were

measured. Elevated levels of lysyl oxidase are a hallmark of fibrosis (Rodriguez et al. 2008). Defects were created in diabetic and non-diabetic mice and on day 7 post-surgery total RNA and total protein were isolated from healing tissues in diabetic and non-diabetic mice. Lysyl oxidase mRNA and enzyme activity levels were assessed using real-time PCR and a fluorometric assay, respectively, as explained in [Materials and methods](#). On day 7 of bone healing, lysyl oxidase mRNA levels in both frontal and parietal bone were increased under diabetic conditions (Fig. 5). Increased lysyl oxidase enzyme activity levels were observed only in the frontal lobes.

Discussion

Bone healing is broadly divided into endochondral and intramembranous ossification. In intramembranous ossification, progenitor mesenchymal cells differentiate into osteoblasts (bone-forming cells) that deposit the bone matrix which is mineralized in time. In endochondral ossification, the progenitor mesenchymal cells differentiate into chondrocytes. The intramembranous bone ossification lacks the transient cartilage tissue formation that occurs in endochondral bone formation (Zuscik and O'Keefe 2009). The mechanism(s) behind impaired bone healing in diabetes has not been fully understood. Two mechanisms have been proposed to explain bone healing disorders in diabetes: (i) reduced chondrogenesis in diabetic bone healing (ii) diminished osteoblast proliferation and differentiation during bone healing in diabetes. The former hypothesis mainly applies to endochondral bone formation and not intramembranous bone formation.

Fig. 3 Histologic analyses indicate an excess formation of fibrotic extracellular matrix in healing calvaria defects in diabetic mice. Diabetic and non-diabetic mice were sacrificed 7 days after defects were created. Skull tissues were fixed in 10 % formalin and decalcified for 1 month. Samples were serially sectioned and these sections were stained with Masson's trichrome or hematoxylin and eosin. Photos were taken at 100× magnification. The *scale bars* indicate 0.2 mm. Images are representative of histological analysis from diabetic and non-diabetic ($n=5$ per group). 70 % of diabetic healing tissues showed either excess of fibrotic matrix or unresolved hematomas

Reduced chondrogenesis in early stages of bone healing was proposed as one the mechanisms of impaired bone healing in diabetes. Ogasawara et al. reported lower levels of type II and X collagens in diabetic bone fracture healing tissue, which suggests reduced cartilage formation (Ogasawara et al. 2008). In addition, studies on diabetic mouse fracture healing showed that the newly formed bone callus is reduced in diabetic mice. Further, the authors reported higher osteoclast numbers at day 16 of healing, and higher levels of cytokines and proteases, such as TNF- α and ADAMTS 4 and 5. Therefore, they suggested that the enhanced removal of cartilage as a function of diabetes results in a reduced scaffold for new bone formation, which can account for reduced callus formation (Kayal et al. 2010). Further studies to determine the mechanisms of enhanced cartilage removal in diabetes suggested that elevated levels TNF- α and ROS in diabetes induce chondrocytes apoptosis via up-regulation of Forkhead Box protein O1 (FOXO1) (Siqueira et al. 2010).

Several in-vitro studies suggested diminished osteoblast function under diabetic conditions. The Manolagas group

reported on the up-regulation of FOXO signaling and down regulation of Wnt signaling in aging mouse bones. Further, the up-regulated FOXO3a in H₂O₂-stimulated uncommitted mesenchymal cells was shown to compete with Wnt signaling. This competition inhibits the Wnt-stimulated differentiation of uncommitted mesenchymal cells into osteoblasts. The authors suggested that elevated levels of ROS in diabetes antagonize Wnt signaling and inhibits osteoblast differentiation (Almeida et al. 2007).

Ogawa et al. reported no significant mineralization changes in differentiated MC3T3 osteoblasts under AGE-stimulation. The authors showed the up-regulation of RAGE, a class of receptors binding to AGEs, in the early stage of differentiation in cells incubated with 22 mM glucose and in the later stage of differentiation when cells were incubated with AGE-BSA. The levels of osteocalcin mRNA on differentiation day 21 and 14 was down-regulated in cells differentiated in the presence of both 22 mM glucose and 300 μ g/ml AGE-BSA. The authors collectively suggested that the combination of AGEs and glucose inhibits osteoblast differentiation most likely via activation of AGE-RAGE signaling (Ogawa et al. 2007).

Fig. 4 Per cent area of bone and fibrotic tissues in healing tissues. Per cent of area of mature bone, and per cent area of hematoma plus fibrosis in each histology slide from diabetic ($n=7$) and non-diabetic control mice ($n=8$) was measured as described in [Materials and methods](#). Data are provided as means $\pm$ SEM, *, $p<0.05$

Studies on bone marrow stromal cells (BMSCs) and primary calvarial osteoblasts showed that a non-toxic dose of reactive oxygen species inhibits the phosphorylation of Runx2-related transcription factor 2 (Runx2) and synthesis of type I collagen in BMSCs. Runx2 is the master transcription factor regulating the genes required for osteoblast differentiation (Komori et al. 1997; Otto et al. 1997; Rodan and Harada 1997). Additionally, it was reported that the inhibition of Erk-dependent NF- κ B rescued the inhibition of phospho-Runx2 by H_2O_2 in both bone marrow stromal cell cultures and calvarial primary osteoblasts. The authors collectively suggested that reactive oxygen species, which are elevated in diabetes, interfere with osteoblast differentiation via an Erk-dependent pathway through the regulation of Runx2, an important transcription factor in osteoblast differentiation (Bai et al. 2004).

All these in-vitro studies taken together indicate that diabetes leads to diminished osteoblast differentiation. However, it is unclear whether diabetes-induced osteoblast dysfunction causes impaired bone healing in diabetes or whether it contributes to the variety of complications in which lead to impaired diabetic bone healing. For example, an excess or insufficient angiogenesis in different tissues has been reported

Fig. 5 Lysyl Oxidase mRNA and lysyl oxidase enzyme activity are up-regulated in calvarial defect healing under diabetic conditions. **a** Data are lysyl oxidase enzyme activity levels in the frontal and parietal healing bone in diabetic and non-diabetic mice. Mice were sacrificed 7 days after defects were made. Lysyl oxidase protein was extracted from three pooled samples per group ($n=3$). Each pooled sample was derived from four mice. Thus 12 mice per group were employed to generate the data. Lysyl oxidase enzyme activities were assessed as explained in [Materials and methods](#), using three replicates per sample $\pm$ BAPN. Data are means $\pm$ SEM, $n=3$; *, $p<0.05$; student's t -test. **b** Data are lysyl oxidase mRNA levels of healing calvarial bone on day 21 after first STZ injection in diabetic and non-diabetic mice which corresponds to 7 days of healing. Total RNA was extracted from three pooled samples per group ($n=3$). Each sample was derived from three mice. Thus a total of nine mice per group were employed. Real time qPCR was employed to measure lysyl oxidase mRNA levels normalized to 18S rRNA with Taqman Probes. Data are means $\pm$ SEM, $n=3$; *, $p<0.05$; student's t -test

in diabetes (Martin et al. 2003). Angiogenesis is the initial stage of bone healing thus potential diabetes-associated complications at this stage would interfere with the later stages of bone healing and eventually with the bone healing in general.

Our group previously reported an AGE-dependent mechanism for diminished diabetic intramembranous bone healing (Santana et al. 2003). Our new studies on calvarial intramembranous bone healing in diabetic mice here reports the excess formation of a non-mineralized fibrotic extracellular matrix in diabetic bone healing, which is potentially due to unresolved hematomas. Impaired angiogenesis in diabetes could be a possible explanation of unresolved hematomas in diabetic bone healing. Platelets are rich in growth factors, particularly CCN2 and TGF- β which are required for early osteoblast proliferation and differentiation, but which inhibit

full differentiation and mineralized extracellular matrix production. Accumulated platelets, as byproducts of unresolved hematomas, could result in elevated levels of CCN2 and TGF- β , in particular. TGF- β up-regulates lysyl oxidase in fibroblasts and osteoblasts, while the role of CCN2 may be principally mitogenic for osteoblasts (Boak et al. 1994; Feres-Filho et al. 1995). TGF- β inhibits later stages of bone formation (Tang and Alliston 2013). The current study shows that lysyl oxidase mRNA and enzyme activity are up-regulated in diabetic calvaria defects. Up-regulation of lysyl oxidase is a hallmark of fibrosis (Kagan 2000). Taken together, we propose that the unresolved hematomas which are a rich source of cytokines and growth factors, tip the balance of cell proliferation in the healing tissue to fibroblast proliferation at the expense of osteoblasts, leading to formation of an excess non-mineralized matrix. Collectively, these events favor the formation of a fibrotic matrix in the healing bone tissue that disrupts normal course of bone healing. Further studies, ideally time course evolution of bone healing under diabetic conditions, are required to further examine this novel hypothesis in impaired intramembranous diabetic bone healing.

Finally, it is of interest that elevated lysyl oxidase enzyme activity was observed only in frontal bones in diabetic mice, while elevated levels of lysyl oxidase mRNA were seen in both bones. Parietal and frontal calvaria bones have a different embryonic origin (Behr et al. 2010; Quarto et al. 2010) and we speculate that the kinetics of enzyme development, or alternatively the stability of lysyl oxidase protein, may differ as a consequence. This notion is supported by data in Fig. 4 in which bone formation in non-diabetic mice appears to be faster in frontal bones compared to parietal bones. We therefore propose that increased lysyl oxidase enzyme activity was not yet observed in the diabetic parietal tissues because lysyl oxidase enzyme activity had not yet fully developed from its elevated mRNA levels. Clearly, further studies, are required to more fully understand balances that may be disrupted by diabetes that result in a fibrotic matrix instead of a normal mineralized matrix in healing intramembranous bone defects. The use of both genetic models of diabetes and insulin supplementation studies of the STZ model will further enhance the understanding of lysyl oxidase regulation and fibrosis in the context of intramembranous bone healing under diabetic conditions.

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