Delayed osteoblast differentiation and altered inflammatory response around implants placed in incisor sockets of type 2 diabetic rats

Authors’ affiliations:
John S. Colombo, Deepak Balani, Alastair J. Sloan, Rachel J. Waddington, Mineralised Tissue Group, Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Heath Park, Cardiff, UK
St John Crean, Faculty of Health & Social Care, University of Central Lancashire, Preston, UK
Joji Okazaki, Department of Removable Prosthodontics and Occlusion, Osaka Dental University, Osaka, Japan

Corresponding author:
Dr Rachel Waddington
Mineralised Tissue Group
Tissue Engineering and Reparative Dentistry
School of Dentistry
Cardiff University
Heath Park
Cardiff CF14 4XY
UK
Tel.: +44 0 29 2074 2609
Fax: +44 0 29 2074 6489
e-mail: waddingtonrj@cardiff.ac.uk

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Abstract
Objective: Central to the process of osseointegration is the recruitment of mesenchymal progenitor cells to the healing site, their proliferation and differentiation to bone synthesising osteoblasts. The process is under the control of pro-inflammatory cytokines and growth factors. The aim of this study was to monitor these key stages of osseointegration and the signalling milieu during bone healing around implants placed in healthy and diabetic bone.

Methods: Implants were placed into the sockets of incisors extracted from the mandibles of normal Wistar and diabetic Goto-Kakizaki rats. Mandibles 1–12 weeks post-insertion of the implant were examined by histochemistry and immunocytochemistry to localise the presence of Stro-1 positive mesenchymal progenitor cells, proliferating cells, osteopontin and osteocalcin, macrophages, pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, and tumour growth factor (TGF)-β1. Image analysis provided a semi-quantification of positively expressing cells.

Results: Histological staining identified a delay in the formation of mineralised bone around implants placed in diabetic animals. Within the diabetic bone, the migration of Stro-1 mesenchymal cells in the healing tissue appeared to be unaffected. However, in the diabetic healing bone, the onset of cell proliferation and osteoblast differentiation were delayed and subsequently prolonged compared with normal bone. Similar patterns of change were observed in diabetic bone for the presence of IL-1β, TNF-α, macrophages and TGF-β1.

Conclusion: The observed alterations in the extracellular presence of pro-inflammatory cytokines, macrophages and growth factors within diabetic tissues that correlate to changes in the signalling milieu, may affect the proliferation and differentiation of mesenchymal progenitor cells in the osseointegration process.
cern. The disease pathology is multi-factorial, influenced by environmental factors, such as obesity, age and lack of exercise, or genetically predisposed individuals. This is in contrast to type 1 diabetes, which is an autoimmune disease affecting insulin production by the β cells of the pancreas. However, both types result in hyperglycaemia and in the context of dental implants, diabetes has been shown to hamper bone formation, significantly impeding osseointegration. The slower attachment of tissue to the implant surface allows a greater chance for bacterial infiltration, infection and sustained inflammation [Valero et al. 2007], leading to a generally poorer outcome for diabetic individuals.

Several histomorphometric studies investigating osseointegration around implants inserted in the femur or tibia of a streptozotocin-induced type 1 diabetes rat model have indicated that bone-to-implant bone healing is impaired [McCracken et al. 2000, 2006; Siqueira et al. 2003; Kwon et al. 2005; Kopman et al. 2005]. Titanium implants have also been placed in the maxillary molar sockets of a type 1 diabetic rat model, where the mineral apposition rate was reduced, although no morphological differences in bone structure were evident once the bone healing process had finished [Shyng et al. 2006]. In contrast, few studies have examined the effect of type 2 diabetes on the osseointegration process, although the same effects of impaired bone formation during early stages of the healing process are reported [Casap et al. 2005, 2008; Hasegawa et al. 2008; Sakai et al. 2008].

Hyperglycaemia, resulting from diabetes, has the potential to affect a number of stages in the bone healing process, such as vascularisation, clot formation, bone matrix synthesis and mineralisation and bone remodelling, although the exact cellular mechanisms are unclear [Valero et al. 2007]. Studies investigating the pathogenesis of osteopenia associated with type 1 diabetes have observed a varied response, with bone and osteoid formation and mineral apposition reduced during early stages of the disease [Goodman & Hori 1984], leading to low bone turnover as a result of lower osteoclast and osteoblast numbers [Shires et al. 1981; Verhaeghe et al. 1989]. Osteopenia is not normally associated with type 2 diabetes [Tuominen et al. 1999]. However, studies using a type 2 diabetic mouse model have demonstrated that diabetic bone exhibited a reduced osteoclast formation and a reduced reparative bone formation, as evidenced by the reduced osteocalcin (OCN) expression and an increase in apoptosis of osteoblasts, following bacterial-induced inflammatory bone loss and reparative bone formation [He et al. 2004]. The alteration in the bone cell behaviour may be associated with the enhanced expression of inflammatory cytokines, where studies have implicated advanced glycosylation end (AGE) products with the induction of cytokine expression mediated via nuclear factor-κB activation [Neumann et al. 1999; El-seweidy et al. 2002].

The aims of the present study were to examine further the expression of specific cellular markers associated with the bone healing process around titanium implant in normal rat bone and bone representative of type 2 diabetes. Titanium implants were inserted into the socket of extracted incisors of normal Wistar and Goto-Kakizaki (GK) rats, which is a spontaneous type 2 diabetes model for non-insulin-dependent diabetes [Goto et al. 1975]. The appearance of cellular and matrix proteins associated with the inflammatory response to injury, recruitment of the mesenchymal progenitor cells, proliferation, osteoblast differentiation and formation of a mineralised matrix were examined at specific time points during the osseointegration period.

Material and methods

Three-week-old male diabetic GK rats and 3-week-old male Wistar rats [Shimizu Laboratory Supplies Co. Ltd, Kyoto, Japan] were used in the study. All animal procedures were performed in accordance to the basic principles of the Osaka Dental University, following approval of the Animal Experiment Committee of Osaka Dental University, conforming to procedures in the Laboratory Animal Facilities, Osaka Dental University. Previously published observations have demonstrated that there was no significant difference in body weight of the diabetic and normal Wistar control groups over a 12-week period [Sakai et al. 2008]. Over this period, the diabetic group was shown to develop hyperglycaemia, with blood glucose and HbA1c levels on sacrifice at 3, 6, 9 and 12 weeks, significantly higher compared with the control group [Sakai et al. 2008]. During the experimental period, animals were fed solid stock food (MR stock, Oriental Yeast Co. Ltd, Tokyo, Japan) and provided water ad libitum.

Titanium alloy (Ti–6Al–4V) screw implants [1.2 mm diameter; 17 mm length: SNK Screwpost Titan®, Dentsply-Sankin K.K., Tokyo, Japan] were inserted into the incisor socket of a lower mandible of each animal as described previously by Sakai et al. (2008). Briefly, the left continuously erupting incisors were trimmed at 14, 11, 7, and 4 days before extraction (under anaesthesia) in order to stimulate eruption and facilitating extraction of the whole incisor. The right incisor was left in situ to allow mastication of food. After extraction, the socket was curetted to remove debris and the majority of the periodontal ligament. Remnants of periodontal ligament lining the alveolar bone remained, providing a source of progenitor cells capable of differentiating into osteoblasts [Shi et al. 2005]. Implants were then immediately placed in the socket, and the rats were left to heal for the following post-operative time points before sacrifice at 1, 3, 6, 9 and 12 weeks. Immediately following sacrifice with excess sodium pentobarbitral, tissues were fixed by perfusion with 10% neutral buffer formalin and mandibles portions containing the implant were dissected out. At each time point, we were able to recover two mandibles containing implants (two animals) for both the diabetic and control group, which were used in subsequent analysis.

Histological examination

Implants were removed, by careful unscrewing, from the mandible portions. After removal of the ramus and condyle together with the soft sounding mucosal tissue, mandibles were then cut into transverse section approximately 2 mm thick using a bone saw and demineralised in 10% formic acid, with agitation, for 72 h. Sections were dehydrated through 70%, 90% and 100% alcohols and cleared with xylene before embedding in paraffin wax. Five micrometre sections were cut, mounted onto poly-l-lysine-coated glass slides (Fisher Scientific UK Ltd, Loughborough, UK) and dried overnight at 60°C. For histological examination, sections were stained with haematoxylin and eosin and then mounted using DPX glue [Raymond A Lamb, East Sussex, UK], before viewing using a microscope (Olympus AX70) and digital camera (Nikon UK Ltd, Surrey, UK) and ACT-1 imaging software.

Immunocytochemistry

At each time point (1, 3, 6, 9 and 12 weeks post-implant placement), cellular proliferation was assessed by the identification of
The presence of mesenchymal progenitor cells migrating to the tissue repair site was observed by immunoreactivity to the cell surface marker Stro-1. Osteoblast differentiation was assessed by the detection of immunoreactivity for osteopontin (OPN) and OCN. The duration of the inflammatory phase was determined following the expression of pro-inflammatory cytokines interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α, the macrophage cellular antigen F4/80 and the tumour growth factor (TGF)-β. Full details on antibodies used are provided in Table 1.

Five micrometres tissue sections, mounted onto poly-L-lysine-coated slides, were prepared as described above. Sections were deparaffinised with xylene for 10 min, rinsed with industrial methylated spirit for 5 min and washed in tap water for 5 min. In order to quench endogenous peroxidase activity, sections were incubated in 3% H2O2 for 10 min.

Immunoreactivity for PCNA, OPN and OCN was visualised using the Vectorstain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK) and the DAB peroxidase kit (Vector Laboratories). Non-specific binding was blocked for 20 min with 3% normal horse serum, prepared in Tris-buffered saline (TBS) (0.15 M NaCl, 1 × 10−3 M Tris-HCl, pH 7.4). Sections were then incubated with the appropriate 1st antibody, diluted in 1% foetal bovine serum (Sigma Aldrich, Dorset, UK) per TBS for 1 h [Table 1]. As negative controls, a non-immunogenic immunoglobulin G (IgG) control antibody (Sigma Aldrich) was substituted for the 1st antibody [used at the same dilution as the 1st antibody] or the 1st antibody was excluded altogether. Sections were washed twice with TBS and then incubated with the biotinylated universal 2nd antibody followed by the avidin/biotinylated horse radish peroxidase complex contained within the Vectorstain Universal Elite ABC kit as per the manufacturer’s instructions. Immunoreactivity was visualised using a DAB substrate detection kit (Vector Laboratories), including nickel to increase sensitivity. Sections were washed under tap water for 5 min, counter stained with 0.1% methyl green for 1 min, with excess stain removed by excessive rinsing with tap water. Sections were then soaked in xylene for 5 min and mounted using DPX glue (Raymond A Lamb).

In order to detect for the cell surface marker Stro-1, which uses an IgM class antibody, sections were first blocked with 5% goat serum (Sigma Aldrich) for 20 min and incubated with the 1st antibody overnight. Negative controls included incubation at this stage with a non-immunogenic IgM (Sigma Aldrich; 1:5 dilution) negative control or omission of the 1st antibody. Sections were washed twice for 5 min with TBS before incubation with biotinylated goat anti-mouse IgM 2nd antibody [Vector Laboratories] for 30 min. Immunoreactivity was detected using the avidin/biotinylated horse radish peroxidase complex contained within the Vectorstain Universal ABC Elite kit, the DAB substrate detection kit and the methyl green counterstain as described above.

For the detection of IL-1, IL-6, TNF-α, TGF-β and F4/80 pan macrophage marker, sections were treated with 24 μg/ml proteinase K (Sigma Aldrich) for 10 min. Following this antigen retrieval step, endogenous peroxidase activity was quenched and immunoreactivity was detected as described for PCNA, OPN and OCN above.

Image analysis

All immunocytochemical sections obtained were viewed under an Olympus AX70 light microscope, with images captured using a Nikon digital camera and ACT-1 imaging software. Five images, representing different areas around the implant site, were captured and analysed using Image Pro Plus image analysis software (version 6; Media Cybernetics Inc., Bethesda, MD, USA). Positively stained cells within the captured images were counted using the software, which allows colours to be manually selected from an image and then automatically counts continuous objects of the same designated colour as “positive” cells. For each of these five images (×20 magnification), positive cells in 5 × 100 μm² random areas were counted. Numerical data were expressed as an average mean ± standard deviation, to reflect the dispersion of the data only. The method provides a semi-quantitative analysis and therefore the results presented are descriptive, rather than representing actual changes in the levels of positive staining cells.

Results

Bone formation around healing implant sockets

Bone healing was observed histologically following haematoxylin and cosin staining (Fig. 1). Sections were analysed from healing sockets along the entire length of the implant, from the site of insertion to the tip of the implant, where the growing root tip had once been. No differences were observed in bone healing along the length of the implant. At week 1, cell-rich soft tissue formation was observed within the socket and around im-

Table 1. Details of primary antibodies and corresponding secondary antibodies used in immunocytochemical analysis

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antibody source</th>
<th>1st Isotype and dilution used</th>
<th>Biotinylated 2nd and dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human Stro-1</td>
<td>National Hybridoma Bank</td>
<td>IgM; Mouse polyclonal; 1:25</td>
<td>Goat anti-mouse IgM; 1:600</td>
</tr>
<tr>
<td>Anti-rat PCNA (PC10)</td>
<td>Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA</td>
<td>IgG; Mouse monoclonal; 1:25</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>OPN (recombinant OPN human origin; FMB-14)</td>
<td>Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA</td>
<td>IgG; Mouse monoclonal; 1:50</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>OCN (amino acid 1–95 mouse OCN; FL-95)</td>
<td>eBioscience, Inc., San Diego, CA, USA</td>
<td>IgG; Rabbit polyclonal; 1:50</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>IL-1β (anti-rat B122)</td>
<td>Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA</td>
<td>IgG; Armenia Hamster polyclonal; 1:10</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>IL-6 (C-terminus rat origin; R-19)</td>
<td>Santa Cruz Biotechnology Inc</td>
<td>Goat polyclonal IgG; 1:10</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>TNF-α (N-terminus mouse origin; L-19)</td>
<td>Santa Cruz Biotechnology Inc</td>
<td>IgG; Goat polyclonal; 1:10</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>TGF-β (C-terminus human origin; v)</td>
<td>Santa Cruz Biotechnology Inc</td>
<td>IgG; Rabbit polyclonal; 1:10</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
<tr>
<td>F4/80 (anti-rat Pan macrophage marker ab15637)</td>
<td>Abcam, Inc., Cambridge, MA, USA</td>
<td>IgG; Mouse monoclonal; 1:40</td>
<td>Vectastain Universal IgG; 1:100</td>
</tr>
</tbody>
</table>

PCNA, proliferating cellular nuclear antigen; Ig, immunoglobulin; IL, interleukin; TNF, tumour necrosis factor; TGF, tumour growth factor; OPN, osteopontin; OCN, osteocalcin.
plants placed into both normal and diabetic bone. There was no evidence of mineralising tissue formation at this time, suggesting that the tissue formed represented granulation tissue. In the normal animal at 3 weeks post-operation, haematoxylin and eosin staining demonstrated the formation of foci of bone-like tissue, which had increased considerably by weeks 9 and 12. In diabetic animals, bone healing around the implants appeared to be delayed, with no bone-like tissue apparent at 3 weeks post-insertion, and noticeably less bone-like tissue formation at 9 weeks compared with implants placed into normal bone. Bone healing in the diabetic animal appeared to have increased substantially by week 12, with proportions of new bone-like tissue only slightly less than that seen in the normal bone.

**Analysis of bone-forming markers**

The temporal formation of bone-like tissue was assessed by immunocytochemistry. Selected images demonstrating differences in bone healing for normal and diabetic animals are shown in Figs 2 and 3. Digital histomorphological analysis, to determine the number of positively expressing cells for each particular marker within the healing bone tissue at all analytical time points examined are also shown to clearly depict the chronological changes in the number of positive staining cells. By measuring the number of positively staining cells [not levels of staining intensity], semi-quantitative analysis for the synthesis of various marker proteins were obtained. Thus, the data are discussed in relation to the chronological patterns of expression observed relevant to migration of cells to the wound healing site, cellular proliferation and differentiation to osteoblasts were obtained. The analysis provides a comparison for trends in diabetic and normal bone healing. The number of Stro-1 cells, demonstrating the presence of mesenchymal progenitor cells migrating into the bone healing site, was observed to be highest at week 1, which then declined steadily with time over the 3- and 9-week post-insertion observation points [Fig. 2a]. No Stro-1-positive cells were observed in either bone type at week 12. No major differences were observed in the number of Stro-1 cells at any of the time points in the diabetic bone healing site compared with normal bone. Cellular proliferation, in response to the healing process and as determined by the PCNA expression, appeared to be delayed in the diabetic-forming bone [Fig. 2b]. PCNA-positive cells were first detectable in normal bone at week 1. Levels had risen at week 3 and were negligible by week 9. In contrast, there was virtually no staining for PCNA at week 1 in diabetic bone. Considerable staining for PCNA-positive cells was evident at week three and unlike normal bone, this continued in the diabetic bone that high cell numbers were still detectable at week 9.

Monitoring of the mesenchymal cells along the osteoblast differentiation pathway was achieved by analysis of OPN and OCN as markers of differentiation [Fig. 3]. Within
normal animals, a peak in the number of OPN and OCN positively expressing cells was observed at week 3 post-insertion of the implant, which appeared reduced to minimal levels by weeks 9 and 12. In the diabetic animals, OPN levels at weeks 1 and 3 rose following a pattern similar to that of normal bone. However, at week 9 the high levels of OPN-expressing cells continued to be observed in the diabetic bone, with an approximately 40-fold increase in positively expressing cells compared with normal. The number of OCN-positive cells present in diabetic bone compared with normal bone was considerably lower at week 3 (approximately threefold reduction observed), but numbers of positively staining cells at week 9 post-insertion in the diabetic tissue were approximately 60 times higher in the diabetic bone, where normal bone had very few positive staining cells. Consideration of expression profiles at each time point and determination of maximal numbers of positively expressing cells indicated a delay in the development of the mature osteoblast cell capable of synthesising mineralised bone.

**Analysis of inflammatory phase during bone healing**

Following immunocytochemical analysis, results from image analysis of cell numbers positively expressing selected pro-inflammatory cytokines, macrophage pan marker and the growth factor TGF-β within the healing bone tissue are shown in Fig. 4. For normal bone healing, a higher number of IL-1β positivity was observed during weeks 1 and 3 with very low levels observed at weeks 6–12. However, in diabetic bone the observed increase in the number of IL-1β-positive cells in the bone-healing site appeared to be delayed, with negligible numbers of positive cells observed at week 1 and the highest numbers apparent at weeks 3–6. For normal bone healing, the number of TNF-α-expressing cells was maintained a near static level for the duration of the 12 weeks. In diabetic bone, higher numbers of positively expressing cells were observed at weeks 3 and 6, which then declined to levels similar to those observed in normal bone at week 9. No or negligible levels, representing one positively expressing cell within the 100 μm² counting area, were observed following immunodetection for IL-6. This was apparent for both diabetic and normal healing bone and graphical results have been omitted. The delay in the appearance of IL-1β- and TNF-α-positive cells in the diabetic bone healing tissue was also mirrored for the analysis of F4/80-positive macrophages and the growth factor TGF-β1. In normal bone healing, the number of F4/80 positively expressing cells was the highest for week 1 with minimal numbers of cells detected over weeks 3–12. In diabetic tissue, the significant appearance of F4/80-positive cells was observed over weeks 3 and 6 and reaching a maximum at week 9. Minimal levels of F4/80-positive cells were observed at week 12. Assessment of the growth factor TGF-β1 indicated a high number of positively expressing cells in normal bone at week 1, which reduced over weeks 3 and 6 to a near static level. In contrast, in diabetic healing bone, TGF-β1-positive cells were only significantly apparent from week 3, and demonstrated slight fluctuations in these levels until week 9. Unlike diabetic bone where the number of TGF-β1 staining cells was 1–2 within the 100 μm² areas, levels of TGF-β1 positively expressing cells were considerably higher at week 12 in
Discussion

Bone healing during osseointegration of titanium implants is now recognised and regularly described to follow a sequential and overlapping sequence of events (Davies 2003). On placement of the implant into the prepared bony socket, haemostasis and clot formation are followed by the recruitment of inflammatory cells to the wound site, collectively providing the source of growth factors, cytokines and chemokines. The signalling milieu promotes the wound healing events associated with fibrolysis and the migration of mesenchymal cells to the site to synthesis a loose connective tissue capable of supporting angiogenesis. Undifferentiated mesenchymal cells migrate into the site, attach, proliferate and differentiate down osteogenic lineages, leading to contact osteogenesis (de novo bone formation on the implant surface) and distance osteogenesis [bone repair on pre-existing bone surfaces].

Within this study, histological examination of the bone-forming tissue indicated that early events associated with bone repair in animals with characteristics of type 2 diabetes were delayed. The recruitment of progenitor cells, as assessed by the numbers of Stro-1-positive mesenchymal progenitor cells identifiable in the tissue repair site was similar for both bone types. However, the proliferative phase, as judged by the PCNA expression [within mesenchymal progenitors and other cells associated with the immune response and angiogenesis], appears delayed in the diabetic bone, with the maximal activity spanning weeks 3 and 9. By comparison, cellular proliferation in normal bone was significant at weeks 1 and 3. These results partially support previous findings by Liu et al. (2007) who similarly assessed cellular proliferation by immunocytochemical staining for the PCNA antigen, and identified a reduction in cellular proliferation during the first 14 days of distraction osteogenesis in Zucker Diabetic Fatty rats as a model of type 2 diabetes. Earlier studies have reported a similar reduction in PCNA expression, and hence cellular proliferation, over the first 7 days of bone fracture healing bone in a diabetic type 1 model (Gebauer et al. 2002; Tyndall et al. 2003). However, by extending the analytical time scale to 12 weeks, we have been successful in identifying a delayed proliferative phase, which in our model is still very active at 9 weeks post-insertion of the implant into diabetic healing bone, although it may be argued that cellular proliferation is also reduced.

Immunocytochemistry also demonstrated that the period of synthesis for the bone matrix proteins, OPN and OCN, marking the differentiation of the osteoblast, was altered in diabetic bone compared with normal bone healing. The period of synthesis of OCN in diabetic bone appear to be delayed, with the highest levels recorded at week 9 as opposed to 3 weeks for normal bone healing. OCN is proposed to represent a marker of the more differentiated osteoblast, where it is proposed to function in the arrestment of bone mineral formation (Ducy et al. 1996) although evidence has suggested a partial role in stimulating bone maturation (Boskey et al. 1998). Its identification within the healing bone tissue in the present study indicates a delay in the development of the osteoblast phenotype in diabetic bone.
By contrast, the period of synthesis of OPN in diabetic bone appeared prolonged, with no major differences in OPN synthesis in diabetic bone evident at weeks 1 and 3, but very high levels detected at week 9 compared with minimal levels in normal bone. OPN is abundantly synthesised by differentiating osteoblasts at early stages of bone formation, where it has been implicated in roles in bone remodelling (Reinholt et al. 1990; Gorski 1992). OPN, however, appears to have a broader role in wound healing. Consequently, it is proposed to modulate chemotaxis and neutrophil recruitment (Wang & Denhardt 2008) and provide an adhesive matrix for cell attachment (Sodek et al. 2006); influence cell-mediated immunity by enhancing T helper type 1 response and B-cell immunoglobulin production (Wang & Denhardt 2008); and provide an anti-apoptotic role for the survival of macrophages, T cells, fibroblastic and pre-osteoblasts and endothelial cells (Denhardt et al. 2001). In the current study, the continued synthesis of OPN at week 9 in the diabetic healing tissue also correlated with a high macrophage presence. OPN has been proposed to have a role in mediating the accumulation of macrophages in adipose tissue as a result of diet-induced obesity (Nomiyama et al. 2007), which can directly promote insulin resistance in adipocytes (Senn et al. 2002; Borst 2004). Of note, our own study does indicate a higher peak of expression for the cytokine TNF-α and higher levels of IL-1β at week 6 in diabetic bone, which is concomitant with the observed increase in macrophage activity, although these cells are unlikely to be the sole source for these cytokines. The elevated levels of pro-inflammatory cytokines may also be a result of other stimulatory mechanisms such as hyperglycaemia (Wilmer et al. 2001; Devanaj et al. 2005) and the increased presence of AGE products from elevated reactive oxygen species activity (Schmidt Stern 2000; Vlassara & Palace 2002; Santana et al. 2003). High levels of pro-inflammatory cytokines are not apparent at week 9, suggesting that the healing tissue is eventually able to resolve the inflammatory response.

A significant finding within this study was a delay in the expression of TGF-β1 at week 1 correlated with TGF-β1 functioning to create a pool of osteoblast progenitor cells and the subsequent decrease in the levels of the growth factor corresponds with the formation of mature osteoblasts capable of synthesising bone. The delayed expression of TGF-β1 in the diabetic healing tissue may, therefore, be a significant change in the signalling milieu leading to prolongation of the cell proliferation, delayed differentiation of osteoblasts and hence a delay in bone formation.

On comparing normal and diabetic bone healing, raised and possibly prolonged levels of the expression of the cytokines IL-1β and TNF-α are also apparent in the diabetic healing tissue. However, caution should be made in determining how much higher this is because the peak of expression in normal bone may have been missed between the sampling points and it may have been shorter in duration. These pro-inflammatory cytokines have a number of effects on osteoblast proliferation and differentiation, but their consequential effects on bone physiology again are dependent upon the concentration and synergistic/antagonistic effects on the osteoblast cell and its stage of differentiation [reviewed by Hughes et al. 2006]. IL-1β has been shown to inhibit osteoblast proliferation and enhance bone formation (Hanazawa et al. 1986; Ohmori et al. 1988), but prolonged exposure can stimulate osteoblast proliferation and inhibit bone formation [Ellies & Aubin 1990]. IL-1β can also stimulate the...
synthesis of IL-6 and TNF-2 by osteoblasts, although other cells, including the immunoregulatory cells, are capable of producing these cytokines [reviewed by Dinarello 1998]. These cytokines relay different effects on osteoblast function and hence bone formation. TNF-2 has established roles in bone pathology, stimulating osteoclast bone resorption [Nanes 2003], while inhibiting osteoblast proliferation and differentiation [Gowen et al. 1988].

To summarise, this study has identified a delay in bone healing around titanium implants placed into diabetic rats. The delay in bone healing appears to be as a result of a prolongation in cellular proliferation and in the synthesis of OPN with consequential effects in delaying the synthesis of OCN. At the same time, this study has also demonstrated, within the healing diabetic bone tissue, an elevation in the expression of pro-inflammatory cytokines and macrophage numbers. The raised levels of pro-inflammatory cytokines are very likely to exert an effect on the mesenchymal progenitor cells and their differentiation towards bone synthesising osteoblasts. The precise mechanism is not apparent because the action of these mediators will be dependent upon the local concentrations and the differential stage of the osteoblast, but it may be speculated that the raised levels of pro-inflammatory cytokine act on mesenchymal progenitors by stimulating proliferation and inhibiting differentiation. Equally, from recent studies investigating adipocyte behaviour in diabetes [Nomiya et al. 2007], proteins such as OPN, synthesised by the mesenchymal pre-osteoblast cells may also have a role in perpetuating the inflammatory status within the healing tissue. Overall, the identification of changes to the signalling milieu in relation to delayed and prolonged presence of growth factors and pro-inflammatory cytokines has highlighted potential targets, which may be therapeutically manipulated to promote bone healing in diabetic individuals.

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References


