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REVIEW

Inflammation as death or life signal in diabetic fracture healing

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Abstract Increased apoptosis of chondrocytes and osteoblasts and prolonged survival of osteoclasts lead to early destruction of callus tissue and impair bone remodeling in fracture healing of diabetic patients. Diabetes is accompanied by an increased inflammatory state, reactive oxgen species (ROS) generation and accumulation of advanced glycation end products (AGEs), a heterogenous group of toxic metabolites that can induce inflammation. Prolonged hyperglycemia and insulin resistance correlate with increased apoptosis rate and, accordingly, the proapoptotic role of several inflammatory mediators, ROS and AGEs has been also documented. In this review we summarize the most recent reports supporting the idea that inflammatory signaling increases chondrocyte and osteoblast death and prolongs osteoclast survival, resulting in impaired bone regeneration in diabetes. Antagonising inflammatory signal pathways and solution of inflammation may deserve greater attention in the management of diabetic fracture healing.

Keywords Diabetes mellitus · Apoptosis · Inflammation · Bone homeostasis · Regeneration

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Introduction

Increased risk of bone fractures is associated with diabetes mellitus [1–3]. The relative risk for fracture in type 1 diabetes mellitus (T1DM) varies between 1.7 and 12.3, while type 2 diabetes mellitus (T2DM) is associated with a 2.8-fold increase of bone fracture risk (reviewed by [4]). Clinical studies also show that diabetes delays fracture healing [1]. In diabetic patients fracture reunion time may be prolonged by 87% [5] and diabetes is associated with a relative risk of 3.4 for complications in osseus healing [6], including delayed reunion, non-union, redislocation or pseudoarthrosis [1].

The reparative phase of bone fracture healing is initiated by proliferation and chondroblastic differentiation of periosteal precursor cells resulting in a hyaline cartilage callus around the wounded bone [7]. At a later stage of healing, the cartilage callus calcifies in the process of enchondral ossification. Imbalances of chondrocyte apoptosis and osteoblast differentiation affect this transition from cartilage to bone [8]. Supernormal osteoclast activity disturbs remodeling of the osseus callus [8]. Insulin insufficiency, hyperglycemia and oxidative stress, hallmarks of both T1DM and T2DM, reduce osteoblast differentiation, increase osteoclast activity, alter apoptosis of chondrocytes and osteoblasts leading to impaired fracture healing in diabetic patients [2, 5, 8–13].

Diabetes is accompanied by an increased inflammatory state [14]. Elevated circulating levels of inflammatory cytokines (e.g., interleukin-6, interleukin-1β, tumor necrosis factor) predispose insulin resistance [15–20]. Hyperglycemia leads to non-enzymatic glycation of intracellular and extracellular proteins with the formation of advanced glycation end products (AGEs), a heterogenous group of toxic metabolites that aggravate inflammation [21]. Recently, the

proapoptotic role of several inflammatory mediators and AGEs has been documented, and prolonged hyperglycemia and insulin resistance have been recognised as correlating with increased apoptosis rate [22–24]. In this review we summarize findings supporting the possibility that diabetes-associated inflammation leads to uncontrolled apoptosis of bone forming cells, resulting in impaired bone regeneration. We provide an overview on the roles governed by proinflammatory cytokines, AGEs, nitric oxide (NO) and prostaglandins in apoptosis of chondrocytes, osteoblasts and osteoclasts, key players in bone regeneration.

Inflammatory mediators induce chondrocyte apoptosis in diabetes

Apoptosis of chondrocytes in diabetes: TNFα carries the scythe

Premature chondrocyte death leads to reduced callus size and imperfect chondroid ossification [9]. Tumor necrosis factor (TNFα) and members of the TNFα receptor family constitute one major pathway initiating cell death. In mice with streptozotocin-induced T1DM, mRNA and protein levels of TNFα along with gene sets related to apoptosis are upregulated in cartilage callus of femoral fractures [8]. TNFα-specific inhibitor reduces chondrocyte apoptosis in diabetic mice, but not in normoglycemic control mice, indicating the proapoptotic role of upregulated TNFα in the early destruction of cartilage tissue [9]. Inhibition of caspase-8 and -9 significantly reduces TNFα induced apoptosis in chondrogenic cells, showing that TNFα activates caspases, main effectors of cell suicide. Recent research has shown that TNFα-induced chondrocyte apoptosis may be mediated by FoxO1, a forkhead family transcription factor [8, 24, 25] (Fig. 1). Forkhead family transcription factors regulate the expression of a number of genes that are crucial for the proliferative status of a cell or are involved in programmed cell death [24, 26]. Their activation depends on the phosphatidylinositol 3-kinase/protein kinase B/Akt pathway, which is activated by cytokines and is essential for development and function of certain immune cells [25]. Silencing FoxO1 using siRNA significantly reduces TNFα induced apoptosis and caspase-3 activity in differentiated chondrocytes as well as caspase-3,-8,-9 and TRAIL (TNFα-related apoptosis inducing ligand) gene transcription in chondrocytic cells in vitro [8]. Moreover, TNFα potentiates the production of NO in chondrocytes, thus it may contribute to apoptosis through NO-mediated cytotoxicity [27]. TNFα also increases chondrocyte susceptibility to cytokine-induced apoptosis [28]. Similarly, TNFα stimulates the production of prostaglandin E (PGE) in chondrocytes [28]. Roles of NO and PGE in chondrocyte apoptosis and dedifferentiation have been extensively studied in osteoarthritis [28, 29], however, their impact on chondrocyte death during early fracture healing in diabetes has not yet been evaluated.

Insulin treatment in T1DM reduces chondrocyte apoptosis and improves femoral fracture healing in diabetic animal models [1, 9], indicating the pivotal role of insulin in normal chondroid ossification and early phase bone regeneration [9, 30, 31]. Insulin exerts direct anabolic effects and promotes chondrocyte maturation in vitro [32]. Insulin administration in vivo normalizes glycemic control and reduces AGE production, thus may also enhance chondrocyte survival indirectly [1, 4]. AGEs provoke TNFα and IL1β production [33, 34] and also induce apoptosis and senescence of differentiating chondrocytes [9, 10]. Production of AGEs in T1DM therefore may be one possible thread linking insulin deficiency, TNFα upregulation and chondrocyte apoptosis [35]. In hyperglycemic conditions the accumulated toxic metabolite AGEs reduce phosphorylation and increase expression and acetylation of FoxO1, leading to disturbed insulin gene transcription [36] and the consequent insulin deficiency further increases AGE deposition and inflammation [37–39].

Proteasome function and chondrocyte death: links from TNFα to NFκB signaling

Tumor necrosis factor also activates the transcription factor NFκB (nuclear factor kappa beta), which is a major regulator of immune responses stimulated by proinflammatory signals [40] (Fig. 1). Activation of NFκB by inflammatory cytokines, such as interleukin-1β (IL1β) and TNFα leads to hypertrophic differentiation of chondrocytes and inhibits chondrogenesis from human mesenchymal stem cells [34, 41, 42]. Interleukin-1β and TNFα also promote β-catenin and cyclooxygenase-2 (COX-2) expression in chondrocytes and aggravate inflammation [34, 41, 42]. Activation of NFκB also mediates the effect of insulin-like growth factor-1 (IGF-1), promoting chondrocyte differentiation and preventing chondrocyte apoptosis [30].

Recent reports suggest that NFκB and TNFα-receptor pathways are negatively regulated by the proteasome system [43, 44]. Proteasome modulates inflammatory signaling and transcription of inflammatory mediators, and therefore plays a role in the solution of inflammation [43]. It has shown that the chymotryptic component of the proteasome is a regulator of osteoblast differentiation and bone formation [45]. Chondrocyte apoptosis may be induced by proteasome inhibition [45]. In cultured rat metatarsal bones the presence of proteasome inhibitor I (PSI), a known inhibitor of the chymotrypsin-like activity of the 20S proteasome particle, suppresses growth plate differentiation and reduces metatarsal linear growth. In

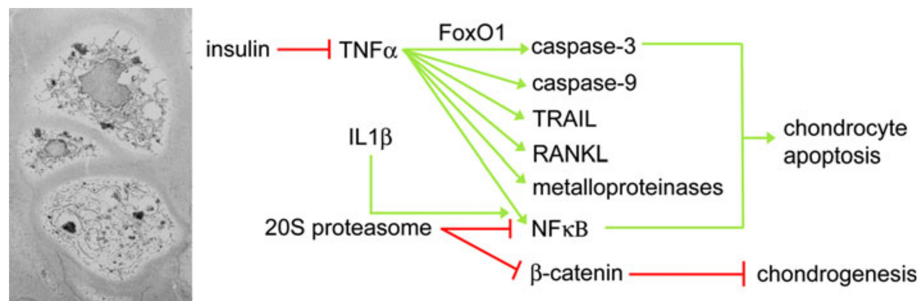

Fig. 1 Inflammatory signals and pathways regulating chondrocyte apoptosis. *Left*: Electron micrograph of hypertrophic chondrocytes. *Right*: Insulin insufficiency is coupled to elevated plasma TNFα and IL1β levels, which may upregulate proapoptotic signaling in chondrocytes. Interference with NFκB and β-catenin signaling affects chondrocyte survival. *TRAIL* tumor necrosis factor-related apoptosis-inducing ligand, *RANKL* receptor activator for NFκB ligand

cultured chondrocytes, PSI increases the expression of β-catenin, a negative regulator of chondrogenesis and reduces the DNA binding of NFκB [41] (Fig. 1). Interestingly, there is an inverse relationship between 20S proteasome levels in red blood cells and body mass index [46]. Plasma ubiquitin and 20S proteasome levels are considered to be novel biomarkers for the risk assessment of the development of human obesity, a predisposing factor of T2DM [46]. Similarly, many proteasome subunits show negative correlation with body mass index in men, however, their role in insulin resistance has not yet been defined [47]. NFκB signaling and its modulation by proteasome can also be a link between insulin resistant conditions, inflammation and premature chondrocyte death.

Diabetes, inflammation and osteoblast apoptosis

AGEs provoke inflammation, cause oxidative stress and kill osteoblasts

AGEs are accumulated in T1DM and T2DM [48, 49] and provoke inflammation through upregulation of TNFα and IL1β in monocytes and macrophages [33, 50]. AGEs induce apoptosis in primary cultures of human or neonatal rat osteoblastic cells or MC3T3-E1 cells in vitro [51]. The apoptotic effect is largely mediated through AGE receptor (RAGE) and an increase in p38 and JNK (c-Jun N-terminal kinase) activity, caspase-8 and caspase-3 activation, but seems to be independent of NFκB pathway (Fig. 2a) [35]. In more differentiated osteoblastic cultures, apoptosis may be enhanced even further by AGEs than in premature osteoblastic cells. AGEs contribute to reactive oxygen species (ROS) generation which may also lead to oxidative stress and cell death [11]. In an animal model of T1DM, AGEs reduce osseus healing, showing their impact in delayed bone regeneration in diabetes [52]. Methylglyoxal (MG), a reactive dicarbonyl compound endogenously produced mainly from glycolytic intermediates, also induces osteoblast cell death, and elevated MG levels in T1DM patients may contribute to impaired bone structure and regenerative ability [53]. Mechanism of MG-induced apoptosis of osteoblasts involves oxidative stress, JNK activation, mitochondrial membrane potential changes, cytochrome *c* release, increased Bax/Bcl-2 protein ratios, and activation of caspase-9, caspase-3 and p21-activated protein kinase 2 (PAK-2) [50]. Similarly to its role in FoxO1 mediated chondrocyte apoptosis, nuclear translocation of NFκB is required for MG-induced osteoblast apoptosis [53].

Although in chondrocytes FoxO1 is required for TNFα-induced cell death, in osteoblasts FoxO1 protects against oxidative stress and apoptosis by upregulation of genes required for scavenging free radicals [54]. Interestingly, mice lacking FoxO1 in osteoblasts show increased β-cell proliferation, insulin secretion, and insulin sensitivity [26]. The ability of osteoblast-specific FoxO1 deficiency to affect metabolic homeostasis is due to an increased expression and bioactivity of osteocalcin, a protein hormone secreted by osteoblasts [26]. Osteocalcin facilitates bone mineralization and also acts as a regulator of glucose metabolism and fat mass [26]. Although serum osteocalcin is associated with elevated fasting plasma glucose in patients with T2DM, the role of osteoblast FoxO1 in altered osteocalcin level and its impact in fracture repair is still undefined [55, 56].

Inflammatory cytokines induce osteoblast apoptosis through nitric oxide

The free radical gas messenger molecule NO, which is produced by various bone cells, may induce osteoblast apoptosis (Fig. 2a). Endothelial NO synthase (eNOS) is constitutively expressed in bone, whereas inducible NOS (iNOS) is expressed only in response to inflammatory stimuli [57]. The eNOS isoform seems to play a key role in regulating osteoblast activity and bone formation [58] and iNOS is required for bone repair in mice [59].

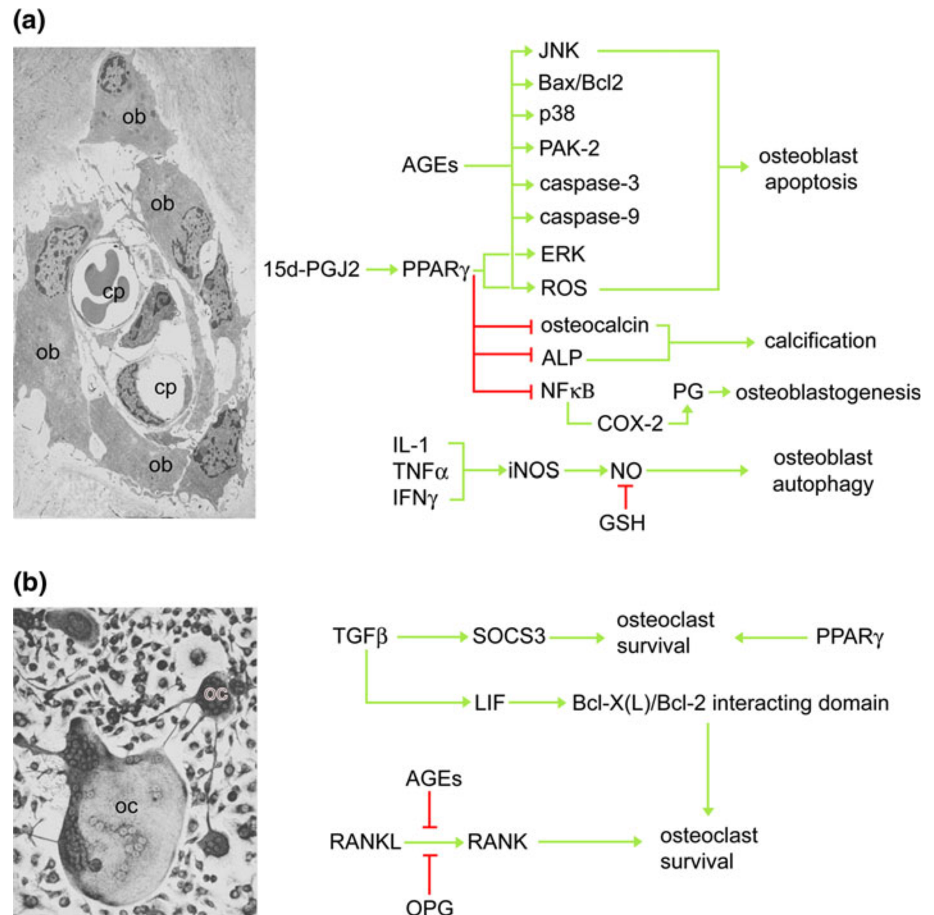

Fig. 2 Inflammation and control of osteoblast and osteoclast apoptosis. **a** *Left*: Electron micrograph of differentiating osteoblasts (*ob*) around a capillary (*cp*) at the transition zone of the callus. *Right*: Effect of inflammation on osteoblast survival. **b** *Left*: Cultured mouse osteoclasts (*oc*). Kindly provided by Dr. L. Fuentes (Madrid). *Right*: Life signals for osteoclasts in diabetes. *JNK* c-Jun N-terminal kinase, *PAK-2* p21-activated protein kinase 2, *ERK* extracellular signal-regulated kinase, *ROS* reactive oxygen species, *ALP* alkaline phosphatase, *GSH* glutathione, *OPG* osteoprotegerin, *LIF* leukemia inhibitory factor, *PG* prostaglandin

Proinflammatory cytokines such as IL1β, TNFα and interferon gamma (IFNγ) cause activation of the iNOS pathway in bone and the elaborated NO potentiates bone loss [60]. In T2DM patients, elevated circulating TNFα levels show direct correlation with vascular iNOS expression, giving a possible link between inflammation and reduced bone mass [61, 62]. High levels of NO lead to oxidative stress and autophagic osteoblast death [63] resulting in impaired ossification. However, the production of AGEs may reduce bioavailability of NO in diabetes [64], and pharmacological NO donors increase bone mass [57], indicating that the mechanisms behind NO-mediated osteoblast apoptosis are still not fully understood.

Prostaglandins in the TNFα-induced osteoblast apoptotic programme

In cultured osteoblasts, TNFα induces liberation of arachidonate from diacylglycerol and increases prostaglandin (PG) synthesis [65]. A PG derivative, the prostaglandin *15*-deoxy-delta *12*,*14*-prostaglandin J2 (15d-PGJ2), induces apoptosis in various cell types and provokes cell death in mouse osteoblastic cell cultures (Fig. 2a). Oxidative injury represents a primary event following 15d-PGJ2 treatment resulting in Akt inactivation, which, in turn, leads to mitochondrial injury and apoptosis [66]. In cultured osteoblasts, treatment with 15d-PGJ2 induces transient activation of ERK1/2 and sustained activation of JNK, causes ROS generation and triggers the mitochondrial apoptotic pathway indicated by enhanced Bax expression, loss of mitochondrial membrane potential, cytochrome *c* release, and caspase-3 activation [67]. Interestingly, 15d-PGJ2 is a natural ligand of peroxisome proliferator activated receptor-γ (PPARγ), one target of anti-diabetic therapy [68–70]. Troglitazone and ciglitazone, synthetic PPARγ ligands, also trigger osteoblast apoptosis [71, 72]. Furthermore, PPARγ ligands inhibit NF-κB associated downstream COX-2 and iNOS signaling [73].

Inflammatory signals prolong osteoclast life

Hyperglycemia was reported to inhibit osteoclast formation, ROS production, caspase-3 activity and macrophage functions linked to RANKL (receptor activator for NFκB ligand), and NFκB pathways [12–14, 74] leading to decreased bone resorption [75]. However, bone resorption may be unaltered [76] or increased [13, 14, 77–79] in T1DM patients. Increased osteoclast survival overshadows

apoptosis in animal models of T2DM [80] and may explain impaired fracture healing in mice with T1DM [9]. Increased osteoclast activity is likely to be the net effect of various diabetes-associated factors, such as elevated levels of saturated fatty acids, low density lipoproteins, prostaglandins and AGEs, which interfere with the RANK (receptor activator for NFκB), NFκB and caspase-3 pathways [13, 78, 81–84]. Increased level of the inflammatory mediator transforming growth factor-β (TGF-β) also promotes osteoclast survival through upregulation of leukemia inhibitory factor and suppressor of cytokine signaling-3 (SOCS3) expression [85] (Fig. 2b). Osteoblasts and other stromal cells of the bone produce RANKL, which binds to RANK and helps osteoclast survival [86] (Fig. 2b). Osteoprotegerin (OPG), a soluble tumor necrosis factor-receptor-like molecule mainly produced in bone and connective tissues, acts as a decoy receptor by binding to RANKL and preventing RANK signaling, consequently inducing osteoclast apoptosis [86, 87] (Fig. 2b). OPG expression and production are regulated by inflammatory cytokines, and elevated plasma OPG levels have been recorded in T1DM and T2DM patients [39, 87–90]. Lower RANKL to OPG ratios have also been found in T1DM [91]. TNFα also promotes osteoclast survival and this anti-apoptotic effect requires induction of osteoclast NO synthesis [92]. TNFα-induced NO synthesis may be a paracrine survival signal for macrophages [92]. In co-cultures of osteoblasts and osteoclasts, however, IL1β, TNFα and IFNγ enhance NO production 50 to 70-fold in osteoblasts, and this excess NO liberation leads to osteoclast death [57, 93].

The anti-inflammatory cytokine interleukin-10 inhibits osteoclastogenesis, through modulation Ca^{2+} mobilization, RANKL-induced expression and nuclear translocation of the transcription factor NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1) [94]. These studies support the idea that proinflammatory signals help osteoclast survival, while anti-inflammatory signaling may limit osteoclast differentiation. Surprisingly, the anti-diabetic and anti-inflammatory thiazolidinediones (TZDs), synthetic ligands of PPARγ, promote osteoclastogenesis through regulation of c-fos expression [95], leading to osteoporotic changes in patients taking TZDs [96]. Mice with PPARγ deletion in osteoclasts develop osteopetrosis characterized with increased bone mass, impaired osteoclast differentiation and compromised RANKL signaling [95].

Conclusion and outlook

Targeting of inflammatory signaling in chondrocytes and osteoblasts, as well as the use of anti-inflammatory therapy, show potential to support fracture healing in diabetic patients. However, clinically relevant substances displaying anti-inflammatory and antidiabetic benefits, such as TZDs, cause unwanted effects on bone homeostasis [95]. Some recent studies have attributed anti-apoptotic role to adiponectin, the major adipose tissue hormone with anti-diabetic effects. Most recently, promising results have been obtained with statins in reduction of osteoclast activity and osteoblast apoptosis. Statins, independently from their lipid-lowering effects, also display anti-inflammatory benefits, and their potential to reduce inflammation and osteoblast death makes them candidate future targets in diabetic fracture management [97, 98]. Similarly, curcumin, which is a potential pharmacological agent in treatment of insulin resistance, also exerts anti-inflammatory and apoptosis-modulating profile [38, 99]. Curcumin inhibits osteoclast survival and protects chondrocytes from apoptosis, showing a strong future potential in combined combating of inflammation, insulin resistance, chondrocyte death and excess osetoclastogenesis [34, 79]. Progression in this field shows the many potential benefits of apoptosis modulation in the treatment of diabetes-associated healing deficit, and anti-inflammatory agents will earn greater attention in the preservation of osteogenic cells in diabetic patients.

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