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Research Article

Factors circulating in the blood of type 2 diabetes mellitus patients affect osteoblast maturation – Description of a novel *in vitro* model

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is one of the most frequent metabolic disorders in industrialized countries. Among other complications, T2DM patients have an increased fracture risk and delayed fracture healing. We have demonstrated that supraphysiological glucose and insulin levels inhibit primary human osteoblasts' maturation. We aimed at developing a more physiologically relevant *in vitro* model to analyze T2DM-mediated osteoblast changes. Therefore, SCP-1-immortalized pre-osteoblasts were differentiated with T2DM or control (non-obese and obese) sera. Between both control groups, no significant changes were observed. Proliferation was significantly increased (1.69-fold), while AP activity and matrix mineralization was significantly reduced in the T2DM group. Expression levels of osteogenic marker genes and transcription factors were altered, e.g. down-regulation of RUNX2 and SP-7 or up-regulation of STAT1, in the T2DM group. Active TGF-β levels were significantly increased (1.46-fold) in T2DM patients' sera. SCP-1 cells treated with these sera showed significantly increased TGF-β signaling (2.47-fold). Signaling inhibition effectively restored osteoblast maturation in the T2DM group. Summarizing our data, SCP-1 cells differentiated in the presence of T2DM patients' serum exhibit reduced osteoblast function. Thus, this model has a high physiological impact, as it can identify circulating factors in T2DM patients' blood that may affect bone function, e.g. TGF-β.

Abbreviations: ACTB, beta-actin; ATF4, activating transcription factor 4; BGLAP, osteocalcin; COL1A1, collagen type I alpha 1; FN1, fibronectin; IBSP, bone sialoprotein 2; IFN-γ, interferon-γ; IL, interleukin; RUNX2, runt-related transcription factor 2; SATB2, special AT-rich sequence-binding protein 2; SP7, transcription factor osterix; SPARC, osteonectin; SPP1, osteopontin or bone sialoprotein 1; STAT1, signal transducer and activator of transcription 1; TGF-β, transforming growth factor-β; TNF-α/β, tumor necrosis factor *α/β*

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Introduction

Diabetes mellitus represents one of the most common metabolic disorders in developed countries with a current prevalence of 230 million patients affected. This number is estimated to increase to 366 million by the year 2030 [1]. Diabetes mellitus is associated with multiple complications such as micro- and macro-vascular pathologies, nephropathy, neuropathy and alterations in bone homeostasis. The latter aspect is referred to as diabetic bone disease. It is associated with an increased fracture risk and subsequently delayed fracture healing and potentially non-union [2,3]. While the bone mineral density (BMD) is decreased in diabetes mellitus type 1 patients, most authors describe an increased BMD in diabetes mellitus type 2 (T2DM) patients [4]. Despite the greater BMD, T2DM patients have an increased fracture risk and impaired fracture healing [2–7]. This paradox of greater BMD with inferior bone quality increasingly challenges orthopedic surgeons. Understanding the alterations of cellular and molecular mechanisms influencing bone homeostasis in T2DM patients is of crucial importance to identify possible treatment strategies in the clinical setting.

Osteoblast differentiation is regulated by a network of interacting transcription factors, with RUNX2 and osterix (*SP7*) being key players. Osterix acts downstream of RUNX2 and is mainly expressed during late osteogenesis [8]. Their target genes are crucial components of the organic matrix such as collagen type I alpha 1 (*COL1A1*), Fibronectin (*FN1*), osteocalcin (*BGLAP*), osteonectin (*SPARC*) and the bone sialoproteins 1 (osteopontin; *SPP1*) and 2 (*IBSP*) [9]. Osteocalcin, osteonectin, bone sialoprotein 1 and −2 levels are associated with the mineralization of bone matrix [10], and thus these proteins exert a major role in cell attachment [11,12]. However, their transcription is not solely controlled by RUNX2. Especially, *BGLAP* gene expression is additionally controlled by the transcription factor *ATF4*. In the promoter region of *BGLAP* the binding site for ATF4 and RUNX2 are located next to each other [9]. Interestingly, both ATF4 and osteocalcin have been shown to regulate the glucose metabolism [13–15]. SATB homeobox 2 (SATB2), an adapter molecule between ATF4 and RUNX2, increases their activity and thus promotes the transcription of the genes *SPP1* and *BGLAP* [16]. Contrary to ATF4 and SATB2, STAT1 inhibits the activity of RUNX2 by inducing conformational changes in the nuclear localization sequence of this transcription factor. Thus *STAT1* knock-out mice display a predominant increase in osteoblast differentiation leading to a significantly higher BMD [17].

In particular, the metabolic effects of poor glycemic control on the osteoblast–osteoclast-balance may depict a key factor in bone homeostasis. In 2012, we have described an *in vitro* model mimicking the onset of T2DM by stimulating primary human osteoblast with supraphysiological concentrations of insulin and/or glucose. In this model, osteoblast function was inhibited by increased transforming growth factor beta (TGF-β) expression and activation in the presence of insulin [18]. TGF-β has been described as the central player in bone homeostasis by inducing recruitment and proliferation of osteoblasts [19]. However, several murine studies suggest that TGF-β may also negatively influence bone structure [20,21]. This is supported by our recent results showing that primary human osteoblasts chronically exposed to TGF-β lose their characteristic functions, *e.g.* formation of organic and inorganic matrix [22].

Although a direct correlation between glucose levels and TGF-β signaling has been described in several cell types [23,24], this cannot be the sole regulator in T2DM associated bone changes. With our earlier described *in vitro* model solely the direct effect of high concentrations of insulin and glucose on primary human was investigated, which was critically discussed as the weakness of the model. Therefore, aim of the present study consisted in establishing a more physiologically relevant *in vitro* cell culture model that allows the identification of factors circulating in the blood that may affect bone function.

Materials and methods

Cell Culture Medium and supplements, as well as ALEXA-fluor labeled secondary antibodies were purchased from Life Technologies (Darmstadt, Germany); TriFast™ and KAPA Fast ready mix was obtained from Peqlab (Erlangen, Germany); Chemicals, *e.g.* the Alk1 inhibitor Sb431542, were obtained from Sigma (Munich, Germany); Human recombinant active TGF-β_1 was obtained from Peprotech (London, UK).

Ethics statement

Blood sampling was conducted in accordance with the Declaration of Helsinki (1964) and its amendments. The study protocol was approved by the hospital's Ethics Committee (Reference: 365/2012B02) and informed consent was obtained from all subjects. Patients with viral and bacterial infections were not included in the study. Additionally, patients with clinically pronounced micro- or macroangiopathy and advanced diabetic nephropathy (creatinine clearance <30 ml/min) were excluded from the study.

Sample population

18 ml serum blood was collected from each donor. Serum blood samples were centrifuged at 1000 g for 10 min. Aliquots of the serum supernatants were stored at $-80\,^{\circ}C$ until further use. Samples were pooled into a T2DM (with an impaired oral glucose tolerance and/or a fasting glucose levels ≥ 126 mg/dl) and two control groups (elderly non-obese $N=13$; young obese $N=14$). The obese control group consisted of obese volunteers being under medical surveillance, as they have a high risk to develop a T2DM. Demographic data are summarized in Table 1.

Culture and differentiation of SCP-1 cells

Human immortalized bone marrow mesenchymal stem cells (SCP-1 cells) serve as basis for our in vitro model. SCP-1 cells were cultured in MEM alpha medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, as described [25,26]. Osteogenic differentiation was induced by supplementing the MEM alpha medium with 5% human serum (from T2DM patients or controls), 100 U/ml penicillin, 100 µg/ml streptomycin, 200 µM L-ascorbate-2-phosphate, 10 mM β-glycerol-phosphate, 25 mM HEPES, 1.5 mM $CaCl_2$ and 100 nM dexamethasone.

Resazurin conversion

Proliferation was measured indirectly by Resazurin conversion, representing mitochondrial activity. Briefly, 1/10 volume of a 0.025% (w/v) Resazurin solution (in DPBS) was added to the cells.

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