EFFECTS OF GLUCOSE CONCENTRATIONS ON CATHEPSIN K AND ALKALINE PHOSPHATASE ACTIVITIES IN PRIMARY HUMAN OSTEOBLASTIC CELL CULTURES

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Summary

Type 1 diabetes mellitus affects bone turnover by interfering with the normal functions of bone cells. Osteoblasts seem to participate to both bone formation and resorption. Their direct bone resorptive effect was discovered recently as osteoblasts were proved to secrete cathepsin K, a lysosomal protease which represents the major cysteine cathepsin in osteoclast and is responsible for bone resorption by digesting the organic extracellular matrix of bone. **Purpose:** Our aim was to explore osteoblast secretion of products involved in bone formation and resorption, in particular of a bone formation enzyme, alkaline phosphatase and a bone resorption enzyme, cathepsin K and to further evaluate if the secretion of these enzymes is influenced by diabetic conditions by testing the effects of different glycemic concentrations on human primary osteoblastic cell cultures. **Materials and methods:** Primary human osteoblastic cell cultures were obtained using femoral head trabecular bone from patients with hip arthroplasty. Third passage subconfluent osteoblasts were exposed for 36 hours to several glucose concentrations: 2.8 mmol/l (hypoglycemia), 5.6 mmol/l (normoglycemia), 11.1 mmol/l (moderate hyperglycemia) and 28 mmol/l (extreme hyperglycemia). Cathepsin K and alkaline phosphatase activities were measured from cell supernatants. **Results:** Alkaline phosphatase activity values ranged between 65.67 ± 9.29 U/l in extreme hyperglycemia and 74.33 ± 13.61 in hypoglycemia group. The difference between groups was tested and a p-value of 0.806 was obtained. The lowest cathepsin K activity was observed in moderate hyperglycemia group (31.04 ± 0.73 pmol/l) and highest in hypoglycemia (35.51 ± 6.97 pmol/l). There existed no significant difference between groups regarding cathepsin K activity (p=0.671). Weak inversely proportional correlations were found between glucose and alkaline phosphatase and cathepsin K levels respectively. A significant association was observed between alkaline phosphatase and cathepsin K. **Conclusions:** Our study sustains the secretion of cathepsin K by osteoblasts and concludes that short time exposure (36 hours) to high- or low-glucose medium seems to have no significant impact on alkaline phosphatase and cathepsin K activities in human osteoblastic cell cultures. It is the first research that studies the effects of glucose on osteoblast-secreted cathepsin K. Further studies are warranted to complete information on the matter. **Keywords:** osteoblast, cell culture, glucose, cathepsin K, alkaline phosphatase

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Introduction

Osteoporosis has received increasing attention from the medical world in the last years. Although primary (postmenopausal and senile) osteoporosis represents a major focus, secondary causes of osteoporosis should also be kept in attention, one of these causes being type 1 diabetes mellitus. This disease affects bone metabolism by interfering with the normal function of bone cells with impact on bone turnover (Wongdee and Charoenphandhu, 2011). Osteoblasts are known as bone forming cells and osteoclasts as bone resorption cells, however their actions are much more complex and their intricate effects conduct to a proper bone turnover. Osteoblasts participate to both bone formation and resorption, in the latter being involved both indirectly and directly. Their indirect action is represented by osteoclast activation mainly through RANKL (receptor activator of nuclear factor κB ligand)-RANK (receptor activator of nuclear factor κB)-OPG (osteoprotegerin) system (Silva and Branco, 2011). Recently, their direct bone resorptive effect was discovered as osteoblasts were proved to secrete cathepsin K (Mandelin et al., 2006). Cathepsin K is a lysosomal protease belonging to the papain-like family and represents the major cysteine cathepsin in osteoclast. It is responsible for bone resorption by digesting the organic extracellular matrix of bone (Lecaille et al., 2008). Its secretion also by osteoblasts represents a proof of the complex actions of these cells in bone remodeling.

Our aim was to explore osteoblast secretion of products involved in bone formation and resorption, in particular of a bone formation enzyme, alkaline phosphatase and a bone resorption enzyme, cathepsin K and to further evaluate if the secretion of these enzymes may be influenced by diabetic conditions by testing the effects of different glycemic (Diagnosticum Inc., Hungary). Values were expressed in U/l.

Materials and methods

Cell cultures

Human primary osteoblastic cell cultures were obtained. According to the protocol of (Pepene et al., 2001), femoral head trabecular bone fragments resulting from hip arthroplasty were placed on culture plates and culture medium was added. Culture medium contained Dulbecco’s Modified Eagle Medium (DMEM) Low Glucose, Pyruvate, no Glutamine, no Phenol Red (Invitrogen) with 10% Fetal Bovine Serum (Sigma-Aldrich), 1% antibiotic Penicillin-Streptomycin-Glutamine (Invitrogen) and Phenol Red (Sigma-Aldrich). Culture plates were incubated at 37°C, 5% CO₂. The culture medium was changed regularly twice a week. After the third passage, when reaching subconfluency, cells were trypsinized, reseeded and treated with glucose in various concentrations.

Glucose concentrations

Cells were treated with D-(+)-Glucose (Sigma-Aldrich) to simulate hypoglycemia, normoglycemia and hyperglycemia (moderate and extreme). 2.8 mmol/l (50.4 mg/dl) glucose was added to create hypoglycemia, 5.6 mmol/l (100.9 mg/dl) for normoglycemia, 11.1 mmol/l (200.002 mg/dl) for moderate hyperglycemia and 28 mmol/l (504.5 mg/dl) for extreme hyperglycemia. Three replicates were created for each glucose concentration. After an exposure of 36 hours, supernatants were collected and determinations were performed.

Alkaline phosphatase and cathepsin K determinations

Alkaline phosphatase activity was determined using a reagent kit for the quantitative determination of alkaline phosphatase activity- DGKC method

Cathepsin K activity was determined quantitatively using an ELISA
kit from Biomedica, Austria. Values were expressed in pmol/l.

**Statistical analysis**

Quantitative data was presented by mean and standard deviation. The differences between independent groups of quantitative data were checked with ANOVA. Then, post-hoc pairwise tests were performed with Tukey Kramer test. To assess the association between two quantitative variables, Spearman correlation coefficient along with its 95% CI, and scatter plots were used. P-values <0.05 were considered significant. The statistical analysis was made in R environment for statistical computing and graphics, version 1.15.1 (R Core Team, 2012).

**Results**

Alkaline phosphatase activity values ranged between 65.67 ± 9.29 U/l in extreme hyperglycemia and 74.33 ± 13.61 in hypoglycemia group. The difference between groups was tested and a p-value of 0.806 was obtained. The lowest cathepsin K activity was observed in moderate hyperglycemia group (31.04 ± 0.73 pmol/l) and highest in hypoglycemia (35.51 ± 6.97 pmol/l). There existed no significant difference between groups regarding cathepsin K activity (p=0.671). Details are presented in Table 1 and Figures 1 and 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mmol/l)</th>
<th>Alkaline phosphatase (U/l) (Means ± SD)</th>
<th>Cathepsin K (pmol/l) (Means ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYPOGLYCEMIA</td>
<td>2.8</td>
<td>74.33 ± 13.61</td>
<td>35.51 ± 6.97</td>
</tr>
<tr>
<td>NORMOGLYCEMIA</td>
<td>5.6</td>
<td>65.67 ± 17.01</td>
<td>34.78 ± 7.56</td>
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<tr>
<td>MODERATE HYPERGLYCEMIA</td>
<td>11.1</td>
<td>73.33 ± 16.17</td>
<td>31.04 ± 0.73</td>
</tr>
<tr>
<td>EXTREME HYPERGLYCEMIA</td>
<td>28</td>
<td>65.67 ± 9.29</td>
<td>31.54 ± 2.80</td>
</tr>
</tbody>
</table>

Table 1. Values of alkaline phosphatase and cathepsin K in different glycemic groups
The differences between each group and the normoglycemic group were tested. No significant differences were observed neither in alkaline phosphatase activity nor in cathepsin K activity. Details are available in Table 2.

We then tested the association between glucose and alkaline phosphatase activity and cathepsin K activity respectively. We found a weak, inversely proportional correlation between glucose and alkaline phosphatase with a Spearman coefficient of -0.15 (95% CI -0.63 -0.35), \( p=0.637 \), an increase in glucose decreased alkaline phosphatase. In respect of cathepsin K the correlation with glucose was weak and inversely proportional, a decrease of cathepsin K being observed with the increase in glucose, Spearman coefficient was -0.17 (95% CI -0.64 -0.61), \( p=0.591 \). Correlations are presented in Figures 3 and 4.

By testing the association between alkaline phosphatase and cathepsin K, an acceptable, directly proportional correlation was found with a Spearman coefficient of 0.37 (95% CI 0.18 - 0.92), \( p=0.232 \): Figure 5.

### Table 2. Comparison of each glycemic group with the normoglycemic one regarding alkaline phosphatase and cathepsin K respectively

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>P values for alkaline phosphatase</th>
<th>P values for cathepsin K</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMOGLYCEMIA-HYPOGLYCEMIA</td>
<td>0.88</td>
<td>1</td>
</tr>
<tr>
<td>NORMOGLYCEMIA-MODERATE HYPERGLYCEMIA</td>
<td>0.91</td>
<td>0.83</td>
</tr>
<tr>
<td>NORMOGLYCEMIA-EXTREME HYPERGLYCEMIA</td>
<td>1</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Discussions

Osteoblast differentiation comprises three major phases: proliferation, matrix maturation and matrix mineralization. Osteoblasts cultured in monolayer low-density cell cultures like in our experiment usually offer a picture of the first two phases. Alkaline phosphatase is a bone formation enzyme secreted by osteoblasts in a membrane attached form (Golub and Boesze-Battaglia, 2007). It is a marker for osteoblast differentiation and points the switch from the proliferation phase to the matrix maturation phase. Its expression is high in the early maturation phase and lowers drastically in the late maturation phase when the expression of other products rises, like osteocalcin. Human primary osteoblasts have a good viability for seven days, after this period cell death starts to be significant. In this period alkaline phosphatase values rise as cell number increases (Jähn et al., 2010).

Glucose effect on osteoblasts was previously evaluated in different exposure times and there exist a few records of its impact on alkaline phosphatase. In our study we found similar alkaline phosphatase activities in different glycemic groups in osteoblast culture supernatants after an exposure of 36 hours to glucose. In previous experiments, studying the effects of short-time exposure of osteoblastic cultures to various glucose concentrations, researchers didn’t reach an unanimous conclusion. The contradictions may be explained either by the different osteoblastic cell lines used and their origin (human or rat) or by the differences in glucose concentrations entitled “high” and the substrates used for determinations (cell lysate or supernatants). A similar result
with ours was obtained by (Wang et al., 2010) as they found similar alkaline phosphatase activities in high glucose (25.5 mmol/l) and normal glucose (5.5 mmol/l) after an exposure of 24 hours. The same study reported increased alkaline phosphatase activity in high glucose after increasing the exposure to 72 hours, effect maintained at 7 days, while (Wu et al., 2012) observed decreased alkaline phosphatase activity in a high concentration of glucose of 22 mmol/l compared to normal (5.5 mmol/l) after 72 hours. Increased alkaline phosphatase activities at 7 days were reported also by (Wang et al., 2010) with 25.5 mmol/l of glucose and (Botolin and McCabe., 2006) with 35.5 mmol/l vs. normal (5.5 mmol/l) while (Garcia-Hernandez et al., 2012) observed decreased alkaline phosphatase activity after seven days exposure to 24 mM of glucose. (Botolin and McCabe, 2006) also studied alkaline phosphatase gene expression and found it increased at 7, 14, 21 and 29 days of exposure to 35.5 mmol/l glucose. As alkaline phosphatase is a dynamic parameter which increases in osteoblast from 1 to 7 days in culture the effect of glucose on it may not be obvious from the beginning and may explain our result at 36 hours exposure. A mechanism that may explain glucose effect on osteoblasts is the activation of PKA (protein kinase A)/ MAPK (mitogen-activated protein kinase)/ ERK (extracellular signal-regulated kinases) pathway (Wang et al., 2010). The hyperosmolarity that glucose induces was excluded as a cause (Botolin and McCabe, 2006; Garcia-Hernandez et al., 2012).

Regarding cathepsin K, we found similar activities in the presence of different glucose concentrations in culture supernatants, so glucose doesn’t seem to influence cathepsin K activity in osteoblast, at least after 36 hours of exposure and in the concentrations that we used. No other studies researched the matter so our study represents a novelty. By our research we also confirm that cathepsin K is secreted also by osteoblasts as previously mentioned only by two studies (Mandelin et al., 2006; Gautschi et al., 2009). One of these studies proved the gene expression, protein presence and activation of cathepsin K in osteoblasts after 24 hours and that cathepsin K levels in culture supernatants are about half the levels in cell lysates. They reported that cathepsin K expression is low during the matrix mineralization phase (cultures analyzed at 14 and 28 days of culture in osteogenic medium to assess mineralization). They also reported that cathepsin K expression in osteoblast doesn’t respect the pattern of another bone resorption enzyme secreted by osteoblasts, collagenase-3 (matrix metalloproteinase-13 MMP-13), the latter being expressed especially in the first part of the mineralization phase (higher in day 14 than in day 28 (Mandelin et al., 2006). The significant correlation that we found between activities of alkaline phosphatase and cathepsin K may also sustain this if cathepsin K is secreted in the same stage of osteoblast development as alkaline phosphatase but studies on different time points are necessary. This observation points out the fact that cathepsin K seems to be secreted in the matrix maturation phase and it can be speculated that cathepsin K may have a role in adjusting bone matrix development by digesting the collagen while it is secreted, in contrast to MMP-13 which acts on a mature mineralized matrix. Cathepsin K involvement in type 1 diabetes was studied on diabetic animals (streptozocin-induced) and its expression and activity was found increased, possibly leading to increased cathepsin K-mediated bone resorption in type 1 diabetes mellitus (Hie et al., 2007).

**Conclusions**

In conclusion, our study is the first one to research the effects of glucose on osteoblast-secreted cathepsin K. Further studies on the matter are needed in order to be able to draw conclusions. Regarding alkaline phosphatase, our study comes to
support previous findings that short exposure of osteoblasts to glucose doesn’t influence alkaline phosphatase activity. Type 1 diabetes mellitus osteoporosis underlying mechanisms remain an open research topic.

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References