Experimental diabetic conditions in primary human osteoblastic cell cultures and effects on osteoblast-derived cathepsin K

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Abstract

The mechanisms underlying bone pathology in type 1 diabetes are still under research. Dysregulation of osteoblast-derived cathepsin K production in type 1 diabetes mellitus could contribute, partly, to the onset of osteopenia associated to this disease. This study aimed to assess the expression of cathepsin K gene and the activity of this enzyme in primary human osteoblastic cell cultures exposed for 24 hours to various concentrations of glucose and insulin, simulating dysglycemic states encountered in patients with type 1 diabetes. RNA was isolated from the cell lysates, reverse transcription was performed and gene expression was assessed by quantitative polymerase chain reaction (qPCR). Cathepsin K activity was determined quantitatively from supernatants. The expression of cathepsin K gene was increased in low glucose and decreased in severe hyperglycemia. A significant inversely proportional association was observed between cathepsin K activity and glucose (Spearman coefficient=-0.71, 95% CI -0.83--0.26). In a multiple regression model, statistically significant positive associations were observed between cathepsin K and insulin (p=0.017) and between cathepsin K and the interaction glucose-insulin (p=0.017). The inversely proportional relationship between cathepsin K and glucose and the positive one between cathepsin K and insulin may be important in the case of insulin therapy induced hypoglycemia which, if occurred repeatedly, may result in increased bone resorption.

Keywords: type 1 diabetes mellitus, insulin, glucose, systemic hormones, osteoblasts, cathepsin K.

1. Introduction

Osteoporosis and type 1 diabetes mellitus (DM) represent two major health concerns. Type 1 diabetes-induced osteoporosis is a chronic complication of utmost importance, all the more so as type 1 diabetic-patients are usually young and bone impairment therefore impacts on the long-term on their physical performance and quality of life.

The mechanisms underlying bone pathology in type 1 DM have been arduously studied and a majority of researchers concluded that decreased bone formation largely accounts for type 1 diabetes-induced osteoporosis [1]. On the other hand, some reports were pleading for an increased bone resorption rate associated to type 1 DM, one study demonstrating increased
expression and activity of cathepsin K (EC 3.4.22.38) - a key bone-resorbing cysteinase - in bone homogenates from streptozotocin-induced diabetic rats [2].

Cathepsin K represents the main collagen resorptive protease secreted by the osteoclast [3]. Nevertheless, cathepsin K and matrix metalloproteinase-13 delivery by osteoblastic cells in the bone microenvironment has been demonstrated recently, thus suggesting participation of the osteoblast in the bone remodeling process [4]. In theory, dysregulation of osteoblast-derived cathepsin K production in type 1 DM could contribute, partly, to inadequate bone formation and onset of osteopenia associated to this disease.

In view of these data, we aimed to evaluate the matrix-modeling function of osteoblasts exposed in vitro to diabetic conditions by assessing cathepsin K gene expression and the protease activity in primary human osteoblastic cell cultures maintained under various concentrations of glucose and insulin, therefore, simulating dysglycemic states encountered in patients with type 1 DM (including glycemic states associated with the Insulin therapy that these patients undergo).

2. Materials and methods

Primary human osteoblastic cell cultures

The cell culture method followed the protocol described by Pepene et al. 2001 [5]. In brief, femoral head trabecular bone fragments, sampled during hip arthroplasty from donors without any endocrine or metabolic disease were dissected, fragmented, thoroughly washed with Phosphate Buffered Saline (PBS), pH = 7.4 and seeded into 9-cm diameter plastic culture dishes. Bone fragments were half-covered with phenol red-free Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, California, USA) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich Chemie, Germany) and 1% penicillin G/streptomycin/glutamine (Invitrogen, California, USA) and incubated at 37°C temperature and 5% CO₂ atmosphere. Culture medium was changed twice a week and cell growth was followed up using an optical microscope. At confluence, osteoblasts were trypsinized and passaged. Third passage sub-confluent cell cultures were used for further experiments.

Experimental diabetic conditions

Glucose (D-(+)-Glucose) and insulin (Insulin, human) were purchased from Sigma-Aldrich Chemie (Germany). Glucose was solubilized in purified water and insulin in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany). Twenty-four hours prior to further experiments, the culture medium was replaced by phenol red-free DMEM supplemented with 10% FCS and 1% penicillin G/streptomycin. Glucose and insulin were mixed in various concentrations with the culture medium (i.e. DMEM with 1% penicillin G/streptomycin) creating four particular types of culture mediums [6, 7], namely obtained by addition of 2.8 mmol/L glucose and 10⁶ pmol/L insulin (Group 1-hypoglycemia), no glucose or insulin (Group 2-control), 5.6 mmol/L glucose and 1 pmol/L insulin (Group 3-well managed diabetes mellitus) and 28 mmol/L glucose and 0.1 pmol/L insulin (Group 4-unbalanced hyperglycemia).

Water and DMSO were used as controls. Four replicates were prepared for each type of medium. Cells were incubated for 24 hours, thereafter supernatants were collected and analyzed.

RNA isolation and reverse transcription

After supernatant removal, cells were prepared for RNA extraction by washing with PBS (Phosphate Buffered Saline). Total RNA was extracted using PeqGOLD Total RNA Kit S-Line (PEQLAB Biotechnologie, Erlangen, Germany). Cells were lysed directly in the culture
plates by addition of 100 μL RNA Lysis Buffer in each well of the 24-well culture plates. The lysates of every 4 wells containing the same type of treated cells were combined, in the end obtaining three replicates of 400 μL cell lysate for each type of diabetic medium treated cells. The lysates were transferred to DNA-removing columns and centrifuged at 12000 g for 1 minute. The flow-through was transferred in 1.5 mL tubes, where an equal volume of 70% ethanol was added. Afterwards, the mixture was transferred to Perfect Bind-RNA columns and centrifuged at 10000 g for 1 minute. After the flow-through was discarded, columns were placed into new tubes and two consecutive washes were performed; eventually, the RNA was eluted in 50 μL RNAse-free water.

Samples for the reverse transcription assessment were stored at -80°C. Reverse transcription was performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, New Hampshire, USA). Eight μL of each RNA sample were mixed with 1 μL oligo (dT)18 primer and 3 μL nuclease-free water and incubated for 5 minutes at 65°C and then chilled rapidly. To each sample, 4 μL 5x Reaction Buffer, 1 μL RiboLock RNase Inhibitor (20u/μl), 2 μL 10 mM dNTP Mix and 1 μL Revert Aid M-MuLV Reverse Transcriptase (200 u/μL) were added to obtain a final volume of 20 μL. The samples were incubated for 60 minutes at 42°C and then for 5 minutes at 70°C.

**Real-time quantitative polymerase chain reaction (qPCR) protocol**

Gene expression was assessed by quantitative polymerase chain reaction (qPCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA). For cathepsin K and alkaline phosphatase, predesigned TaqMan Gene Expression Assays (Life Technologies, California, USA) containing primers and Minor Groove Binder (MGB) probes were used, for cathepsin K, Mm00484039_m1 and for alkaline phosphatase, Hs01029144_m1. Beta-actin was employed as a housekeeping gene. Beta-actin gene expression was determined using TaqMan β-actin Control Reagents (Life Technologies, California, USA) containing β-actin Probe (FAM™ Probe), β-actin Forward Primer, and β-actin Reverse Primer.

The PCR plate contained the samples in triplicate. For cathepsin K and alkaline phosphatase, each sample consisted of a 20 μL mixture: 10 μL TaqMan Universal Master Mix with Uracil-N glycoslyase (UNG; Life Technologies, California, USA), 1 μL assay, 7 μL PCR-Grade Water (Jena Bioscience, Germany) and 2 μL DNA 5 ng/μL concentration. For β-actin, the samples (volume of 20 μL) contained 10 μL TaqMan Universal Master Mix with UNG, 2 μL Forward Primer, 2 μL Reverse Primer, 2μL β-actin Probe, 2 μL PCR Grade Water and 2 μL DNA 5 ng/μL concentration. The qPCR was run on 50°C for 2 minutes then 95°C for 10 minutes then 45 amplification cycles (95°C for 15 seconds and 60°C for 1 minute).

The ΔΔCT method (threshold cycle [CT] represents the PCR cycle where the linear part of the PCR curve intersected the threshold) was used to perform qPCR analysis. ΔCT, ΔΔCT and relative quantification (RQ) were calculated. ΔCT of each sample represented the difference between the CT of the target gene (cathepsin K or alkaline phosphatase) and the CT of the housekeeping gene (β-actin). RQ (determined as 2 -ΔΔCT) was calculated in order to see how many times the target gene is more (RQ>1) or less (RQ<1) expressed in a sample then in the reference sample.

**Laboratory assays**

Alkaline phosphatase (AP) activity was quantitatively determined with a kit obtained from Diagnosticum Inc., Hungary according to the Deutsche Gesellschaft für Klinische Chemie (DGKC) kinetic method.

Cathepsin K activity was measured in culture medium supernatants quantitatively using an ELISA kit purchased from Biomedica Gruppe, Vienna, Austria. The sensitivity of the Romanian Biotechnological Letters, Vol. 19, No. 5, 2014...
Data analysis

Replicates were analyzed as groups for each particular medium. Mean and standard deviation were used to describe quantitative data. In order to analyze the differences between independent groups of quantitative data, ANOVA test was applied and post-hoc pairwise analyses were performed using Tukey Kramer test. Associations between two quantitative variables were evaluated using Spearman correlation coefficient, 95% CI and scatter plots. Statistically significant p-values were considered when the two-tailed p was ≤0.05. Multiple linear regressions models were built with cathepsin K (respectively alkaline phosphatase) as dependent variable and glucose and insulin as explanatory variables, with and without interaction between the two. For statistical analysis, R environment for statistical computing and graphics, version 1.15.1 was used [8].

3. Results

Osteoblastic cell cultures exposed to a culture medium with no addition of glucose or insulin, were considered as reference group. The relative expression compared to the reference group (RQ values) of the cathepsin K and alkaline phosphatase genes for each group of treated osteoblasts was determined. Testing the differences between cell groups, no significant differences in mRNA levels were observed neither regarding cathepsin K, nor alkaline phosphatase (Table 1).

Table 1. qPCR cathepsin K - and alkaline phosphatase - mRNA levels, respectively assessed in primary human osteoblastic cells exposed to experimental diabetic conditions. Data are expressed as mean ± SD. RQ value represents the relative expression compared to the reference group (Group 2). Non-parametric distributed variables were log-transformed prior to analysis. *p = 0.245, **p = 0.227 by ANOVA.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Simulated glycemic state</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (pmol/L)</th>
<th>Cathepsin K&lt;sup&gt;a&lt;/sup&gt; (RQ)</th>
<th>Alkaline phosphatase&lt;sup&gt;b&lt;/sup&gt; (RQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Hypoglycemia</td>
<td>2.8</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.25 ± 0.22</td>
<td>1.18 ± 0.07</td>
</tr>
<tr>
<td>Group 2</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Group 3</td>
<td>Well managed diabetes mellitus</td>
<td>5.6</td>
<td>1</td>
<td>1.75 ± 1.27</td>
<td>0.80 ± 0.55</td>
</tr>
<tr>
<td>Group 4</td>
<td>Unbalanced hyperglycemia</td>
<td>28</td>
<td>0.1</td>
<td>0.56 ± 0.37</td>
<td>0.54 ± 0.42</td>
</tr>
</tbody>
</table>

However, as tendency, the expression of both genes was increased in low glucose culture conditions and decreased in severe hyperglycemia. The association between cathepsin K gene expression and glucose was tested and although the association was not significant, an inversely proportional relationship was observed (r = -0.8, 95% CI -0.996 - 0.697, p = 0.2). Regarding the association between cathepsin K gene expression and insulin, an insignificant positive association was found (r = 0.8, 95% CI -0.697 - 0.996, p = 0.2). Alkaline phosphatase gene expression had near-significant negative association with glucose (r = -0.9758).
Mean values of the enzymatic activities of cathepsin K and alkaline phosphatase for each cell group are presented in Fig. 1.

Differences between groups with regard to the cathepsin K levels and alkaline phosphatase activity were tested using ANOVA and a statistically significant p-value was observed only in the case of cathepsin K (p = 0.016). In order to assess the differences in cathepsin K values between osteoblasts cultured under conditions expressing various dysglycemic states, groups were paired for analysis. In comparison to Group 2 (Fig. 1), the concentration of cathepsin K was significantly lower in supernatants from cultures exposed to moderate hyperglycemia (p = 0.041 Group 3 vs. Group 2) as well as borderline decreased in supernatants from osteoblastic cells exposed to severe hyperglycemia (p = 0.064 Group 4 vs. Group 2). Low glucose culture conditions appeared not to affect cathepsin K secretion in the culture medium of osteoblastic cells in comparison to group 2 (p = 0.997 Group 1 vs. Group 2); nonetheless, cathepsin K were higher when compared to moderate hyperglycemic culture conditions although the difference was statistically non-significant (p = 0.054 Group 1 vs. Group 3).

The association between cathepsin K and glucose in culture supernatants was tested and a significant, inversely proportional correlation was observed (r = -0.71, 95% CI -0.83--0.26, p = 0.009). No significant association was found between cathepsin K and insulin (r = -0.02, 95% CI -0.53-0.85, p = 0.946).

A multiple linear regression model having culture medium cathepsin K as dependent variable and glucose and insulin as explanatory ones showed no significant associations (data not shown). Another multiple regression model was then created, where, besides glucose and insulin,
insulin alone, their interaction was also introduced as an explanatory variable. This model revealed statistically significant positive associations between cathepsin K and insulin ($p = 0.017$) and between cathepsin K and the interaction glucose-insulin ($p = 0.017$) (Table 2).

Table 2. Multiple linear regression testing the association between cathepsin K as dependent variable and glucose, insulin and glucose-insulin interaction as explanatory ones.

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>95% CI</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>0</td>
<td>(-0.24 - 0.24)</td>
<td>0.987</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>8.296</td>
<td>(1.9 - 14.69)</td>
<td>0.017</td>
</tr>
<tr>
<td>Glucose-insulin interaction</td>
<td>-2.963</td>
<td>(-5.25 - -0.68)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

In summary, lower levels of cathepsin K activity were observed in osteoblastic cultures exposed to elevated glucose concentrations in the culture medium. In agreement to that, an inversely proportional association was found between cathepsin K and glucose. A positive relation was proven between cathepsin K and insulin.

The same tests were also applied for alkaline phosphatase but with no significant results.

4. Discussions

The osteoblast is known as a bone formation cell however recent evidence indicates its capacity to participate to bone resorption. An in vitro study showed the ability of these bone-forming cells to resorb the type 1 collagen matrix remained undigested in bone pits after osteoclasts were removed, cleaning bone surface before producing new one [9]. Another study went further and investigated the mechanism of this process and demonstrated osteoblastic secretion of resorptive enzymes including cathepsin K [4].

Cathepsin K is a lysosomal cysteine protease having the unique ability to cleave the triple helix of collagen in multiple locations and make it available for further degradation [10]. In osteoblasts, cathepsin K expression accompanies that of alkaline phosphatase as time frame; in the early differentiation phases of the osteoblastic lineage however mesenchymal stem cells with osteogenic differentiation express higher levels of cathepsin K during the first days and while these start to fall towards the end of the first and during the second week, alkaline phosphatase values rise [11].

Animal studies found an increased expression of cathepsin K in bones of streptozotocin-induced diabetic animal models of type 1 DM [2, 12]. Another study, assessing the case of type 2 DM found decreased expression of cathepsin K in osteoclasts cultured under high glucose and high insulin medium [13].

One major finding of the present study was the significant inverse proportional correlation observed between cathepsin K and glucose. Likewise, a previous experiment of ours, where osteoblastic cells were exposed only to various glucose concentrations in the culture medium revealed an inverse proportional association between cathepsin K and glucose, although not statistically significant [14]. We hypothesized that the addition of insulin to the culture media may increase the strength of the relationship between cathepsin K and glucose in vitro through interaction with glucose, as it is known that insulin promotes glucose uptake in cells. Therefore, we included the glucose-insulin interaction among the
explanatory variables of a multiple regression model. This regression model revealed a positive association between insulin and cathepsin K.

Possible explanations for these results may be sustained either by the general effect of glucose and insulin on osteoblasts, or by their direct effects on cathepsin K expression, protein synthesis or activation. Glucose is known to suppress osteoblast differentiation [15], whereas insulin has a positive effect on osteoblasts proliferation and differentiation, increasing collagen synthesis and alkaline phosphatase production and inhibiting apoptosis [1]. If these general effects on osteoblasts would have explained our results, one can assume that significant changes would have been expected also regarding alkaline phosphatase activity between experimental cell groups, but in its case we observed no significant results.

Reviewing the literature we found that glucose might influence cathepsin K indirectly through a multitude of mechanisms. Hyperglycemia up-regulates c-Jun, a transcription factor belonging to AP1 family [15] and cathepsin K gene promoter contains an AP1 binding site [16]. It also increases p38 phosphorylation [17] and cathepsin K expression is regulated by p38 mitogen-activated protein kinase (MAPK) [16]. Hyperglycemia enhances IL-10 expression [18], a cytokine able to block the synthesis of cathepsin K in most cells [16]. Although not significant, in this experiments, cathepsin K mRNA levels were 1.8-1.85-fold lower when osteoblastic cultures were exposed to moderate and extreme hyperglycemia. Moreover, hyperglycemia determines an acid pH and increases oxidative stress [18], which may further inhibit the activity of cathepsin K [16].

Other two cathepsins also encountered in osteoblast, cathepsin B and D, respectively, were studied in primary cultures of rat mesangial cells exposed to high glucose and an inhibitory effect of glucose on their expression was found [19]. This fact, considered together with our results, may indicate that glucose could have a role in suppressing production of proteolytic enzymes [19].

5. Conclusions

In conclusion, our study comes to present a bone metabolic change which may be encountered in patients with type 1 DM. The inversely proportional relationship between cathepsin K and glucose and the positive one between cathepsin K and insulin may be important in the case of insulin therapy induced hypoglycemia which, if occurred repeatedly, may result in increased bone resorption.

Acknowledgements

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