Measurement of [^{18}F]-fluorodeoxyglucose incorporation into human osteoblast—An experimental method

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Abstract An evaluation of human osteoblast metabolism usually involves measurements of the by-products of bone matrix elaboration. The assessment of glycolytic activity of osteoblasts is not a standard tool in most of the reports, but might be of value by providing a direct indicator of cellular metabolism. Measurement of the incorporation of [^{18}F]-fluorodeoxyglucose, which is not further degradable following its conversion into glucose-6-phosphate during glycolysis and is trapped in this form within the cells, can be used as an effective research tool for estimation of osteoblast metabolism. In order to estimate the [^{18}F]-fluorodeoxyglucose incorporation we used cultured human osteoblast-like cells. Following incubation of the culture samples in a glucose free medium with 5 μCi [^{18}F]-fluorodeoxyglucose we measured the radioactivity of the cell fraction, as a percent from the initial dose, and

compared to the incorporation values in cells treated by protoporphyrine IX (10^{-5} M), an endogenous pro-apoptotic agent. To compare the response of [^{18}F]-fluorodeoxyglucose incorporation studies, following treatment of cells with the protoporphyrine IX, to other experimental cell metabolism evaluation methods, we performed a parallel comparison of alkaline phosphatase activity, which is a standard measurement tool of osteoblast metabolism, in the control and treatment groups. A narrow range of 0.22–1.36% of [^{18}F]-fluorodeoxyglucose incorporation per million cells was found. Additionally in the protoporphyrine IX treated cells a significant 62% decrease of [^{18}F]-fluorodeoxyglucose incorporation was observed ($p < .05$). A parallel significant decrease in alkaline phosphatase activity ($p < .001$) was found in the cells treated by the protoporphyrine IX. Therefore we suggest that the presented method of [^{18}F]-fluorodeoxyglucose incorporation measurement can be utilized as an effective research tool for estimation of the cellular glycolytic activity in human osteoblast-like cells in vitro.

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Introduction

In the research of osteoblast metabolism the usual measurement variables are related to the different

steps in the bone matrix elaboration pathway, e.g. cellular alkaline phosphatase activity, osteocalcin secretion, interstitial collagen concentration etc, or to the measurements of cellular DNA and to the RNA contents relevant to the matrix elaboration. Interestingly the mitochondrial energy consumption evaluation is not a standard tool for evaluation of an osteoblast metabolism. Clearly the energy consumption evaluation of osteoblast is a valuable information in the research of bone metabolism. Furthermore, studies on glucose utilization by osteoblast might be of a special importance in view of the previously reported interrelation between insulin availability and the development of osteoporosis in vivo (Bouillon 1990) and its anabolic effect on osteoblasts in vitro (Levy et al. 1986; Thomas et al. 1995). Therefore we suggest that the evaluation of osteoblast metabolic activity should be an important part of bone metabolism research. For this purpose we present a method for evaluation of osteoblast glucose consumption by utilizing a measurement of cellular [^{18}F]-fluorodeoxyglucose incorporation under controlled conditions.

Glucose is incorporated into cells by several mechanisms including slow diffusion, active transport in certain epithelial cells, and by a rapid facilitated transfer via the plasma membrane glucose transporters (GLUT) family (Pauwels 2000). The intracellular glucose is converted by one of the 4 types of the enzyme *hexokinase* (each type is specific to a specific cell type), into glucose-6-phosphate. This energetically irreversible reaction takes place on the mitochondrial membrane by utilizing ADP, which is pumped from the mitochondria through the voltage dependent anion channel (VDAC), under regulation of insulin or insulin-like growth factors (Golshani-Hebroni and Bessman 1997). The produced glucose-6-phosphate is then available for the further metabolism through the glycolysis and the pentose phosphate shunt. Since the *hexokinase* mediated glucose conversion into glucose-6-phosphate is an irreversible rate limiting step of glucose incorporation into glycolysis pathway, it might be a good indicator of glucose incorporation rate into cell for estimation of its energetic consumption.

For this purpose in the previous studies cellular incorporation of [$2\text{-}^3\text{H}$] deoxyglucose (HDOG) was measured, since HDOG following its procession by *hexokinase*, is not susceptible to further utilization by

the glycolysis enzymes and is trapped intra-cellularly. Its concentration therefore provides a stable and reliable indicator of glucose consumption and glycolysis rate (Thomas et al. 1995).

[^{18}F]-fluoro-2-deoxy-D-glucose (FDG), a positron emitter labeled glucose analogue, is incorporated into a living cell by the same enzymatic and transport pathways as glucose, and similarly to the HDOG, is trapped intra-cellularly (Brock et al. 1997). This important characteristic of the [^{18}F]-fluorodeoxyglucose is the basis of its use for estimation of cellular glucose consumption, i.e. cellular metabolic activity, in the live tissue and of its clinical use for PET scanning. The short half life of [^{18}F] ($t_{1/2} = 109$ min) and its current wide availability makes [^{18}F]-fluorodeoxyglucose advantageous for clinical and research use over previously described HDOG with $t_{1/2}$ of 4497 days (Brock et al. 1997; Macmahon 2006).

Several studies have reported measurement of cellular [^{18}F]-fluorodeoxyglucose incorporation in different cell types but osteoblast incorporation of [^{18}F]-fluorodeoxyglucose has not been assessed before (Slosman et al. 1993; Haberkorn et al. 1994). We present a method of [^{18}F]-fluorodeoxyglucose incorporation measurement into human osteoblast for the use as a research tool for the estimation of osteoblast metabolic activity.

Methods

Cell line

In this study we used osteoblast-like cells of human origin originated from cancellous bone from distal femur. The cancellous bone samples were taken from the disposable bone tissue during osteoarthritic knee arthroplasty. Chips of cancellous bone, 2–3 g in total, were incubated in DMEM with heat-inactivated fetal calf serum (10%), 20 mM HEPES buffer, 2 mM L-glutamine, 100 μM ascorbate-2-phosphate, 10 nM dexametazone, 50 U ml^{-1} penicillin, 150 $\mu\text{g ml}^{-1}$ streptomycin at 37 °C in humidified atmospheric environment of 95% air with 5% CO_2 (v:v) for 20 days. Human osteoblast-like cells grew out from the chips as adherent to the plastic culture plates until confluence. The human bone cell cultures obtained by this method have been shown previously to express

osteoblast-like characteristics (Gundle et al. 1998). These cells were passaged into plastic cell culture flasks for further experiments. Each flask was seeded with equal number of cells.

Experimental culture samples

Nine samples with cultured osteoblast-like cells were kept as controls and in additional six samples protoporphyrine IX at 10^{-5} M (Sigma) was added. Protoporphyrine IX in high concentration is known to have a mitochondrial apoptosis effect (Azarashvili et al. 2007) probably by changing mitochondrial membrane permeability with their subsequent release of the pro-apoptotic factors that are situated in the mitochondrial inner space. Therefore we used this component for a comparison model of cells that will not be able to keep the labeled [^{18}F]-fluorodeoxyglucose in their damaged mitochondria.

Incorporation study

The cells from each culture sample were washed with PBS and put in a glucose free medium; $5 \mu\text{Ci}$ [^{18}F]-fluorodeoxyglucose (FDG) were then added to the cultures; the cells were incubated at room temperature for 45 min; the cells were then washed with PBS to eliminate free FDG; the cells were then removed from the flasks by trypsinisation, counted and suspended in PBS. The suspended cells were centrifuged at 200 g; the supernatant was discarded; the cells pellets and a standard solution were counted in a γ counter (Atomic Products Corporation, Atomlab, New York USA). The [^{18}F]-fluorodeoxyglucose incorporation was determined as a percent of the initial dose and normalized to one million cells (Slosman et al. 1993).

Alkaline phosphatase activity assay

In order to co-estimate the influence of protoporphyrine IX on FDG incorporation into human osteoblast-like cell with another measure of the cell metabolism we measured the alkaline phosphatase activity in both groups of these cells, controls and exposed to the protoporphyrine IX. Alkaline phosphatase was chosen

for this purpose as a general marker of matrix elaboration by osteoblasts (Siffert 1951).

Alkaline phosphatase activity was determined in lysed cell culture samples, after incubation with P-nitrophenyl phosphate substrate, by 410 nm wavelength spectrophotometry (Bessey 1946). The alkaline phosphatase activity in each culture sample was normalized to a mean cell count from three $\times 70$ microscopic magnification fields.

Statistical analysis

The incorporation values of the FDG into cells and alkaline phosphatase activity in the cells in the control cultures and in the cells exposed to protoporphyrine IX were compared by *t*-test. *P* value below 0.05 was accepted as significant (Bourke 1985).

This experimental protocol was approved by the institutional Ethical Committee.

Fig. 1 A narrow range of FDG cellular incorporation is found. The FDG incorporation into cells is affected by a proapoptotic agent, protoporphyrine IX

Fig. 2 A similar to the FDG cellular incorporation response is found by alkaline phosphatase activity measurements in osteoblast-like cells with and without treatment by protoporphyrine IX

Results

In culture samples of human osteoblast-like cells the [^{18}F]-fluorodeoxyglucose incorporation was demonstrated in a narrow range of incorporation fractions, 0.22–1.36% (mean 0.88%, and standard deviation 0.25%) of an initial dose of [^{18}F]-fluorodeoxyglucose per million cells (Fig. 1). Exposure of the cultured osteoblast-like cells to protoporphyrine IX caused a significant decrease in [^{18}F]-fluorodeoxyglucose incorporation ($p < .05$, decrease of 62% in mean incorporation, Fig. 1). Similarly the alkaline phosphatase activity in the cells treated with protoporphyrine IX decreased significantly, i.e. decrease of 87% of the mean value of alkaline phosphatase activity per cell ($p < .001$, Fig. 2).

Discussion

Pharmacological and toxicological research studies on human osteoblast may raise research hypotheses concerning its metabolic response to certain chemical exposure. In most of current studies the experimental tools for estimation of osteoblast metabolic response involve measurements of the by-products of bone matrix elaboration. Measurement of glucose consumption as an index of osteoblast metabolic activity evaluation might be of importance as well, providing direct expression of cellular metabolism. In the present study we show that [^{18}F]-fluorodeoxyglucose incorporation into human osteoblast can be measured effectively, providing consistent results of a relatively narrow range of incorporation values. Additionally we show that the incorporation of FDG into osteoblasts is sensitive to an intervention into its metabolic pathways, e.g. interruption of the normal mitochondrial function, following exposure of cells to the protoporphyrine IX. We also observed the expected significant decrease in alkaline phosphatase activity, which is a characteristic marker of the osteoblast metabolism, with a parallel significant decrease in [^{18}F]-fluorodeoxyglucose incorporation.

Therefore we conclude that measurement of [^{18}F]-fluorodeoxyglucose incorporation into osteoblasts can be effectively utilized as a research tool for

evaluation of human osteoblast metabolism in physiological environment and following a pharmacological challenge.

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