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Expression of glutamyl aminopeptidase by osteogenic induction in rat bone marrow stromal cells

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Abstract

Glutamyl aminopeptidase (GluAP, EC 3.4.11.7, ENPEP) is a 130-kDa homodimeric zinc metallopeptidase which specifically cleaves the N-terminal glutamate or aspartate residue of peptidic substrates such as cholecystokinin-8 or angiotensin (Ang) II, *in vitro*. We used a DNA microarray hybridization (Genechip Rat Expression Array 230A, Affymetrix Inc., Santa Clara, CA, USA) to demonstrate that GluAP was upregulated in osteogenic induced rat bone marrow stromal cells (BMSCs). To compare the expression of GluAP in the osteogenic differentiation and non-osteogenic differentiation of rat BMSCs *in vitro*, the cells were osteogenic induced *in vitro*. We also performed an MTT assay, alkaline phosphatase assay, alizarin red staining, and an immunohistochemical analysis to determine the osteogenic differentiation of BMSCs. The expression of GluAP was examined by real-time polymerase chain reaction (PCR). The real-time PCR results showed that GluAP was upregulated in osteogenic differentiated BMSCs *in vitro*, suggesting that GluAP may be correlated with the osteogenic differentiation of BMSCs.

Keywords: GluAP; EMPEP; Bone marrow stromal cells; Osteogenic induction; Gene expression

1. Introduction

A special feature of tissue engineering is to regenerate a patient's own tissues and organs that are entirely free of poor biocompatibility and low biofunctionality as well as the risk of severe immune rejection. Owing to these outstanding advantages of such tissues, tissue engineering is often considered as an ultimately ideal medical treatment. To regenerate new tissues, biomedical engineering utilizes three basic tools: cells, scaffolds, and growth factors (Ikada, 2006). Multipotent bone marrow stromal cells have several characteristics that make them potentially useful for approaches using cells and gene therapy (Vacanti and Bonassar, 1999), and have been widely explored in skeletal tissue engineering applications. Mahesh and coworkers have used an established bone defect model to demonstrate the feasibility of using large volumes of autologous BMSC transplants to close critical-sized calvarial defects, and confirmed that the extent of bone formation could be reliably estimated using non-invasive techniques (Mankani et al., 2006). Phenotypically, BMSCs have been found to be capable of differentiating into bone, cartilage, muscle, adipose, and neural tissue elements (Owen and Friedenstein, 1988; Kopen et al., 1999; Ferrari et al., 1998; Bennett et al., 1991; Pereira et al., 1998). There are a range of proteins that play key roles in the proliferation and differentiation of cells. These proteins are endogenously secreted in the body by cells themselves (autocrine) or as a result of communication with the surrounding cells (paracrine). These proteins are called cell growth factors or simply growth factors. Genes correlated with the osteogenic differentiation of BMSCs such as BMPs, Runx2, Osterix, LRP5 have been studied (Krane, 2005). Although a considerable amount of

Abbreviations: GluAP, glutamyl aminopeptidase; DMSO, dimethyl sulfoxide.

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data is available describing BMSCs, there is still a lack of information regarding the molecular mechanisms that govern their differentiation. Therefore, to clarify the complexity of their molecular network expression, the detection of more osteo-related genes is required.

In a previous study, we used DNA microarray hybridization (Genechip Rat Expression Array 230A, Affymetrix Inc., Santa Clara, CA, USA) to demonstrate that 12 genes (Pla2g2a, Expi, Pcp4, Cx3cl1, GluAP, Mmp9, Lcn2, Il10, Fcna, Fmo1, and two undefined genes) were upregulated while one undefined gene was downregulated in osteogenic induced BMSCs. GluAP was one of the several genes that showed obvious changes in its expression patterns. According to these previous results, we hypothesized that GluAP might correlate with the osteogenic differentiation of BMSCs.

GluAP (EC 3.4.11.7, aminopeptidase A, ENPEP) is a 130-kDa homodimeric zinc metallopeptidase which specifically cleaves the N-terminal glutamate or aspartate residue of peptidic substrates such as cholecystokinin-8 or angiotensin (Ang) II, *in vitro* (Wilk and Healy, 1993; Nagatsu et al., 1970). GluAP is strongly expressed in peripheral tissues such as intestinal and renal epithelial cells, and in the vascular endothelium (Lojda and Gossrau, 1980), and is also localized in brain nuclei expressing Ang II type-1 receptors (Zini et al., 1997). In addition, GluAP is found on stromal cells in the bone marrow and the thymic cortex, and on subpopulations of cells in the ovary and placenta (Li et al., 1993; Mizutani et al., 1981). GluAP appears to have a role in the catabolic pathway of the renin–angiotensin system (Ahmad and Ward, 1990). The amino terminal amino acid aspartyl residue of angiotensin II can be removed by GluAP to yield the less active angiotensin III. Thus, GluAP is also defined as an angiotensinase, and is implicated in the regulation of blood pressure by affecting circulating angiotensins. Zini et al. (1997) reported the distribution of GluAP in brain nuclei involved in the control of cardiovascular functions, and in the pituitary. The cell membrane-bound and soluble forms of GluAP in serum have already been identified (Bausback et al., 1988). The soluble form of GluAP secreted by placental cells may be an indicator of its placental function, and it is believed to play a role in controlling blood pressure during pregnancy (Yamada et al., 1988; Okuyama et al., 1991). GluAP may also regulate autocrine and paracrine signals by proteolytic activation or inactivation of specific regulatory peptides (Stefanovic et al., 1992). Although widely distributed, GluAP expression on hemopoietic cells is restricted to the early stages in B lineage differentiation. Enhanced GluAP expression is found on pre-B and immature B cells that are virally transformed, generated in long term bone marrow cultures, or stimulated with IL-7, a well-known facilitator of early B cell development in mice (Cooper et al., 1986; Namen et al., 1988; Welch et al., 1990; Whitlock et al., 1987; Sherwood and Weissman, 1990). However, Lin and coworkers generated a mouse model of GluAP deficiency. They showed that, surprisingly, mice homozygous for the GluAP null mutation exhibited normal B and T cell developments, indicating that GluAP activity was not essential for these lymphoid differentiation pathways (Lin et al., 1998).

Recently, few studies have been carried out on the correlation between GluAP and osteogenic BMSCs. Since microarray hybridization revealed different expression patterns of GluAP between undifferentiated BMSCs and osteogenic induced BMSCs, it is necessary to determine whether the expression of GluAP is involved in the osteogenic differentiation of BMSCs. Therefore, we used quantitative real-time PCR as our main method to further investigate the differentiation mechanisms of BMSCs; this information is valuable for gene therapy and skeletal tissue engineering.

2. Materials and methods

2.1. Collection and culture of BMSCs

BMSCs were obtained from the femurs of 2-month-old male Sprague–Dawley rats (145–180 g) according to the protocols of Tohill et al. (2004), with some modifications. All animal works were approved by the Animal Care and Use Committee of the Sichuan University. Cells from marrow aspirates suspended in α-MEM (Gibco, USA), supplemented with 10% fetal bovine serum (FBS, SIGMA), 100 U/mL penicillin, and 100 μg/mL streptomycin (SIGMA) were seeded in tissue culture flasks, and incubated at 37 °C, and with 5% CO_2. The culture medium was changed every 3 days. Red blood cells and other non-adherent cells were removed with the medium waste. After 7 days, the 80% confluent culture (P0) was trypsinized with 0.25% trypsin (Gibco), and reseeded. The resultant cells were designated as P1. The adherent cells were further propagated for three passages.

Osteogenic induction was performed to half the amount of the P3 cells, while the other half of the cells were considered as control. For the osteogenic induction, 10 mmol/L β-glycerophosphoric sodium, 10−8 mol/L Dex (dexamethasone), 10 mmol/L VitD3 (vitamin D3, Gibco), 50 mg/L L-ascorbic acid, 100 U/mL penicillin and streptomycin (SIGMA) were added to the previous culture medium. Thereafter, all cells (osteogenic induced and control) were cultured as described above.

2.2. Proliferation assay

The MTT assay was performed to measure the proliferation of osteogenic induced BMSCs (Mosmann, 1983). BMSCs were first seeded in a 96-well microplates at a density of 2×10^4 cells per well containing 200 μL culture medium. The cells were cultured for 8 h before the MTT assay. Then, 20 μL MTT (5 mg/ml) were added to each well, and the microplates were further incubated at 37 °C. After 4 h incubation, 200 μL DMSO were added to each well. Absorbance was read using an HTS 7000 Plus Bio Assay reader (PE, USA) at 570 nm.

2.3. Alkaline phosphatase assay

A colorimetric assay was performed for the multilayered cells using an alkaline phosphatase (ALP) assay (Merit Choice