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Expression and functional significance of osteocalcin splicing in disease progression of hematological malignancies

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Abstract

The aim of this study was to investigate the expression of osteocalcin (OCN) splicing variants in hematological malignancies. We analysed bone marrow obtained from two patients with chronic myeloid leukemia (CML), seven patients with other myeloproliferative diseases (MPD) and four patients with acute myeloid leukemia (AML). RT-PCR analyses were performed in order to assess and quantify spliced (OCNs) and unspliced (OCNu) mRNA, the associated transcription factors (AML1 and AML3) as well as c-KIT which is a marker for activated stem cells. Our data indicate that OCNs mRNA and OCN protein is expressed in c-KIT positive neoplastic stem cells in hematological malignancies.

Keywords: Osteocalcin, c-KIT; Hematological malignancy; Runt-domain transcription factors

1. Introduction

OCN has originally been defined as marker for mature osteoblasts – cells involved in bone formation. The protein itself is a small (MW 6.9 kDa) molecule and represents the most abundant non-collagenous protein in bone. It has α-helical domains forming a tightly packed charged molecule that coordinates Ca^{2+} at the surface of the hydroxyapatite-like lattice of bone mineral crystals. The fact that no OCN deficient persons have been found to date suggests a crucial role of OCN for human life and development, but the dimension of its action has yet to be completely defined.

OCN may function as a matrix signal in the recruitment and differentiation of bone-resorbing cells [1] and studies of murine long-term bone marrow cultures with rat bone OCN indicate that OCN promotes osteoclastic differentiation of a stroma-free subpopulation of hematopoietic progenitors in the presence of GM-CSF [1]. These bone-resorbing cells are derived from hematopoietic stem cells, an indication that OCN may influence the differentiation of this stem cell pool as well [2]. Recently, OCN has been shown to enhance bone remodelling, a process depending on the number and activity of both osteoclasts and osteoblasts [3].

In addition to bone-related tissues, OCN is synthesised by vascular smooth muscle cells [4] and its mRNA is also expressed in megakaryocytes and peripheral blood platelets, which possibly contribute to the OCN levels in blood and the regulation of bone turnover [5].

Recent data demonstrated that activated HSC (hematopoietic stem cells) from hematologic malignancies, as characterised by KIT, also express the osteocalcin protein (OCN) [6].

There is a species dependent difference in the regulation of this protein between human, rats and mice: in human and rats OCN expression is strongly increased by 1,25-dihydroxy-Vitamin D3 while in mice the transcription is attenuated by this vitamin [7]. Thyroid hormones, however, increase the

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transcription of OCN from the mouse promoter [8,9] but inhibit the transcription from the human promoter (Wihlidal, unpublished results). The expression of OCN is tightly linked to the maturation process of osteoblasts, whereas during the proliferation and synthesis phase of osteoblasts the OCN expression is suppressed [10]. In mice there are three very homologous genes, in human and rat, there is only one gene. Furthermore, in humans, splicing variants were described. Apart from previously defined OCN splicing variants in several tissues, hematopoietic cells and cells of isolated bone marrow also express an unspliced variant OCNu which is characterised by intron retention [11] resulting in trace amounts of a truncated protein (resulting from a stop codon in the first intron) with no apparent function.

Based on observations that hematopoietic precursors reside closely to endosteal surfaces, it was hypothesised that mesenchymal stem cells and osteoblasts play a central role in hematopoiesis, and it has been shown that osteoblasts produce many factors essential for survival, renewal and maturation of hematopoietic stem cells [12] and vice versa. Similarities in gene regulation of OCN and c-KIT proteins may support this hypothesis. Recent experiments [13] showed that Imatinib mesylate (IM) a drug targeting c-KIT and PDGF-R tyrosine kinases inhibits osteogenic differentiation and proliferation of mesenchymal stem cells.

Both, OCN and c-KIT expression are regulated by Runt-domain transcription factors: AML1 (Runx1) and AML3 (Runx2) bind to the same response element [14,15]. AML1 and AML3 transcription factors regulate differentiation of both, hematopoietic and mesenchymal stem cells, respectively.

Aim of this study was to characterise the expression of OCN and its splicing-variants in normal and malignant cells of hematopoietic origin and to compare their expression with that of c-KIT, as well as the transcription factors AML1 and AML3 to test the hypotheses that leukemic blast cells may display features of a stem cell candidate common to hematopoiesis and mesenchymal progeny and/or may mimic osteoblastic properties in order to escape the immune defence.

2. Materials and methods

Frozen spare samples from routine diagnostics of bone marrow and peripheral blood (PBSC stem cells) from a previous project [16] were used in this study. Bone marrow samples were obtained from four patients with AMLs, one patient with CML at blast crisis, one CML patient in chronic phase and seven patients with MPD, as well as stem cells from peripheral blood and bone marrow obtained from healthy donors. In an attempt to find out whether the presence of OCN expressing stem cells is associated with disease status, sequential bone marrow samples from a patient with AML (A1) were analysed at six time points including diagnosis, first remission (months 4 and 5 after diagnosis), second remission (month 17 after diagnosis) and relapse (months 29 and 41 after diagnosis). As control, cells from the HL60 cell line were used.

Analyses were carried out from bone marrow collected in EDTA tubes. After separation of plasma, mononuclear cells were enriched by density gradient centrifugation using Ficoll–Hypaque separation medium (density according to fraction index: 1.077) and washed several times in phosphate buffered saline (PBS). Aliquots of 1–10 million cells were frozen in 4 M Guanidine-isothiocyanate for preparation of mRNA and cDNA-synthesis.

mRNA was analysed from at least 1 million mononuclear blood cells (MNC). Isolation of mRNA and preparation of cDNA were performed according to standard procedures using commercially available kits (ROCHE Diagnostics).

PCR conditions in block cycler PCR (Perkin-Elmer; Norwalk, CT or Eppendorf Mastercycler Gradient) were the same for all assays (30 s 95 °C/30 s 52 °C to 62 °C/30 s 72 °C, 35 cycles). PCR products were analysed on 1% agarose gels.

The level of gene expression was measured by relative and absolute quantitative real time PCR (RTQPCR). For absolute quantification, a standard curve relates to the PCR signal of the input copy numbers of a defined template (cloned amplimer, termed as external standard), while the quantification measures. Relative quantification was adequate to study differences in gene expression. This was done by calculating the number of copies of the analysed gene per 100 copies of β-actin, which showed the lowest standard deviations per amount of cDNA and is therefore used as internal standard [17,18].

RTQPCR was carried out using a LightCycler™ System (Roche), which allows amplification and detection (by fluorescence) in the same tube, using a kinetic approach. The methodology of quantitative PCR has been described in detail elsewhere [19–21]. Dilutions of 200–2 ng of cDNA were used in each assay. For OCN, only OCNs was quantified. Details of primers and PCR conditions are presented in Table 1.

For immunocytochemistry, cultured cells from the HL60 cell line or mononuclear cells from blood or bone marrow of patients and controls were fixed in suspension by overnight incubations at 4 °C in a 3% solution of formaldehyde in neutral PBS. Cytospins from these cellular suspensions were applied on adhesive glass slides (Starfrost). Slides were dehydrated with ethanol according to standard procedures and air-dried at room temperature.

Air-dried bone marrow smears of the A1-patient were retrieved from the archive of the 3rd Medical Department of Hanusch Hospital. Following fixation in a 3% solution of formaldehyde in PBS, slides were dehydrated with ethanol according to standard procedures and air-dried at room temperature.

To determine the co-expression of c-KIT and OCN in single cells, we performed a double staining procedure using anti c-KIT (104D2), a mouse monoclonal antibody obtained from Santa Cruz Biotechnology (USA), and anti-osteocalcin (bone Gla9), a polyclonal antibody (rabbit) obtained from