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Modulation of cyclin D1 expression in human tumoral parathyroid cells: Effects of growth factors and calcium sensing receptor activation

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Abstract

The study investigated cyclin D1 regulation by growth factors and calcium sensing receptor (CaSR) in human tumoral parathyroid cells. Basic fibroblast and epidermal growth factors increased cyclin D1 and phosphorylated extracellular signal-regulated kinases (pERK1/2) levels that were both efficiently inhibited by CaSR agonists. By contrast, in growth factors-free medium cyclin D1 levels were either unaffected or stimulated by CaSR activation independently from ERK1/2 pathway. Transforming growth factor β (TGFβ) reduced cyclin D1 levels in the majority of tumors, this effect being not influenced by CaSR activation and menin expression levels. In conclusion, in parathyroid tumors cyclin D1 expression was modulated by growth factors and CaSR activation. These data further support the oncogenic role of cyclin D1, which resulted to be target for stimulation by bFGF and EGF and inhibition by CaSR and TGFβ signalling in the parathyroid.

Keywords: Cyclin D1; Menin; Parathyroids; Calcium sensing receptor

1. Introduction

Cyclin D1 is a key sensor and integrator of extracellular signals, such as growth factors and hormones, in early to mid-G1 phase. It modulates local chromatin structure of genes involved in regulation of cell proliferation and differentiation through binding both the cyclin-dependent kinases and hystone acetylase and deacetylases [1]. Moreover, cyclin D1 forms physical associations with several transcription factors or transcriptional coregulators [1]. Several evidence point to a primary role of cyclin D1 in parathyroid cell growth and PTH secretion. Cyclin D1 was initially cloned and recognized as an oncogene in the development of the parathyroid tumors [2]. In fact, a subset of parathyroid adenomas shows cyclin D1 overexpression,

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that in a few cases is due to clonal rearrangement that justaposes PTH gene regulatory region with cyclin D1 gene [2–6]. When targeted to parathyroid gland, cyclin D1 overexpression is sufficient to induce parathyroid adenoma in transgenic mice [7]. Experimental evidences suggested that cyclin D1 interacts with the products of tumor suppressor genes involved in parathyroid tumorigenesis, such as the product of the multiple endocrine neoplasia type 1 (Men1) gene menin [8] and parafibromin [9].

Parathyroid targeted overexpression of cyclin D1 has been shown to be associated with a down-regulation of the calcium sensing receptor (CaSR) and a shift to the right of the calcium-PTH set-point in transgenic mice [10,11]. As shown in CaSR-transfected cell line and in animal and human parathyroid cells, CaSR mediates the inhibition of PTH secretion through the modulation of intracellular cAMP and calcium levels and the activation of several kinases, including the extracellular signal-regulated kinases (ERK1/2) [12–14]. CaSR also appears involved in the control of parathyroid cell proliferation as suggested by the presence of parathyroid hyperplasia in patients carrying inactivating mutations of CaSR gene and in knock-out mice [15,16]. However, the mechanisms responsible for the inhibitory effect of CaSR on parathyroid growth are uncertain.

In this study we investigated the effects of CaSR activation on cyclin D1 expression in human tumoral parathyroid cells in the presence of different growth factors and the involvement of ERK1/2 pathway in this process.

2. Materials and methods

2.1. Cultures of human parathyroid cells

Ten single adenomas and 4 hyperplasia obtained from patients affected with sporadic primary hyperparathyroidism were studied. Tissues removed at surgery were in part placed in sterile medium for cell culture and in part snap frozen in liquid nitrogen and stored at −80 °C until analysis. The study was approved by the local ethical committee and informed consent was obtained from each patient. Dispersed parathyroid cells were prepared by 2-h collagenase (2 mg/ml) digestion at 37 °C, as previously described [17].

2.2. Determination of cyclin D1 protein and phospho-ERK1/2 levels

Dispersed cells (10^6 cells/well) were starvested by 16 h-incubation in HAM-F10 (containing 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}), supplemented with 0.5% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (1%) in a humidified atmosphere of 5% CO_2 at 37 °C, as previously described [13,18]. Subsequent incubation with various reagents were performed in HAM-F10 + 0.1% bovine serum albumin (BSA) at 37 °C for 8 h, based on preliminary experiments carried out in parathyroid cells at 4, 8, 16 and 24 h showing the maximal cyclin D1 stimulation by 10% FCS at 8 h. Incubation with different stimuli was stopped placing the cells on ice. The medium was removed and cells were treated with 200 μl ice-cold lysis buffer in the presence of protease and phosphatase inhibitors, as previously described [19]. Cyclin D1 was selectively immunoprecipitated from 100 μg of total proteins by the specific mouse monoclonal anti-cyclin D1 antibodies (Novocastra Laboratories Ltd, New Castle upon Tyne, UK) and the resulting immunoprecipitates of 36 kD were measured by western blotting using the same antibody diluited 1:200, as previously described [10,20]. The expression of glyceraldeyde-3-phosphate dehydrogenase (GAPDH) by a mouse monoclonal anti-GAPDH antibody (Ambion, Ltd, Huntingdon, Cambridgeshire, UK) was used as an internal control of protein loading, as previously described [10]. For the determination of total and phosphorylated ERK1/2 levels, Western blot analysis of cell lysates and of total protein from parathyroid tissues was performed using anti-p44/42 ERK and anti-phospho-p44/42 ERK polyclonal antibodies diluited 1:1000 (New England BioLabs, Inc., Beverly, MA), as previously described [13]. The membranes were finally incubated with a secondary rabbit antibody conjugated to horseradish peroxidase (diluited 1:2000), and treated with chemiluminescent substrate and enhancer (Pico Western Detection Kit, New England Biolabs, Beverly, MA, USA) and exposed to X-ray film. Experiments were repeated at least twice. The densitometric readings of the resulting bands were evaluated using an Epson imaging densitometer and the TotalLab v2.01 program.

2.3. Determination of CaSR and menin proteins expression

Twenty micrograms of total proteins extracted from parathyroid tumors using Cell Lysis Buffer (New England Biolabs, Beverly, MA, USA) supplemented with 200 mM PMSF and protease and phosphatase inhibitors, were separated by SDS–PAGE at 10% for menin and at 7.5% for the CaSR, and transferred to nitrocellulose filters, as previously described [10,17]. The filters were subsequently incubated for 3 h with an affinity purified goat polyclonal antibody raised against the C-terminal region of the menin protein of human origin (Santa Cruz Biotechnology, Inc., CA, USA) diluited 1:200, and with a polyclonal antibody raised against a peptide of the CaSR [17]. The following membrane treatment and densitometric readings were carried out as in Section 2.2.