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Induction of type 1 iodothyronine deiodinase expression inhibits proliferation and migration of renal cancer cells

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ABSTRACT

Type 1 iodothyronine deiodinase (DIO1) regulates peripheral metabolism of thyroid hormones that control cellular proliferation, differentiation and metabolism. The significance of DIO1 in cancer is unknown. In this study we hypothesized that diminished expression of DIO1, observed in renal cancer, contributes to the carcinogenic process in the kidney.

Here, we demonstrate that ectopic expression of DIO1 in renal cancer cells changes the expression of genes controlling cell cycle, including cyclin E1 and E2F5, and results in inhibition of proliferation. The expression of genes encoding collagens (*COL1A1*, *COL4A2*, *COL5A1*), integrins (*ITGA4*, *ITGA5*, *ITGB3*) and transforming growth factor-β-induced (*TGFBI*) is significantly altered in renal cancer cells with induced expression of DIO1. Finally, we show that overexpression of DIO1 inhibits migration of renal cancer cells.

In conclusion, we demonstrate for the first time that loss of DIO1 contributes to renal carcinogenesis and that its induced expression protects cells against cancerous proliferation and migration.

1. Introduction

Thyroid hormones (TH), thyroxine (T4) and 3,5,3′ triiodothyronine (T3) regulate key cellular processes such as proliferation, differentiation, apoptosis, and metabolism. Most of TH effects are executed by T3 whose actions are mediated by specific nuclear receptors that bind to regulatory regions of target genes and regulate their expression in a ligand-dependent manner. The intracellular availability of T3 is controlled by iodothyronine deiodinases which catalyse selective removal of iodine from iodothyronines (Visser and Peeters, 2012). Iodothyronine deiodinases are membrane bound proteins encoded by three genes: *DIO1*, *DIO2*, and *DIO3*. The two latter enzymes are best characterized. DIO2 contributes to the production of T3 due to deiodination of T4 at 5′ position, while DIO3 is a T3 inactivating enzyme and removes iodine at position 5 of the iodothyronine. DIO1 is a unique enzyme that catalyses deiodination at both (5′ and 5) positions of iodothyronines and thus may contribute to production as well as degradation of T3. However, the physiological significance of DIO1 remains controversial. Basing on studies performed on knockout mice devoid of *DIO* genes it was suggested that DIO1 is a scavenger enzyme that enables re-use of iodine recovered from deiodinated iodothyronines (Schneider et al., 2006).

The expression and activities of DIO enzymes are disturbed in cancers, including cancers of thyroid, skin, and kidney (Piekielko-Witkowska and Nauman, 2011). Furthermore, it was demonstrated that DIO3 actively contributes to carcinogenesis. Enhanced DIO3 expression in basal cell carcinoma (BCC) and in colon cancer reduces T3-dependent signalling and promotes cancerous proliferation. In contrast, inhibition of DIO3 expression in cancer cells decreases proliferation with concomitant induction of differentiation and apoptosis in cell cultures and/or xenograft mouse models (Dentice et al., 2007, 2012).

The three major sites of physiological DIO1 expression are liver,

Abbreviations: BCC, basal cell carcinoma; DIO1, iodothyronine deiodinase type 1; DIO2, iodothyronine deiodinase type 2; DIO3, iodothyronine deiodinase type 3; ECM, extracellular matrix; RCC, renal cell carcinoma; rT3, 3,3′,5′-triiodothyronine; T3, 3,5,3′ triiodothyronine; T4, thyroxine; TH, thyroid hormones; TRs, thyroid hormone receptors.

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kidney, and thyroid. In contrast, in the most common type of kidney cancer, renal cell carcinoma (RCC), the expression and activity of DIO1 enzyme are barely detectable (Pachucki et al., 2001; Master et al., 2010). In this study we hypothesized that reduced DIO1 expression may contribute to carcinogenic process in renal cells. To verify this hypotheses we stably transfected two human RCC-derived cell lines with DIO1 coding plasmid and checked their proliferative, adhesive, and migratory properties. This is the first study showing that DIO1 actively influences carcinogenesis by introducing changes in expression of genes involved in proliferation and migration and inhibiting both pathological processes.

2. Materials and methods

2.1. Cell lines

KIJ265T and KIJ308T were obtained from Mayo Foundation for Medical Education and Research (Tun et al., 2010) and cultured as described previously (Boguslawska et al., 2014). Cells were transfected as previously described (Wojcicka et al., 2014) with pcDNA3-DIO1 plasmid (van der Deure et al., 2009) or with an empty vector, and selection of stably transfected cells was performed. Briefly, 24 h after transfection, medium was renewed, and on the following day selective medium containing 500 μg/ml of Geneticin (Thermo Fisher Scientific, Rockford, IL, USA) was added. Cells were cultured in selective medium for 3 weeks and medium was changed every 3 days. The selected clones were further cultured in complete medium. Expression of DIO1 was determined by qPCR and Western blot.

For T3 supplementation experiments, KIJ265T and KIJ308T cells were cultured in medium supplemented with charcoal stripped FBS (Sigma-Aldrich, St. Louis, MO, USA) for 7 days. Next, cells were seeded on 12-well plates at density 5×10^4 per well. After 24 h the medium was renewed and 100 nM T3 or vehicle was added. The medium was renewed again after 24 h, then cells were cultured for the next 24 h and RNA was isolated using GeneMATRIX Universal RNA Purification Kit (EURx, Gdansk, Poland).

For rT3 or T4 supplementation experiments, KIJ265T cells stably transfected with pcDNA3-DIO1 or empty plasmid were seeded on 12-well plates at a density of 5×10^4 cells per well and cultured in medium without phenol red supplemented with charcoal stripped FBS (Sigma-Aldrich). The medium was renewed after 24 h and 48 h. Following 72 h from seeding of the cells rT3 (Sigma-Aldrich), T4 (Henning, Berlin, Germany), or vehicle was added at specified concentrations. At 24 h after hormone supplementation, RNA was isolated using GeneMATRIX Universal RNA Purification Kit (EURx).

2.2. RNA isolation and cDNA synthesis

RNA from cell lines was isolated using GeneMATRIX Universal RNA Purification Kit (EURx). Reverse transcription was performed on 100 ng of RNA using Revert Aid H Minus First Strand cDNA Synthesis Kit and random hexamers (Thermo Fisher Scientific). Reverse transcription for RT2 Profiler Extracellular Matrix and Adhesion Molecules PCR Array analysis (SABioscience/Qiagen, Frederick, MD, USA) was performed according to manufacturer's instructions.

RNA from RCC tissue samples (clear cell pathology) and matched-paired control samples with no signs of tumour infiltration was obtained from the RNA Bank deposited at the Department of Biochemistry and Molecular Biology at Centre of Postgraduate Medical Education. The use of RCC tissue samples was approved by the local Bioethical Committee (no. 18/PB/2012 and no. 75/PB-A/2014).

2.3. Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed using primers and probes given in Supplementary Table S1 and SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) or TaqManUniversal Master MiX II with UNG (Thermo Fisher Scientific), according to manufacturer's instructions. The expression of reference genes used for normalization was analysed using Normfinder.

Analysis of genes involved in adhesion and ECM-remodelling consisted of two steps. Firstly, pooled cDNA from three independent cell culture experiments was subjected to analysis using Human Extracellular Matrix & Adhesion Molecules RT^2 Profiler PCR Array (SABioscience/Qiagen) according to manufacturer's instructions. Then, genes whose expression: 1) was statistically significantly changed by at least 15% between cells overexpressing DIO1 and control cells transfected with empty vector; and 2) was known from published studies for its involvement in the pathology of RCC, were selected for manual validation in each cDNA sample separately using primers given in Supplementary Table S1 and SYBR Green I Master (Roche Diagnostics).

2.4. Protein isolation and western blot

For protein analysis 1.25×10^5 cells were seeded on 6 well plates. After 72 h the cells were trypsinized and proteins were isolated using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) with 0.5 mM PMSF and protease inhibitor cocktail (Roche Diagnostics), as previously described (Boguslawska et al., 2016). The obtained supernatants were used for analysis of protein concentration with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and stored at −70 °C in aliquots. For E2F2, E2F5, cyclin E1, ITGA5, TGFBI and DIO1 Western blotting, 10 μg of protein extract was resolved by 10% SDS-PAGE. After electrophoresis the proteins were transferred onto nitrocellulose membranes that were subsequently blocked overnight at 8 °C in 5% non-fat milk or BSA (for TGBI) in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 7.6). The membranes were washed three times in TBST for 10 min at RT, and incubated overnight at 8 °C with anti-E2F2 (ab138515, 1:1000 dilution, Abcam, Cambridge, UK), anti-E2F5 (ab22855, 1:1000, Abcam, Cambridge, UK), anti-cyclin E1 (ab71535, 1:2000, Abcam), anti-ITGA5 (ab150361, 1:1000, Abcam), anti-TGFBI (#5601, 1:1000, Cell Signalling Technology, Danvers, MA, USA) or anti-DIO1 antibody (Kuiper et al., 2003) in TBST buffer with 5% non-fat milk or BSA (for TGFBI). After washing 3 times for 10 min with TBST, the membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated goat anti-rabbit antibody diluted at 1:10,000 in TBST (Dako Inc, Glostrup, Denmark). Next, the membranes were washed three times with TBST, and the proteins were detected using Supersignal West Pico Chemiluminescent Substrate or SuperSignal™ West Dura Extended Duration Substrate (E2F5, TGBI, DIO1) (Thermo Fisher Scientific) according to standard procedures. Protein expression was normalized to β-actin as described previously (Master et al., 2010).

2.5. Analysis of cellular proliferation, migration, and adhesion

Cell proliferation was analysed using Cell Proliferation ELISA, BrdU (colometric) (Roche Diagnostics), according to manufacturer's instructions. 1×10^4 cells were seeded on 96-well plate (cat. no. 6005181, ViewPlate 96 TC, Perkin Elmer, Waltham, Massachusetts, USA) and after 72 h 10 μl of BrdU labeling solution was added to each well. Next, after 2 h of incubation medium was removed, cells were incubated with FixDenat solution for 30 min, then FixDenat was removed, and anti-BrdU POD-solution was added. Following 90 min of incubation, the cells were washed 3 times with PBS, and