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Comparative effects of transforming growth factor beta isoforms on redox metabolism in thyroid cells

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ABSTRACT

Introduction: Transforming growth factor beta (TGF-β) regulates thyroid function and growth. However, tumoral thyroid cells became resistant to this factor as they undifferentiated. Little is known about the effects of TGF-β isoforms. We compared the role of redox metabolism in the response to TGF-β isoforms between non tumoral and tumoral thyroid cells.

Methodology and results: Differentiated rat thyroid cells (FRTL-5) and human thyroid follicular carcinoma cells (WRO) were treated with the three isoforms of TGF-β. TGF-β isoforms stopped cell cycle at different steps; G1 for FRTL-5 and G2/M for WRO. The three isoforms decreased cell viability and increased ROS accumulation in both cell lines. These effects were more pronounced in FRTL-5 than in WRO, and the isoform β1 was more potent in ROS production than the other two. TGF-β isoforms decreased total glutathione, catalase expression and it activity in both cell lines. Only in FRTL-5 the lipid peroxidation was demonstrated. Moreover, TGF-β1 decreased glutathione peroxidase and mitochondrial superoxide dismutase mRNA expression and increased mitochondrial ROS in FRTL-5, but no in WRO. Pretreatment with selenium increased glutathione peroxidase activity and decreased ROS production in WRO treated with TGF-β isoforms. Furthermore, selenium partially reversed the effect of TGF-β isoforms on cell viability only in WRO cells. The knockdown of endogenous NOX4 significantly reduced the TGF-β1 effect on cell viability in WRO but no in FRTL-5.

Conclusion: TGF-β disrupted the redox balance and increased ROS accumulation in both cell lines. FRTL-5 cells showed reduced antioxidant capacity and had a greater sensitivity to TGF-β isoforms, while WRO cells were more resistant. This observation provides new insights into the potential role of TGF-β in the redox regulation of thyroid cells.

1. Introduction

The three isoforms of Transforming Growth Factor beta (TGF-β) are part of a family of multifunctional cytokines which regulate many physiological functions, such as cell proliferation, differentiation, migration, apoptosis, and immune reactions (Massagué, 2012). In normal epithelial cells they act as tumor suppressors. However, they may have a tumor promoter effect at late stages of tumor evolution, stimulating uncontrolled growth, metastasis and epithelial-mesenchymal transition (EMT) (Biere and Moses, 2006). In cells of mesenchymal origin TGF-β causes an increase in cell proliferation and has been related to the induction of fibrosis (Massagué, 2012).

In the thyroid gland it has been shown that TGF-β1 is synthesized by the thyrocyte and inhibits both cell proliferation and function under normal conditions (Tsushima et al., 1988; Colletta et al., 1988; Taton et al., 1993). TGF-β1 synthesis is stimulated by TSH and iodine excess in cell cultures (Yuasa et al., 1992). This result has led to postulate that these cytokines participate in thyroid regulation (Pisarev et al., 2009). The antiproliferative action of TGF-β1 in thyroid tissue decreases as the cancer becomes more undifferentiated. Several mechanisms have been proposed to explain the

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"scape phenomenon", but none has provided a universal cause (Massagué, 2012). In some instances, mutations in the TGF-β receptors, inactivating mutations in SMAD2 and 4, modifications in the AKT/PI3K pathway have been reported in thyroid tumors (Lazzereschi et al., 2005; Matsuo et al., 2010). On the other hand, it has been shown, that under certain circumstances, TGF-β1 becomes protumorigenic (Riesco-Eizaguirre et al., 2009). In fact, several studies with human thyroid samples showed an increased expression of this protein in pathological thyroid tissue (Morosini et al., 1996; Matoba et al., 1998; Kimura et al., 1999).

Increased levels of reactive oxygen species (ROS) are associated with many human diseases. The thyroid cells are exposed to ROS due to its increase during hormonogenesis. The potentially damaging effects of ROS are limited by the antioxidant systems and the location of the DUOX/TPO system at the apical surface of the thyrocyte (Björkman and Ekholm, 1984). When the balance between the production and the degradation of ROS is not properly regulated within the cell, the increased oxidative species can cause morphological and functional damage (Song et al., 2007). Moreover, the excessive accumulation of ROS may cause DNA damage, resulting in mutations which can promote cancer development. In fact, oxidative stress seems to be involved in the pathogenesis of thyroid autoimmune diseases and thyroid cancer (Ohye and Sugawara, 2010).

There is evidence that TGF-β stimulates ROS production in different cell types. The increase of ROS caused by TGF-β would be associated to various cellular events such as EMT, fibrosis, apoptosis and cell senescence, depending on the cell type (Junn et al., 2000; Proell et al., 2007; Yoon et al., 2005).

Several lines of evidence have shown that TGF-β1 can trigger numerous effects on thyroid cells. However, little is known about the effects of TGF-β isoforms on redox balance in cells where the peroxide has a central role. In the present study we have analyzed the influence of the three isoforms of TGF-β in human thyroid follicular carcinoma cells (WRO) and differentiated rat thyroid cells (FRTL-5). We have compared the response of both cell lines by determining parameters such as cell cycle, proliferation, viability, ROS generation and its degradation and the role of selenium in reversing these effects. The aim of this study was to investigate if the cellular context, in terms of antioxidant status, is decisive on the sensitivity to TGF-β thyroid cells.

2. Materials and methods

2.1. Cell culture

The differentiated rat thyroid cells (FRTL-5) were cultured in Dulbecco's modified Eagle's medium (DMEN) (Gibco) supplemented with 5% fetal bovine serum (FBS; Natocor, Argentina), and six hormones mixture (6H: 1 nM hydrocortisone, 5 μg/mL transferrin, 10 ng/mL somatostatin, 10 ng/mL glycl-L-histidyl-L-lysine acetate, 1 mUI/mL TSH and 10 μg/mL insulin; Sigma) (Ambesi-Impiombato et al., 1980). The human thyroid carcinoma cells (WRO) were grown in RPMI-1640 medium (Gibco) supplemented with 10% FBS. Cells were harvested with TRYPSIN (0.25 trypsin 0.25%-EDTA 0.02% solution), seeded on 24 and 96 well plates or in Tissue-Culture 60 mm and 100 mm dishes for experimental purposes. All cells were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and were kept in humidified atmosphere of 5% CO2 at 37 °C.

2.2. Cell viability

Cell viability was assessed by the MTT assay (Green et al., 1984). FRTL-5 cells were seeded into 96-well plates at a density of 2×10^3 cells/well. After 2 days, the medium was changed and cells were treated with Human Recombinant TGF-β1, TGF-β2 and TGF-β3 (Sigma) at different concentrations. WRO cells were seeded into 96-well plates at a density of 1×10^3 cells/well. After 1 day, the medium was changed and cells were treated with TGF-β isoforms. Following incubation with TGF-β isoforms during 3 days, MTT (Sigma) was added to the medium at a concentration of 0.5 mg/mL. After 3 h the MTT solution was carefully removed and the purple crystals were solubilized using 150 μL DMSO per well. The absorbance was measured at 540 nm (Spectra Microplate Reader) and was calculated as percentage of absorbance with respect to control cells. Alternatively, cells were seeded into 60 mm dishes and treated with TGF-β isoforms (5 ng/mL) for 3 days. The number of viable cells was counted in a hemocytometer (Trypan Blue exclusion test) and the average cell number from four experiments by duplicated were determined.

2.3. Flow cytometric analysis of cell cycle distribution

Analysis was performed using a flow cytometer (BD FACSCalibur). Cell cycle distribution was determined by staining of cell nuclei with propidium iodide (0.05 mg/mL). Cells were detached from dishes by the addition of 0.25% trypsyn-0.02% EDTA and fixed in absolute ethanol. The percentage of cells in different phases of cell cycle was analyzed using the ModFit LT cell cycle analysis software. For the computer analysis, only signals from single cells were considered (10,000 cells/assay).

2.4. Analysis of Caspase-3 activity

Caspase-3 activity was determined with the Caspase-3 assay kit (Sigma CASP-3-C; Sigma-Aldrich), according to the manufacturer's instructions. This assay is based on the spectrophotometric detection of the Ac-DEVD-p-nitroaniline (pNA) substrate after cleavage. Cells were grown in 100 mm dishes, treated with TGF-β isoforms and harvested in lysis buffer (50 mM HEPES, 5 mM CHAPS, 5 mM DTT, pH 7.4). Lysates were clarified by centrifugation at 15,000xg for 10 min at 4 °C, and clear lysates containing 200 μg proteins were incubated with caspase-3 substrate at 37 °C for 3 h. The concentration of pNA released from the substrate was calculated from the absorbance values at 405 nm. The activity, expressed as picomoles of pNA per minute per milliliter, was calculated with a pNA calibration curve. A positive control of caspase-3 and an inhibitor of caspase-3 (200 mM inhibitor Acetyl-Asp-Glu-Val-Asp-al [AcDEVD-CHO]) were added to the plate.

2.5. Apoptosis assay by nuclear morphology

At the indicated times after TGF-β isoforms treatment, the cells were washed with PBS, and changes in cell morphology were examined by staining slides with the Hoechst dye 33,342 (5 μM/mL in PBS) reagent, during 10 min at room temperature. After staining, the slides were dried thoroughly, rinsed in PBS, and nuclear morphology was observed using a fluorescence microscope, Olympus BX50 F-3 (Olympus Optical Co, LTD, Japan). The percentage of apoptotic cells was determined by counting the number of nuclei showing chromatin condensation and fragmentation characteristic of apoptosis after observing a total of at least 200 cells.

2.6. Measurement of intracellular ROS level

The intracellular ROS level was measured using a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma). DCFH-DA is a nonpolar compound that is readily diffused into cells, where it is hydrolysed to the nonfluorescent polar derivative DCFH

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