Dysregulation of secretion of CXC α -chemokine CXCL10 in papillary thyroid cancer: modulation by peroxisome proliferator-activated receptor- γ agonists

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Abstract

In papillary thyroid carcinomas (PTCs), oncogenes activate a transcriptional program including the upregulation of CXCL10 chemokine, which stimulates proliferation and invasion. Furthermore, peroxisome proliferator-activated receptor- γ (PPAR γ) activators thiazolidinediones (TZDs) modulate CXCL10 secretion in normal thyroid follicular cells (TFC), and inhibit PTC growth. Until now, no study has evaluated the effect of cytokines on CXCL10 secretion in PTCs, nor the effect of PPAR γ activation. The combined effects of interferon γ (IFN γ) and tumor necrosis factor α (TNF α) stimulation on CXCL10 secretion in primary cells from PTCs and TFC were tested. Furthermore, the effect of PPAR γ activation by TZDs, on CXCL10 secretion and proliferation in these cell types was studied. In primary cultures of TFC and PTCs CXCL10 production was absent under basal conditions; a similar dose-dependent secretion of CXCL10 was induced by IFN γ in both cell types. TNF α alone induced a slight but significant CXCL10 secretion only in PTCs. The stimulation with IFN γ +TNF α induced a synergistic CXCL10 release in both cell types; however, a secretion more than ten times higher was induced in PTCs. Treatment of TFC with TZDs dose-dependently suppressed IFN γ +TNF α -induced CXCL10 release, while TZDs stimulated CXCL10 secretion in PTCs. A significant antiproliferative effect by TZDs was observed only in PTCs. In conclusion, a dysregulation of CXCL10 secretion has been shown in PTCs. In fact, a CXCL10 secretion more than ten times higher has been induced by IFN γ +TNF α in PTCs with respect to TFC. Moreover, TZDs inhibited CXCL10 secretion in TFC and stimulated it in PTCs. The effect of TZDs on CXCL10 was unrelated to the significant antiproliferative effect in PTCs.

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Introduction

Chemokines are chemoattractant cytokines that regulate the trafficking and activation of leukocytes and other cell types under a variety of inflammatory and noninflammatory conditions (Luster 1998).

Over the past few years, studies have increasingly shown that chemokines play an important role in several aspects of tumor progression. Tumor cells express functional chemokine receptors, which can sustain proliferation, angiogenesis and survival, and promote organ-specific localization of distant metastases.

Chemokine expression in human malignancies is associated with a leukocyte infiltration favoring the establishment of immune escape mechanisms. Relevant studies on preclinical testing of cancer therapies based on interference with the cancer chemokine network have been published (Ruffini et al. 2007).

CXC chemokines lacking the N-terminal ELR motif, such as interferon γ (IFN γ)-inducible protein 10 (IP-10/CXCL10), monokine induced by IFN γ (Mig/CXCL9) and IFN γ inducible T cell α -chemoattractant (ITAC/CXCL11), do not act as chemoattractants for neutrophils (Dewald et al. 1992, Loetscher et al. 1996, Mackay 1996), but attract activated T cells (Taub et al. 1993, Mackay 1996).

In the last few years, experimental evidence has accumulated supporting the concept that IFN γ -inducible chemokines (CXCL9, CXCL10 and CXCL11) and their receptor, CXCR3, play an important role in the initial stage of autoimmune disorders involving endocrine glands (García-López et al. 2001, Romagnani et al. 2002, Kemp et al. 2003, Antonelli et al. 2004, 2005). The fact that, after IFN γ stimulation, endocrine epithelial cells secrete CXCL10, which is able to induce the chemotaxis of type 1 T helper lymphocytes, that in turn secrete IFN γ , thus perpetuating the autoimmune process, strongly supports the concept that chemokines play an important role in endocrine autoimmunity (Antonelli et al. 2006).

In papillary thyroid carcinomas (PTCs), rearrangements of the RET receptor (RET/PTC) and activating mutations in the BRAF or RAS oncogenes lead to the upregulation of the CXCL10 chemokine, which in turn stimulates proliferation and invasion (Melillo et al. 2005). Other studies have obtained similar results (Puxeddu et al. 2005).

Furthermore, it has been shown recently that high basal levels of Toll-like receptor 3 (TLR3) and noncanonical WNT5a RNA are present in PTCs cell lines consistent with their overexpression and colocalization in PTC cells *in vivo*, and that the basally expressed TLR3 are functional in PTC cells, as evidenced by the ability of double-strand RNA (polyinosine–polycytidylic acid) to increase significantly the levels of the end product of TLR3 signaling CXCL10 (McCall et al. 2007).

Recently, the presence of peroxisome proliferator-activated receptor- γ (PPAR γ) has been demonstrated in thyroid tissue, and PPAR γ has been shown to be involved in the modulation of inflammatory responses (Antonelli et al. 2006). In fact, the treatment of normal thyroid follicular cells (TFC) with a pure PPAR γ activator, rosiglitazone (RGZ), at near-therapeutic

doses, significantly inhibited IFN γ -stimulated CXCL10 secretion, strongly suggesting that PPAR γ might be involved in the regulation of IFN γ -induced chemokine expression in human thyroid autoimmunity (Antonelli et al. 2006).

Moreover, PPAR γ expression appears important in thyroid cancerogenesis and tumor progression, in fact PPAR γ is considered a tumor suppressor gene. In follicular thyroid carcinomas, the PAX8/PPAR γ fusion oncogene appears to suppress the activity of the wild-type gene (Kroll et al. 2000). The antitumor effect of PPAR γ agonists is probably due to transactivating genes that regulate cell proliferation, apoptosis and differentiation. Agonists of PPAR γ had an antiproliferative action in six cell lines, originated from patients with papillary thyroid cancer which expressed PPAR γ , inducing apoptosis (Ohta et al. 2001), significantly inhibited tumor growth and prevented distant metastasis of thyroid cancer in nude mice. Furthermore, it has been shown that troglitazone significantly inhibited cell growth by cell cycle arrest and apoptotic cell death (Park et al. 2005). More recently, we have shown in primary cultured human anaplastic and papillary dedifferentiated thyroid cancers cells the antiproliferative effect of PPAR γ agonists thiazolidinediones (TZDs; Antonelli et al. 2008a,b).

Until now, to our knowledge, no data are present in the literature about the regulation by IFN γ and tumor necrosis factor α (TNF α) of CXCL10 secretion in PTC, nor of PPAR γ activators effect on these chemokines.

The aims of this study were firstly, to test the effect of IFN γ stimulation on the secretion of the CXC α -chemokine CXCL10 in primary cultures of cells obtained from PTC thyrocytes, in comparison with normal thyroid tissue; and secondly, to assess the effect of PPAR γ activation on CXCL10 secretion in these cell types, correlating this effect to the antiproliferative effect of PPAR γ activators.

Materials and methods

Patients source for thyroid tissue

Surgical thyroid tissue were obtained from six patients with PTC at the time of surgery. In addition, normal thyroid tissue was obtained from six patients (four undergoing parathyroidectomy, two to laryngeal intervention). The diagnosis was established on commonly accepted clinical, laboratory, and histological criteria. Immunohistochemistry showed the presence of expression of TSH receptor, thyroperoxidase, thyroglobulin and sodium/iodide symporter.

The study subjects gave their informed consent to the study, which was approved by the local ethical committee.

Microdissection and DNA extraction were performed using conventional methods previously described (Antonelli *et al.* 2008a, 2009).

Detection of BRAF mutation by PCR single strand conformation polymorphism and direct DNA sequencing were performed using conventional methods previously described (Antonelli *et al.* 2008a, 2009).

Thyroid follicular cells

Thyrocytes were prepared as previously reported (García-López *et al.* 2001, Antonelli *et al.* 2006). The specimens were minced with scissors and digested by collagenase (1 mg/ml; Roche) in RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, MD, USA) for 1 h at 37 °C. Semi-digested follicles were removed, sedimented for 2 min, washed, and cultured in RPMI 1640 medium supplemented with 10% v/v FBS (fetal bovine serum; Seromed, Biochrom, Berlin, Germany), 2 mM glutamine, and 50 µg/ml penicillin/streptomycin at 37 °C and 5% CO₂ in plastic 75 cm² flasks (Sarstedt, Verona, Italy).

CXCL10 secretion assays

For CXCL10 secretion assays, 3000 cells were seeded onto 96-well plates in growth medium. After 24 h, the growth medium was removed, cells were accurately washed in PBS, and incubated in phenol red and serum-free medium. Cells were incubated (24 h) with IFN γ (R&D Systems, Minneapolis, MN, USA; 500, 1000, 5000, 10 000 U/ml) and 10 ng/ml TNF α (R&D Systems), alone or in combination. The concentration of TNF α was selected in preliminary experiments to yield the highest responses. After 24 h, the supernatant was removed and kept frozen at –20 °C until CXCL10 assay.

To investigate the effect of PPAR γ activators on IFN γ -induced chemokine secretion, cells were stimulated (24 h) with IFN γ (1000 U/ml) and TNF α (10 ng/ml) in the absence or presence of increasing concentrations (0, 1, 10, 20 µmol/l) of the pure PPAR γ agonists, RGZ (Glaxo, Welwyn, UK), or pioglitazone (Alexis Biochemicals, Lausen, Switzerland), and conditioned medium were assayed by ELISA for CXCL10 concentrations. All experiments were repeated three times with the different cell preparations.

Cell cultures and TZDs treatment

Cultures of thyrocytes were treated (24 h) with 10 or 20 µmol/l, RGZ or pioglitazone. Control cultures were grown (24 h) in the same medium containing vehicle (absolute ethanol, 0.47% v/v) without RGZ, or pioglitazone. Some cultures were examined by phase contrast microscopy using an Olympus IX50.

For quantitation of total protein in cell preparations, lysis and homogenization were performed and the sample was immediately assayed for its protein concentration by conventional methods.

Cell viability test

The number of viable cells was evaluated by a viability and proliferation assay, based on the cleavage of tetrazolium salts added to the culture medium (Cell Proliferation Reagent WST-1; Roche; Antonelli *et al.* 2008a,b,c, 2009).

The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample, which leads to an increase in the amount of formazan dye formed, that directly correlates to the number of metabolically active cells in the culture (Antonelli *et al.* 2008a,b,c, 2009). The formazan dye produced by metabolically active cells was quantified by a scanning multiwell spectrophotometer (ELISA reader) by measuring the absorbance of the dye solution at 450 nm. Cells were seeded in a 96 wells microtiter plate at a concentration of 35 000 cells/ml in a final volume of 100 µl in each well. Then, they were incubated for 48 h with PPAR γ activators, in a humidified atmosphere (37 °C, 5% CO₂).

After the incubation period, 10 µl of the Cell Proliferation Reagent WST-1 were added to 100 µl of culture medium in each well and the absorbance of the samples was measured at 450 nm against the control (the same cells without any treatment) using a microtiter plate (ELISA) reader. The same volume of culture medium and Cell Proliferation Reagent WST-1 (10 µl of Cell Proliferation Reagent WST-1/100 µl of culture medium) was added into one well, in order to use this background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader.

The absorbance was measured again after 1 and 2 h.

The measured absorbance of blank was subtracted from control and treatments and the control was normalized to 100% for each assay; treatments were expressed as % of the control.

The experiments were conducted in triplicate for each sample.

Proliferation assay: cell counting

Since MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] measures mitochondrial cell activity and it has already been demonstrated that there is not always a direct relationship with cell number, the proliferation was evaluated using cell number counting (Gutiérrez-Cañas et al. 2003), as well. Cells were seeded at a density of 13 000 cells per well in 24-well tissue culture plates in medium supplemented with 10% v/v FBS with or without the indicated factors (Antonelli et al. 2008a,b,c, 2009).

The medium was changed every other day. After 72 h in an atmosphere of 5% CO₂ at 37 °C, cells were detached from plates by incubation with 500 ml PBS containing 100 mg trypsin and 1 mmol/l EDTA. Cells were counted using a hemocytometer.

ELISA for CXCL10

CXCL10 levels were measured in culture supernatants using commercially available kits (R&D Systems). The mean minimum detectable dose was 1.67 pg/ml for CXCL10; the intra- and inter-assay coefficients of variation were 3.0 and 6.9%. Samples were assayed in duplicate. Quality control pools of low, normal or high concentration for all parameters were included in each assay.

Quantitative real-time PCR for TLR3 and WNT5a

Total RNA from the cells was extracted with the RNeasy Mini reagent kit according to the manufacturer's recommendations (Qiagen). TaqMan Reverse Transcription Reagents kit and Universal PCR SYBR Green were from Applied Biosystems (Foster City, CA, USA). Quantitative PCR human reference total RNA was purchased from Stratagene (La Jolla, CA, USA). Primers for the WNT5A (NM_003392.3; forward: CAGGACGGTGTACAA-CCTGG, reverse: CTTCTCCTTCAGGGCATCAC), for TLR3 (NM_003265.2; forward: TCAAATT-AAAGAGTTTTCTCCAGG, reverse: GCTTCTCTGT-AAGGCTGGGA) and for GAPDH (forward: TGCACCACCAACTGCTTAGC and reverse: GGCATGGACTGTGGTCATGAG) were designed using Primer Blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Total RNA (400 ng) was reverse transcribed using TaqMan Reverse Transcription Reagents kit. Reverse transcription (RT) was

performed in a final volume of 80 µl containing 500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl (pH 8.3), 5.5 mM MgCl₂, 500 µM of each dNTP, 2.5 µM random examers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Multiscribe Reverse Transcriptase. The RT reaction was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 3 min. Measurement of gene expression was performed by quantitative real-time PCR (TaqMan). For each sample, 12.5 ng of cDNA were added to 10 µl of PCR mix containing each primer set and 1 × Universal PCR SYBR Green. The samples were then subjected to 40 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s in the ABI Prism 7700 Sequence Detector (Applied Biosystems). The amount of target, normalized to the endogenous reference GAPDH and relative to a calibrator (quantitative PCR human reference total RNA), was given by the 2^{-ΔΔC_t} (Livak & Schmittgen 2001).

Apoptosis determination: Hoechst uptake

PTC cells were seeded in a 96-wells microtiter plate at a concentration of 35 000 cells/ml in a final volume of 100 µl in each well. Then, cultures were incubated for 48 h with PPARγ activators (10 or 20 µmol/l, RGZ or pioglitazone), in a humidified atmosphere (37 °C, 5% CO₂).

After 48 h of treatments, the cells were stained with 5 µg/ml of Hoechst 33342 for 10 min at 37 °C. At the end of the incubation time, adherent cells were collected and analyzed using a hemocytometer under a fluorescence microscope. Cells that incorporated the Hoechst dye and showed typical morphological apoptotic features, such as chromatin condensation, were considered apoptotic cells according to Schmid et al. (2007).

The apoptosis index (ratio between apoptotic and total cells) × 100, was calculated. Data were analyzed by one-way ANOVA with Newman-Keuls multiple comparisons test.

Apoptosis determination: annexin V binding assay

The cells were plated in Lab-tekII Chamber Slide System (Nalge Nunc International, Roskilde, Denmark), treated with 10 or 20 µmol/l, RGZ or pioglitazone for 48 h. At the end of the incubation time, the cells were stained using the Annexin V-FIT Fluorescence Microscopy Kit (BD Biosciences, San Jose, CA, USA) following manufacturer's instruction, and observed under the fluorescence microscope (Leica). Images were captured by an online Leica DFC320 camera.

Nuclear extracts preparation

PTC and TFC cells were seeded in cell culture dishes at a concentration of 200 000 cells/ml in a final volume of 10 ml. Then, cells were treated (1 h) with IFN γ (1000 U/ml) and TNF α (10 ng/ml) in the absence or presence of 10 or 20 μ mol/l RGZ or pioglitazone.

Nuclear extracts (nucleosol) were prepared according to the protocol of [Andrews & Faller \(1991\)](#), with some modifications.

All steps were performed on ice, with ice-cold reagent. Treated and untreated cells were collected in PBS and centrifugated at 1000 *g* for 2 min. The pellets were resuspended with buffer B (HEPES 10 mM pH 7.9, KCl 10 mM, MgCl₂ 2 mM and EDTA 200 μ M, Triton X-100) 1%, with protease inhibitors (leupeptin 4 μ g/ml, aprotinine 1 μ g/ml and phenylmethylsulphonyl fluoride (PMSF) 1 mM) and phosphatase inhibitors (NaF 50 mM, Na₃VO₄ 0.1 mM and β -glycerolphosphate 20 mM), left for 30 min on ice and centrifugated subsequently for 1 min at 10 000 *g*.

For the nuclear lysis, samples were incubated in agitation at 4 °C for 20–30 min in extraction buffer (HEPES 10 mM pH 7.9, NaCl 600 mM, 2MgCl₂ 1 mM and EDTA 200 μ M, glycerol 25%) with protease inhibitors (leupeptin 4 μ g/ml, aprotinine 1 μ g/ml and PMSF 1 mM) and phosphatase inhibitors (NaF 50 mM, Na₃VO₄ 0.1 mM and β -glycerolphosphate 20 mM) and then centrifugated at 20 000 *g* for 30 min. The supernatants were retained for use in the DNA binding assay.

Protein concentration was determined by the BCA protein assay (Sigma).

Electrophoretic mobility shift assay

DNA binding reactions were performed in a 30 μ l reaction volume with 20 μ g of nuclear protein, double-stranded biotin-labeled probe (canonical NF- κ B binding sequence: 5' TTGGCAACGGCAGGG-GAATCCCCCTCTCCTTA 3'; Primm, Milan, Italy), and acetylated BSA in a Binding Buffer 5 $\times$ (glycerol 20%, MgCl₂ 5 mM, EDTA 2.5 mM, dithiothreitol (DTT) 2.5 mM, NaCl 250 mM, Tris-HCl 50 mM, pH 7.5 and poly (dI-dC) 0.25 mg/ml). Binding reactions were allowed to proceed for 30 min on ice and samples were loaded onto nondenaturing 6% polyacrylamide gels. After electrophoresis, the gel was transferred on nylon membrane Hybond N (Amersham Biosciences Europe GmbH). The membrane was washed with SSC 2 $\times$ (NaCl 0.3 M and Na citrate 0.03 M) for 5 min, then left to dry to the air and exposed to u.v. for 45 s. Blocking step was then performed incubating the membrane 'overnight' at room temperature with

a solution composed of Dendhart's 10 $\times$ (BSA 0.25%, Ficoll 400 0.25%, polivinilpirolidone 0.25%, SSC 6 $\times$, SDS 0.1%).

Detection of the biotinylated sequence was performed by using a chemiluminescence method with 'Phototope – Star Detection kit' (New England BioLabs, Inc., Ipswich, MA, USA) following the manufacturers instruction. Briefly, biotinylated DNA was detected on a membrane support by first exposing the membrane to streptavidin. Next, biotinylated alkaline phosphatase is added, resulting in the creation of a conjugate between the alkaline phosphatase and the DNA on the membrane. In final step, CDP – Star reagent was added. The acquisitions of the images were carried out with Chemi Doc (Bio-Rad Laboratories, Inc).

For competitor studies, a 100-fold excess of not-biotinylated probes were preincubated at room temperature for 10 min.

Immunoblotting

Papillary thyroid cancer cells were seeded in cell culture dishes at a concentration of 200 000 cells/ml in a final volume of 10 ml. Then, cells were treated (24 h) with IFN γ (1000 U/ml) and TNF α (10 ng/ml) in the absence or presence of 10 or 20 μ mol/l RGZ or pioglitazone.

At the end of the treatments, cells were washed with PBS and collected in lysis buffer (25 mM HEPES, pH 7.7, 400 mM NaCl, 0.5% Triton X-100, 1.5 mM MgCl₂, 2 mM DTT), protease inhibitors (leupeptin 4 μ g/ml, aprotinine 1 μ g/ml and PMSF 1 mM) and phosphatase inhibitors (NaF 50 mM, Na₃VO₄ 0.1 mM and β -glycerolphosphate 20 mM).

SDS-PAGE was performed essentially using the method of [Laemmli \(1970\)](#), with 5% acrylamide for the stacking gel and 12% for the separating gel. The proteins were transferred to nitrocellulose sheets (Bio-Rad Laboratories) following the methods of [Towbin *et al.* \(1979\)](#). Immunodetection was performed employing anti p-extracellular signal-regulated kinases (ERK) 1/2 and anti ERK1/2 (Cell Signaling, Danvers, MA, USA) and was revealed by peroxidase-labeled anti-rabbit IgG (Sigma) and BM Chemiluminescence Blotting Substrate (POD; Roche). The acquisitions of the images were carried out with Chemi Doc (Bio-Rad Laboratories, Inc).

Data analysis

Values are given as mean $\pm$ s.d. for normally distributed variables, otherwise as median and (inter-quartile range). Mean group values were compared by using one-way ANOVA for normally distributed

variables, otherwise by the Mann–Whitney *U* or Kruskal–Wallis test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni–Dunn test.

Results

In primary normal TFC cultures, CXCL10 was undetectable in the supernatant. IFN γ dose-dependently induced the CXCL10 secretion (Fig. 1A), whereas TNF α alone had no effect. However, the combination of TNF α and IFN γ had a significant synergistic effect on the CXCL10 release (Fig. 2A). Treatment of thyrocytes with RGZ, added at the time of IFN γ and TNF α stimulation, dose-dependently inhibited CXCL10 release (Fig. 3A). A similar effect was observed with pioglitazone (Fig. 4A). RGZ or pioglitazone alone had no effect and did not affect cell vitality or total protein content (data not shown).

Figure 1 CXCL10 release from normal thyroid follicular cells (A) and from papillary thyroid cancer cells (B) was absent under basal conditions (0) and was significantly stimulated by increasing doses of IFN γ ($P < 0.0001$ by ANOVA). The pattern of IFN γ stimulation was similar in normal thyrocytes and in papillary thyroid cancer cells. Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less versus 0, and †not significantly different from the preceding dose by Bonferroni–Dunn test.

Figure 2 The combination of TNF α and IFN γ (1000 U/ml) had a significant synergistic effect on CXCL10 (A) release from normal thyroid follicular cells ($P < 0.001$ by ANOVA). The combination of TNF α and IFN γ (1000 U/ml) had a significant synergistic effect on CXCL10 release from papillary thyroid cancer cells, too ($P < 0.001$ by ANOVA) (B). The effect of TNF α and IFN γ on papillary thyroid cancer cells is more than ten times higher than in normal thyroid cells ($P < 0.001$ by ANOVA). Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less.

The data obtained with TFC from normal thyroid tissue were different from those obtained from PTC patients. In PTC thyrocytes, CXCL10 was undetectable in the supernatant. IFN γ dose-dependently induced the CXCL10 release (Fig. 1B); the pattern of IFN γ stimulation was similar to that observed in normal thyrocytes. TNF α alone in PTC cells had a slight but significant effect (with TNF α 10 ng/ml; CXCL10, 26 ± 15 pg/ml, versus 0 in controls; $P < 0.001$, by ANOVA). The combination of TNF α and IFN γ had a significant synergistic effect on the CXCL10 secretion (Fig. 2B). The synergistic effect of TNF α and IFN γ was significantly stronger on the CXCL10 secretion in PTC cells than in normal thyrocytes ($P < 0.001$; by ANOVA).

Treatment of PTC thyrocytes with RGZ, added at the time of IFN γ and TNF α stimulation, dose-dependently stimulated CXCL10 release (Fig. 3B). A similar effect was observed with pioglitazone (Fig. 4B). RGZ or pioglitazone alone had no effect

Figure 3 Increasing doses of rosiglitazone (10, 20 μ mol/l) inhibit CXCL10 (A) release from thyroid follicular cells stimulated with IFN γ (1000 U/ml) and TNF α (10 ng/ml), but stimulated CXCL10 release in papillary thyroid cancer cells (B) ($P < 0.01$ by ANOVA). * $P < 0.05$ or less versus 0, and **significantly different from the preceding dose by Bonferroni–Dunn test.

and did not affect cell vitality or total protein content (data not shown).

The results of WST-1 assay in normal TFCs with 10 and 20 μ mol/l RGZ showed a slight but not significant reduction of proliferation with respect to the control both at 1 h (data not shown) and at 2 h (Fig. 5A). Cells counting confirmed the above mentioned results. In control TFCs the cell number, at 2 h, was $19\,350 \pm 580/100\ \mu$ l, per well; after the treatments, the cell number was (at 2 h from the start of tetrazolium reaction) $17\,635 \pm 588$ (91%, with respect to the control) with RGZ 10 μ mol/l; $17\,211 \pm 591$ (89%) with RGZ 20 μ mol/l. The results of WST-1 assay in PTC cells showed a slight but significant reduction of proliferation with respect to the control with

10 and 20 μ mol/l RGZ at 1 h (data not shown) and at 2 h (Fig. 5B). Cells counting confirmed the above mentioned results at 2 h. In control PTC the cell number was $17\,830 \pm 1710/100\ \mu$ l per well; $14\,978 \pm 629$ (84%) with RGZ 10 μ mol/l; $12\,841 \pm 2321$ (72%) with RGZ 20 μ mol/l.

Similarly, the results of WST-1 assay in normal TFCs with 10 and 20 μ mol/l pioglitazone showed a slight but not significant reduction of proliferation with respect to the control both at 1 h (data not shown) and at 2 h (Fig. 6A). Cells counting confirmed the above mentioned results. In control TFCs the cell number, at 2 h, was $19\,520 \pm 650/100\ \mu$ l, per well; after the treatments, the cell number was: (at 2 h from the start of tetrazolium reaction) $17\,954 \pm 665$ (92%, with respect to the control) with pioglitazone 10 μ mol/l;

Figure 4 Increasing doses of pioglitazone (10, 20 μ mol/l) inhibit CXCL10 (A) release from thyroid follicular cells stimulated with IFN γ (1000 U/ml) and TNF α (10 ng/ml), but stimulated CXCL10 release in papillary thyroid cancer cells (B) ($P < 0.01$ by ANOVA). Bars are mean $\pm$ s.e.m. * $P < 0.05$ or less versus 0.

Figure 5 The results of WST-1 assay (at 2 h from the start of tetrazolium reaction) (mean $\pm$ S.E.M. of all samples) showed a slight but not significant reduction of proliferation with respect to the control with rosiglitazone (after 24 h of incubation) 10 or 20 μ mol/l in normal thyroid follicular cells (A), while a significant ($P < 0.01$; ANOVA) reduction of proliferation was observed in papillary thyroid cancer cells (B). Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less versus 0.

16 820 $\pm$ 690 (90%) with pioglitazone 20 μ mol/l. The results of WST-1 assay in PTC cells showed a slight but significant reduction of proliferation with respect to the control with 10 and 20 μ mol/l pioglitazone at 1 h (data not shown) and at 2 h (Fig. 6B). Cells counting confirmed the above mentioned results at 2 h. In control PTC the cell number was 17 815 $\pm$ 839/100 μ l, per well; 14 786 $\pm$ 502 (83%) with pioglitazone RGZ 10 μ mol/l; 14 071 $\pm$ 499 (79%) with pioglitazone 20 μ mol/l.

Apoptosis index was determined in PTC thyrocytes by the Hoechst methods: cells incorporating the dye and showing typical morphological features were considered as apoptotic. Both control and cells treated with RGZ (10 and 20 μ mol/l for 48 h) were stained with fluorescent Hoechst dye. The percentage of apoptotic cells increased markedly in a dose-dependent manner: after treatment with RGZ 10 μ mol/l, 11.8% of the cells were apoptotic and such a percentage increased up to more than 19% with RGZ 20 μ mol/l ($P < 0.001$; by ANOVA; Fig. 7). At the pioglitazone

lower dose 8% of the cells were apoptotic, and this percentage increased up to more than 10% with pioglitazone 20 μ mol/l ($P < 0.01$; by ANOVA). Annexin V was used to further confirm the induced cell apoptosis (merged panels, Fig. 7).

In order to explain the inhibition exerted by PPAR γ agonists on CXCL10 secretion, the effect of TZDs on NF- κ B activation in the PTC cells has been evaluated, by electrophoretic mobility shift assay (Fig. 8). The untreated PTC cells showed a constitutive activation of NF- κ B (lane 1–2) and the treatment with IFN γ and TNF α enhanced the DNA binding activity of NF- κ B (lane 3–4). The treatments with RGZ or pioglitazone (20 μ mol/l) significantly reduced the IFN γ and TNF α activation of NF- κ B (lane 5–6 and 7–8 respectively) and, although to a less extent, even the treatment with the lower dose (10 μ mol/l) inhibited the nuclear translocation of such transcription factor (lane 9 and 10). Normal TFC did not show a constitutive activation of NF- κ B, which was induced by IFN γ and TNF α , and inhibited by PPAR γ agonists (data not shown).

Figure 6 The results of WST-1 assay (at 2 h from the start of tetrazolium reaction) (mean $\pm$ S.E.M. of all samples) showed a slight but not significant reduction of proliferation with respect to the control with pioglitazone (after 24 h of incubation) 10 or 20 μ mol/l in normal thyroid follicular cells (A), while a significant ($P < 0.01$; ANOVA) reduction of proliferation was observed in papillary thyroid cancer cells (B). Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less versus 0.

Figure 7 Apoptosis in papillary thyroid cancer cells treated with rosiglitazone 10 (ROSI10) and 20 $\mu\text{mol/l}$ (ROSI20) for 48 h. Apoptosis index was determined by Hoechst staining (see Materials and methods). The percentage of apoptotic cells increased markedly in a dose-dependent manner: after treatment with RGZ 10 $\mu\text{mol/l}$, 11.8% of the cells were apoptotic and such a percentage increased up to more than 19% with RGZ 20 $\mu\text{mol/l}$ ($P < 0.001$; by ANOVA). Data are expressed as means $\pm$ s.d. ($n = 5$). Data were analyzed by one-way ANOVA with Newman–Keuls multiple comparisons test and with a test for linear trend ($P < 0.001$, for ROSI20 versus ROSI10 or control; $P < 0.001$ for ROSI10 versus control). The merged panels show a representative example of annexin V binding assay in treated and control cells.

As far as the MAP-kinase ERK1/2, there were no significant differences neither in the level of ERK1/2 phosphorylation or in the amount of not-phosphorylated protein expression between untreated and treated PTC cells (data not shown).

The ^{V600E}BRAF mutation was observed in three PTC. The results about the stimulation with IFN γ and/or TNF α on the chemokines secretion, the modulation of chemokines secretion by RGZ and/or pioglitazone, and of the inhibition of proliferation by PPAR γ activators, obtained in PTC from tumors with ^{V600E}BRAF mutation, were similar to those from tumors without BRAF mutations (data not shown).

TLR3 and WNT5a mRNAs were detectable in all primary cells derived from papillary carcinomas. According to real time RT-PCR results, the TLR3 expression versus the reference gene (GAPDH) ranges from 0.8 to 1.2, while the expression of WNT5a from 4.15 to 4.90.

Discussion

The results of the present study demonstrate that: firstly, both IFN γ and TNF α (synergistically with IFN γ) dose-dependently induce an exaggerated release of CXCL10 by primary thyrocytes from PTC, with respect to normal TFC; and secondly,

the PPAR γ agonists, RGZ and pioglitazone, exert a dose-dependent inhibition of proliferation of PTC cells, but (in contrast to normal TFC) have stimulatory effect on CXCL10 release.

Our data are in agreement with those found in other studies that demonstrate an overexpression of CXCL10 in PTC. In PTCs, rearrangements of the RET receptor (RET/PTC) and activating mutations in the BRAF or RAS oncogenes are mutually exclusive. It has been shown that the three proteins function along a linear oncogenic signaling cascade in which RET/PTC induces RAS-dependent BRAF activation and RAS- and BRAF-dependent ERK activation. Adoptive activation of the RET/PTC–RAS–BRAF axis induced cell proliferation of thyroid follicular cells. Gene expression profiling revealed that the three oncogenes activate a common transcriptional program in thyroid cells that includes upregulation of the CXCL1 and CXCL10 chemokines, which in turn stimulate proliferation and invasion (Melillo *et al.* 2005).

Furthermore, RET/PTC-induced gene expression in thyroid PCCL3 cells reveals early activation of genes involved in regulation of the immune response, among them CXCL10 (Puxeddu *et al.* 2005).

Figure 8 Electrophoretic mobility shift assay (EMSA) of papillary thyroid cancer cells nuclear extracts: (A) lane 1–2, constitutive NF- κ B binding activity in papillary thyroid cancer cells; lane 3–4, treatment with 'IFN γ (1000 U/ml) and TNF α (10 ng/ml)' (IFN γ + TNF α); lane 5–6, treatment with IFN γ + TNF α and pioglitazone (20 $\mu\text{mol/l}$); lane 7–8, treatment with IFN γ + TNF α and rosiglitazone (20 $\mu\text{mol/l}$); lane 9, treatment with IFN γ + TNF α and rosiglitazone (10 $\mu\text{mol/l}$); lane 10, treatment with IFN γ + TNF α and pioglitazone (10 $\mu\text{mol/l}$). The untreated PTC cells showed a constitutive activation of NF- κ B (lane 1–2) and the treatment with IFN γ + TNF α enhanced the DNA binding activity of NF- κ B (lane 3–4). The treatments with RGZ or pioglitazone (20 $\mu\text{mol/l}$) significantly reduced the IFN γ + TNF α activation of NF- κ B (lane 5–6 and 7–8 respectively) and, although to a less extent, even the treatment with the lower dose (10 $\mu\text{mol/l}$) inhibited the nuclear translocation of such transcription factor (lane 9 and 10). (B) This figure shows the competition test (nuclear extracts were preincubated with a 100-fold molar excess of unlabeled probe): lane 1, constitutive NF- κ B in control cells; lane 2, treatment with IFN γ + TNF α ; lane 3, treatment with IFN γ + TNF α and pioglitazone (20 $\mu\text{mol/l}$); lane 4, treatment with IFN γ + TNF α and rosiglitazone (20 $\mu\text{mol/l}$); lane 5, treatment with IFN γ + TNF α and pioglitazone (10 $\mu\text{mol/l}$); lane 6, treatment with IFN γ + TNF α and rosiglitazone (10 $\mu\text{mol/l}$).

The results of our study confirm that TLR3 and WNT5a are expressed in primary cells derived from PTC (McCall *et al.* 2007, Schweppe *et al.* 2008). WNT5a levels in PTC cells may be related to high TLR3 levels and signaling. The basally expressed TLR3 are functional in PTC cells as evidenced by the ability of double-strand RNA (polyinosine-polycytidylic acid) to increase significantly the activity of transfected NF- κ B and IFN β luciferase reporter genes and the levels of two end products of TLR3 signaling, IFN β and CXCL10. This may explain, at least in part, the mechanism of the induction of CXCL10 secretion by cytokines in PTC (McCall *et al.* 2007).

In our study, the synergistic effect of TNF α and IFN γ on CXCL10 release was more than ten times higher in PTC cells than in normal thyrocytes, confirming that the genetic abnormalities underlying the neoplastic transformation in PTC lead to an overexpression of CXCL10, that is functionally expressed in an exaggerated response of the chemokine under the influence of the stimulatory cytokines IFN γ and TNF α .

The modest but significant increase of CXCL10 after stimulation with TNF α alone may also be due to the above mentioned genetic abnormalities.

In the last few years, it has been increasingly shown that chemokines play an important role in tumor progression and chemokine receptors can sustain proliferation, angiogenesis and survival, and promote organ-specific localization of distant metastases. The expression of chemokines in human malignancies is associated with a leukocyte infiltration and with immune escape mechanisms. Important studies have been conducted on the preclinical testing of cancer therapies based on interference with the cancer chemokine network. For example, the blockade of agents and antibodies against CXCR4 prevent the establishment of metastasis; in mouse models, overexpression of selected chemokines causes tumor infiltration by distinct leukocyte subsets, resulting in tumor regression and tumor-specific immunity generation; moreover, chemokines have also been successfully used as carriers and/or adjuvants for cancer vaccines (Ruffini *et al.* 2007, Yang *et al.* 2008).

The cancer chemokine network is a multifaceted therapeutic target. CXCL10 chemokine stimulates proliferation and invasion in PTC (Melillo *et al.* 2005); in this view, agents that prevent CXCL10 production or action should be tested in PTC refractory to traditional treatments.

PPAR γ has recently been shown to modulate inflammatory responses in many cell types, such as

endothelial cells (Marx *et al.* 2000, Gosset *et al.* 2001, Schaefer *et al.* 2005), dendritic cells (Gosset *et al.* 2001), and in other kinds of cells (Yang *et al.* 2008). Furthermore, we have recently shown (Antonelli *et al.* 2006) that treatment of TFC, orbital fibroblasts or preadipocytes with a pure PPAR γ activator, RGZ, significantly inhibited IFN γ -stimulated CXCL10 secretion, strongly suggesting that PPAR γ might be involved in the regulation of IFN γ -induced chemokine expression in human thyroid autoimmunity and Graves' ophthalmopathy. The results of our study confirm the above mentioned results in normal TFC, but first demonstrate that treatment of PTC cells with two pure PPAR γ activators, RGZ and pioglitazone, at near-therapeutical doses, increased the IFN γ -stimulated CXCL10 secretion, strongly reinforcing the hypothesis that PPAR γ might be involved in the regulation of IFN γ -induced chemokine expression in human thyroid cells (Schaefer *et al.* 2005).

With regard to the mechanism of action on chemokine secretion, PPAR γ activators may act in different ways: either, decreasing CXCL10 promoter activity and inhibiting protein binding to the two NF- κ B sites (Marx *et al.* 2000); or, TZDs reduce CXCL10 protein levels in a dose-dependent manner at concentrations (nanomolar) that did not affect mRNA levels or NF- κ B activation (Schaefer *et al.* 2005). More recently, it has been shown that the TZD effect is not only mediated by activation of the NF- κ B and Stat1 classic pathways, but also involves a rapid increase in phosphorylation and activation of ERK1/2 (Lombardi *et al.* 2008).

Our results in PTC cells show that NF- κ B is basally activated in PTC cells and that TZDs are able to inhibit the NF- κ B activation, in agreement with the effect observed by Marx *et al.* (2000). This result parallels the effect of TZDs on PTC proliferation, but is opposed to the effect of TZDs on CXCL10 secretion, suggesting that other pathways are involved in the chemokine modulation. Regards MAP-kinase ERK1/2, we were unable to show any significant differences either in the level of ERK1/2 phosphorylation or in the amount of not-phosphorylated protein expression between untreated and treated thyroid cells, in contrast to the results obtained in endothelial cells (Lombardi *et al.* 2008).

It can be hypothesized that, while PPAR γ activators have a physiologically inhibitory role on chemokine secretion, the derangement of the pathways regulating CXCL10 expression may lead to an unexpected stimulatory response in PTC.

PPAR γ are members of a superfamily of nuclear hormone receptors (Grommes *et al.* 2004). Activation

of PPAR γ isoforms elicits both anti-neoplastic (Grommes *et al.* 2004, Garcia-Bates *et al.* 2008) and anti-inflammatory effects (Antonelli *et al.* 2006) in several types of mammalian cells. Recently, it has been shown that agonists of PPAR γ induce apoptosis and exert antiproliferative effects on human papillary carcinoma cells (Ohta *et al.* 2001), prevent distant metastasis of BHP18–21 tumors in nude mice *in vivo* (Ohta *et al.* 2001), and induce redifferentiation in thyroid cancer cell lines (Klopper *et al.* 2004, Philips *et al.* 2004, Frohlich *et al.* 2005, Park *et al.* 2005, Aiello *et al.* 2006), demonstrating effective therapeutic agents for the treatment of patients with thyroid cancer that fail to respond to traditional treatments (Klopper *et al.* 2004, Philips *et al.* 2004, Frohlich *et al.* 2005, Park *et al.* 2005). Moreover, the expression of PPAR γ gene and protein were examined in five human anaplastic cancer cell lines (Hayashi *et al.* 2004). The five cell lines showed higher levels of the PPAR γ gene and protein expression than papillary thyroid cancer, and PPAR γ agonists down regulated their invasive potential (Hayashi *et al.* 2004). These results have been confirmed by other studies (Park *et al.* 2005).

Furthermore, we have recently shown in primary cultured human anaplastic and papillary dedifferentiated thyroid cancer cells the antiproliferative effect of PPAR γ agonists TZD (Antonelli *et al.* 2008a,b).

The results of our study fully confirmed that PPAR γ agonists are not able to exert an inhibitory role in normal TFC, while demonstrate a significant antiproliferative effect of TZDs in PTC primary cell cultures. Interestingly, the antiproliferative effect of PPAR γ activators in PTC is dissociated from the ability to inhibit the IFN γ -stimulated CXCL10 secretion: PPAR γ activators increase the IFN γ and TNF α stimulated CXCL10 secretion in PTC, in contrast to that observed in normal TFC, where an inhibitory role of TZDs was observed. The discrepancy between the stimulatory effect of TZDs on CXCL10 secretion and the inhibitory role on PTC proliferation is probably due to the different pathways involved.

In fact, TZDs mediate growth inhibition through induction of apoptosis. Our results confirm that both RGZ and pioglitazone are able to induce apoptosis in PTC cells. Even with regard to thyroid carcinoma, there is controversy on the induction of apoptosis by TZD in relation to the proto-oncogene, *c-myc*, or cell cycle inhibitor protein, p27 (Ohta *et al.* 2001, Martelli *et al.* 2002). Other findings suggested that PPAR γ agonists should induce apoptosis in thyroid carcinoma cell lines through up-regulation of Bax protein (Hayashi *et al.* 2004).

BRAF provides crucial signals for proliferation of thyroid carcinoma cells spontaneously harboring the ^{V600E}*BRAF* mutation and, therefore, *BRAF* suppression might have therapeutic potential in ^{V600E}*BRAF*-positive thyroid cancer (Salvatore *et al.* 2006, Benvenega 2008). Our results show that PPAR γ agonists act both in PTC with or without ^{V600E}*BRAF* mutation, suggesting that the effect of TZD is mediated by other mechanisms; furthermore, no significant effect of ^{V600E}*BRAF* mutation was observed on chemokine secretion. However, owing to the small number of patients (three with and three without *BRAF* mutation) further studies in larger series will be needed.

In conclusion, the present study demonstrates that IFN γ and TNF α (synergistically) induce a huge release of CXCL10 by primary thyrocytes from PTC. Since CXCL10 stimulate proliferation and invasion, agents that prevent CXCL10 production or action should be tested in PTC refractory to traditional treatments. A discrepancy between the stimulatory effect of TZDs on CXCL10 secretion and the inhibitory role on PTC proliferation is shown.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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