The paramount role of cytokines and chemokines in papillary thyroid cancer: a review and experimental results

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Abstract

Our study demonstrates that (C-X-C motif) ligand 9 and 11 (CXCL9, CXCL11) chemokines were absent basally in non-neoplastic thyroid (TFC) and papillary thyroid carcinoma (PTC) cells. Interferon (IFN)γ induced the chemokine secretion in TFC and PTC, while tumor necrosis factor (TNF)α induced it only in PTC. IFNγ+TNFα induced a synergistic chemokines release in PTC, and at a lower level in TFC. Peroxisome proliferator-activated receptor (PPAR)γ agonists suppressed dose-dependently IFNγ+TNFα-induced chemokine release in TFC, while stimulated it in PTC. PPARγ knocking down, by RNA interference technique in PTC cells, abolished the effect of PPARγ agonists on chemokines release. In PTC cells, PPARγ agonists reduced proliferation, and CXCL9 or CXCL11 (100 and 500 pg/mL) reduced proliferation and migration ($P < 0.01$, for all). In conclusion, in PTC cells: (a) IFNγ+TNFα induced a marked release of CXCL9 and CXCL11; (b) PPARγ agonists stimulated CXCL9 and CXCL11

secretion, while inhibited proliferation; (c) CXCL9 and CXCL11 inhibited proliferation and migration. The use of CXCL9 or CXCL11 as antineoplastic agents in PTC remains to be explored.

Highlights

• IFNγ and IFNγ+TNFα induce dose-dependently CXCL9 (and less CXCL11) in PTC cells.

• Rosi and Pio dose-dependently inhibit the PTC cells proliferation.

• Rosi and Pio (at variance of normal TFC) stimulate CXCL9 or CXCL11 secretion.

• CXCL9 or CXCL11 induce a significant antiproliferative effect in PTC cells.

• Chemokines induced by IFNγ (CXCL9 or CXCL11) inhibit migration in PTC cells.

Keywords

CXCL9
CXCL11
Papillary thyroid cancer
PPARγ
CXCL10

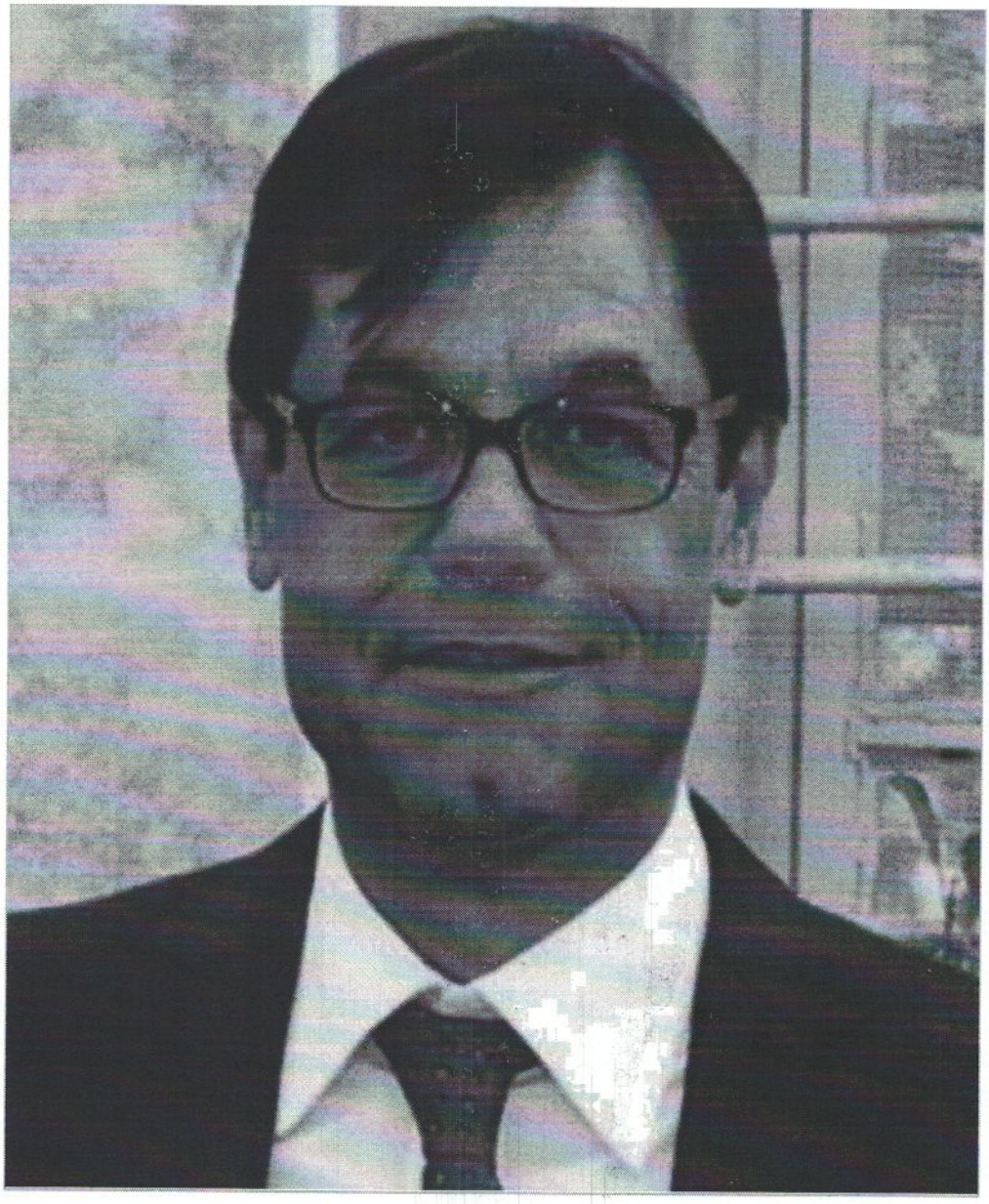
Take-home messages:

• The treatment with IFNγ+TNFα induces a marked release of CXCL9, and to a lesser extent of CXCL11, by primary thyrocytes from PTC. A discrepancy is present between the stimulatory role of PPARγ agonists on CXCL9 and CXCL11 release and the inhibitory effect on PTC proliferation. Furthermore, CXCL9 and CXCL11 are able to inhibit the proliferation and migration of primary PTC cells.

• The use of CXCL9 or CXCL11 as antineoplastic agents in PTC remains to be explored.

Introduction

Chemokines play a paramount role in tumor progression, angiogenesis, and metastasis [1, 2, 3, 4]. In papillary thyroid carcinoma (PTC), rearrangements of the RET receptor (RET/PTC) and activating mutations in BRAF or RAS oncogenes activate a common transcriptional program in thyroid cells that includes upregulation of the (C-X-C motif) ligand 10 (CXCL10) chemokine, which in turn stimulates proliferation and invasion [5]. Moreover, RET/PTC-induced gene expression shows an early activation of the genes involved in CXCL10 regulation in thyroid PCCL3 cells [6]. More recently, we have shown that a more than ten times higher CXCL10 secretion has been induced by interferon (IFN)γ+ tumor necrosis factor (TNF)α in PTCs in comparison to non-neoplastic thyroid follicular cells (TFC) [7]. Few other studies evaluated IFNγ-inducible chemokines in thyroid cancer [8, 9, 10]. However, to our knowledge, no study has evaluated CXCL9 and CXCL11 chemokines in thyroid cancer.



Recently, the presence of peroxisome proliferator-activated receptor (PPAR)γ has been reported in thyroid tissue [11, 12], and it has been shown that treating TFC cells with PPARγ activators, at near-therapeutical doses, IFNγ-stimulated CXCL10, CXCL9, and CXCL11 secretion was significantly inhibited [11, 12, 13, 14, 15, 16, 17]. Furthermore, recent data show that PPARγ are expressed in cell lines from patients with PTC [18, 19]. Agonists of PPARγ had an antiproliferative action in PTC cells, inducing apoptosis, and inhibited cancer cell growth preventing distant metastasis of thyroid carcinoma in nude mice [18, 19]. Moreover, we have recently shown the antiproliferative effect of PPARγ agonists in primary

cultured human anaplastic and papillary dedifferentiated thyroid cancer cells [20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33].

Furthermore, it has been shown that PPARγ agonists inhibited CXCL10 release in TFC and induced it in PTCs [7].

Tumoral cells, leukocytes, and platelets associated with the tumor produce several cytokines (IFNγ, TNFα) and chemokines [4, 24]. Expression studies showed an elevated expression of inflammatory molecules in PTCs, suggesting a proinflammatory relationship between thyroiditis and thyroid cancer [24]. Cytokines may have different effects on the tumor, being able to maintain the invasive phenotype in some studies [24]. However, other studies have shown that T helper 1 (Th1)-type cytokines, such as IFNγ and TNFα, increase the sensitivity of neoplastic thyrocytes to Fas ligand and TNF-related apoptosis-inducing ligand, inducing apoptosis [25].

Up to now, no data have been reported in literature CXCL9 and CXCL11 regulation in PTC by IFNγ and TNFα, nor of PPARγ agonists effect on these chemokines, or the effect of these chemokines on PTC proliferation.

In this study, we aimed to (a) test the effect of IFNγ and TNFα stimulation on the release of the CXC-α-chemokines, CXCL9 and CXCL11, in primary cell cultures established from PTC, with respect to non neoplastic thyroid tissue; (b) assess the effect of PPARγ activation on IFNγ-inducible CXCL9 and CXCL11 secretion and on proliferation in these cell types; (c) evaluate the effect of CXCL9 and CXCL11 on proliferation and migration of PTC thyrocytes.

Materials and methods

Thyroid tissue samples

Thyroid tissues were collected from 5 PTC patients during thyroidectomy and non-neoplastic tissues from 5 subjects (3 undergoing parathyroidectomy, 2 to laryngeal intervention). The accepted laboratory, histological and clinical criteria were used to establish the diagnosis [7].

Tumors were classified according to the thyroid malignancy World Health Organization classification and staged by the sixth edition of Tumor,

Node, Metastasis (TNM) staging (variant: classic, 4; follicular, 1) (pTNM; T1mN1, T3N1, T2N0, T1N1a, T2N0) [24].

The expression of sodium/iodide symporter (NIS), thyroperoxidase (TPO), thyrotropin (TSH) receptor, and thyroglobulin (Tg) was shown by immunohistochemistry.

The ethical committee of the University of Pisa approved the study, and the participating subjects gave their informed consent.

Microdissection and DNA extraction were performed according to conventional methods earlier described [26], such as detection of *BRAF* mutation by PCR-single strand conformation polymorphism (PCR-SSCP) and direct DNA sequencing [26].

Thyroid follicular cells

Thyrocytes were established as reported previously [7].

The specimens were minced with scissors and digested by collagenase (1 mg/mL; Roche Diagnostics, Almere, The Netherlands) in RPMI 1640 (Gibco, ThermoFisher Scientific, Waltham, MA, USA) for 1 h at 37 °C. Then, the cells were centrifuged for 2 min and seeded in medium RPMI 1640 with 50 mg/mL penicillin/streptomycin, 2 mM glutamine, and 10% *v/v* fetal bovine serum (Biochrom–Merck Millipore, Germany), and maintained at 37 °C and 5% CO_2.

Immunocytochemistry revealed the absence of cell contamination reporting the expression of TSH receptor, TPO, Tg, and NIS in the cultured cells [7].

CXCL9 and CXCL11 secretion assays

For CXCL9 and CXCL11 secretion assays, 3000 cells were seeded into 96-well plates in growth medium. After 24 h, growth media were removed, cells were washed in PBS (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and maintained in phenol red and serum-free medium. Cells were treated with IFNγ (R&D Systems, Minneapolis, MN, USA; 500, 1000, 5000, 10,000 IU/mL) and 10 ng/mL TNFα (R&D Systems), alone or in combination, for 24 h [11, 12, 13]. The

concentration of TNFα (10 ng/mL) was selected in preliminary experiments (with TNFα 0, 1, 5, 10 ng/mL) to yield the highest responses. After 24 h, supernatants were removed and kept frozen at − 20 °C until CXCL9 or CXCL11 assay.

To investigate the effect of PPARγ agonists on IFNγ-induced chemokine secretion, cells were treated for 24 h with IFNγ (1000 IU/mL) and TNFα (10 ng/mL) with/without increasing concentrations (10 or 20 μM) of the PPARγ agonists, pioglitazone (Pio; Alexis Biochemicals, Lausen, Switzerland) or rosiglitazone (Rosi; GlaxoSmithKline, Welwyn, UK). Culture media were tested for CXCL9 and CXCL11 concentrations by ELISA. The results have been normalized for the final cell number after 24 h.

siRNA transfection

To elucidate if the effects of the PPARγ ligands were mediated by their receptor, we decided to perform a PPARγ knocking down, by using RNA interference technique in PTC cells. Twenty-four hours before transfection, 1×10^5 cells were plated in a 6-multiwell plate in the growth medium without antibiotics, to obtain 50% confluence. PPARγ specific (Silencer Validated ID5636) and a scrambled negative control (Silencer Negative Control #1) oligonucleotides (60 nM) (Ambion, ThermoFisher Scientific, Waltham, MA, USA) were used to perform a 24-h transfection using Lipofectamine™ 2000, according to the manufacturer's instructions (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) [27, 28].

Cells were maintained for 24 h with oligonucleotides and TaqMan analysis was performed at different times from oligonucleotide removal to verify silencing efficacy and its time dependence.

For chemokine expression and secretion experiments, cells were starved after the oligonucleotide removal and stimulated 24 h with IFNγ (1000 IU/mL) plus TNFα (10 ng/mL), in the presence or absence of 20 μM Rosi or Pio.

Cell proliferation assays

We have tested the antiproliferative effects of PPARγ agonists, or of increasing concentrations of CXCL9 or CXCL11 (0, 100, 500 pg/mL;

Sigma-Aldrich) using:

(1) A proliferation assay [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (used in the MTT assay) assay (Cell Proliferation Reagent WST-1; Roche Diagnostics)] [29], as previously described [7, 20, 22]. Cells were plated (35,000 cells/mL in 100 μL/well in a 96-well microtiter plate) and treated with PPARγ agonists, or CXCL9 or CXCL11 (100 and 500 pg/mL), for 48 h, at 37 °C and 5% CO2. Then, 10 μL of WST-1 was added to 100 μL/well and absorbance was measured at 450 nm in comparison to the control (the same not treated cells), by a microtiter plate (ELISA) reader.

To have a background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader, 10 μL of WST-1 plus 100-μL culture medium were added to one well. The absorbance was evaluated also after 1 and 2 h, from the beginning of tetrazolium reaction. Blank absorbance was subtracted from the ones of control and treatments, and treatments were expressed as a percentage of the control, normalized to 100%.

The experiments were carried out for three times, and the inhibition of proliferation was calculated as the mean (vs. control) for each sample.

(2) Cell counting. MTT assay evaluates mitochondrial cell activity, which is not always directly correlated with the cell number. For this reason, proliferation was evaluated also using the cell counting, as previously described [20, 22, 30].

Cell migration assay

Cell migration was determined using Transwell Permeable Supports (Corning Life Sciences, NY, USA), according to manufacturer instructions [31, 32], with minor modifications [33]. Intracellular fluorescence was evaluated using a 96-well plate reader (ELISA reader; at 485 nm for excitation and 520 nm for emission).

A standard curve with different cell concentrations was prepared for each assay to convert the fluorescence values to the number of migrated cells.

ELISA for CXCL9 and CXCL11

CXCL9 and CXCL11 levels were measured in culture supernatants, using commercially available kits (R&D Systems), as earlier reported [13].

Real-time (RT-PCR) for toll-like receptor 3 (TLR3) and wingless type member 5A (WNT5a)

The RNeasy Mini reagent kit was used to extract the total RNA from cells, according to the manufacturer's recommendations (Quiagen, Italy). RT-PCR for *TLR3* and *WNT5a* were performed as previously reported [7]. TaqMan Reverse Transcription Reagents kit, used to reverse transcribed 400 ng of total RNA [7], and Universal PCR SYBR Green were purchased from Applied Biosystems (Life Technologies, Foster City, CA, USA). Quantitative PCR human reference total RNA was from Stratagene (La Jolla, CA, USA). Primers for the WNT5a~~A~~ (NM_003392.3; forward: CAGGACGGTGTACAACCTGG, reverse: CTTCTCCTTCAGGGCATCAC), for TLR3 (NM_003265.2; forward: TCAAATTAAAGAGTTTTCTCCAGG, reverse: GCTTCTCTGTAAGGCTGGGA) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)~~GAPDH~~ (forward: TGCACCACCAACTGCTTAGC and reverse: GGCATGGACTGTGGTCATGAG) were delineated by Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast /).

The amount of target, normalized to the endogenous reference ~~glyceraldehyde 3-phosphate dehydrogenase (~~GAPDH~~)~~ and relative to a calibrator (Quantitative PCR human reference total RNA), was expressed as the $2^{-\Delta\Delta CT}$ [7].

Apoptosis determination—Hoechst 33342 staining

PTC cells were plated (35,000 cells/mL in 100 μL/well in a 96-well microtiter plate) and treated for 48 h with PPARγ activators (10 or 20 μM, Rosi or Pio), at 37 °C and 5% CO_2.

Then, cells were stained with Hoechst 33342 (Sigma-Aldrich), as previously described [7].

The apoptosis index (ratio between apoptotic and total cells) × 100 was calculated.

Apoptosis detection—flow cytometry

For quantification of apoptosis, PTC cells were grown at a density of 50–60% confluence in 100-mm culture dishes and treated with 10 and 20 μM Rosi or Pio. The cells were trypsinized, washed with PBS, and processed for labeling with FITC-conjugated Annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). The labeled cells were analyzed by flow cytometry (FACS Calibur; BD Biosciences). The cellular status was defined as the following: unstained cells were classified as 'live'; cells stained for annexin V only were 'early apoptotic'; cells stained for both annexin V and PI were 'late apoptotic'; and cells stained for PI only were 'dead'. Apoptotic cells were the sum of early and late apoptotic cells [34, 35].

Nuclear extract preparation and electrophoretic mobility shift assay

PTC and TFC cells were plated (200,000 cells/mL in 10 mL in cell culture dishes) and treated with IFNγ (1000 IU/mL) and TNFα (10 ng/mL) in the absence or presence of 10 or 20 μM Rosi or Pio for 1 h. Nuclear extracts were prepared and processed for EMSA, as previously described [36].

Immunoblotting

PTC cells were plated (200,000 cells/mL in 10 mL in cell culture dishes) and treated for 24 h with IFNγ (1000 IU/mL) and TNFα (10 ng/mL) with/without 10 or 20 μM Rosi or Pio. Then, cells were processed as previously described [7]. Immunoblotting was conducted with anti p-extracellular-signal-regulated kinase (ERK)1/2 and anti ERK1/2 (Cell Signaling) as previously described [7].

Statistical analysis

Values are given as mean ± SD for normally distributed variables (in text), or mean ± SEM (in figures), otherwise as median and [interquartile range]. The experiments were repeated three times with the cells from each donor. The mean of the experiments in the five specimens from different donors, for normal and PTC samples, is reported. One-way ANOVA for normally distributed variables, or Mann-Whitney *U* or Kruskal-Wallis test were used to compare mean group values and χ^2 test to compare proportions. Post hoc comparisons on normally distributed

variables were carried out using the Bonferroni-Dunn test. Data about apoptosis were analyzed by one-way ANOVA with Newman–Keuls multiple comparison test.

Results

ELISA for CXCL9 and CXCL11

In primary normal thyrocyte cultures, CXCL9 and CXCL11 were undetectable in the supernatant. IFNγ induced dose-dependently the CXCL9 and CXCL11 release (CXCL9: 0, 38 ± 15, 67 ± 18, 181 ± 38, 193 ± 45, pg/mL; respectively, with IFNγ 0, 500, 1000, 5000, 10,000 IU/mL; ANOVA, $P < 0.001$) (CXCL11: 0, 44 ± 19, 131 ± 31, 190 ± 47, 294 ± 58, pg/mL, respectively, with IFNγ 0, 500, 1000, 5000, 10,000 IU/mL; ANOVA, $P < 0.001$), whereas TNFα alone had no effect. However, the combination of TNFα (with TNFα 0, 1, 10 ng/mL) and IFNγ (with IFNγ 0, 500, 1000 IU/mL) had a significant (ANOVA, $P < 0.001$) synergistic effect on the CXCL9 and CXCL11 secretion (data are shown for IFNγ 1000 IU/mL plus TNFα 10 ng/mL; Fig. 1a, c, respectively).

Fig. 1

The combination of IFN-γ (1000 IU/mL) and TNF-α (10 ng/mL) significantly and sinergistically increased the secretion of CXCL9 (**a**) or CXCL11 (**c**) in non-neoplastic thyrocytes ($P < 0.001$, ANOVA, for both). The treatment with IFN-γ plus TNF-α significantly and sinergistically increased the secretion of CXCL9 (**b**) or CXCL11 (**d**) secretion from PTC cells, too ($P < 0.001$, ANOVA, for both). The effect of IFN-γ and TNF-α on CXCL9 in PTC cells is significantly higher than in non-neoplastic thyrocytes ($P < 0.001$, ANOVA), while the effect on CXCL11 is slightly, but not significantly higher than in non-neoplastic thyroid cells. The experiments were repeated 3 times with the cells from each donor. The mean of the experiments in the five specimens from different donors, for normal and PTC samples, is reported. Bars are mean ± SEM *$P < 0.05$ or less vs. IFN-γ by Bonferroni-Dunn test

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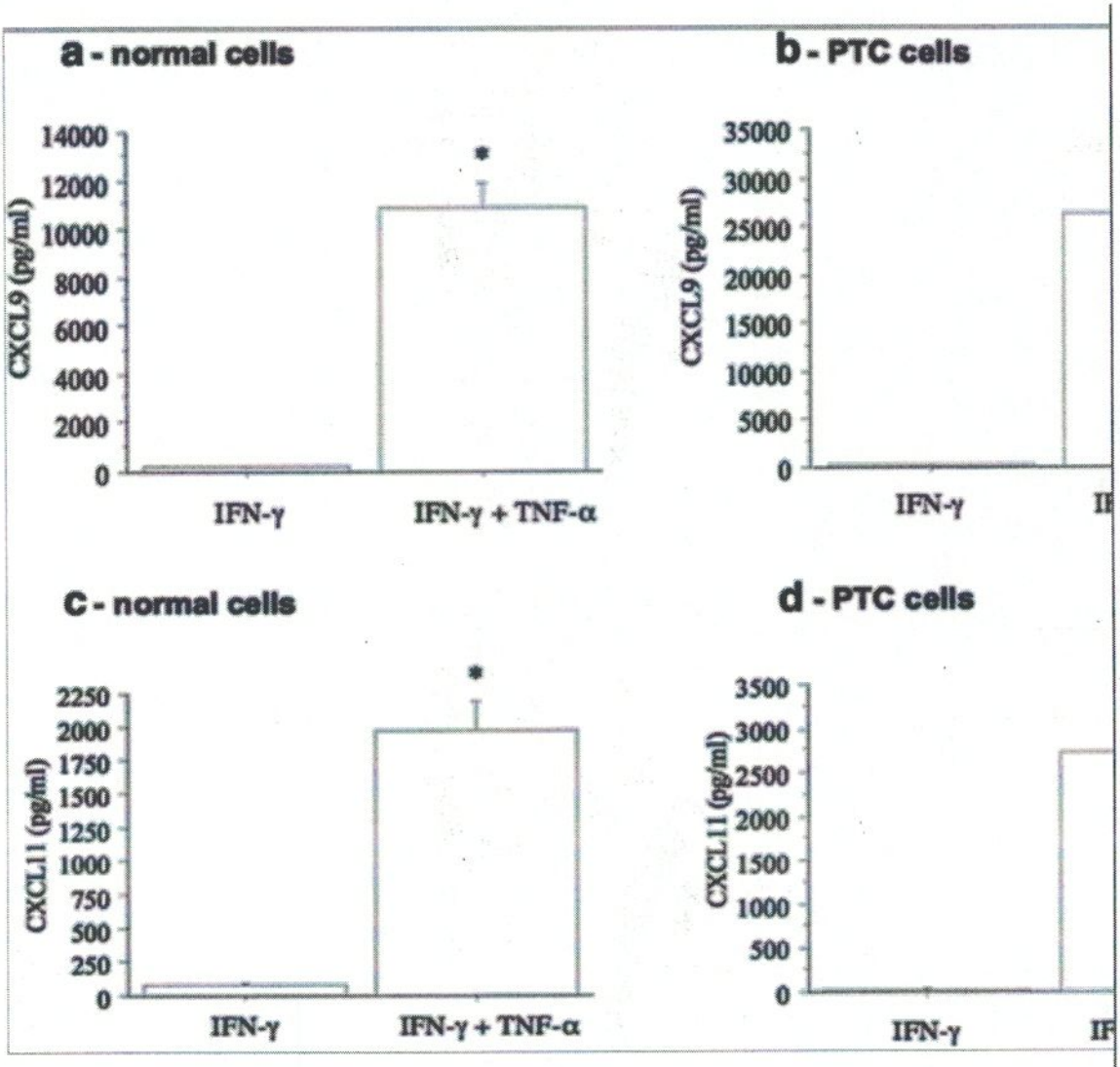


In primary thyrocytes, cultures from PTC, CXCL9, and CXCL11 were undetectable in the supernatant. IFNγ induced dose-dependently the CXCL9 and CXCL11 release (CXCL9: 0, 29 ± 12, 71 ± 21, 184 ± 37, 175 ± 41 pg/mL, respectively, with IFNγ 0, 500, 1000, 5000, 10,000 IU/mL; ANOVA, $P < 0.001$) (CXCL11: 0, 35 ± 15, 111 ± 18, 169 ± 37, 278 ± 41 pg/mL, respectively, with IFNγ 0, 500, 1000, 5000, 10,000 IU/mL; ANOVA, $P < 0.001$); the pattern of IFNγ stimulation was similar to the one present in normal thyroid cells. In PTC cells, TNFα alone had a slight but significant effect (with TNFα 10 ng/mL; CXCL9 39 ± 44 pg/mL; CXCL11 41 ± 17 pg/mL; $P < 0.01$, ANOVA, for both, in comparison with undetectable basal values).

However, the combination of TNFα (with TNFα 0, 1, 10 ng/mL) and IFNγ (with IFNγ 0, 500, 1000 IU/mL) had a significant (ANOVA, $P < 0.001$)

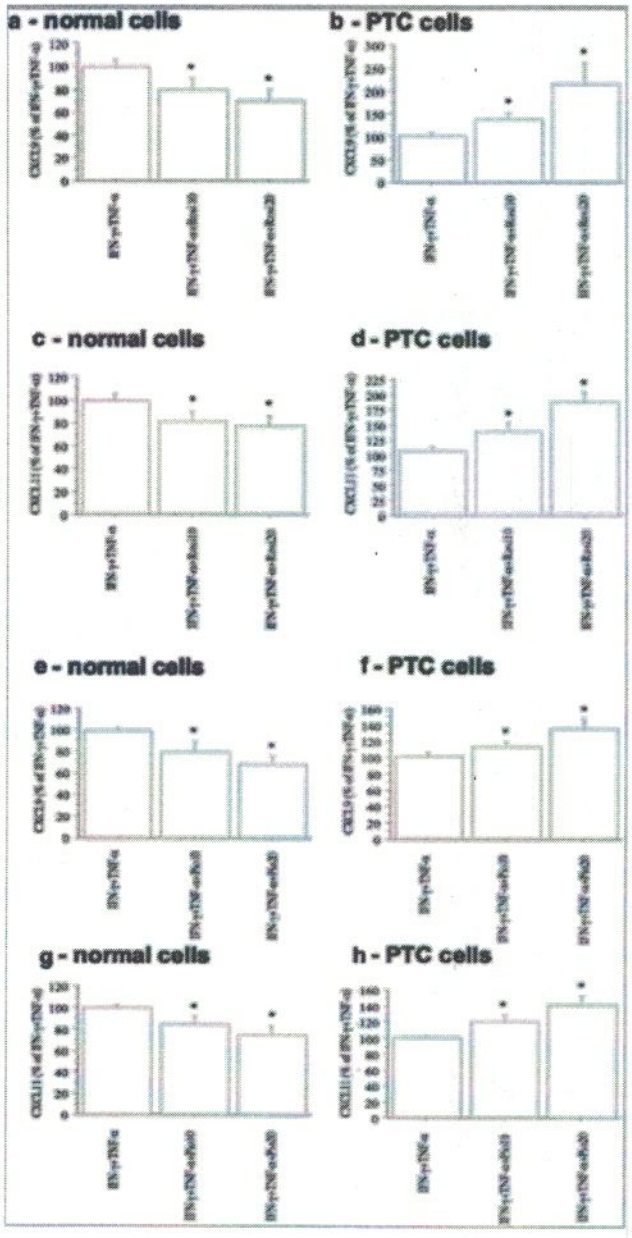
synergistic effect on the CXCL9 and CXCL11 secretion (with TNFα 10 ng/mL plus IFNγ 500 IU/mL: CXCL9, 8811 ± 983 pg/mL; CXCL11: 1084 ± 376 pg/mL, respectively) (data are shown for IFNγ 1000 IU/mL plus TNFα 10 ng/mL; Fig. 1b, d, respectively).

The synergistic effect on CXCL9 secretion by TNFα and IFNγ was significantly higher in PTC than in normal thyrocytes ($P < 0.001$, ANOVA, for both), while it was slightly, but not significantly, higher on CXCL11 secretion in PTC than in TFCs.

Treatment of normal thyroid cells with Rosi, in presence of IFNγ plus TNFα, inhibited dose-dependently CXCL9 and CXCL11 secretion (Fig. 2a, c). A similar effect was observed with Pio (Fig. 2e, g). Treatment of PTC thyrocytes with Rosi had no effect on basal production of CXCL9 and CXCL11 that remained undetectable, but in presence of IFNγ plus TNFα, stimulated dose-dependently CXCL9 and CXCL11 release (Fig. 2b, d). A similar effect was observed with Pio (Fig. 2f, h). The results in thyrocytes from non-neoplastic thyroid tissues were statistically different from those in PTCs ($P < 0.001$).

Fig. 2

Increasing doses of rosiglitazone (Rosi; 10, 20 μM) inhibited CXCL9 (**a**) and CXCL11 (**c**) secretion from thyrocytes treated with IFN-γ (1000 IU/mL) and TNF-α (10 ng/mL) ($P < 0.001$, ANOVA, for both), but stimulated CXCL9 (**b**) and CXCL11 (**d**) release in PTC cells ($P < 0.01$, ANOVA, for both). Bars are mean ± SEM *$P < 0.05$ or less vs. IFN-γ+TNF-α by Bonferroni-Dunn test. Increasing doses of pioglitazone (Pio; 10, 20 μM) inhibited CXCL9 (**e**) and CXCL11 (**g**) secretion from thyrocytes treated with IFN-γ (1000 IU/mL) and TNF-α (10 ng/mL) ($P < 0.001$, ANOVA, for both), but stimulated CXCL9 (**f**) and CXCL11 (**h**) release in PTC cells ($P < 0.01$, ANOVA, for both). The experiments were repeated 3 times with the cells from each donor. The mean of the experiments in the five specimens from different donors, for normal and PTC samples, is reported. Bars are mean ± SEM *$P < 0.05$ or less vs. IFN-γ+TNF-α by Bonferroni-Dunn test



To elucidate if the effects of the PPARγ ligands were mediated by their receptor, we performed a PPARγ knocking down, by using siRNA in PTC cells (siRNA-PTC). TaqMan analysis of PPARγ expression levels in silencing conditions confirmed that RNA interference technique was able to significantly blunt PPARγ mRNA (Fig. 3a, b) at least up to 24 h from oligonucleotide removal. The effect of PPARγ knocking down through siRNA on Rosi and Pio chemokine modulation was investigated. CXCL9 and CXCL11 were stimulated by the combination of IFNγ and TNFα in siRNA-PTC. Treatment of siRNA-PTC with Rosi or Pio (20 μM) had no effect on basal production of CXCL9 and CXCL11 that remained

undetectable, and when added at the time of IFNγ and TNFα stimulation, did not affect CXCL9 and CXCL11 release (Fig. 3c, d) (with respect to IFNγ and TNFα) suggesting that the increase of both chemokines observed in PTC cells was specifically induced by PPARγ activation.

Fig. 3

PPAR-γ knocking down in PTC cells. **a** PTC cells were treated with small interfering RNA specific for PPAR-γ (siRNA-PTC) or with scrambled oligonucleotides (scrambled) for 24 h and then were subjected to TaqMan analysis of PPAR-γ at different times (T: 0, 6, 24 h) from oligonucleotide removal, to verify silencing efficacy. Statistical significance vs. respective scrambled controls $^{\circ}P < 0.01$, $^{*}P < 0.001$ ($n = 3$). **b** After transfection, siRNA-PTC were starved and stimulated for 24 h with IFN-γ (IFN; 1000 IU/mL) + TNF-α (TNF; 10 ng/mL), in the presence or absence of 20 μM rosiglitazone (ROSI) or pioglitazone (PIO), to furtherly verify silencing efficacy. Statistical significance vs. respective scrambled controls $^{\circ}P < 0.01$, $^{*}P < 0.001$ ($n = 3$). **c**, **d** Treatment of siRNA-PTC with rosiglitazone (ROSI; 20 μM) or pioglitazone (PIO; 20 μM) had no effect on basal production of CXCL9 and CXCL11 that remained undetectable, and when added at the time of IFN-γ (IFN) and TNF-α (TNF) stimulation, did not affect CXCL9 and CXCL11 release (with respect to IFN-γ+TNF-α), suggesting that the increase of both chemokines observed in PTC cells was specifically induced by PPAR-γ activation

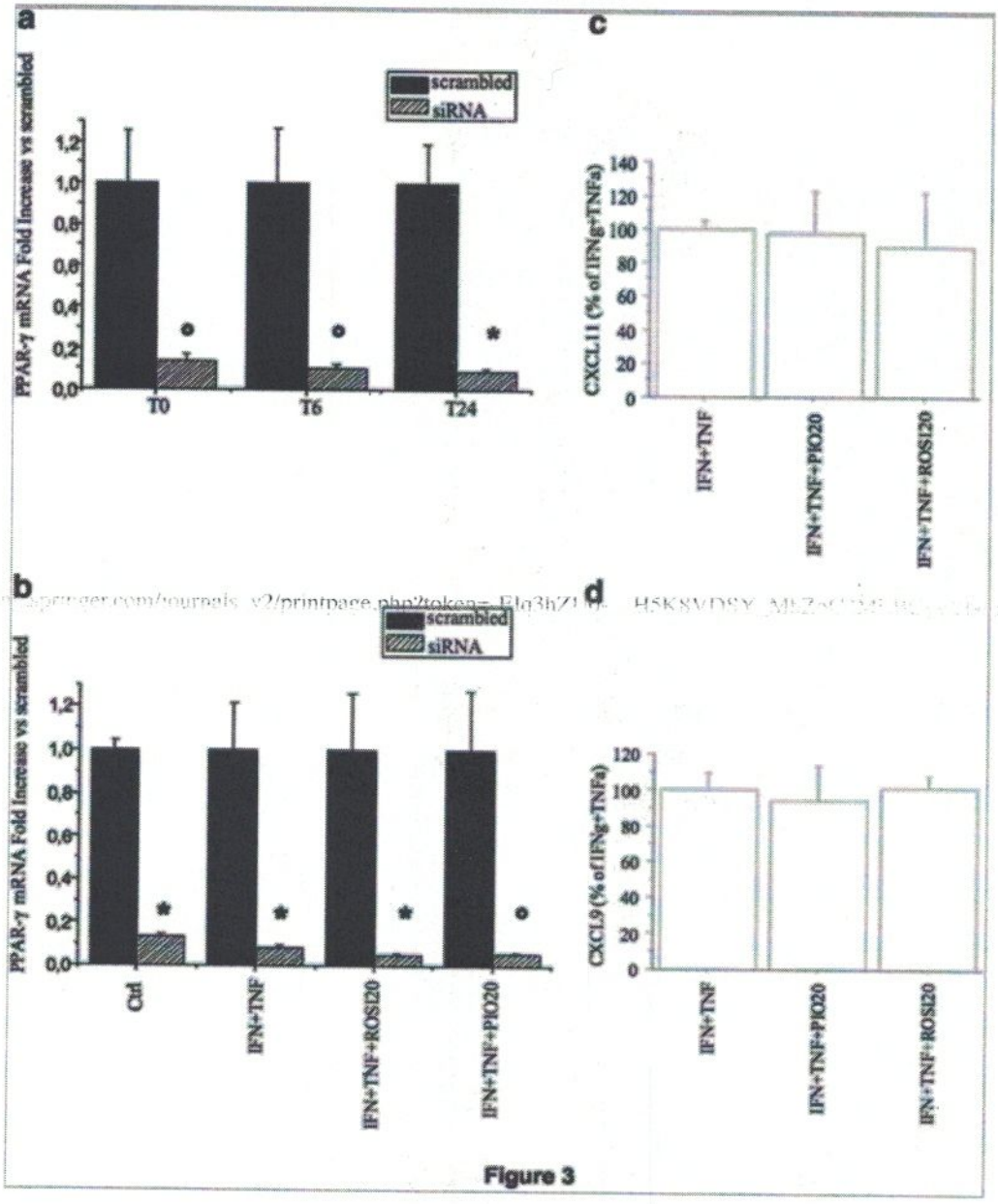


Figure 3

Cell proliferation assay-PPARγ agonists

In normal TFCs, WST-1 results (at 1 h and at 2 h) with 10 and 20 μM Rosi were slightly, but not significantly different in comparison to the control, and the cell counting confirmed the above reported data (at 1 h, 91% of control with Rosi 10 μM, 89% of control with Rosi 20 μM; at 2 h, 89% with Rosi 10 μM, 87% with Rosi 20 μM). In PTC cells, WST-1 results showed a slight, but significant decrease in proliferation with 10 and 20 μM Rosi at 1 h and at 2 h (vs. control) ($P < 0.01$, ANOVA, for both) (at 1 h, 88% of control with Rosi 10 μM, 81% of control with Rosi

20 μM; at 2 h, 84% of control with Rosi 10 μM, 79% of control with Rosi 20 μM). The cell counting confirmed the above mentioned results at 2 h. In PTC, the cell number was 18,236 ± 710/100 μL, per well; 15,669 ± 1358 (86%) with Rosi 10 μM; 14,356 ± 1435 (79%) with Rosi 20 μM ($P < 0.01$, ANOVA, for both).

In normal TFCs, WST-1 results (at 1 h and at 2 h) with 10 and 20 μM Pio were slightly, but not significantly different (vs. control), and the cell counting confirmed the above reported data (at 1 h, 96% of control with Pio 10 μM, 93% of control with Pio 20 μM; at 2 h, 94% with Pio 10 μM, 90% with Pio 20 μM).

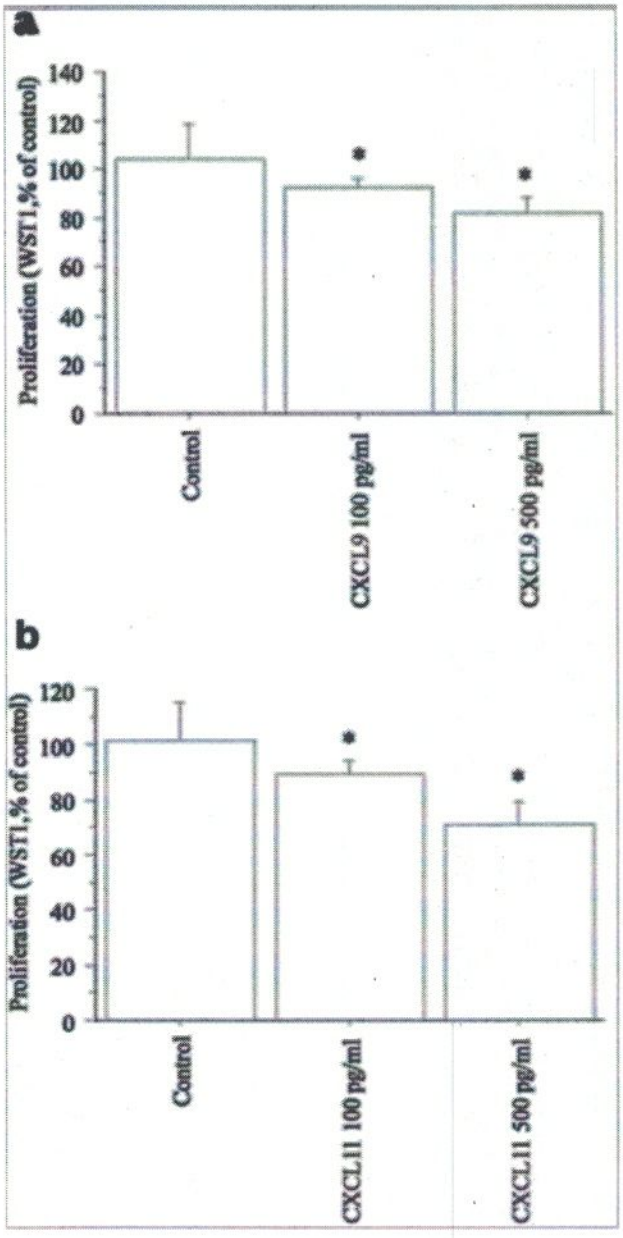
In PTC cells, WST-1 results with 10 and 20 μM Pio were slightly, but significantly different in comparison to the control at 1 h and at 2 h ($P < 0.01$, ANOVA, for both) (at 1 h, 90% of control with Pio 10 μM, 79% of control with Pio 20 μM; at 2 h, 85% of control with Pio 10 μM, 76% of control with Pio 20 μM. In PTC, the cell number was 18,675 ± 809/100 μL, per well; 15,789 ± 1215 (85%) with Pio 10 μM; 13,952 ± 1563 (75%) with Pio 20 μM ($P < 0.01$, ANOVA, for both).

Cell proliferation assay-CXCL9 and CXCL11

WST-1 results in PTC cells treated with 100 and 500 pg/mL CXCL9 showed a significant reduction of proliferation with respect to the control at 1 h and at 2 h ($P < 0.005$, ANOVA, for both) (Fig. 4a). The combination of CXCL9 (500 pg/mL) with Rosi 20 μM showed an additive effect on PTC cell proliferation (reducing proliferation to 59%, with respect to control; $P < 0.01$, ANOVA).

Fig. 4

WST-1 assay (at 2 h from the beginning of tetrazolium reaction) showed a significant reduction of proliferation in primary cells from PTC ($P < 0.005$, ANOVA) treated with 100 and 500 pg/mL CXCL9 (**a**) or 100 and 500 pg/mL CXCL11 (**b**), with respect to the control (control). The experiments were repeated three times with the cells from each donor. The mean of the experiments in the five specimens from different donors is reported. Bars are mean ± SEM $*P < 0.05$ or less vs. Control by Bonferroni-Dunn test



The results of WST-1 assay in PTC cells treated with 100 and 500 pg/mL CXCL11 showed a significant reduction of proliferation with respect to the control at 1 h and at 2 h ($P < 0.005$, ANOVA, for both) (Fig. 4b). The combination of CXCL11 (500 pg/mL) with Rosi 20 μM showed an additive effect on PTC cell proliferation (reducing proliferation to 51%, with respect to control; $P < 0.01$, ANOVA).

The cell counting confirmed the above mentioned results at 2 h. In PTC, the cell number was 18,532 ± 798/100 μL, per well; 10,985 ± 1114 (59%) with CXCL9 (500 pg/mL) and Rosi 20 μM; 9558 ± 1205 (52%) with

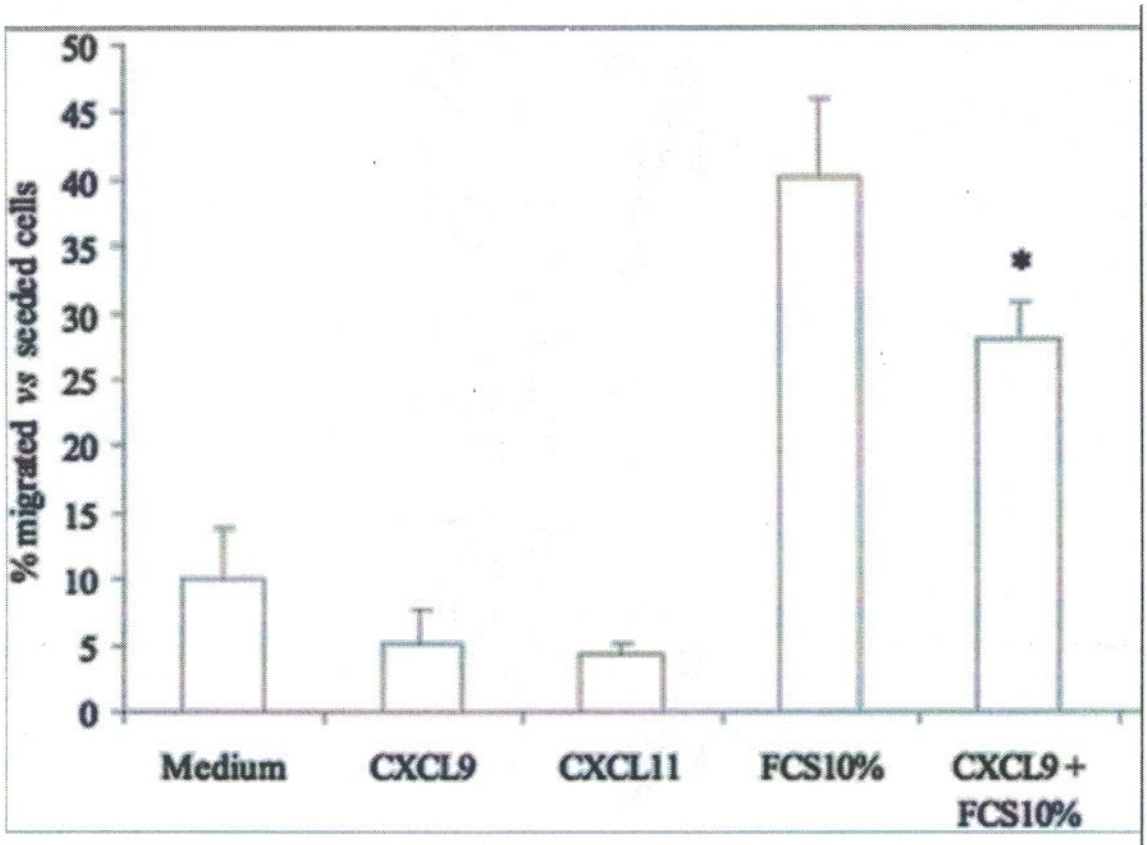
CXCL9 (500 pg/mL) and Pio 20 μM.

Migrations assays-CXCL9 and CXCL11

Experiments were performed in order to evaluate the possible effects of CXCL9 and CXCL11 on cell migration. As shown in Fig. 5, both compounds were able to reduce significantly cell migration induced by FCS 10% *v/v* when used at 500 pg/mL for 20 h. Indeed, a reduction, even if not statistically significant, was also observed in basal cell migration in absence of chemoattractant.

Fig. 5

CXCL9 or CXCL11 (500 pg/mL) inhibit the migration of primary cells from PTC ($P < 0.005$, ANOVA), with respect to FCS10% (medium = serum-free medium; CXCL9 = medium+CXCL9 500 pg/mL; CXCL11 = medium+CXCL11 500 pg/mL; FCS10% = medium+FCS10%; CXCL9 + FCS10% = medium+FCS10% + CXCL9 500 pg/mL; CXCL11 + FCS10% = medium+FCS10% + CXCL11 500 pg/mL). Indeed, a reduction, even if not statistically significant, was also observed in basal cell migration in absence of chemoattractant. The experiments were repeated three times with the cells from each donor. The mean of the experiments in the five specimens from different donors is reported. Bars are mean ± SEM $*P < 0.01$ vs. FCS10%, $°P < 0.001$ vs. FCS10% by Bonferroni-Dunn test



BRAF and chemokines

The ^{V600E}*BRAF* mutation was observed in three PTCs (RET/PTC rearrangements and RAS mutations were absent in all samples). The results about the stimulation with IFNγ and/or TNFα on the chemokines secretion, the modulation of the chemokines secretion and of the inhibition of proliferation by PPARγ activators, obtained in PTC from tumors with ^{V600E}*BRAF* mutation were similar (Mann-Whitney *U* test) to those from tumors without *BRAF* mutations (data not shown).

Apoptosis determination

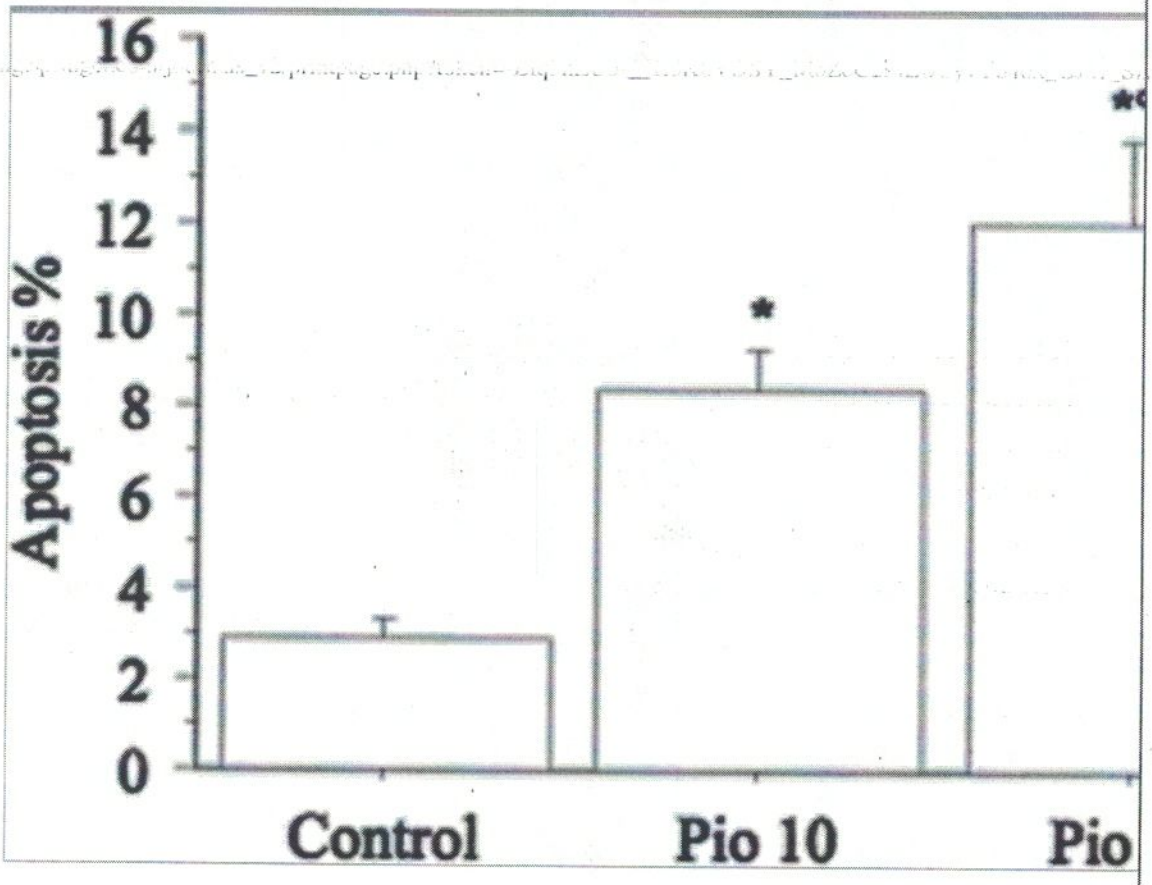
The percentage of apoptotic (Hoechst test) PTC cells increased dose-dependently: after treatment with Pio 10 μM, 7.6% of the cells were apoptotic and this percentage increased up to 10.1% with Pio 20 μM, with respect to the control (about 1.5%) ($P < 0.001$; by ANOVA).

Using an annexin V–FITC dye, compared with vehicle-treated cells, treatment with 10 and 20 μM Pio induced a significant increase of apoptosis in PTC cells (Fig. 6). After treatment with Rosi 10 μM, 12.1%

of the cells were apoptotic and 21.3% with Rosi 20 μM ($P < 0.01$, ANOVA).

Fig. 6

Apoptosis in PTC cells treated with pioglitazone 10 (Pio10) and 20 (Pio20) μM for 48 h. Using an annexin V–FITC dye, compared with vehicle-treated cells, treatment with 10 and 20 μM pioglitazone induced a significant increase of apoptosis in PTC cells ($P < 0.001$, ANOVA). Data are expressed as means ± SD ($n = 5$). One-way ANOVA with Newman–Keuls multiple comparison test and a test for linear trend were used to analyze the results. *$P < 0.001$ vs. control; °$P < 0.001$ vs. Pio10



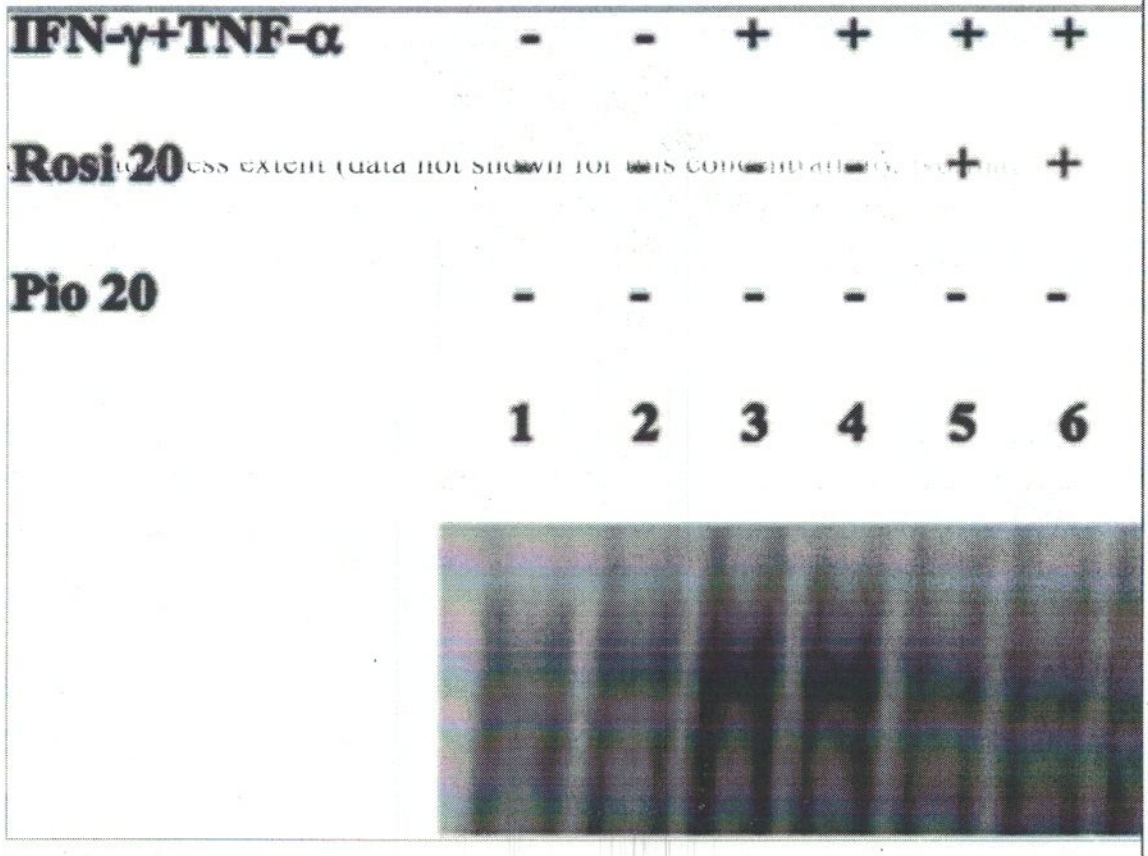
EMSA

The effect of PPARγ agonists on NF-kB activation in the PTC cells was evaluated, by EMSA (Fig. 7), to explain the effect exerted by PPARγ agonists on CXCL9 and CXCL11 secretion. Not treated control PTC cells constitutively expressed NF-kB activation (lanes 1–2) and the stimulation

with IFNγ and TNFα increased the DNA binding activity of NF-kB (lanes 3–4). Rosi or Pio (20 μM) significantly decreased the IFNγ+TNFα stimulation of NF-kB (lanes 5–6 and 7–8, respectively) and also the treatment with Rosi or Pio 10 μM inhibited NF-kB nuclear translocation, even if to a less extent (data not shown for this concentration). Normal TFCs did not express constitutively the NF-kB activation, which was induced by IFNγ+TNFα, and inhibited by PPARγ agonists (data not shown).

Fig. 7

EMSA assay in PTC cell nuclear extracts: not treated PTC cells constitutively express NF-kB activation (lanes 1–2) and the treatment with IFN-γ+TNF-α increased the DNA binding activity of NF-kB (lanes 3–4). Rosiglitazone or pioglitazone (20 μM) significantly decreased the IFN-γ+TNF-α activation of NF-kB (lanes 5–6 and 7–8, respectively)



Immunoblotting

Considering treated and not treated PTC cells, no significant

discrepancies in the level of the mitogen-activated protein kinase (MAPK) ERK1/2 phosphorylation or in the amount of not-phosphorylated protein expression were present (data not shown).

RT-PCR for TLR3 and WNT5a

TLR3 and WNT5a mRNAs were detectable in all PTC primary cultures. The TLR3 expression vs. the reference gene (GAPDH) ranges from 0.9 to 1.3, and WNT5a from 4.2 to 4.9, by real-time RT-PCR.

Discussion

The present study demonstrates that (a) both IFNγ and IFNγ+TNFα induce dose-dependently an exaggerate release of CXCL9 and, at a lower level, of CXCL11 in PTC primary cells, in comparison to normal TFC; (b) the PPARγ ligands, Rosi and Pio, dose-dependently inhibit PTC cell proliferation, but (differently from normal TFC) stimulate CXCL9 or CXCL11 secretion; (c) CXCL9 or CXCL11 induce a significant antiproliferative effect in PTC cells, and inhibit migration.

The involvement of Th1 chemokines in autoimmunity is well known, while their role in PTC is under evaluation [9, 10, 37, 38].

Our results agree with the ones published by other studies demonstrating an overexpression of another CXC chemokine (CXCL10) in PTC. In PTC, *RET/PTC* and activating mutations in the *BRAF* or *RAS* oncogenes activate a common transcriptional program in thyroid cells, which includes upregulation of the CXCL1 and CXCL10 chemokines, which in turn stimulate proliferation and invasion [5]. Furthermore, it has been shown that a more than ten times higher CXCL10 secretion has been induced by IFNγ+TNFα in PTCs with respect to TFC [7]. High basal levels of TLR3 and WNT5a RNA have been reported in PTC cell lines in accord with their overexpression and colocalization in PTC cells in vivo. In PTC cells, the basal expression of TLR3 is functional, and it is able to rise CXCL10 final levels [8]. Our results agree that TLR3 and WNT5a are expressed in primary PTC cells [7, 8, 39] and WNT5a levels could be related to the increased TLR3 levels and signaling. This could partly clarify the mechanism of the stimulation of CXCL9 or CXCL11 release by cytokines in PTCs.

We show a significantly stronger synergistic effect of IFNγ and TNFα on CXCL9, than CXCL11, secretion in PTC cells than in normal thyroid cells, suggesting that the genetic alterations at the basis of the neoplastic transformation in PTC cause an overexpression of CXCL9, resulting in an abnormal chemokine response after the stimulation by IFNγ and TNFα. The moderate but significant increase of CXCL9 and CXCL11 after the treatment with TNFα could be caused by the above mentioned genetic alterations, too. Interestingly, the response of CXCL9 to TNFα and IFNγ is about ten times stronger than that of CXCL11, suggesting a leading role of CXCL9 in this process.

The results of our study first show that treating PTC cells with the two PPARγ ligands, Rosi and Pio, at near-therapeutical doses, the IFNγ-induced CXCL9 and CXCL11 release increased. These results are in agreement with those published in one of our studies that showed that PPARγ agonists inhibited CXCL10 secretion in TFC and induced it in PTC [7] and strongly reinforce the hypothesis that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in human thyroid cells [40, 41, 42, 43].

To elucidate whether the effects of the PPARγ ligands were mediated by their receptor, we performed a PPARγ knocking down, by using RNA interference technique in PTC cells. Treatment of siRNA-PTC thyrocytes with Rosi or Pio (20 μM) did not affect CXCL9 and CXCL11 release (after IFNγ and TNFα stimulation), suggesting that the increase of both chemokines observed in PTC cells was specifically induced by PPARγ activation.

PPARγ activators may act through different mechanisms of action on chemokine secretion: (1) decreasing CXCL10 promoter activity and inhibiting protein binding to the two NF-kB sites [40, 44]; (2) reducing CXCL10 protein levels dose-dependently at nanomolar concentrations [42]; (3) increasing phosphorylation and activation of ERK1/2 [27, 28, 45].

The results of our study in PTC demonstrate that NF-kB is constitutively activated and PPARγ agonists inhibit the NF-kB activation, according to Marx et al. [40]. This is in agreement with the effect of PPARγ agonists

on PTC proliferation, but in opposition with the one exercised on CXCL9 or CXCL11 secretion, leading to hypothesize that other pathways are implicated in the chemokine modulation. Considering the MAPK ERK1/2 in treated or not treated thyrocytes, no significant discrepancies in the level of ERK1/2 phosphorylation or not-phosphorylated protein expression were reported, differently from what observed in endothelial cells [27].

We can suppose that while PPARγ activators physiologically inhibit chemokine release, the derangement of the pathways at the basis of CXCL9 or CXCL11 expression could cause an unexpected stimulation in PTC.

Our study demonstrates a significant antiproliferative effect of PPARγ agonists in primary PTC cells, in agreement with the results of other studies [7, 13, 18, 22, 27, 46, 47, 48, 49, 50]. Of note, in PTC, the antiproliferative effect of PPARγ agonists is not associated with the capacity of inhibiting the IFNγ-induced CXCL9 and CXCL11 release. PPARγ agonists increase the IFNγ+TNFα-induced CXCL9 and CXCL11 release in PTC, differently from what reported in normal TFC, in which PPARγ agonists have an inhibitory role. This discrepancy is probably caused by the different involved pathways.

In fact, PPARγ agonists modulate cell growth, inducing apoptosis, as confirmed by our data, that report that both Pio and Rosi induce apoptosis in PTC cells. Also considering thyroid cancer, a debate is present about the possible induction of apoptosis by PPARγ agonists in relation to proto-oncogene, *c-myc*, or cell cycle inhibitor protein p27 [51], or up-regulation of Bax protein [46].

Recently, it has been shown that Rosi was associated with an increased risk of stroke, heart failure, and all-cause mortality in elderly patients [52], and the European Medicines Agency (EMA) recommended on September 2010 that Rosi be suspended from the European market. More recently, EMA extended review of safety to Pio [53]. Even if these arguments cannot be automatically translated in oncological field, they do not actually advice PPARγ agonists for the therapy of PTC.

Our study first shows that CXCL9 and CXCL11 chemokines are able to

inhibit the proliferation and migration of primary PTC cells. Our results are in agreement with the results of other studies showing that CXC chemokines (CXCL9, CXCL10, and CXCL11) inhibit endothelial cell proliferation [4]. Furthermore, antineoplastic effects of CXCL9, in murine carcinoma cells [54, 55], and of CXCL11, in lymphoma and breast cancer [56, 57], have been shown.

The mechanisms leading to the inhibition of PTC proliferation and migration remain to be clarified.

The induction, by IFNγ and TNFα, of CXCL9 in PTC cells may have two main antineoplastic effects: (a) the recruitment of Th1 lymphocytes in the neoplastic area, that are able to produce an antitumoral activity; (b) a direct inhibition of the PTC proliferation and migration. The use of CXCL9 and CXCL11 as antineoplastic agents in PTC remains to be explored.

Conclusions

Our study demonstrates that the treatment with IFNγ+TNFα induces a marked release of CXCL9, and to a lesser extent of CXCL11, by primary thyrocytes from PTC. It has been shown a discrepancy between the stimulatory role of PPARγ agonists on CXCL9 and CXCL11 release and the inhibitory effect on PTC proliferation. Furthermore, our study first shows that CXCL9 and CXCL11 are able to inhibit the proliferation and migration of primary PTC cells; the use of CXCL9 or CXCL11 as antineoplastic agents in PTC remains to be explored.

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