Interferon- γ inhibits integrin-mediated adhesion to fibronectin and survival signaling in thyroid cells

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Abstract

Hashimoto's thyroiditis is the most frequent autoimmune disorder, characterized by the presence of a large lymphocytic infiltration and secretion of inflammatory cytokines in the thyroid. Infiltrating lymphocytes and cytokines play a pivotal role in the progression of HT, characterized by the progressive destruction of the normal follicular architecture of the gland and death of follicular cells, ending with loss of thyroid function. Integrins are plasma membrane receptors for the cell–extra-cellular matrix components, with both structural and signaling functions. Integrin-mediated fibronectin (FN) binding is necessary for the correct function and survival of thyroid follicular cells. The purpose of this study was to determine the effect of interferon- γ (IFN- γ) stimulation on integrin expression and signaling in the thyroid cell. Cytotoxicity,

integrin expression, cell adhesion to FN, and FN-stimulated ERK and AKT phosphorylation were determined in a normal human thyroid cell line treated with IFN- γ . IFN- γ induced apoptosis and reduced the expression of the integrin $\alpha v \beta 3$. Integrin-mediated cell adhesion to FN was strongly impaired. Similarly, FN-stimulated ERK and AKT phosphorylation were inhibited. In conclusion, our study in a thyroid cell model demonstrates that IFN- γ induces apoptosis and inhibits the expression of the integrin $\alpha v \beta 3$, reducing cell adhesion to FN and the succeeding outside-in signaling. These results suggest that integrins mediate the cytotoxic effect of IFN- γ and are involved in the destructive mechanism of autoimmune thyroiditis.

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Introduction

Chronic lymphocytic thyroiditis, also known as Hashimoto's thyroiditis (HT), is the most common autoimmune disease, with a prevalence of 5–10% in the general population (Tunbridge *et al.* 1977, 1981). Auto-antibodies to thyroid antigens and lymphocyte infiltration on histology are the hallmark of HT (Stassi *et al.* 2000, Caturegli *et al.* 2007). A transient modest hyperthyroidism and a small goiter can be the first clinical findings of this disease until the gradual loss of thyroid function appears with hypothyroidism. The reduced thyroid function is due to the progressive destruction of the normal follicular architecture of the gland and death of follicular cells. T helper type 1 (Th1) is the main subset of lymphocytes present in HT and has a crucial role in the pathogenesis of the disease by secreting inflammatory cytokines such as interferon- γ (IFN- γ ; Salgame *et al.* 1991, Romagnani 1994, Carter & Dutton 1996, Pala *et al.* 2000, Mazziotti *et al.* 2003, Santaguida *et al.* 2011). The role of IFN- γ in the pathogenesis of thyroid damage in HT is supported by the evidence of intra-thyroidal cytokine

secretion and by experimental evidences in cells in culture and in animal models (Liblau *et al.* 1995, Roura-Mir *et al.* 1997, 2005, Stassi *et al.* 2000, 2001).

Integrins are a group of cell-surface heterodimers characterized by a common $\beta 1$ chain non-covalently associated with a distinctive α subunit (Hemler *et al.* 1987). The members of this family are receptors for the components of the extra-cellular matrix (ECM), involved in cell–ECM interactions. Their level of expression undergoes quantitative and qualitative changes upon differentiation, neoplastic transformation, and hormone stimulation (Dedhar 1989, Heino & Massague 1989, Plantefaber & Hynes 1989, Wilkins *et al.* 1991, Vitale *et al.* 1994, 1995). The $\alpha \beta$ integrin complex binds ECM by its extracellular domain and interacts with cytoskeletal proteins by its intracellular domain, contributing to the stability of the tissue architecture (Otey *et al.* 1990). Some integrins are components of a subcellular structure called focal adhesion, where they interplay with regulatory proteins (Luna & Hitt 1992). Integrin binding to the ECM generates multiple intracellular signals that contribute to the regulation of many cell processes, including differentiation,

growth, and survival (Mortarini *et al.* 1992, Pasqualini & Hemler 1994). The integrin expression profile is cell-type-dependent and is modulated by a number of factors, including cell-to-cell contact, extracellular soluble factors, and tumor transformation. Normal thyroid cells have a limited integrin expression repertoire ($\alpha 3\beta 1$ and $\alpha v\beta 3$) that changes upon cell-to-cell contact and tumor transformation (Vitale *et al.* 1993, 1994, 1995, Illario *et al.* 2003). Integrin activation by fibronectin (FN) has important biological effects in the thyroid cell, regulating cytoskeletal organization and stimulating cell proliferation and survival (Vitale *et al.* 1998, Di Matola *et al.* 2000). The aim of this study was to investigate whether integrins mediate the cytotoxic effect exerted by IFN- γ on thyroid cells. For this purpose, we investigated the effect of IFN- γ on cytotoxicity, integrin expression, adhesion to FN, and FN-stimulated signaling in a human thyroid cell model. We found that IFN- γ reduces the expression of the integrin $\alpha v\beta 3$ and strongly inhibits the FN-dependent intracellular signalings, modulating cell proliferation and survival.

Materials and Methods

Cell cultures

The TAD2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells, was generously donated by Dr T F Davies (Mount Sinai Hospital, New York, NY, USA) and cultured in a 5% CO₂ atmosphere at 37 °C in DMEM (4.5 g glucose) and 10% FCS. The TAD2 cell line has the same integrin expression profile and FN-stimulated signaling of normal human thyroid cells in culture (Vitale *et al.* 1997, Illario *et al.* 2003). Medium was changed every 3–4 days. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free PBS with 0.05% trypsin. When needed, the cells were serum starved in 0.5% BSA and DMEM for 12–18 h before stimulation. To obtain a FN or BSA coating, cell culture plates were filled with the appropriate FN (Collaborative Research, Bedford, MA, USA) or BSA (Sigma) dilution in PBS. After overnight incubation at 4 °C, FN was removed, and the plates were washed with PBS and stored at 4 °C.

Antibodies to integrins and flow cytometric analysis

All the antibodies specific for single integrin subunits or heterodimers were mouse monoclonals purchased from Santa Cruz Biotechnology, Inc. Fluorescein-conjugated anti-mouse IgG was purchased from Ortho (Raritan, NJ, USA). Cells harvested from cell cultures by trypsin/PBS were incubated with MABs for 1 h at 4 °C in PBS and 0.5% BSA (BSA/PBS), washed in the same buffer, and incubated again with the second fluorescein-conjugated antibody for 30 min at 4 °C. Cells were resuspended in BSA/PBS and analyzed by flow cytometry. Single cell suspensions were analyzed by a

FACScan (Becton Dickinson, Mountain View, CA, USA). Forward scatter vs side scatter analysis was performed on a logarithmic scale using a high forward threshold to cut off cellular and collagen debris. Cytofluorimetric estimation of DNA cell content was performed as described (Illario *et al.* 2003). Floating cells were collected, washed in cold PBS, added to adherent cells, and trypsinized. Cells were washed again in PBS and fixed in 70% cold ethanol for 30 min. Ethanol was removed by two PBS washes, and cells were incubated in PBS, 50 μ g/ml propidium iodide, 10 μ g/ml ribonuclease A, and deoxyribonuclease-free overnight at 4 °C. Cells were then analyzed by flow cytometry using a FACScan.

Cell attachment assay

The assay was performed in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA). The wells were filled with 100 μ l of the appropriate dilution in PBS of FN (Collaborative Research). After overnight incubation at 4 °C, the plates were washed with PBS, filled with 100 μ l 1% heat-denatured BSA, and incubated for 1 h at room temperature. Then, plates were washed and filled again with 100 μ l/well PBS, 0.9 mM CaCl₂, and 0.5 mM MgCl₂ containing 2×10^4 cells. After 30 min at 37 °C, plates were gently washed three times with PBS, and the attached cells were fixed with 3% paraformaldehyde for 10 min followed by 2% methanol for 10 min and finally stained with 0.5% crystal violet in 20% methanol. After 10 min, plates were washed with tap water, the stain was eluted with a solution of 0.1 M sodium citrate, pH 4.2, in 50% ethanol, and the absorbance at 540 nm was measured by a spectrophotometer. Where indicated, the cells were coincubated with 500 μ g/ml of integrin/FN-binding inhibitory peptide RGSP (Gly-Arg-Gly-Asp-Ser-Pro) or the ineffective peptide RGE (Gly-Arg-Gly-Glu-Ser-Pro), Telios (San Diego, CA, USA). All experiments were performed in quadruplicate.

Western blot

The cells were lysed in Laemmli buffer (125 mM Tris (pH 6.8), 5% glycerol, 2% SDS, 1% 2 β -mercaptoethanol, and 0.006% bromophenol blue) and resolved by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane (Immobilon P, Millipore Corp., Bedford, MA, USA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine, and after three washes, the membranes were incubated for 1 h at 4 °C with mouse monoclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4 °C with a HRP-conjugated anti-mouse secondary antibody. After a final wash, protein bands were detected by an ECL system (Amersham Pharmacia Biotech). Mouse MABs to ERK and phosphorylated ERK, Akt and phospho-threonine-308-Akt, caspase-3, and PARP were from Santa Cruz Biotechnology, Inc.

Figure 1 IFN- γ modulates integrin expression. TAD2 cells cultured at low confluence were treated for 3 days with IFN- γ (10 ng/ml), harvested by mild trypsinization, and incubated with MABs specific for single integrin subunits ($\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$) or whole receptors ($\alpha 5\beta 1$ and $\alpha v\beta 3$) followed by the secondary fluorescein-conjugated antibody. The relative fluorescences were measured by flow cytometry as described in Materials and Methods section. The expression of each integrin etherodimer or single subunit is reported as relative fluorescence index: mean of experimental mean fluorescence/control mean fluorescence of three independent primary cultures. * $P=0.048$; ** $P=0.039$; *** $P=0.0017$ by Student's *t*-test.

Statistical analysis

Results are presented as the mean $\pm$ s.d. Statistical analysis was performed using the Student's *t*-test or by two-way ANOVA. The level of significance was set at $P<0.05$. Statistical analyses were conducted using SPSS 18.0 (IBM Corporation).

Results

IFN- γ modulates the expression of FN receptors of the integrin family in TAD2 cells

All the experiments were performed with TAD2 cells cultured at 75% confluence. The cells were treated for 3 days with IFN- γ , then integrin expression was assessed by flow cytometry with specific antibodies for the subunits $\beta 1$, $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, or the heterodimers $\alpha v\beta 3$ and $\alpha 5\beta 1$ (Fig. 1). The expression of the $\alpha 3$ and $\beta 1$ subunits was increased by IFN- γ treatment. More robust was the effect of IFN- γ on the $\alpha v\beta 3$ heterodimer, whose expression was reduced by 76.6%.

IFN- γ inhibits thyroid cell adhesion to FN

To investigate whether the adhesion of thyroid cells to FN was changed by IFN- γ , TAD2 cells were treated with the cytokine for 3 days and a cell attachment assay was performed (Fig. 2). Cell adhesion in the presence of the RGDSP peptide, a specific inhibitor of integrin binding to FN, was reduced by 84.7%, demonstrating the major role of integrin receptors in the adhesion to immobilized FN. IFN- γ treated cells showed a remarkable reduction of attachment to FN. The reduction of integrin-dependent attachment (CTRL + RGE – CTRL + GRDSP/IFN- γ + RGE – CTRL + GRDSP) in

10 and 50 μ g/ml FN adhesion assays was 71 and 65% respectively. Two-way ANOVA assessment of effects of IFN- γ indicated a significant difference between IFN- γ + RGE and CTRL + RGE (F -ratio = 5.38, $P=0.045$), a significant difference among FN concentrations (F -ratio = 10.78, $P=0.009$), and a significant interaction (F -ratio = 17.7, $P<0.001$). Moreover, two-way ANOVA for comparison of RGE to RGDSP indicated a significant difference between CTRL + RGE and CTRL + RGDSP (F -ratio = 10.73, $P=0.010$), a significant difference among FN concentrations (F -ratio = 4.88, $P=0.049$), and a significant interaction (F -ratio = 17.7, $P<0.001$). This data are consistent with the reduction of $\alpha v\beta 3$ expression and/or reduced affinity of $\alpha 3\beta 1$ to FN induced by IFN- γ .

Effect of IFN- γ on the cell cycle and survival

The effect of IFN- γ on the cell cycle was evaluated in TAD2 by flow cytometry analysis. TAD2 cells were plated onto FN-coated plates and cultured for 3 days in the presence of IFN- γ or left untreated. Then, all the cells, both floating in the medium and adherent, were collected, stained with propidium iodide, and analyzed by FACScan (Fig. 3A). The number of cells in the G0–G1 phase decreased from 49% in untreated cells to 38 and 34% after 10 and 100 ng/ml IFN- γ treatment respectively. The number of hypodiploid cells

Figure 2 Effect of IFN- γ on thyroid cell adhesion to FN. Ninety-six-well microtiter plates were coated with soluble FN at 10 or 50 μ g/ml and saturated with heat denatured BSA. TAD2 cells were untreated (CTRL) or treated with IFN- γ , 10 ng/ml for 3 days. A total of 2×10^4 thyroid cells were seeded in the wells, with RGE or RGDSP peptides, and the plates were incubated at 37 °C for 30 min. Attached cells were measured as described in Materials and Methods section. Data are reported as the mean $\pm$ s.d. of quadruplicate experiments. RGE and RGDSP, 500 μ g/ml. Two-way ANOVA analysis for IFN- γ : IFN- γ + RGE vs CTRL + RGE (F -ratio = 5.38, $P=0.045$), among FN concentrations (F -ratio = 10.78, $P=0.009$), interaction (F -ratio = 17.7, $P<0.001$). Two-way ANOVA for comparison of RGE to RGDSP: CTRL + RGE vs CTRL + RGDSP (F -ratio = 10.73, $P=0.010$), among FN concentrations (F -ratio = 4.88, $P=0.049$), interaction (F -ratio = 17.7, $P<0.001$).

Figure 3 DNA cell content of IFN- γ treated cells. (A) TAD2 cells were plated onto 10 μ g/ml FN-coated plates and cultured for 3 days in the presence of 10 or 100 ng/ml IFN- γ or left untreated. Then, both adherent and suspended cells were collected, stained with propidium iodide, and analyzed by FACSscan. (B) The cells were treated as in (A) for 6 days. S.D.s were <15%. A0, hypodiploid cells. Experimental point vs 0 point: * P <0.05, ** P <0.005 by Student's t -test.

increased from 2.9 to 11% and 14%. The presence of a large number of DNA-loosing cells prevented distinguishment between S and G2-M phases, which appeared merged. By 6 days of treatment, the effect of IFN- γ was massive, about 20% of the cells were detached and up to 39.5% of the cells (both floating and adherent) were hypodiploid (Fig. 3B). These data demonstrate a time- and concentration-dependent cytotoxicity of IFN- γ .

IFN- γ induces apoptosis in thyroid cells

Caspase-3 cleavage and PARP fragmentation were investigated by western blot to assess the occurrence of apoptosis (Fig. 4). TAD2 cells were treated for 3 days with IFN- γ and analyzed, demonstrating that the hypodiploid cells were due to loss of fragmented DNA following apoptosis.

Effect of IFN- γ on FN-induced signaling

To determine whether IFN- γ affects the Ras/Raf/MEK/ERK and phosphatidyl-inositol 3 kinase (PI3K)/AKT signaling, thyroid cells were untreated or treated with IFN- γ for 3 days, starved from serum overnight, and plated

onto immobilized BSA or FN. ERK phosphorylation was achieved by serum stimulation. After 30 min, the cells were lysed and phosphorylated Akt or ERK were evidenced by western blot (Fig. 5). Adhesion to FN stimulated both Akt and ERK phosphorylation. The treatment with IFN- γ strongly inhibited FN-induced phosphorylation of both kinases.

Discussion

IFN- γ , TNF- α , and IL2 can modulate the expression of β 1 integrins, and therefore change the cell-ECM interaction in some cell types including fibroblasts, endothelial cells, and lymphoid cells (Pirila & Heino 1996). It has been proposed that adhesion molecules are involved in the maintenance of autoimmunity. Some cytokines induce upregulation of α 1 β 1, enhancing the persistence of inflammatory cells in the extralymphatic tissues and the in-site production of cytokines (Ben-Horin & Bank 2004). However, the effects of inflammatory cytokines on thyroid integrin expression and function remained poorly investigated (Marazuela *et al.* 1997). Our study demonstrates that IFN- γ has a cytotoxic effect and modulates integrin expression and function in the TAD2 cell line. This cell line was demonstrated to be a reliable model to study cell-ECM interactions as it has the same integrin expression profile and FN-stimulated signaling of normal human thyroid cells in culture (Vitale *et al.* 1997, 1998, Illario *et al.* 2003). Indeed, some of the experiments of this study have been performed with identical results in thyroid primary cultures (Russo *et al.* 2012). All the experiments were performed with TAD2 cells cultured at 75% confluence to normalize integrin expression, which is downregulated by cell-to-cell contact (Vitale *et al.* 1995). Thyroid cells are polarized epithelial cells, whose basal plasma membrane adheres to ECM components of the basal membrane.

Figure 4 Western blot analysis of caspase-3 and PARP in IFN- γ treated cells. TAD2 cells were plated onto 10 μ g/ml FN-coated plates and cultured for 3 days in the presence of 10 or 100 ng/ml IFN- γ or left untreated. Cell lysates were analyzed by western blot with antibodies to caspase-3 and PARP.

Figure 5 Stimulation of AKT and ERK phosphorylation by adhesion to FN. TAD2 cells were untreated or treated with IFN- γ 10 ng/ml for 3 days. The cells were starved from serum overnight, harvested by mild trypsinization, and seeded in serum-free medium onto BSA-coated plates (BSA) or 50 μ g/ml FN-coated plates (FN), or untreated plates in the presence of 10% FCS for 30 min. The cells were lysed and the proteins were analyzed by western blot with antibodies anti-total (AKT and ERK) or anti-phosphorylated AKT ERK (pAKT and pERK).

Impaired adhesion to FN modifies outside-in signaling, inhibits cell proliferation, and triggers a programmed cell death known as 'anoikis' (Vitale *et al.* 1997, 1998, 1999, Di Matola *et al.* 2000, Illario *et al.* 2003, 2005). The adhesion assays, with the highly specific integrin/FN-binding inhibitor RGDSP, demonstrated that thyroid cell adhesion to FN was almost exclusively mediated by β 1 integrins. Therefore, α 3 β 1 and α v β 3, the only β 1 integrins expressed, are the adhesion molecules involved in thyroid cell adhesion to FN. While α 3 β 1 is localized on the entire plasma membrane facing the basal membrane and has mainly a structural function, α v β 3 is localized in the focal adhesions and mediates FN outside-in signaling. Inhibition of FN/ α v β 3 binding and the succeeding signaling have important consequence for the cell, including inhibition of proliferation and induction of apoptosis. IFN- γ induced a modest upregulation of α 3 β 1 expression and a more evident downregulation of α v β 3 in TAD2 cells. The remarkably impaired adhesion to FN might be due to the more relevant reduction of α v β 3/FN interaction. However it cannot be excluded that IFN- γ possibly reduced the binding affinity of one or both the receptors. Indeed, the affinity of integrin receptors for their ligands is cell-type-dependent and modulated by different factors (Wilkins *et al.* 1991). The cytotoxic effect of IFN- γ was an apoptotic process, as demonstrated by the caspase-3 and PARP cleavage. This cytokine generates a pleiotropic signaling with multiple effects. It has been previously demonstrated that impairment of α v β 3 adhesion to FN induces apoptosis in thyroid cells (Vitale *et al.* 1998, Illario *et al.* 2003). Although a direct involvement of this integrin in the cytotoxic effect of IFN- γ was not demonstrated in our study, it can be speculated that the inhibition of MAPK and PI3K/AKT signaling is involved in the apoptotic process induced by this cytokine. Indeed, the inhibition of the AKT survival pathway, the same of many growth factors necessary for cell survival, makes the thyroid cell more sensitive to damaging agents and thus might

contribute to the cytotoxicity of IFN- γ or of other damaging mechanisms active in the HT. In conclusion, our study in a thyroid cell model demonstrates that IFN- γ induces apoptosis and inhibits the expression of the integrin α v β 3, reducing cell adhesion to FN and the succeeding outside-in signaling. These results suggest that integrins mediate the cytotoxic effect of IFN- γ and play a role in the destructive mechanism of autoimmune thyroiditis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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