EXPERIMENTAL STUDY

Interferon- γ down-regulates claudin-1 and impairs the epithelial barrier function in primary cultured human thyrocytes

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Abstract

Objective: Proinflammatory cytokines are known to affect the follicular epithelium in autoimmune thyroid disease. Here we investigated the effect of interferon- γ (IFN- γ) on the barrier function of primary cultured human thyrocytes.

Design: Graves' thyroid follicle segments were cultured as a tight and polarised monolayer on the filter of a bicameral chamber, thereby allowing the *in vivo* epithelial characteristics to be maintained.

Methods: Transepithelial electrical resistance was measured with a Millicell ERS ohmmeter. The tight junction proteins claudin-1 and occludin were analysed by immunofluorescence and Western blotting. Cell morphology was studied by transmission electron microscopy.

Results: Thyrotrophin (TSH; 1 mU/ml) promoted the development of a tight epithelium monitored as a persistent increase in the transepithelial resistance to about $800 \Omega \cdot \text{cm}^2$. IFN- γ (100 U/ml), on the other hand, decreased the resistance to $60\text{--}150 \Omega \cdot \text{cm}^2$ after 48 h. In IFN- γ -treated cells the expression of claudin-1, but not that of occludin, was decreased along with a diminished intracellular and cell surface immunostaining. In addition, claudin-1 was disrupted at cell–cell contacts. IFN- γ also caused profound cell shape changes and a multilayered cellular organisation, without ultrastructural or biochemical (caspase-3 activity) signs of cytotoxicity. TSH was unable to counteract the effects of IFN- γ .

Conclusions: IFN- γ destroys the barrier function of filter-cultured human thyroid epithelial cells. The loss of barrier involves down-regulation and an altered distribution of claudin-1. This novel effect of IFN- γ on target cells in thyroid autoimmunity might be of pathophysiological relevance to the exposure of hidden autoantigens.

European Journal of Endocrinology 149 215–221

Introduction

Interferon- γ (IFN- γ) is a pleiotropic cytokine that exerts multiple roles in the regulation of inflammatory and immune responses (1). As well as stimulating immune cells, IFN- γ also has important direct effects on other cells such as epithelia. For example, IFN- γ regulates the major histocompatibility complex (MHC) class II-dependent immune cell recognition of, for example, viral antigens expressed by host cells, and exaggerates an autoimmune reaction by inducing HLA-DR expression in target cells. In the thyroid this may potentially promote autoantigen presentation (2).

Thyroid follicles consist of a single layer of highly polarised epithelial cells that constitute a barrier between the follicular lumen, the storage place of thyroglobulin, and the extrafollicular space. A prerequisite for maintaining the barrier function and apical/basolateral polarity of epithelia is the presence

of tight junctions that prevent paracellular leakage and lateral diffusion of membrane proteins and lipids. For this purpose, tight junctions consist of a number of proteins that assemble in a regulated manner (3). In the thyroid, the epithelial barrier is known to be very tight (4) thus preventing inappropriate release of thyroglobulin to the circulation. In addition, thyroid tight junctions likely contribute to the restricted localisation of the thyrotrophin (TSH) receptor and thyroperoxidase to the basolateral and apical domains of the cell surface, respectively.

Proinflammatory cytokines have been found to negatively affect epithelial barrier functions *in vitro* in experiments mainly conducted on cell lines (5–8). We have previously shown (9, 10) that interleukin-1 α (IL-1 α) severely impairs tight junctions in primary cultured human thyrocytes obtained from Graves' thyroid tissue, and suggested that this may provide a possible mechanism for release and exposure of otherwise

hidden thyroid autoantigens to the immune system. In this study, we have investigated the effect of recombinant IFN- γ on thyroid epithelial properties of human thyrocytes cultured as a monolayer in a bicameral chamber. Cells exposed to the cytokine were analysed for transepithelial electrical resistance, expression and localisation of tight junction proteins and ultrastructural morphology.

Materials and methods

Isolation and culture of human thyrocytes

Human tissue was obtained from patients operated on for Graves' disease ($n = 8$). As described earlier (4), minced tissue was digested with 0.25 mg/ml collagenase (Worthington, Freehold, NJ, USA) in the presence of 0.1 mg/ml trypsin inhibitor type I-S and 2 μ g/ml DNase I (both purchased from Sigma Chemical Co., St Louis, MO, USA). In order to remove connective tissue, the cell suspension was filtered through nylon filters with diminishing pore size. Blood cells and thyroid-infiltrating lymphocytes were to a major extent excluded by repeated washings and centrifugations at 600 r.p.m. for 5 min of the follicle preparation. The isolated segments of ruptured thyroid follicles were either frozen in fetal calf serum (Gibco, Paisley, UK) containing 10% DMSO (Sigma) and stored at -80°C before use, or seeded directly on a plastic support or on a permeable filter pre-coated with 0.3 mg/ml collagen S type I (Roche Diagnostics Co., Indianapolis, IN, USA) (pore size 0.4 μm), of Transwell inserts (no. 3413; Corning Costar Co., Acton, MA, USA). The cells were cultured in 5% CO_2 at 37°C in Eagle's Minimum Essential Medium supplemented with 5% fetal calf serum, fungizone (2.5 $\mu\text{g}/\text{ml}$), penicillin (200 U/ml) and streptomycin (200 U/ml) (all cell culture reagents from Gibco). Subconfluent or confluent thyrocytes were stimulated with human recombinant IFN- γ (10–1000 U/ml; Roche), IL-1 α (100 U/ml; Roche) or tumour necrosis factor- α (TNF- α , 10 ng/ml; Roche), in the presence or absence of bovine thyroid-stimulating hormone (TSH, 1 mU/ml; Sigma).

Transepithelial electrical resistance

The barrier function of the thyroid epithelial monolayer was estimated by measuring the transepithelial electrical resistance with a Millicell ERS ohmmeter (Millipore, Bedford, MA, USA). The values were corrected for the background resistance measured across filter without cells.

Western blot analysis

Subconfluent thyrocytes grown in 35 mm Petri dishes (Falcon, BD Biosciences, Stockholm, Sweden) were stimulated with cytokines for 48 h and thereafter

lysed with modified sample buffer containing 0.5 mol/l Tris-HCl pH 6.8 and 2% w/v SDS. The protein concentration was determined with the Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. Solubilised proteins were separated by SDS-PAGE (4–20%) and thereafter transferred to 0.45 μm nitrocellulose membranes in a mini trans-blot cell (Bio-Rad Laboratories AB, Sundbyberg, Sweden). After pre-incubation with 5% milk in Tris-buffered saline containing 0.1% Tween 20, pH 7.6, to reduce unspecific antibody binding, the membrane was incubated for 1 h with a polyclonal primary antibody against claudin-1 (Zymed Laboratories Inc., San Francisco, CA, USA, cat. no. 71-7800; 1:500) and, after stripping, relabelled with an anti-occludin antibody (Zymed; 1:1500). Anti- β -actin (Sigma; 1:2000) was used to confirm equal loading. Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse antibodies (DAKO A/S, Glostrup, Denmark; 1:1000) were used for detection of immunoreactivity. Peroxidase activity was visualised by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK), and the staining intensity was quantified by densitometric scanning on a GS-700 Imaging densitometer (Bio-Rad) using the Molecular Analyst software for calculations.

Immunofluorescence

Human thyrocytes cultured on the filter of a bicameral chamber or on chamber slides (Falcon, BD Biosciences) were fixed in ice-cold ethanol for 20 min followed by washing with PBS pH 7.0 and pre-incubation with blocking buffer containing 5% milk, 0.1% gelatin and 7.5% sucrose in PBS and with avidin-biotin blocking reagents (Vector Laboratories Inc., Burlingame, CA, USA). The cells were then incubated with primary antibodies against claudin-1 (Zymed, cat. no. 71-7800 and 51-9000; 1:100) and occludin (Zymed; 1:400) for 1 h followed by incubation with a biotinylated donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech; 1:400) for 30 min. Bound antibodies were detected by incubation with FITC-streptavidin (Amersham Pharmacia Biotech; 1:300) for 30 min. Filters cut out from the inserts were mounted with Vectashield (Vector) on glass slides (Falcon, BD Biosciences), and examined and photographed in a Nikon Microphot FXA epifluorescence microscope equipped with a QLC100 confocal laser scanning module (VisiTech International, Sunderland, UK).

Electron microscopy

The cultures were fixed in 2.5% glutaraldehyde in 0.05 mol/l sodium cacodylate for 30 min followed by washing with 0.05 mol/l ice-cold Tris-HCl pH 7.6 and 0.15 mol/l sodium cacodylate. The cells were

postfixed in 1% osmium tetroxide for 30 min, dehydrated in ethanol series and embedded in Agar 100 epoxy resin (Link Nordiska, Upplands Väsby, Sweden). Ultrathin sections cut perpendicular to the cell layer were contrasted with uranyl acetate and lead citrate and analysed in a Zeiss 902 transmission electron microscope.

Caspase-3 activity

Caspase-3 proteolytic activity, used as a marker for apoptosis, was determined with the use of a fluorogenic peptide substrate, essentially as described by Andersson *et al.* (11). Briefly, confluent thyrocytes cultured on the filter of a bicameral chamber were exposed to IFN- γ (100 and 1000 U/ml) or IL-1 α (100 U/ml) for 24 h, or incubated with staurosporine (10 μ mol/l; Sigma) for 9 h, and then frozen at -20°C . Thawed samples were solubilised in a buffer containing 0.2% 3[3-cholaminopropyl diethylammonio]-1-propane sulphonate (CHAPS) after which the caspase-3 substrate DEVD-AMC (Calbiochem, La Jolla, CA, USA) was added. The fluorescence of the cleavage product was measured in a microplate spectrofluorometer (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA, USA). Caspase-3 activity levels were normalised to protein concentration determined by the BCA protein assay (Pierce Biotechnology).

Results

IFN- γ impairs the human thyroid epithelial barrier function

In conformity with previous observations (4), addition of TSH (1 mU/ml) to confluent human thyrocytes cultured on the filter of a bicameral chamber resulted in a 70% increase in transepithelial electrical resistance, amounting to 550–800 $\Omega\cdot\text{cm}^2$ in different experiments, after 24 h and onwards (Fig. 1). In contrast, after stimulation with IFN- γ (100 U/ml) for 24–48 h there was a gradual decrease in resistance to 60–150 $\Omega\cdot\text{cm}^2$ (Fig. 1). The level of reduced barrier function was similar to that previously reported for IL-1 α -treated thyrocytes and reflecting paracellular leakage not only to ions but also to molecules including thyroglobulin (9). Lower concentrations of IFN- γ (10 U/ml) also caused a decrease in transepithelial electrical resistance (data not shown), but this was not a consistent finding. The IFN- γ -induced loss of resistance was only slightly less pronounced in cultures co-stimulated with TSH (Fig. 1).

IFN- γ counteracts thyroid epithelial organisation

We have previously shown that filter-cultured human thyrocytes form a rather disorganised cell layer unless

Figure 1 Effect of IFN- γ on transepithelial electrical resistance (TER) in filter-cultured human thyrocytes. Confluent thyrocytes were stimulated with IFN- γ (100 U/ml) in the absence (●) or presence (■) of TSH (1 mU/ml) for 48 h. TSH (□) promotes a high TER. Data are expressed as percentage of untreated controls (○). Means $\pm$ S.D. ($n = 3$).

TSH is present (4). In conformity with this, the TSH-stimulated cultures largely consisted of a monolayer of polarised cells (Fig. 2A) that were connected by typical tight junctions at the apical extremity of the intercellular space (Fig. 2B). After treatment with IFN- γ , the cell layer changed to a multilayered appearance also in the presence of TSH (Fig. 2C). There were no general ultrastructural changes of the tight junctions, but occasionally discrete dilations of the plasma membrane contact were observed (Fig. 2D). The IFN- γ -treated cultures did not show any ultrastructural signs of necrosis or apoptosis. Lack of apoptosis was further confirmed by analysis of caspase-3. In contrast to the response to the proapoptotic agent staurosporine, neither IFN- γ (100–1000 U/ml) nor IL-1 α (100 U/ml) were able to stimulate caspase-3-dependent proteolysis (Fig. 3).

IFN- γ reduces the expression and alters the localisation of claudin-1

The pronounced action of IFN- γ on the thyroid barrier prompted us to investigate the possible effect of IFN- γ on tight junction proteins. Western blot analysis demonstrated a 75% decrease of claudin-1 in subconfluent IFN- γ -treated cells regardless of whether or not TSH was present (Fig. 4). In contrast, IL-1 α or TNF- α increased the expression of claudin-1 about three times in comparison with the control levels (Fig. 4). The reduced claudin-1 expression after IFN- γ treatment was accompanied by a decreased intracellular and cell surface immunostaining (Fig. 5A, B). Occludin, another

Figure 2 Electron micrographs of filter-cultured human thyrocytes exposed to TSH and IFN- γ . (A, B) TSH stimulation (1 mU/ml) for 72 h. The cells form a polarised monolayer, and the apical cell surface facing the upper medium exhibits numerous microvilli (A). The tight junctions apically located in the intercellular cleft display closely apposed membranes (B, arrow). (C, D) Co-stimulation with TSH and IFN- γ (100 U/ml) for 72 h. The cells are flat and elongated and extend on top of each other as a multilayer (C). The tight junctions are partly dilated (D, arrows). Scale bars: A, C = 2 μ m, B, D = 0.5 μ m.

tight junction protein, did not show corresponding changes in protein expression after IFN- γ or other cytokine treatments (Fig. 4); only IL-1 α consistently reduced the expression of occludin. The immunofluorescent pattern of occludin was also not changed in cells exposed to IFN- γ (Fig. 5D) in comparison with untreated cells (Fig. 5C). In confluent thyrocytes grown on the filter of a bicameral chamber the inhibitory effect of IFN- γ on claudin-1 expression was less pronounced (data not shown). However, the localisation of the protein was altered. In TSH-stimulated cells claudin-1 showed a smooth and linear distribution along the entire

Figure 3 Caspase-3 activity in confluent filter-cultured thyrocytes exposed to cytokines for 24 h. The values of caspase-3 activity in cultures stimulated with IFN- γ (100 and 1000 U/ml) and IL-1 α (100 U/ml) are below the threshold level for significant degradation (dotted lines indicate range of background). Staurosporine (10 μ mol/l) is used as a positive control of caspase-3-dependent apoptosis. Means $\pm$ S.D. ($n = 3$).

Figure 4 Effects of cytokines on claudin-1 and occludin expression in subconfluent human thyrocytes. After 3 days of culture, the cells were exposed to IFN- γ (100 U/ml), IL-1 α (100 U/ml) or TNF- α (10 ng/ml), in the absence or presence of TSH (1 mU/ml), for an additional 48 h. The upper panel shows Western blots of claudin-1 and occludin (data obtained from relabelled gel). The lower panel illustrates the densitometric quantification of claudin-1 and occludin after normalisation to the corresponding protein levels in untreated cultures (set to 1), and to the simultaneous expression of β -actin used as a loading control (data not shown).

cell-cell contacts (Fig. 6A), but when IFN- γ was added together with TSH claudin-1 obtained an irregular and discontinuous staining pattern (Fig. 6B).

Discussion

The ability of thyroid epithelial cells to maintain a tight barrier and thereby seclude the follicular lumen and its content from the interstitial space is functionally important. The reason is that the high luminal concentrations of iodide and the thyroid prohormone

Figure 5 Immunolocalisation of claudin-1 (A, B) and occludin (C, D) in subconfluent human thyrocytes. (A) Claudin-1 shows a punctate perinuclear staining and a linear fluorescence at cell–cell contacts in untreated cells. (B) Cells exposed to IFN- γ (100 U/ml) for 48 h have a reduced intracellular and a weaker cell surface localisation of claudin-1. (C, D) The distribution and localisation of occludin are not changed upon exposure to IFN- γ for 48 h (D) compared with the untreated cells (C). Scale bar = 10 μ m.

Figure 6 Immunolocalisation of claudin-1 in confluent human thyrocytes. (A) Cells stimulated with TSH (1 mU/ml) for 72 h. Claudin-1 outlines the entire cell borders without interruptions. Very little intracellular staining is observed. (B) Cells exposed to both TSH and IFN- γ (100 U/ml) for 72 h. Claudin-1 immunoreactivity is partly lacking at the cell–cell contacts (arrows), and patches of immunofluorescence that might be located intracellularly are frequent (arrowheads). Scale bar = 5 μ m.

thyroglobulin are essential for a proper iodination and regulated release of thyroid hormones. Moreover, in view of the strong immunogenic properties of thyroglobulin (12), inappropriate leakage of luminal content might trigger and perpetuate an autoimmune reaction. In autoimmune thyroid disease the inflammatory process is considered partly to be mediated by infiltrating T-helper 1 cells that release IFN- γ . A direct pathogenic effect of this cytokine has recently been demonstrated in transgenic mice specifically over-expressing IFN- γ in the thyroid (13). The present data show that IFN- γ impairs the epithelial barrier function in human thyroid epithelial cells in culture. Concomitantly, the expression of claudin-1, an important

member of the tight junction protein complex, is decreased and the junctional localisation of the protein is altered. These effects suggest a possible mechanism for a facilitated exposure of autoantigens (thyroglobulin and thyroperoxidase) normally hidden in the follicular lumen, which may operate in conjunction with IFN- γ -induced aberrant HLA-DR expression to promote the pathogenesis of autoimmune thyroid disease.

The *in vivo* follicular organisation of thyroid tissue can be imitated *in vitro* by using the two-chamber culture system, in which the transepithelial electrical resistance as a measure of epithelial barrier function is easy to record. The demonstrated drop in resistance most often to less than 100 $\Omega \cdot \text{cm}^2$ in filter-cultured human thyrocytes exposed to IFN- γ for 24–48 h resembles the action of IL-1 α , that in the same culture system previously has been shown to cause an increased paracellular permeability to macromolecules as large as thyroglobulin (9). IFN- γ also induced drastic cell shape changes as revealed by electron microscopy; the thyrocytes lost the polarised appearance, became elongated and were partly reorganised into a multi-layer. In addition, the tight junctions were separated at discrete locations along the cell boundaries. It is likely that this response corresponds to the disrupted follicular organisation recently observed in transgenic mice over-expressing IFN- γ in the thyroid (13). The targeted expression of IFN- γ leads to hypothyroidism to which down-regulation of the sodium iodide symporter likely contributes (13). Given the importance of follicular integrity to thyroid function, i.e. maintenance of a high luminal concentration of iodide and thyroglobulin, the present findings suggest another means by which IFN- γ may impair thyroid hormone production. Importantly, a high concentration of TSH was unable to counteract the effects of IFN- γ . On the contrary, IFN- γ blocked the TSH-stimulated increase in transepithelial resistance. Thus, IFN- γ exerts a dominant negative effect on several mechanisms that govern and regulate the epithelial properties of thyroid cells.

An impaired barrier function could potentially be due to apoptotic or necrotic lesions within the cultured thyroid epithelium, and therefore cytotoxic effects possibly occurring after cytokine stimulation have to be considered. In this study, we analysed the level of caspase-3 activation, known to be a very sensitive indicator of caspase-dependent apoptosis. Caspase-3-mediated proteolysis could not be detected after stimulation with IFN- γ even at concentrations as high as 1000 U/ml, and the same was found for IL-1 α -treated cells. Moreover, no ultrastructural signs of cytotoxicity such as organelle vacuolisation, blebbing and disintegration of membranes, or nuclear condensation were encountered. It is likely therefore that loss of barrier function is not secondary to cytokine-mediated cytotoxicity. Similar observations were recently reported by van den Hove *et al.* (14) in studies on nitric oxide as mediator of IL-1 α and IFN- γ interactions.

The maintenance of an epithelial barrier is dependent on the functional interplay of several tight junction proteins (15). In humans, the many members of the claudin family play pivotal roles in tight junction structure and function in different cell types (16). Claudin-1 is known to be present in tight epithelia but absent in leaky epithelia (17). The importance of claudin-1 became obvious when fibroblasts normally lacking tight junctions were demonstrated to form networks of tight junction-like strands when transfected with claudin-1 (18). Moreover, epithelial cells over-expressing claudin-1 display an increased trans-epithelial electrical resistance and a more restricted paracellular permeability (19). This is further illustrated in claudin-1 knockout mice that soon after birth die of dehydration due to a severely impaired epidermal barrier (20).

We have previously shown that claudin-1 is expressed in thyrocytes (21). The present results demonstrate that claudin-1 is strongly reduced by IFN- γ regardless of TSH stimulation. In addition, we find that the junctional distribution of claudin-1 is disrupted in cultures that show a decreased transepithelial resistance in response to IFN- γ . A similar redistribution of claudin-1 was not obvious in IFN- γ -treated subconfluent cells, which merely had a reduced staining intensity of claudin-1 both at the cell-cell contacts and intracellularly. The reduced intracellular pool of claudin-1 is likely related to the strong inhibition of its biosynthesis. That this was not encountered in confluent and growth-arrested cells is explained by the fact that these cells before treatment had almost no intracellular claudin-1 staining. Conceivably, this may be due to a reduced requirement of newly synthesised proteins in mature tight junctions. It is well known from studies on other epithelial cells that the assembly of tight junction proteins during neoformation of the junctional complex are differently regulated than the mechanisms maintaining and stabilising already existing junctions (22). Altogether, the present observations suggest that altered expression and localisation of claudin-1 may play a direct role in the loss of the epithelial barrier in IFN- γ -treated thyrocytes.

Occludin was the first identified integral membrane protein of tight junctions (23). Although the precise role of occludin in tight junctions remains to be established, there are several reports indicating that its expression and interactions with other proteins in the junctional complex are regulated (16). Recently, IFN- γ was found to down-regulate occludin promoter activity in a human intestinal cell line (24), suggesting that occludin-dependent functions are negatively affected. However, we could not find any signs of altered level or distribution of occludin that accompanied the claudin-1 changes in thyrocytes exposed to IFN- γ . In contrast, in IL-1 α -treated cells the expression of occludin was decreased and, similar to previous observations in IL-1 β -treated astrocytes (25), at the same time the

claudin-1 level was paradoxically increased. The functional implications of these changes are presently unknown. Nevertheless, collectively these observations illustrate that cytokines exert distinct effects on tight junction proteins and that the response may differ between cell types. In human thyroid epithelial cells claudin-1 is specifically targeted by the IFN- γ receptor signalling pathway.

In summary, the present findings point to a new mechanism by which IFN- γ could promote autoimmune inflammation in the human thyroid gland. Loss of barrier function, probably involving claudin-1 dysregulation, might facilitate an inappropriate exposure of luminal autoantigens to immunoreactive tissue macrophages and infiltrating lymphocytes. Conversely, transmigration of leukocytes and entry of auto-antibodies to the follicular lumen known to occur in autoimmune thyroid disease might also be supported by leaky tight junctions due to local cytokine action(s).

Acknowledgements

This study was supported by the Swedish Medical Research Council (grant no. 537). We would like to thank Dr S Jansson and colleagues at the Department of Surgery, Sahlgrenska University Hospital, Göteborg for providing human thyroid tissue. Excellent technical assistance by Therése Carlsson, Yvonne Josefsson and Gunnel Bokhede is greatly appreciated. Part of this work was presented in preliminary form at the 27th Annual Meeting of the European Thyroid Association in Warsaw, Poland, 2001.

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Received 15 October 2002

Accepted 12 June 2003