Thyrotropin receptor and leukocyte adhesion molecules in autoimmune thyroid disease: a study of their gene expression by Northern blot analysis and in situ hybridization*

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In order to characterize the role of leukocyte-activating antigens and other immunological parameters in autoimmune thyroid disease, mRNA levels of intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1, E-selectin), invariant chain (II) and the thymic hormone thymosin β4 (Tβ4) were investigated in 18 human thyroid glands, including eight Graves’ thyroids, two Hashimoto’s thyroids, two endemic goiters and six healthy controls. Northern blot analysis showed that in autoimmune thyroid disease, expression of ICAM-1 and Tβ4 was correlated to transcript levels of II, whereas in the healthy controls, expression of Tβ4, ICAM-1 and ELAM-1 was low or nearly absent. ELAM-1 and TSH receptor (TSH-R) expression, the latter serving as a thyroid specific marker, was increased in some diseased glands but showed no relation to the immunological parameters mentioned above. Localization of the specific mRNAs by in situ hybridization demonstrated a cell-specific expression of TSH-R (thyrocytes), ELAM-1 (vascular endothelial cells) and Tβ4 (cells of hematopoietic origin). In contrast, transcripts of II and ICAM-1 were found in thyrocytes, leukocytes and endothelial cells. Our results implicate a coordinate expression of ICAM-1, Tβ4 and II in autoimmune thyroid disease, yielding distinct cellular expression patterns. Differential expression of ICAM-1, II and the TSH-R in thyroid epithelial cells indicates active regulatory events within the thyrocyte.

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Cellular adhesion molecules have been of major interest in the pathogenesis of acute and chronic inflammation in recent years (for a review, see Ref. 1). Adhesion of leukocytes to endothelium is the first step in localizing circulating cells at an inflammatory site that, subsequently, is the place of further leukocyte activation and antigen presentation. Endothelial leukocyte adhesion molecule 1 (ELAM-1 or E-selectin) is an inducible factor from the selectin superfamily, mediating selective adhesion of neutrophils and monocytes to endothelial cells (2), and is involved also in the diapedesis of memory (CD45RO+ T cells (3). Intercellular adhesion molecule 1 (ICAM-1), an integral membrane glycoprotein of the immunoglobulin superfamily, is expressed on a variety of hematopoietic and non-hematopoietic cells, including B and T cells, fibroblasts, keratinocytes and endothelial cells, and the level of expression on these cells can be upregulated by various cytokines (4).

Autoimmune thyroid disease (ATD) is characterized by lymphocytic infiltration of the thyroid gland and the appearance of autoantibodies against thyroid-specific antigens, such as the TSH receptor (TSH-R), thyroglobulin (TG) and thyroid peroxidase (TPO). Therefore, we speculated that expression of adhesion molecules may facilitate lymphocyte migration into autoimmune inflammatory sites, which, in addition to the presentation of autoantigens to the immune system, may be a prerequisite for the induction and maintenance of ATD. Furthermore, hormone-like peptides that regulate cellular functions such as cell locomotion, chemotaxis and phagocytosis may be involved in the autoimmune processes. Thymosin β4 (Tβ4) is a 5-kD polypeptide first isolated from an extract of calf thymus and exhibits

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such functions by regulating actin assembly (5). Invariant chain (Ii) is an indicator of MHC-II expression and directs MHC-II molecules to the endocytic compartment of peptide binding (6, 7).

Using northern blot analysis and in situ hybridization, we investigated gene expression of ELAM-1, ICAM-1, Tß4, and Ii in healthy and diseased thyroid glands. In order to obtain indirect evidence about the
Fig. 1. (A) Northern blot analysis of TSH receptor (TSH-R), invariant chain (Ii), thymosin β4 (Tß4), intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule 1 (ELAM-1) gene expression in 13 different human thyroid glands. 20 µg (each lane) of total RNA isolated from thyroid fragments was applied to nylon membranes and hybridized with 32P-labeled cDNA probes. The mRNA sizes were estimated relative to a 9.5 kb ladder (BRL, USA) in an adjacent lane of which the 7.5, 4.4, 2.4 and 1.5 kb fragment is depicted. Ethidium bromide staining of the agarose gel served as a control for the integrity of RNA samples before transfer. Probing of the filter with 28S rRNA allowed calibration of autoradiographic signals for potential loading differences (e.g. TSH-R mRNA/28S rRNA ratio). (B) Northern blot analysis of TSH-R, Ii, Tß4, ICAM-1 and ELAM-1 gene expression in six healthy thyroid glands (five additional normal tissues to healthy gland AB of (A)). Northern blot analysis was performed as described for (A).
Fig. 2.
Fig. 2. Autoradiographs of healthy, Graves’ and Hashimoto’s thyroid tissue hybridized in situ with 35S-labeled antisense cRNA probes synthesized from TSH receptor (TSH-R), thymosin β4 (Tβ4), invariant chain (Ii), endothelial leukocyte adhesion molecule 1 (ELAM-1) and intercellular adhesion molecule 1 (ICAM-1) cDNA fragments cloned into a Bluescript vector (pBS KS II+). Cryostat sections of a Graves’ (A) and Hashimoto’s (B) thyroid gland hybridized for TSH-R mRNA (x36). As in healthy tissue (not shown), only thyroid follicles stain positive for TSH-R transcripts. Hybridization of Tβ4 in healthy (C, x36), Graves’ (D, x90) and Hashimoto’s (E, x36) thyroid glands. Only non-thyrocytes stain positive for Tβ4 mRNA. Signals are much more abundant in autoimmune thyroid disease (ATD) than in healthy tissue. Hybridization of Ii in healthy (F) and Hashimoto’s (G) thyroid glands (x90). In addition to interstitial cells, thyocytes are a source of Ii transcripts. Hybridization of ELAM-1 in Graves’ (H) and Hashimoto’s (I) thyroid glands (x36). Endothelial leukocyte adhesion molecule 1 mRNA is restricted to vascular endothelial cells and is much more abundant in ATD than in healthy tissue. Hybridization of ICAM-1 in Graves’ (K and L) and Hashimoto’s (M) thyroid glands. Different cell types stain positive for ICAM-1 transcripts including thyocytes (K, x90), interstitial (M, x90) and endothelial cells (L, x36). No signals were found in two healthy thyroid glands studied.

potential impact of these adhesion molecules on thyroid-specific gene activity, we also studied expression levels of the TSH-R gene.

Patients and methods

After approval of the local ethics committee, thyroid tissue was obtained from a total of 18 Patients (eight Graves’ disease, two Hashimoto’s disease, two endemic goiters and six patients who underwent surgery of the parathyroid glands. In order to facilitate surgical preparation of the thyroid gland, Graves’ patients received 400 mg of iodide intravenously (Endojodin®; Bayer, Germany) once a day for 4 days before surgery. Prior to iodide application, patients have been treated with varying concentrations of antithyroid drugs with or without thyroid hormones. Total T1, total T4, thyroxine-binding globulin (TBG) (by RIA; Ciba Corning, Germany) and TSH (by ILMA; Ciba Corning, Germany) were measured routinely in all patients prior to surgery, indicating that thyroid hormone levels were within or above the normal range. In most of the patients, TSH-R antibodies (by RRA; Trak®, Henning Berlin, Germany) and antibodies against TG and TPO (by ELISA; Elias, Germany) were measured in addition.

Northern blot analysis: preparation of probes

Human TSH-R. A 3500 bp 3’-terminal EcoRI fragment of the human TSH-R cDNA (Heldin N-E and Westermark B, unpublished data) covering 1500 bp of the approximately 2400 bp long complete coding sequence published by other investigators (8–12) was used as a probe.

Invariant chain. The Ii probe consisted of 100, 300 and 900 bp PstI fragments of human invariant chain cDNA
(13) (kindly provided by Dr N Koch, Heidelberg, Germany). This probe was chosen carefully in order to avoid hybridization with TG transcripts, because other sequences of the II gene are known to show a high homology to the repetitive elements in the TG gene (14).

Thymosin β4. A 650 bp EcoRI fragment of rat Tβ4 cDNA (15) (kindly provided by Dr M Atkinson, Munich, Germany) was used as a probe.

**Intercellular adhesion molecule 1.** The probe was a 1846 bp XbaI fragment of human ICAM-1 cDNA (16) (British Biotechnology).

**Endothelial leukocyte adhesion molecule 1.** The probe was a 2719 bp XbaI fragment of human ELAM-1 cDNA (2) (British Biotechnology).

A 32P-labeled oligo 28S rRNA probe was used as a calibration standard, as described previously (17).

**Northern blot analysis**

Total RNA was isolated from thyroid fragments that had been frozen in liquid nitrogen immediately after surgical removal. Total RNA was extracted following the method as described by Chirgwin and co-workers (18). For northern blot analysis, 20 μg of total RNA per lane were denatured and fractionated on 1.5% agarose/2.2 mol/l formaldehyde gels, transferred to Zetaprobe® nylon filters (Bio-Rad, Germany), hybridized and washed as described previously (17). Nylon filters were stripped and rescreened.

 Autoradiographic signals were quantified by laser scanning densitometry (Pharmacia LKB, Germany) and compared to signals of oligo 28S rRNA in order to calibrate for potential RNA loading differences. Values of healthy thyroid “AB” were adapted arbitrarily as unit values.

**In situ hybridization**

Cryostat sections, 8 μm thick, mounted on sterilized slides pretreated with 3-aminopropyl-triethoxysilane (Sigma, St Louis, MO) were fixed in 4% (w/v) paraformaldehyde in phosphate buffer saline (pH 7.4) and acetylated to reduce non-specific hybridization (19). The cRNA probes were generated from the following cDNA fragments, which were cloned into a Bluescript vector. TSH-R: 450 bp HindIII fragment coding for a part of the extracellular domain of the TSH-R. II: 160 bp Apal–HindIII fragment (160–320). This fragment did not show any homology to TG either. Tβ4: 650 EcoRI full length cDNA. ICAM-1: 580 bp PstI fragment (921–1501). ELAM-1: 562 bp XbaI–PstI fragment (1–562). Probes were labeled with [35S]UTP to a specific activity of approximately 10⁹ cpm/μg using an in vitro transcription kit (Stratagene, Germany). The hybridization mixture contained 50% formamide, 0.75 mol/l NaCl, 0.025 mol/l PIPES, 0.025 mol/l EDTA (pH 6.8), 5× Denhardt’s 10 mmol/l dithiothreitol, 0.2 × SDS, 250 mg/l ssDNA, 250 mg/l yeast tRNA, 10% (w/v) dextran sulfate and a cRNA probe (0.1 mg/l). Hybridization proceeded with 40 μl of probe at 50°C overnight. The non-hybridized single-stranded RNA probe was then digested with RNase A (40 mg/l) in ribonuclease buffer (0.5 mol/l NaCl, 10 mmol/l TRIS (pH 8.0), 1 mmol/l EDTA) at 37°C for 30 min. Slides were washed at increasing temperatures in SSC of decreasing concentrations, and finally twice at 60°C in 0.1 × SSC for 15 min. The slide preparations were autoradiographed by dipping into autoradiography emulsion (LM 1, Amersham, Germany) that was diluted 1 : 1 with 0.6 mol/l ammonium acetate. Exposure proceeded at 4°C in light-tight boxes. Slides were developed with D19 developer (Kodak, USA) and counter-stained with hematoxylin. Photographs were made on a Zeiss Axiosmat photomicroscope (Zeiss, Germany).

**Results**

**Northern blot analysis**

Northern blot analysis showed that expression of the TSH-R gene was variable in all 18 thyroids investigated (Fig. 1). In comparison to healthy thyroid gland AB, some diseased thyroids expressed higher TSH-R mRNA levels (e.g. Graves’ 1 to 5 and 7, and endemic goiter 1) while others showed lower or absent levels. Matching TSH-R transcript levels of healthy thyroid AB to five additional healthy thyroids showed that expression was in the same order of magnitude in three glands while two other healthy controls (KK and IM) showed slightly higher TSH-R mRNA levels.

Gene expression of II, Tβ4, ICAM-1 and ELAM-1 also was found to exhibit a variation of mRNA levels (Fig. 1A). Invariant chain, Tβ4 and ICAM-1 displayed a comparable order of transcript levels being highest in those thyroid glands where transcript levels of the TSH-R were low (e.g. Hashimoto’s 1). Although mRNA levels of II also showed some differences in the six healthy thyroid glands, no correlation between II and Tβ4 or ICAM-1 could be identified in these tissues (Fig. 1B).

Expression of ELAM-1 was characterized by only sparse hybridization signals in four Graves’ and in both Hashimoto’s and was almost absent in all the normal thyroid glands.

**In situ hybridization**

In situ hybridization (Fig. 2) showed that expression of the TSH-R mRNA was restricted to the thyrocyte in all of the thyroid glands investigated. In healthy tissue (three glands studies by in situ hybridization) and in fragments of Graves’ thyroid glands TSH-R mRNA was...
TSH-R intense distributed thyroid almost Hashimoto’s individual healthy Hashimoto’s thyroiditis numbers and therefore be expressing lymphocytic (interstitium) out discussion more a few clones, diapedesis Discussion Expression Intercellular T/34 thyrocytes source in one diseased healthy cells tissue, of healthy cells showed only the blood infiltration same degree positive only ICAM-1-expressing follicular lymphocytes positive for II and ICAM-1 could be identified in healthy tissues, indicating that different regulatory mechanisms may be active in the healthy state. Indeed, expression of II in the six healthy thyroid investigated seemed to occur independently of expression of other leukocyte adhesion molecules (ICAM-1, ELAM-1), implying that expression of MHC-II is needed not only for T-cell activation but also for induction of T-cell energy (against self-peptides) (23). Additional signals provided by B7, for example, might be needed to initiate the immune attack and activate the cascade of autoimmune events, such as expression of ICAM-1 (23).

So far, little is known about the 5kD thymic polypeptide Tβ4, which is believed to regulate cellular functions such as cell locomotion, chemotaxis and phagocytosis by regulating actin assembly (5). Interestingly, expression of Tβ4 was paralleled by II and ICAM-1 transcript levels only in ATD, suggesting that expression of Tβ4 may be regulated by the same cytokines that induce gene expression of these two surface molecules in the autoimmune context. Further investigations into regulation and function of Tβ4 are needed to prove this hypothesis.

With the help of in situ hybridization, we could demonstrate that thyroid follicular cells expressed II and ICAM-1 in both Graves’ and Hashimoto’s thyroiditis. However, some differences were observed regarding other cell types: most infiltrating mononuclear cells were positive for II, while only a few expressed ICAM-1. In contrast, higher numbers of endothelial cells were found to be positive for ICAM-1 than for II mRNA.

Because the rate of transcription is the major determinant of cell surface expression of these molecules (16, 20), we may assume that our data reflect II and ICAM-1 protein. Northern blot analysis of II, ICAM-1, Tβ4 and TSH-R (Fig. 1) reveals that expression of the
immunological parameters of cell adhesion is not correlated positively to TSH-R as a thyroid-specific functional parameter. Rather, as described previously (17), we detected a tendency towards an inverse relation in ATD. This phenomenon might be due in part to destruction of functional thyroid follicular cells by infiltrating lymphocytes, but also may reflect regulatory events within the thyrocyte itself. The latter suggestion is supported by our finding that thyroid follicular cells are able to express genes of MHC class II (such as Ii) and other leukocyte-activating antigens such as ICAM-1.

Interestingly, in situ hybridization detected TSH-R mRNA only in thyrocytes and not in lymphocytes, despite the high sensitivity of this method. However, these findings are not necessarily in contrast to a recent observation by Francis and co-workers (24) who, with the help of reverse transcription polymerase chain reaction, demonstrated TSH-R transcripts in lymphocytes, whereas the less sensitive method of northern blot analysis failed to detect TSH-R mRNA. This suggests the presence of only low, if any, copy number TSH-R mRNA in lymphocytes.

Expression of ELAM-1 was not correlated to mRNA levels of II or ICAM-1 and was expressed exclusively on vascular endothelial cells. These results implicate that ELAM-1 is not induced by the same cytokines that regulate the expression of ICAM-1 and II (i.e. mainly IFN-γ); this is supported by in vitro data of Pober (25) and Graber (26), who demonstrated stimulation of ELAM-1 expression in human endothelial cell monolayers by interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) but not by IFN-γ. In contrast to our data, Miyazaki and co-workers did not detect ELAM-1 in any type of cell, including thyrocytes, endothelial cells and mononuclear cells (27).

Conflicting data also were reported by this group on the expression of ICAM-1: ICAM-1 expression could not be detected on thyrocytes, either in Graves’ thyroid glands or in the control (27).

In contrast, a positivity for ICAM-1 on thyroid follicular cells was observed by Bagnasco and co-workers (28) in Hashimoto’s thyroiditis specimens but not on thyrocytes of Graves’ glands or normal tissue. Finally, Weetman et al. were able to demonstrate ICAM-1 on thyroid follicular cells in areas of lymphocytic infiltration in patients with Graves’ disease and Hashimoto’s thyroiditis (21, 22). Thus, our data confirm these studies by Weetman and co-workers. In all studies including the one presented, ICAM-1 expression could be detected in endothelial cells in ATD.

In summary, we have demonstrated that II, Tβ4, ICAM-1 and ELAM-1 are expressed at different levels in the thyroid gland. Transcript levels of the TSH-R gene also are variable. While expression of TSH-R and ELAM-1 is confined to thyrocytes and vascular endothelial cells, respectively, different cell types (including thyroid follicular cells) express II and ICAM-1. The comparable transcript levels of II, ICAM-1 and Tβ4 in ATD indicate a co-regulated expression of these genes, implying similar gene activation pathways in a state of autoimmune.

References


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