Summary. To elucidate the role of Th2 cytokines in autoimmune thyroid diseases, we have studied by immunohistochemistry the expression of two Th2 ligand/receptor systems (CD30-L/CD30 and IL-6/IL-6R) in goitrous Graves’ disease (GD) and Hashimoto’s thyroiditis (HT).

A total number of 50 nodular goiters (NG), including 10 GD showing a lymphoid aggregate grade I, 30 HT 8 of which had a lymphoid aggregate of grade I, 12 of grade II and 10 grade III, and 10 colloid goiters have been evaluated. In addition, 5 normal thyroids were included in the study as controls.

Reactivity of ligand and cognate receptor of both CD30-L/CD30 and IL-6/IL-6R pathways was observed in a greater proportion of GD, compared to HT (P<0.005). In HT, the expression of CD30-L/CD30 system was detected more frequently than IL-6/IL-6R (P<0.05) and showed an inverse correlation with the grade of lymphoid aggregate, whereas IL-6/IL-6R correlated positively with lymphocyte infiltration (P<0.05).

Based on our results concerning a dominance of Th2 cytokines in GD, we postulate that CD30-L/CD30 and IL-6/IL-6R systems could play a major role in the pathogenesis of GD. However, the expression of CD30L/CD30 and IL-6/IL-6R in HT suggests that Th2 mechanisms are involved also in tissue damage of HT. The two systems could contribute to drive the autoimmune response skewing toward a Th2 phenotype and this appears to be correlated with the lymphoid aggregate grade.

Key words: CD30 ligand, IL-6, CD30, IL-6 receptor, Autoimmunity, Thyroid, Immunohistochemistry

Introduction

The CD30 ligand (CD30-L) and the cognate receptor CD30 belong to the tumor necrosis factor (TNF)/nerve growth factor (NGF) ligand and receptor superfamilies, respectively (Durkop et al., 1992; Falini et al., 1992; 1995; Smith et al., 1993). The interleukin 6 (IL-6) is a pleiotropic cytokine; its receptor (IL-6R) includes an 80-kD IL-6-binding subunit called IL-6Rα and a gp130/IL-6ß subunit which is responsible for the signal transduction and stabilization of the α-chain ligand complex (Hibi et al., 1990; Hirano, 1998). Both CD30-L and IL-6 are able to regulate cellular growth (Gruss et al., 1994; Blood, 1994; Yokomuro et al., 2000) upon interaction with the corresponding receptor (Kishimoto et al., 1992; Smith et al., 1993) and their effects are correlated to the expression levels (Jones, 1994; Gruss et al., 1996). In addition, they are synthesized by Th2 cells, which play a pivotal role in the autoimmune compartment (Hirano et al., 1998; Croft, 2003).

In thyroid tissue, we have previously reported that the expression of CD30-L/CD30 and IL-6/IL-6R is different in the benign and malignant nodules, suggesting a different role of the two signaling systems in benign and malignant cellular proliferation (Trovato et al., 2001, 2003; Ruggeri et al., 2002). However, the role of these two systems in autoimmune thyroid diseases (AITD) has not been fully elucidated yet. IL-6 expression has been previously evaluated in Graves’ disease (GD) as well as Hashimoto thyroiditis (HT) (Grubeck-Loebenstein et al., 1989; Zheng et al., 1991; Paschke et al., 1994; Watson et al., 1994; Kayser et al., 1995; Ajjan et al., 1996), but no data are currently available regarding the expression of these signals in AITD associated with nodular goiters (NG). Since CD30-L/CD30 and IL-6/IL-6R are involved in mechanisms of both cell proliferation and autoimmunity, we wished to evaluate the expression of these four molecules in goitrous GD and HT, compared to NG not associated with AITD.
Materials and methods

Tissue collection

Thyroid tissue specimens were retrieved from the archives of the Department of Human Pathology, University of Messina, Italy. They included 5 normal thyroids harvested during autopsy and 50 thyroid surgical samples taken from 50 patients who had undergone thyroidectomy for large NG. The 50 patients were recruited at the Endocrinology Unit of the University of Messina, and included: 10 patients with NG associated with GD (6 females and 4 males; the mean age ± SD at the time of thyroidectomy was 43±12); 30 patients with NG associated with HT (25 females and 5 males; mean age ± SD: 52±8); 10 patients with NG and no clinical, laboratory or ultrasonographic evidence of AITD (9 females and 1 male; mean age ± SD: 55±13). All patients were euthyroid at the time of the thyroidectomy. The GD patients had been rendered euthyroid with antithyroid drugs (thiamazole) prior to surgery by a 6-12 months treatment course. The GD or HT lesions were classified at the histological diagnosis according to the criteria proposed by Doniach & Roitt (1976; Li Volsi, 1990). The GD or HT lesions were classified at the histological diagnosis according to the criteria proposed by Doniach & Roitt (1976; Li Volsi, 1990). The GD and HT lesions were studied paired with the associated nodules.

Thyroid tissues were fixed in 4% formalin and routinely processed through graded alcohol and xylene to paraffin wax. Haematoxylin-eosin (H&E) stained sections of each specimen were performed prior to immunohistochemistry. In each H&E section the intra-glandular inflammatory lymphoid aggregates have been evaluated. A lymphoid aggregate was defined as including, at least, 150 lymphocytes and a variable number of plasma cells per high-power field. When this type of lymphoid infiltration was arranged into well-developed follicular centers with central macrophage-like cells showing a large, clear cytoplasmic appearance, it was identified as a lymphoid aggregate with germinal center. The lymphoid aggregates were graded as follows: 0 = no lymphoid aggregate or at least one single, small lymphoid aggregate without germinal center in each section; I = occasional, usually small lymphoid aggregates with rare or absent germinal centers in each section; II = several, usually mixed, small and large lymphoid aggregates with some germinal center in each section; III = numerous, large lymphoid aggregates with frequent germinal centers in each section.

Immunohistochemistry

Serial sections of the selected blocks were cut at five-micrometers for the immunohistochemical studies. Immunohistochemistry was performed, separately, by mouse monoclonal antibodies (MAb) raised against human CD30-L (h-CD30L-Fc type II, 1:100, Genzyme, Cambridge, MA, USA) and CD30 (or Ki-1 antigen) (clone Ber-H2, 1:100, Dako, Carpinteria, CA, USA), respectively, and by goat MAb raised against human IL-6 and IL-6-R, respectively, (1:100, Sigma, St. Louis, MO, USA, respectively), using the biotin-streptavidin-peroxidase method (LSAB kit from Dako Corporation, Carpinteria, CA).

Antigen retrieval technique as described by Gown et al. (1993) was carried out. Tissue sections were deparaffinized in xylene and rehydrated in alcohol. Then, the endogenous biotin was inactivated by addition of 0.05% (v/v) solution of streptavidin in phosphate-buffered saline (PBS) and the endogenous peroxidase activity was quenched by adding 0.3% (v/v) solution of 3% H2O2/methanol for 30 min. The slides, placed in 10 mM citrate buffer adjusted to pH 6.0 with 2 M sodium hydroxide, were microwaved for 15 min (Whirlpool AVM 300, power set at 500 watts). Microwave exposure was broken into three equal time periods and, at the end of the first cycle, 50 ml of distilled water was added to the slide holder to prevent loss of fluid from boiling. Staining was obtained with the LSAB system (kit from Dako). 3,3’-diaminobenzidine (DAB, Sigma) activated with 0.05% hydrogen peroxide was used to develop the end reactions. Sections were counterstained with Mayer’s haematoxylin, dehydrated and mounted. Specificity of the binding was assessed either by omitting the primary antisera or by replacing the primary antisera with normal mouse or goat serum. In each of these conditions, no staining was evident. In addition, an immunoabsorption test was performed to confirm the specific immunoreactivity of each MAb. Specimens of Hodgkin lymphoma were used as positive controls for the CD30-L and CD30 immunoreaction, while specimens of colic mucosa showing inflammatory bowel disease were used as positive controls for the IL-6 and IL-6R immunoreaction. For the evaluation of the results, the following criteria were used: (i) number of positive cases; (ii) number of reactive epithelial, stromal and lymphoid cells per case: the count of the number of reactive cells was based on evaluation of 1000 cells/case, using 50x magnification; (iii) sub-cellular location of the staining: cytoplasm and/or membrane. Based on the proportion of stained cells, we scored the reaction value as low (1-10% stained cells), moderate (11-30%) and high (>31%).

Histological and immunohistochemical evaluations were done twice and blindly by two different pathologists (M.T., G.B.) with an inter-observer concordance of nearly 100%. Where minimal inter-observer discrepancies were present, the mean value was considered as the result.

Statistical analysis

Differences between proportions were analyzed by χ² test with Yates’ correction for continuity. The association between two variables was analyzed by the linear regression analysis. The level of statistical significance was set at P<0.05.
Results

Histopathology

All 50 NG showed histological features of colloid nodules (CN). Most of them were characterized by large colloid filled follicles, built of small flat or cuboidal follicular cells with dark nucleus and eosinophilic cytoplasm, even associated with multicellular microfollicles. Others were collected in solid buds showing few tiny lumina or, apparently dropping off the epithelial layer of cystically dilated follicles, resulted in a papilliferous structure called “Polster di Sanderson”. In all 50 CN no intranodular lymphoid aggregates were observed (grade 0) (Tables 1 and 2).

The histomorphological hallmark of GD was a marked hyperplasia with well-developed papillae associated with a multifocal lymphocytic thyroditis. The papilla, without fibrous stromal axis, were lined by follicular cells with a basally located dark nucleus and columnar or cuboidal/tall cytoplasm. All 10 GD showed lymphoid aggregates of grade I (Table 1).

The HT showed small follicles with scarce, dense, pink colloid, delimitated by cuboidal follicular cells with dark nucleus and eosinophilic cytoplasm. In most cases, oncocytic metaplasia was evident and consisted of

| Table 1. Lymphoid aggregate grade and expression of the CD30-L/CD30 and IL-6/IL-6R systems in epithelial cells* of normal thyroids, colloid nodules and Graves’ disease. |
|------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| SPECIMENS                    | Lymphoid aggregate grade | CD30-L/CD30 SIGNAL | IL-6/IL-6R SIGNAL |
|                              |                  | Ligand            | Receptor          | Ligand            | Receptor          |
|                              |                  | Staining score    | Staining score    | Staining score    | Staining score    |
|                              |                  | Low | Moderate | High  | Low | Moderate | High  | Low | Moderate | High  |
| Normal thyroid (n = 5)       | 0                | 0/5 | 0        | 0     | 0/5 | 0        | 0     | 0/5 | 0        | 0     |
| Colloid nodules (n = 10)     | 0                | 0/10 | 0        | 0     | 0/10 | 0        | 0     | 0/10 | 0        | 0     |
| Grave’s disease (n = 10)     | I                | 10/10 | 3        | 7     | 0/10 | 6        | 4     | 0/10 | 6        | 4     |
| Colloid nodules associated with Grave’s disease (n = 10) | 0 | 0/10 | 0        | 0     | 0/10 | 0        | 0     | 0/10 | 0        | 0     |

Lymphoid aggregate grade and expression of the CD30-L/CD30 and IL-6/IL-6R systems in epithelial cells of normal thyroids, colloid nodules and Graves’ disease. *: The proportion of positive cells was calculated based on evaluation of 1000 epithelial cells using 50x magnification. Semiquantitative grading of immunostained cells distribution was scored as low (1-10% stained cells), moderate (11-30%) and high (>31%) as specified in Materials and Methods.

| Table 2. Lymphoid aggregate grade and expression of the CD30-L/CD30 and IL-6/IL-6R systems in epithelial cells of Hashimoto’s thyroditis and colloid nodules. |
|------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| SPECIMENS                    | Lymphoid aggregate grade | CD30-L/CD30 SIGNAL | IL-6/IL-6-R SIGNAL |
|                              |                  | Ligand            | Receptor          | Ligand            | Receptor          |
|                              |                  | Staining score    | Staining score    | Staining score    | Staining score    |
|                              |                  | Low | Moderate | High  | Low | Moderate | High  | Low | Moderate | High  |
| Hashimoto’s thyroditis (n= 30) | 12/30 | 3 | 6 | 3 | 12/30 | 7 | 5 | 0 | 30/30 | 13 | 11 | 6 | 4/30 | 4 | 0 | 0 |
| I (n= 8)                     | 6/8 | 1 | 3 | 2 | 6/8 | 3 | 3 | 0 | 8/8 | 4 | 3 | 1 | 0/8 | 0 | 0 | 0 |
| II (n= 12)                   | 4/12 | 1 | 2 | 1 | 4/12 | 3 | 1 | 0 | 12/12 | 7 | 3 | 2 | 2/12 | 2 | 0 | 0 |
| III (n= 10)                  | 2/10 | 1 | 1 | 0 | 2/10 | 1 | 1 | 0 | 10/10 | 2 | 5 | 3 | 2/10 | 2 | 0 | 0 |
| Colloid nodules associated with Hashimoto’s thyroditis (n = 30) | 0 | 0/30 | 0 | 0 | 0 | 0/30 | 0 | 0 | 0 | 10/30 | 10 | 0 | 0 | 0/30 | 0 | 0 | 0 |

Lymphoid aggregate grade and expression of the CD30-L/CD30 and IL-6/IL-6R systems in epithelial cells of Hashimoto’s thyroditis and colloid nodules. *: The proportion of positive cells was calculated based on evaluation of 1000 epithelial cells using 50x magnification. Semiquantitative grading of immunostained cells distribution was scored as low (1-10% stained cells), moderate (11-30%) and high (>31%), as specified in Materials and Methods.
follicular cells with oxyphil cytoplasm while, occasionally, clear metaplasia was seen and consisting of follicular cells with clear nucleus and eosinophilic cytoplasm. Lymphoid aggregates were graded as I (8/30 or 27%), II (12/30 or 40%) or III (10/30 or 33%) \((\chi^2=16.2, P<0.001 \text{ vs GD})\) (Table 2).

**Immunohistochemistry**

No expression of CD30-L/CD30 could be detected in any normal thyroids and CN (Tables 1 and 2). IL-6 expression was observed in 20% of the normal thyroids and in 30% of the CN, but all cases were IL-6R, negative, confirming our previous data (Ruggeri et al., 2002). In both normal thyroids and in CN, IL-6+ immunoreaction was always low and it was detected only in the cytoplasm of epithelial cells. No differences in the expression of the two signals were observed between CN associated or not associated to AIDT.

All GD expressed the CD30-L/CD30/IL-6/IL-6R (Table 1), while the contemporary expression of these four proteins was seen in only 4/30 (13%) of the HT \((\chi^2=21.1, P<0.001 \text{ vs GD})\) (Table 2).

When comparison between GD and HT is limited to the grade I lymphoid infiltration (because so were all GD), then the proportion of CD30-L+/CD30+/IL-6+/IL-6R+ cases was 100% in GD and 0% in HT \((\chi^2=14.2, P<0.001)\). The simultaneous expression of the two ligand/receptor systems was observed in HT with a greater extent of lymphoid infiltration, and precisely in 17% of HT cases with grade II and in 20% with grade III infiltration.

As mentioned above, in GD, the immunoexpression of each ligand was invariably associated with the respective receptor. In contrast, only 40% of the HT co-expressed CD30-L and CD30 \((\chi^2=8.62, P=0.003 \text{ vs GD})\) and even fewer (13%) co-expressed IL-6 and IL-6R \((\chi^2=21.09, P<0.001 \text{ vs GD})\). In HT, the 40% rate was greater than the 13% rate. The four HT expressing IL-6 and IL-6R were invariably CD30-L+/CD30+. Interestingly, in HT the extent of lymphoid infiltrate was related inversely with the proportion of CD30-L+/CD30+ cases but directly with the proportion of IL-6+/IL-6R+ (Fig. 1).

In both GD and HT lesions, CD30-L+, CD30+, IL-6+ or IL-6R+ immunoreaction was similar and concerned primarily the epithelial cells. In particular, CD30-L+ immunoreaction was moderate, CD30+ was low as well as IL-6+ and IL-6R+ (Tables 1 and 2). In the reactive GD and HT epithelial cells, CD30-L+ and CD30+ immunostaining was detected in the membrane and cytoplasm (Panel A and B of Figures 2 and 3, respectively) and IL-6+ and IL-6R+ immunostaining was confined to the cytoplasm (Panel C and D of Figures 2 and 3, respectively).

In all GD and HT showing CD30-L+/CD30+/IL-6+/IL-6R+ immunostaining, low immunoreaction was observed for CD30, IL-6 and IL-6R in the cytoplasm of stromal cells (data not shown). In HT with a lymphoid aggregate grade III, low immunoreaction was recognized for IL-6 in the cytoplasm of lymphocytes and for CD30-L and CD30 in the cytoplasm of lymphoid elements with large-clear cytoplasm (macrophages-like cells) included in germinal centers (Panel A and B of Figure 3).

**Discussion**

In the present study, we report that the members of two signaling pathways, namely CD30-L/CD30 and IL-6/IL-6R are expressed in GD and HT lesions, suggesting a role of these pathways in these two diseases. On the contrary, CN arising in the context of AIDT don’t express CD30-L, CD30 and IL-6R and this pattern is also visible in CN not associated with AIDT. These data indicate that CD30-L/CD30 and IL-6/IL-6R signaling pathways are not involved in the growth of CNs, even when CNs are associated with AIDT.

Rather, the expression of these signals in AIDT appears to be related with the mechanisms of the
autoimmune response. It should be considered that, even if both CD30-L and IL-6 belong to Th2 cytokines, they produce different effects on autoimmune processes. CD30-L/CD30 signal protects the organs against autoimmunity through the regulation of the balance Th1/Th2 response. This system induces a positive regulation of T cells by expansion of the Th2 cell subset and suppression of Th1 response (Croft, 2003). Instead, the deregulated production of IL-6 and its receptor is implicated in the pathogenesis of autoimmune diseases by inhibition of autoreactive T-cell apoptosis (Kallen, 2002).

The difference in the relative amounts of Th1/Th2 cytokines triggers the onset of GD or HT. In particular, GD is induced by the TSH receptor stimulating autoantibodies, whose production is likely to depend on Th2 cells function (Burman et al., 1985; Weetman, 2003). Th1 cytokines, on the other hand, facilitate cell-

---

**Fig. 2.** Graves’ disease. Immunoreactive CD30-L (A) and CD30 (B) were detected in tissues from Graves’ disease. Representative positive cases are shown: positive thyrocytes appear brown. The CD30-L and CD30 immunostaining is located on the membrane and cytoplasm of follicular cells (arrow). Note the absence of CD30-L and CD30 immunoreactions in adjacent CN. IL-6 (C) and IL-6R (D) immunoreactions were detected in epithelial cells (brown deposits in cytoplasm) from GD lesions. The IL-6 and IL-6R reactivity is located in the cytoplasm of epithelial cells (arrow). A, x 130; B, x 300; C, x 150; D, x 250
mediated immunity and delayed-type hypersensitivity response in destructive processes of HT (Phenekos et al., 2004). More recently, Harii et al. (2005) reported that TLR-3 protein (a Toll-Like Receptor involved in innate immune responses) is overexpressed in HT, but not in GD, and TLR-3 downstream signals “may be important in the pathogenesis of Hashimoto’s thyroiditis and in the immune cells infiltrates”. TLR-3 preferentially acts through INF regulatory factor (IRF)-3 and causes the synthesis and release of type I IFNs (Th1-related cytokines), but it also signals through nuclear factor kB (NK-kB), that involves MAPK and produces various cytokines (e.g. TNF-alfa and IL-6, Th2-related cytokines). So, Th2 response seems to play a role also in the pathophysiology of HT, in line with previous data from Chiovato et al showing that anti-microsomal autoantibodies are able to induce damage of follicular cells (Chiovato et al., 1993).

Our study indicates that CD30-L, CD30, IL-6 and IL-6R are expressed in AIDT, but the expression of the two signaling systems differs in GD and HT. Further, in HT we have observed that the lymphoid aggregate grade is correlated inversely with the expression of CD30-L/CD30 and positively with IL-6/IL-6R. The expression of CD30-L/CD30/IL-6/IL-6R occurs in a greater proportion of GD with respect to HT. This finding is consistent with the specific characteristic of GD to be prominently a Th2 autoimmune disease while HT is referred to as a Th1 disease. In line with other studies (Chiovato et al., 1993; Okumura et al., 1997), the expression of the two Th2-related systems observed in HT suggests that not only Th1 but also Th2 responses

---

**Fig. 3.** Hashimoto's thyroiditis. **A and B:** CD30-L (A) and CD30 (B) immunoreactions in HT lesions. Representative positive cases are shown. The CD30-L and CD30 immunostaining is located on the membrane and cytoplasm of follicular cells (arrow). Positive thyrocytes appear brown. Note a CD30-L and CD30 reactivity even in lymphoid aggregates defined as grade III (arrows). **C and D:** IL-6 (C) and IL-6R (D) immunoreactions in HT lesions. The IL-6 and IL-6R immunostaining is detected in the cytoplasm of follicular cells (brown deposits in cytoplasm) as indicated by the arrow. Note IL-6 reactivity (indicated by arrows) in lymphoid aggregates grade III. A, B, x 130; C, x 150; D, x 250
could be involved in immunological mechanisms leading to the disease. Moreover, the major expression of the CD30/CD30-L signal with respect to IL-6/IL-6R leads us to suppose that it is the CD30-L/CD30 signal that may contribute to skewing of the immune response toward a Th2 phenotype in HT. This type of consideration is supported by the counterregulatory activity of CD30+ cells as part of a homeostatic response that attempts to control inflammation and tissue damage in Th1-driven diseases (Gerli et al., 2001).

All GD expressing CD30-L/CD30/IL-6/IL-6R showed a lymphoid aggregate grade I. The HT reactive to CD30-L/CD30/IL-6/IL-6R had a lymphoid aggregate grade II or III, while in HT with a lymphoid aggregate grade I the expression of IL-6R was absent. This finding leads us to hypothesize that the lack of expression of IL-6R in HT with a lymphoid aggregate grade I could favor a Th1 environment, while the co-expression of both IL-6 and IL-6R in GD with lymphoid aggregate grade I might contribute to Th2 response.

The possible explanation of the striking difference in IL-6 and IL-6R in HT with respect to GD could be given, in our opinion by the different lymphoid aggregate in the two AIDTs, based on the tenuous but significant correlation.

Another result of our study is the correlation of the two signalling systems expressions with the degree of lymphocytic infiltration. In fact, the CD30-L/CD30 immunoreaction decreases in HT cases showing a lymphoid aggregate grade II or III with respect to grade I, while the IL-6/IL-6R signal is more frequently expressed in HT cases with lymphoid aggregate grade II or III. These data induce us to consider that in HT the lymphocytes infiltrate could down-regulate the epithelial expression of CD30-L/CD30 and up-regulate that of IL-6/IL-6R.

In conclusion, we postulate that CD30-L/CD30 and IL-6/IL-6R systems could play a major role in the pathogenesis of GD. However, the two systems are expressed also in HT, suggesting that Th2 mechanisms are involved in tissue damage of HT. The two systems could contribute to drive the autoimmune response skewing toward a Th2 phenotype and this appears to be correlated with the lymphoid aggregate grade.

Recently, CD30 has been proposed as a target in the immunotherapy of some malignancies (Falini et al., 1995; Schnell et al., 2002; Matthey et al., 2004). Moreover, anti-CD30L antibodies have been tested, able to block the effects of CD30L/CD30 interaction (Del Prete et al., 1995). In AIDT, CD30 and CD30-L may be an unexplored therapeutic potential aimed at modulating the Th1/Th2 balance driving the immune system toward the development of a protective anti-autoimmunity response.

References


Immunol. 16, 249-284.


Accepted October 26, 2005