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# Tie-2 Is Overexpressed by Monocytes in Autoimmune Thyroid Disorders and Participates in Their Recruitment to the Thyroid Gland

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**Context:** The angiopoietin/Tie system seems to have an important role in the pathogenesis of inflammatory diseases. Although Tie-2 is mainly expressed by endothelium, it is also detected in monocytes, which participate in the development of angiogenic and inflammatory phenomena.

**Aim:** The aim was to study the expression and function of Tie-2 and their ligands, angiopoietin-1 (Ang-1) and Ang-2, in thyroid glands and monocytes from patients with autoimmune thyroid disease (AITD).

**Design:** We studied the expression of Tie-2, Ang-1, and Ang-2 by immunohistochemical techniques in surgical thyroid tissues from 17 patients with Graves' disease, 8 with Hashimoto's thyroiditis, and 3 healthy glands. In addition, we explored the expression and function of Tie-2 in peripheral blood monocytes from 17 patients with Graves' disease, 11 with Hashimoto's thyroiditis, and 14 healthy controls.

**Results:** We found that the expression of Ang-1, Ang-2, and Tie-2 was up-regulated in thyroid glands from AITD patients. Flow cytometry, immunofluorescence, ELISA, and RT-PCR analyses confirmed the synthesis and release of Ang-1, Ang-2, and Tie-2 by thyroid follicular cells (TFC) from AITD patients. In addition, these patients showed increased levels of Tie-$2^+$ monocytes in the peripheral blood, which exhibited an enhanced chemotactic response to Ang-2 or autologous TFC.

**Conclusions:** Our data suggest that the Ang/Tie-2 system, through the participation of blood vessels, inflammatory cells, and TFC, may have an important role in the recruitment of monocytes to the thyroid gland and the pathogenesis of the tissue damage seen in AITD. **(*J Clin Endocrinol Metab* 94: 2626–2633, 2009)**

Human autoimmune thyroid diseases (AITD), which mainly include Hashimoto's thyroiditis (HT) and Graves' disease (GD), are characterized by reactivity to self-thyroid antigens with a variable level of chronic inflammation and gland hyperplasia (1). Thus, whereas HT is a destructive inflammatory disease of the thyroid gland, GD is mainly characterized by hyperplasia of the gland and neovascularization with a variable grade of inflammation (2, 3). In GD, the thyroid enlargement and hyperfunction are accompanied by an important increase in blood flow (4). During the course of AITD, endothelial cells (EC) of regional postcapillary venules allow the extravasation of leukocytes to thyroid gland in response to chemotactic molecules, mainly proinflammatory cytokines, contributing to the inflammatory and angiogenic phenomena (3, 5–7).

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Abbreviations: AITD, Autoimmune thyroid disease; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; EC, endothelial cell; $FT_4$, free $T_4$; GD, Graves' disease; HT, Hashimoto's thyroiditis; HUVEC, human umbilical vein ECs; mAb, monoclonal antibody; MCP-1, monocyte chemotactic protein-1; TFC, thyroid follicular cell(s); Tg-Ab, antithyroglobulin antibody; TPO-Ab, antithyroperoxidase antibody; TSHR-Ab, anti-TSH receptor antibody; VEGF, vascular endothelial growth factor.



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Angiogenesis, the growth of new blood vessels from preexisting vasculature, occurs in different tissues under physiological (embryogenesis, wound repair) (2, 8, 9) and pathological conditions (cancer, inflammatory diseases) (8–11). Angiogenesis is regulated by a delicate balance of paracrine signals, which include proinflammatory cytokines and growth factors that promote and regulate EC proliferation and migration, matrix remodeling, recruitment of pericytes and stabilization of neovessels (8, 9). Among the molecules that exert an important regulatory effect on angiogenesis are the angiopoietins (Ang-1 and Ang-2), the tyrosine kinase receptor Tie-2 family, and the vascular endothelial growth factor (VEGF) (12–14). Angiopoietins are expressed by endothelial and some epithelial cells (15), and their synthesis is up-regulated by proinflammatory cytokines, hypoxia, and cellular stress (16). Ang-1 has been identified as a major ligand of Tie-2, resulting in a downstream activation of the phosphatidylinositol-3-kinase/Akt pathway, which promotes EC survival (12–14); on the other hand, Ang-2 binding to Tie-2 induces vasculature regression through the destabilization of the interactions among endothelium, basement membrane, and perivascular extracellular matrix (12–14, 17, 18). However, when VEGF is present, Ang-2 has a marked angiogenic effect (19). Therefore, Ang-2 seems to exert an important role in physiological or pathological processes that involve vascular network remodeling. The tyrosine kinase receptor Tie-2 (also called Tek) is expressed by EC (20) as well as by some hematopoietic cells such as $CD34^+$ stem cells and $CD14^+$ monocytes (21, 22). It has been shown that Tie-2-expressing monocytes are recruited into inflamed or neoplastic tissues, where they promote angiogenesis (23).

Recent studies have implicated Tie-2 and their ligands as well as VEGF in the pathogenesis of inflammatory and autoimmune diseases (24–29). In this regard, we have recently reported that GD patients show increased serum levels of soluble Tie-2 and Ang-2, pointing to a role of the Ang/Tie-2 system in the development of angiogenesis and inflammation in AITD (30). The aim of this study was to assess the possible involvement of Tie-2 and their ligands Ang-1 and Ang-2 in the interactions between thyrocytes and monocytes and its possible role in the recruitment of monocytes to the thyroid gland in AITD.

## Patients and Methods

### Individuals

All patients included in the study fulfilled the classification criteria for AITD (1), and a complete thyroid work-up was performed in them, including history and physical examination. Before treatment, all GD patients were hyperthyroid, whereas all those with HT were hypothyroid. Fourteen age- and sex-matched healthy subjects, in whom the presence of thyroid disorders was specifically excluded, were included as controls.

In all cases, a written informed consent was obtained, and this study was approved by the local Hospital Bioethical Committee.

### Samples

Peripheral blood samples were obtained from 28 patients, 11 with HT and 17 with untreated GD (Table 1). In addition, 25 surgical thyroid tissues were obtained from other patients with GD (n = 17) and HT (n = 8). All these GD patients had relapsed after antithyroid drug treatment and were euthyroid under carbimazole therapy at the time of surgery. GD patients also received preoperative inorganic iodine. Likewise, normal thyroid tissues (n = 3) were obtained from unaffected glands of patients undergoing parathyroidectomy. Thyroid samples from two patients with nontoxic goiter were also studied. All thyroid specimens were frozen, embedded in OCT medium (Ames; Miles Laboratories, Elkhart, IN), and stored at −80 C. In addition, thyroid specimens were fixed and embedded in paraffin.

All samples were taken in accordance with the regulations and approval of the Institutional Review Board of Hospital Universitario de la Princesa.

**TABLE 1.** Clinical features of AITD patients

| | HT | GD | Reference range |
|---|---|---|---|
| n | 11 | 17 | |
| Males/females (n) | 4/7 | 5/12 | |
| Age (yr) | 35.0 ± 16.68 | 36.11 ± 17.22 | |
| TSH (U/liter) | 16.38 ± 2.54 | 0.10 ± 0.35 | 0.3–4.94 |
| $FT_4$ (ng/dl) | 0.84 ± 0.21 | 2.20 ± 1.14 | 0.7–1.48 |
| TPOAb (U/ml) | 839.55 ± 936.48 | 381.0 ± 661.66 | <2.0 |
| Tg-Ab (U/ml) | 696.27 ± 1356.09 | 147.0 ± 366.39 | <2.0 |
| TSHR-Ab (U/liter) | 8.71 ± 10.31[a] | 22.26 ± 16.51 | <1.0 |

Values are given as the mean ± SD.

[a] Only two patients had TSHR-Ab titers above normal levels (11.0 and 30.9 U/liter); in the other nine patients, TSHR-Ab levels were <1.0 U/liter. These antibodies were determined by an ELISA test that does not discriminate between stimulatory and blocking antibodies.

### Laboratory evaluation

Serum free $T_4$ ($FT_4$) (Amerlex $FT_4$ RIA kit; Trinity Biotech, Co. Wicklow, Ireland) and TSH (Diagnost hTSH; Boehring Co., Marburg, Germany) concentrations, and titers of anti-TSH receptor antibody (TSHR-Ab) (DRG Instruments GmbH, Marburg, Germany), antithyroglobulin antibody (Tg-Ab) (ImmunoCAP Thyroglobulin Kit; Phadia AB, Uppsala, Sweden), and antithyroperoxidase antibody (TPO-Ab) (ImmunoCAP Thyroid Peroxidase Kit; Phadia AB) were measured as previously described (30).

### Cell isolation

Thyroid follicular cells (TFC) were isolated as described (31). Briefly, thyroid specimens were minced, digested with collagenase (1.0 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN), and passed through a steel mesh. Then, cells were washed, and cultured in DMEM (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Seromed Biochrom, Berlin, Germany), 2 mM L-glutamine (BioWhittaker), and penicillin (100 U/ml)/streptomycin (100 $\mu$g/ml) in plastic flasks (Flow Labs, McLean, VA). Cell viability was assessed by trypan blue dye exclusion, and it was always higher than 95%.

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (1.077 g/ml; Biochrom AG, Berlin, Germany) cushions. Monocytes were isolated from peripheral blood mononuclear cells by positive selection in an autoMACS separator using anti-CD14 antibodies conjugated to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of monocytes was confirmed by staining with a fluorescein isothiocyanate-conjugated antihuman CD14 (BD Biosciences, San Jose, CA) monoclonal antibody (mAb) and flow cytometry analysis (FACSCalibur; Becton Dickinson, San Jose, CA), and it was always greater than 95%.

### Immunohistochemistry

Tissue sections were immunostained as previously described (32). Briefly, endogenous peroxidase was blocked with 4% $H_2O_2$ in methanol for 10 min, and then Fc receptors were blocked with goat or rabbit serum. Then, sections were immunostained with the indicated antibodies and

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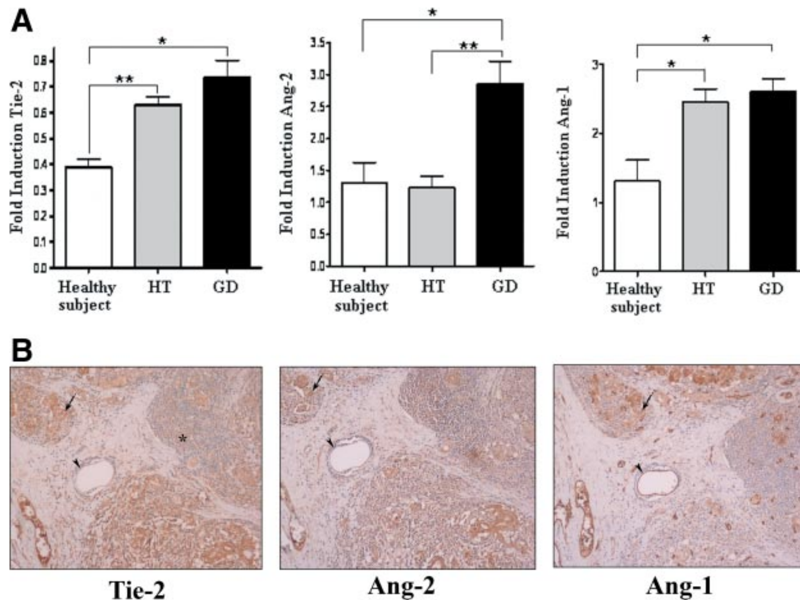


**FIG. 1.** Expression of Tie-2 and their ligands in thyroid glands from AITD patients and healthy controls. A, cDNA was synthesized from total RNA of thyroid tissues and then subjected to PCR with specific primers for Tie-2, Ang-2, and Ang-1, as stated in *Patients and Methods*. Data from 17 GD and 8 HT patients and 3 healthy controls are shown. Data correspond to the arithmetic mean and SD of gene expression (densitometric value) referred to the level of expression of the constitutive gene HPRT-3. B, Immunohistochemical staining of Tie-2 and their ligands Ang-1 and Ang-2 in thyroid tissue from a representative GD patient. Paraffin sections were immunostained for Tie-2, Ang-1, or Ang-2, as stated in *Patients and Methods*. Tie-2 immunostaining was detected on vascular endothelium (*arrowhead*), TFC (*arrow*), and inflammatory cell infiltrate (*asterisk*), whereas Ang-2 immunostaining was observed in ECs (*arrowhead*), and TFC (*arrow*). Ang-1 expression was detected in both ECs (*arrowhead*) and TFC (*arrow*). Original magnification ×125.

the proper horseradish peroxidase-conjugated secondary antibodies (Envision Dako Cytomation, Copenhagen, Denmark). Finally, sections were developed with 3,3′-diaminobenzidine (Envision Dako Cytomation) and counterstained with Carazzi′s hematoxylin.

## RT-PCR

Total RNA was extracted from thyroid tissues, freshly isolated monocytes, cultured TFC, and human umbilical vein ECs (HUVEC) and was reverse transcribed as previously reported (30), using the following specific primers: Ang-1 sense, 5′-GAT GGA CAC AGT CCA CAA CC-3′; and antisense, 5′-ATT CCT TCC AGC CTC TTT GG-3′; Ang-2 sense, 5′-CTG CAA GTG CTG GAG AAC ATC ATG G-3′; and antisense, 5′-GTG TTC CAA GAG CTG AAG TTC-3′; Tie-2 sense, 5′-TGT TCC TGT GCC ACA GGC TG-3′; and antisense, 5′-CAC TGT CCC ATC CGG CTT CA-3′; and histone-3 sense, 5′-AAA GCCGCT CGC AAG AGT GCG-3′; and antisense, 5′-ACT TGC CTC CTG CAA AGC AC-3′. Finally, RT-PCR products were electrophoresed using 1× TAE (Tris acetate-EDTA buffer solution) buffer on 2% agarose gel containing ethidium bromide (Bio-Rad, Hercules, CA) and visualized with a UV transilluminator. Bands were quantified by using a computer-assisted Kodak Analysis System (Kodak, Rochester, NY), and results were normalized according to the value of the HPRT-3 band. The expected sizes of Ang-1, Ang-2, and Tie-2 amplicons were 269, 253, and 317 bp, respectively.

## Flow cytometry analysis

Cell surface expression of Tie-2 was assessed on purified monocytes, after Fc receptor blocking with normal human Ig, by double-staining with phycoerythrin-conjugated antihuman Tie-2 (R&D Systems, Wiesbaden-Nordenstadt, Germany) and anti-CD14-fluorescein isothiocyanate mAbs, or the corresponding isotype-matched irrelevant mAbs. For detection of intracellular expression of Ang-1 and Ang-2, cells were permeabilized, fixed, and then incubated with a polyclonal antihuman Ang-1 or Ang-2 antibodies (R&D Systems), followed by a donkey antigoat IgG F(ab′)2-Alexa Fluor 488 (Invitrogen, San Diego, CA) secondary Ab. Cell analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson).

## Immunofluorescence analysis

TFC were allowed to grow until confluence on fibronectin-coated dishes in DMEM/F12 nutrient mixture (Life Technologies, Inc., Gaithersburg, MD). In addition, freshly isolated monocytes were allowed to adhere to poly-L-lysine coated dishes in RPMI 1640 medium. Then, cells were fixed with 2% formaldehyde, washed with PBS, permeabilized with 0.05% Triton X-100 at 37 C, blocked with 4% BSA, and immunostained with antibodies specific for Tie-2, Ang-1, Ang-2, or cytokeratin and an Alexa Fluor-488 or Alexa Fluor-555-labeled secondary reagent. Finally, slides were analyzed with an epifluorescence Leica microscope (Leica, Wetzlar, Germany), and images were obtained using a Leica DMR camera employing the software QFish.

## ELISA

TFC were stimulated in the presence or absence of recombinant bovine TSH for 72 h. Then supernatants were harvested and levels of Ang-1 and Ang-2 were determined by ELISA as previously described (30).

## Migration assays

Chemotaxis assays were performed on Transwell chambers with 5.0-$\mu$m pore size inserts (Corning, Corning, NY). Lower chambers were filled with RPMI 1640 culture media, supplemented with 0.2% human serum albumin, and with or without recombinant human Ang-1 or Ang-2 at 100 ng/ml (R&D Systems). Monocytes ($5 \times 10^5$ in 100 $\mu$l) were poured into the upper chamber, and after 2 h of incubation at 37 C, filters were removed, fixed, and stained with 4′-6-diamidino-2-phenylindole. Cells that had migrated and were attached to the lower side of the membrane were counted per field with an epifluorescence microscope. Control conditions included cells pretreated with 5.0 $\mu$g/ml of neutralizing anti-Tie-2 mAb (Santa Cruz Biotechnology, Inc., Heidelberg, Germany or the addition of monocyte chemotactic protein-1 (MCP-1) (CCL2) at 100 ng/ml (R&D Systems) to the lower chamber. In additional experiments, TFC were grown until confluence in the lower chamber, and then monocytes were added to the upper chamber. In these assays, control conditions included the addition of neutralizing anti-Ang-1 and anti-Ang-2 mAbs (Santa Cruz Biotechnology, Inc.) to the cell culture of TFC. Results were expressed as the percentage of cell migration referred to the baseline conditions (medium alone = 0%).

## Statistical analysis

Data are shown as the arithmetic mean ± SD or the median and interquartile range, as appropriate. Significance of differences between groups was determined by one-way ANOVA test and *post hoc* comparisons (Bonferroni's test). Mann-Whitney *U* and Wilcoxon tests were used to analyze two unrelated and related samples. Analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA) software. $P < 0.05$ was considered as statistically significant.

# Results

## Tie-2 and their ligands Ang-1 and Ang-2 are overexpressed in thyroid glands from AITD patients

In a previous study, we described increased levels of Tie-2 mRNA in glands from two GD patients compared with healthy

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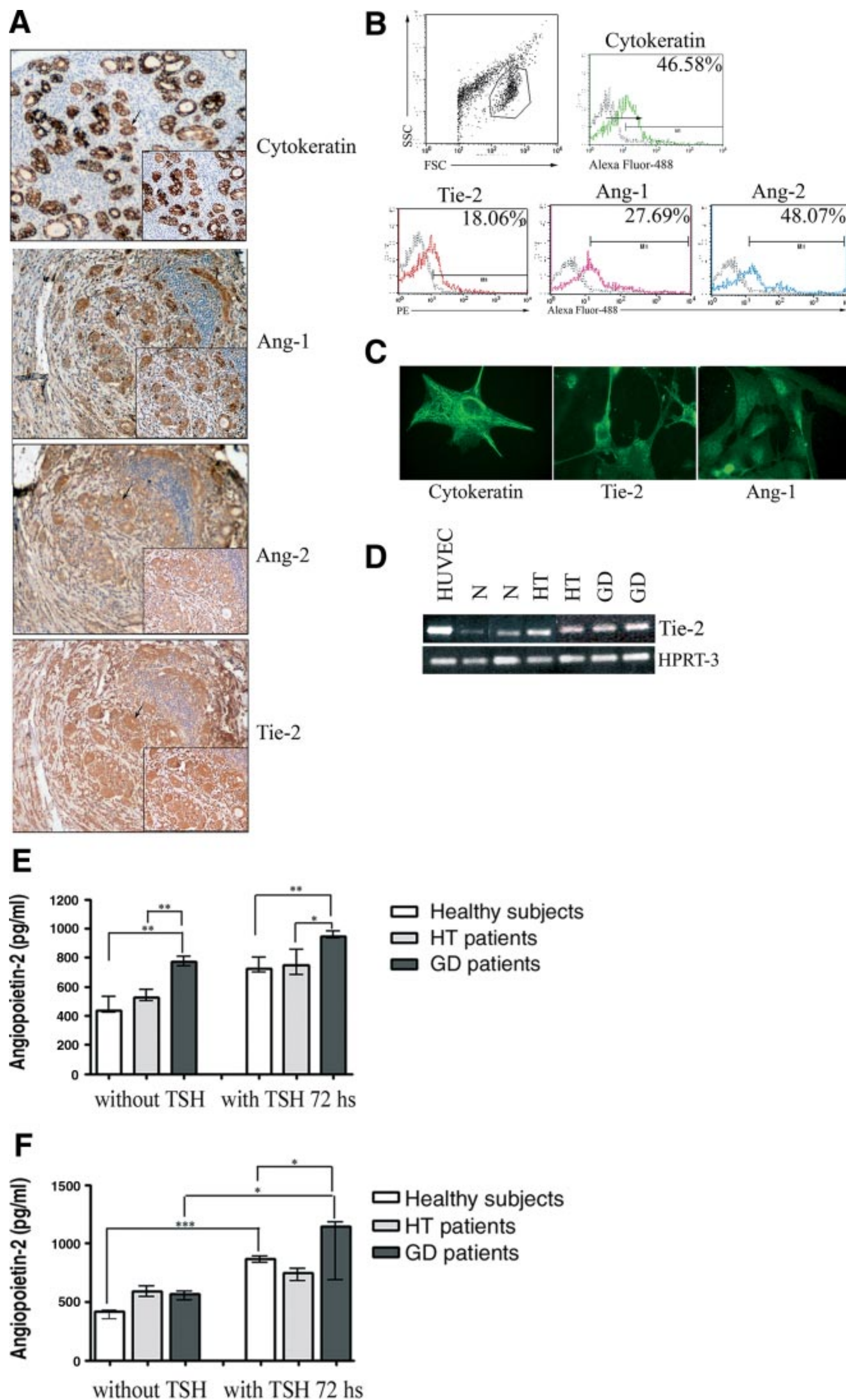


**FIG. 2.** Comparison of the expression and secretion of Tie-2 and their ligands Ang-1 and Ang-2 by isolated TFC. A, Serial immunostaining of proangiogenic molecules and cytokeratin in TFC (*arrow*) of thyroid glands. (Original magnification, ×250; insets, ×500.) B, Isolated TFC were immunostained for Tie-2, Ang-1, and Ang-2 and analyzed by flow cytometry. *Gray line histograms* correspond to the isotype-matched Ab control, and *green*, *red*, *pink*, and *blue line histograms* to cytokeratin, Tie-2, Ang-1, and Ang-2 staining, respectively. Data shown are two replicate experiments performed in TFC from a representative GD thyroid gland. Percentages of marker-positive cells are indicated. C, Thyrocytes from a GD thyroid were plated on a 5 $\mu$g/ml fibronectin-coated dish, fixed, and immunostained for cytokeratin (*left*), Tie-2 (*middle*), and Ang-1 (*right*). D, Tie-2 mRNA was detected by RT-PCR with specific primers, as stated in *Patients and Methods*. Results from HUVEC (positive control), TFC from two healthy thyroid tissues (N), TFC from two HT thyroid glands (HT), and TFC from two GD thyroid glands (GD) are shown. E and F, Effect of exogenous TSH on the *in vitro* release of angiopoietins by thyrocytes. Ang-1 and Ang-2 were quantified in cell culture supernatants of TFC from control thyroid (n = 3), HT (n = 4) and GD (n = 5) stimulated or not with TSH at d 3, as stated in *Patients and Methods*. Experiments were performed in duplicate, and data correspond to the median and interquartile range. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

controls (30). In this work, we further assessed the expression of Tie-2 in glands from AITD patients. We therefore analyzed Tie-2, Ang-1, and Ang-2 mRNA levels in thyroid glands from 17 GD patients, eight HT patients, and three healthy controls by RT-PCR. We found a significantly increased expression of Tie-2 in both GD and HT thyroid tissues when compared with normal thyroid glands or nontoxic goiter ($P < 0.05$; Fig. 1A, *left panel*, and data not shown). A significantly increased Ang-2 expression was also found in GD thyroid glands when compared with both normal and HT thyroid glands ($P < 0.05$; Fig. 1A, *middle panel*). In the case of Ang-1 mRNA levels, a significant enhancement in GD and HT specimens was detected, compared with normal thyroid glands or nontoxic goiters ($P < 0.05$; Fig. 1A, *right panel*, and data not shown).

We then assessed, at the protein level, the expression of Ang-1, Ang-2, and Tie-2 in thyroid glands from AITD patients by immunohistochemistry. Tie-2 staining was observed on TFC, ECs, and mononuclear cells and macrophages (Fig. 1B, *left panel*, *arrow*, *arrowhead*, and *asterisk* for TFC, ECs, and mononuclear cells, respectively). In the case of Ang-2, expression was also seen on both ECs and TFC (Fig. 1B, *middle panel*, *arrowhead* and *arrow*, respectively). Finally, Ang-1 expression was detected on ECs and TFC (Fig. 1B, *right panel*, *arrowhead* and *arrow*, respectively).

### Isolated TFC express Tie-2, Ang-1, and Ang-2 and are able to secrete Ang-1 and Ang-2

To confirm that TFC express Tie-2, Ang-1, and Ang-2, we performed immunohistochemistry, flow cytometry, immunofluorescence, cell culture, and RT-PCR assays on isolated TFC from AITD patients and controls. The expression of Tie-2 and their ligands by TFC was further supported by serial immunostainings with the anticytokeratin mAb (Fig. 2A, *arrow*). Flow cytometry analysis confirmed that freshly isolated TFC from GD and HT patients and healthy controls expressed Tie-2, Ang-1, and Ang-2 (Fig. 2B). Immunofluorescence microscopy analysis confirmed Tie-2 expression by TFC and indicated its location at the cell surface (Fig. 2C). In contrast, Ang-1 and Ang-2 were mainly located at the TFC cytoplasm (Fig. 2C, and data not shown). RT-PCR analysis showed higher levels of Tie-2 in TFC from GD glands compared with HT and control glands (Fig. 2D). In addition, we found that TFC spontaneously released *in vitro* Ang-2 (Fig.

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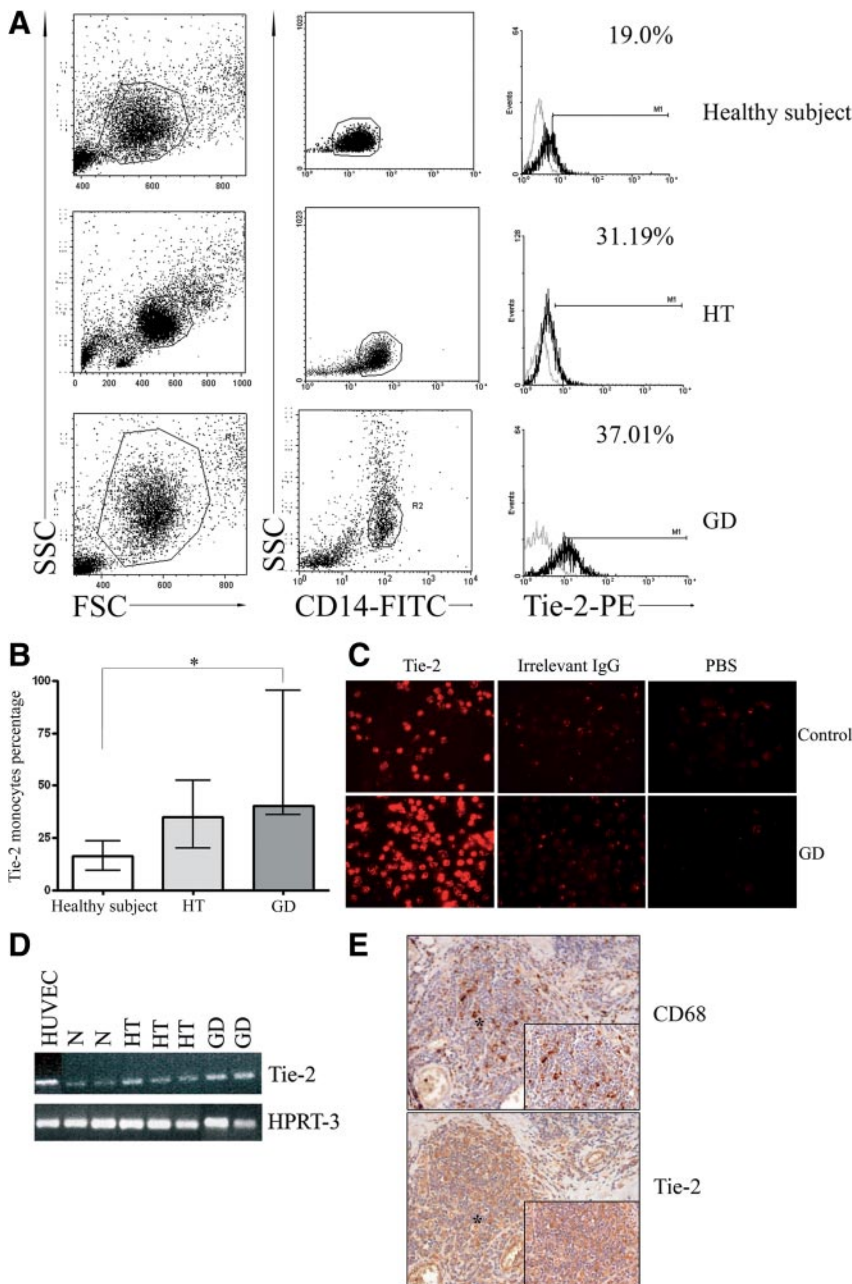


**FIG. 3.** Overexpression of Tie-2 by monocytes from patients with GD. A, Flow cytometry analysis of Tie-2 expression by monocytes from 11 HT, 17 GD patients, and 14 healthy controls. *Gray line histograms* correspond to the negative control, and *black line histograms* to Tie-2 staining. Representative results from a healthy subject and a HT and a GD patient are shown. Percentages of positive cells are indicated. B, Tie-2 expression by monocytes from AITD patients and healthy individuals. Data are shown as the median and interquartile range. C, Immunofluorescence analysis of Tie-2 expression by freshly isolated monocytes from a healthy subject and a GD patient. Intracellular staining for Tie-2 is shown. D, Detection of mRNA transcripts for Tie-2 in monocytes from AITD patients and healthy individuals by RT-PCR analysis. Tie-2 expression by HUVEC (positive control), monocytes from two healthy subjects (N), monocytes from three HT patients (HT), and monocytes from two GD patients (GD) are shown. E, Serial immunostaining of Tie-2 and CD68 in macrophages (*asterisks*) from a GD thyroid gland. *, $P < 0.05$. (Original magnification, ×250; insets, ×500).

2E). This Ang-2 secretion was significantly higher in GD patients when compared with controls and HT patients ($P < 0.05$). As expected, TSH stimulation led to a significant induction of Ang-2 release in all groups ($P < 0.05$), which was significantly higher in GD compared with healthy controls and HT (Fig. 2E) ($P < 0.05$). In contrast, although TFC from all groups showed a constitutive secretion of Ang-1, no significant differences were found among them ($P > 0.05$). After TSH stimulation, there was a significant induction of Ang-1 release in all groups, which was also significantly higher in GD patients when compared with controls and HT patients ($P < 0.05$; Fig. 2F). Because we found enhanced levels of Ang-1 mRNA in GD thyroid glands (see above), these data suggest that Ang-1 is synthesized in the thyroid gland from patients with AITD by different cell types, including TFC. Our data also suggest that TSH (or stimulatory anti-TSHR-Abs) has a relevant role in the induction of Ang-1 synthesis by TFC.

## Enhanced expression of Tie-2 by peripheral blood monocytes from GD patients

We also examined the expression of Tie-2 by peripheral blood monocytes and macrophages in thyroid tissues. Flow cytometry analysis showed that the fraction of $CD14^+$ monocytes that expressed Tie-2 was significantly augmented in GD patients compared with healthy subjects (38.72 and 19.0%, respectively; $P < 0.05$; Fig. 3, A and B). Although there were no significant differences, the proportion of Tie-$2^+$ monocytes in HT patients tended to be higher than in healthy subjects (35.1 and 19.0%, Fig. 3, A and B). These results were confirmed by immunofluorescence microscopy and RT-PCR analyses, which showed the synthesis of Tie-2 by peripheral blood monocytes from GD patients, and the presence of high levels of Tie-2 mRNA transcripts in these cells (Fig. 3, C and D). Finally, serial immunostaining of thyroid sections with the anti-CD68 mAb confirmed the presence of Tie-$2^+$ macrophages in GD and HT glands (Fig. 3E, and data not shown). Preliminary results indicated that macrophages expressing Tie-2 are more abundant in GD compared with HT (data not shown).

## Angiopoietins induce chemotaxis of monocytes from GD patients

Because it has been reported that Ang-2 can induce chemotaxis of monocytes (22), we examined the *in vitro* effect of Ang-2 and Ang-1 on the migration of monocytes from GD and HT patients and healthy controls. We found that peripheral blood monocytes from GD patients showed a significant chemotactic response to recombinant Ang-2 ($P < 0.05$; Fig. 4). In contrast, we did not observe a significant chemotactic effect of Ang-2, at the concentration tested, on peripheral blood monocytes from healthy subjects and HT patients. Accordingly, monocyte migration to Ang-2 was significantly higher in GD than in HT patients ($P < 0.05$). The che-

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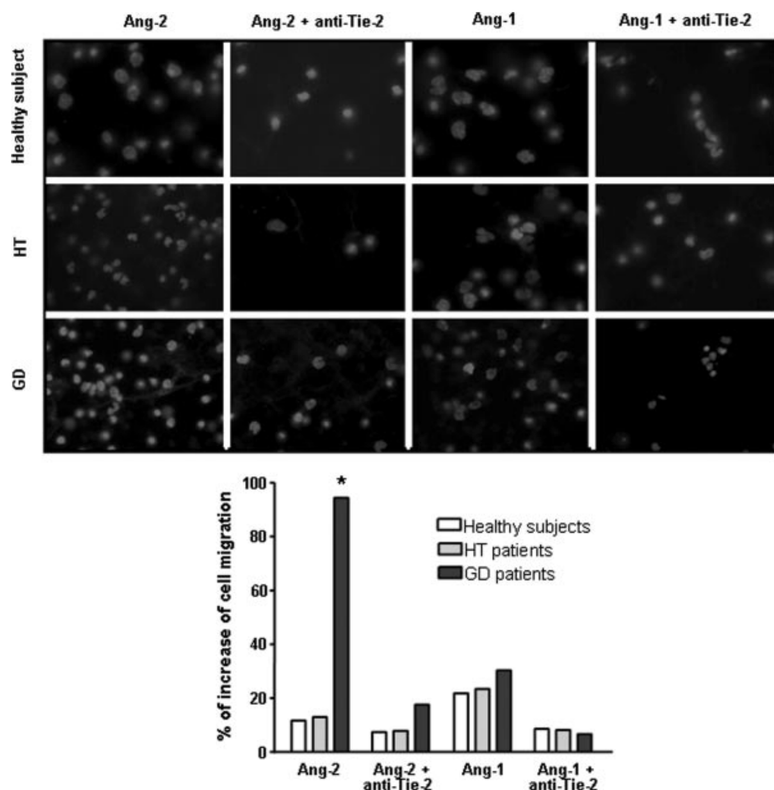


**FIG. 4.** Chemotactic effect of Ang-1 and Ang-2 on monocytes from AITD patients and healthy controls. Monocytes were seeded on the upper chamber of a 5-$\mu$m pore size membrane Transwell plate, and in the lower chamber, MCP-1, Ang-1, or Ang-2 was added to the medium at 100 ng/ml. In addition, blocking anti-Tie-2 mAb was used to inhibit migration. Then, cell migration was assessed by immunofluorescence microscopy, as stated in *Patients and Methods*. Immunofluorescence images (*top*) and the percentage of increase in cell migration (*bottom*) from a representative experiment out of 10 performed in duplicate are shown. Mean group values were compared by one-way ANOVA. *, $P < 0.05$.

motactic effect of Ang-2 on cells from GD patients was similar to that induced by the chemokine CCL2 (MCP-1) (data not shown). On the other hand, no significant differences were observed in the chemotactic response to Ang-1 in the three groups studied ($P > 0.05$; Fig. 4). As expected, the chemotactic responses to Ang-2 and Ang-1 were abolished when the assays were performed with monocytes previously incubated with a blocking anti-Tie-2 antibody, but not with an irrelevant IgG (Fig. 4, and data not shown).

### TFC induce migration of autologous monocytes

The synthesis of Ang-1 and Ang-2 by TFC from AITD suggested a possible role of these cells in the recruitment of peripheral blood monocytes to the inflamed gland. We therefore tested the *in vitro* ability of TFC to induce chemotaxis of autologous peripheral blood monocytes. We found that peripheral blood monocytes from GD patients showed a significant chemotactic response to TFC compared with medium alone ($P < 0.05$; Fig. 5). Moreover, monocytes from GD patients showed a significantly higher chemotactic response to TFC compared with healthy controls and HT patients ($P < 0.05$; Fig. 5). This chemotactic response was almost abolished when monocytes were pretreated with a blocking anti-Tie-2 antibody ($P < 0.05$) or when assays were performed in the presence of neutralizing anti-Ang-2 mAbs ($P < 0.01$). As expected, monocyte migration toward TFC was not affected by an irrelevant control IgG (data not shown).

## Discussion

The pathogenesis of AITD involves the loss of immune tolerance to thyroid self-antigens, with the generation of an immune response toward these molecules. In the case of GD, anti-TSHR antibodies induce the release of thyroid hormones and the hyperplasia of the gland. However, this condition is also usually accompanied by a variable degree of inflammatory cell infiltrate. On the other hand, HT is mainly characterized by a prominent inflammatory phenomenon with progressive destruction of the thyroid gland. In both cases, it has been proposed that angiogenesis may have a relevant role in their pathogenesis because this phenomenon is involved in tissue hyperplasia and inflammation (4). Therefore, we have hypothesized in this work that the Ang-1, Ang-2/Tie-2 system could be involved in the pathogenesis of AITD. In this regard, in the present study we have found an increased expression of Tie-2 and their ligands Ang-1 and Ang-2 in thyroid tissue from patients with GD. In addition, patients with HT show an enhanced synthesis of Ang-1. This expression was not restricted to vascular cells, and it was also detected in TFC, both in thyroid tissue specimens and in isolated or cultured thyrocytes. There was also a significant increase in Tie-2 expression in peripheral blood monocytes

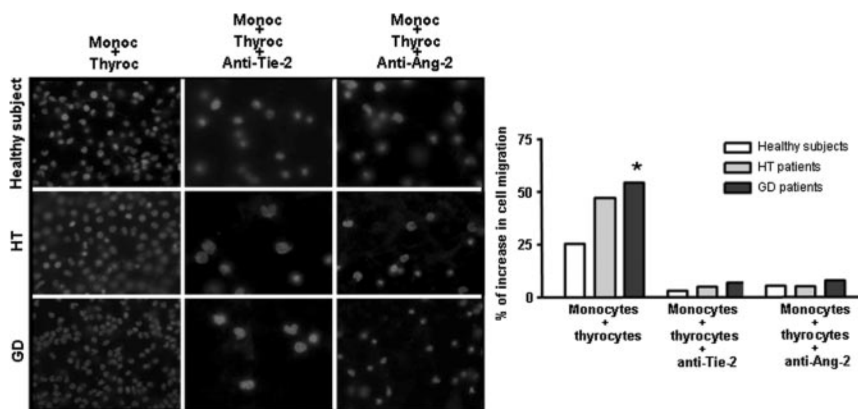


**FIG. 5.** Ang-2 release by thyrocytes is involved in recruitment of autologous monocytes into the thyroid glands of GD. TFC were allowed to grow to confluence on the lower chamber of 5-$\mu$m pore size membrane Transwell plates, and then monocytes were seeded on the upper chamber in the presence or not of blocking anti-Tie-2 or anti-Ang-1/Ang-2 mAbs. Finally, cell migration was determined by immunofluorescence microscopy, as stated in *Patients and Methods*. Immunofluorescence images (*left*) and the percentage of increase in cell migration (*right*) from a representative experiment out of three performed in duplicate are shown. Mean group values were compared by one-way ANOVA. *, $P < 0.05$.

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from AITD patients, and these cells were attracted *in vitro* by Ang-2 or autologous cultured TFC. All of these data support the theory that these angiogenic molecules and their membrane receptor could be involved in the tissue damage seen in AITD, mainly in GD.

Up-regulation of the Ang/Tie-2 system has been reported in different conditions, mainly neoplastic and inflammatory diseases. In this regard, it has been described that Tie-2 is the main mediator of the angiogenic phenomenon induced by TNF-$\alpha$ in the synovial membrane of patients with rheumatoid arthritis (33). Other conditions in which it has been described that the Ang/Tie-2 system is up-regulated include chronic viral hepatitis (10, 34) and psoriasis (26). In the latter condition, it is of interest that the therapeutic effect of the TNF-$\alpha$ blockade with the chimeric antibody infliximab is associated with down-regulation of Tie-2 expression in skin (35). In this regard, it seems evident that the neoformation of blood vessels is closely related to chronic inflammation and that the abundance of postcapillary venules facilitates the extravasation and homing of great numbers of different subsets of bloodstream leukocytes to the injured tissue. All of these data suggest that it is feasible that the high expression of Ang/Tie-2 found in AITD thyroids is induced, at least in part, by high levels of proinflammatory cytokines, and that in turn, angiopoietins might favor the synthesis of these cytokines as well as the tissue infiltration by inflammatory cells. On the other hand, our data suggest that methimazole therapy does not affect the increased synthesis of Ang/Tie-2. However, it is conceivable that this drug could exert an immunomodulatory effect on monocytes or TFC because it has been reported for other leukocyte subsets (36, 37).

The enhanced expression of angiopoietins and Tie-2 by TFC in glands from AITD patients is of interest. This finding is in agreement with several reports on the expression of Tie-2 by non-ECs, including some bone marrow-derived and different neoplastic cells. In addition, angiopoietin synthesis has been detected in different cell types, including epithelial cells (38). Furthermore, Tie-2, Ang-1, and/or Ang-2 have been previously detected in human TFC from thyroid tumors and adenomatous goiter as well as in thyroid rat cell lines (11, 27, 39). Therefore, our data confirm that TFC seems to be an important element in the Ang/Tie-2 system in the thyroid gland and suggest that these cells may exert a relevant role in the induction of angiogenesis under inflammatory conditions. The synthesis of VEGF by TFC (40, 41) and the enhanced levels of this molecule seen in some GD patients (30) further support the latter possibility. In addition, it is conceivable that the synthesis/expression of Ang/Tie-2 by TFC also participates in the activation and proliferation of these cells through an autocrine/paracrine mechanism (42). However, this point remains as an interesting possibility to be investigated.

An interesting observation of this study was the increased Tie-2 expression by peripheral blood monocytes from AITD patients and their enhanced chemotactic response to Ang-2. In this regard, the enhanced expression of Tie-2 by peripheral blood monocytes from GD patients and their increased chemotaxis toward angiopoietins suggest the possible involvement of these molecules in the recruitment of peripheral blood monocytes to the diseased gland. This is an interesting possibility because monocytes, mainly through their differentiation into activated macrophages, play an important role in the tissue damage seen in AITD. In fact, in the nonobese diabetic mouse model, only when monocytes, macrophages, and dendritic cells are attracted to the thyroid gland is the AITD precipitated (43). As stated above, the possible source of the angiopoietins in the thyroid gland that would contribute to attracting the bloodstream monocytes corresponds, at least in part, to the TFC.

The increased expression of Tie-2 by monocytes from patients with GD, but not from HT, is of interest. Although the factors that modulate the proportion of Tie-2$^{+}$ monocytes as well as the level of synthesis of this receptor by these cells have not been described (21, 23), it is tempting to speculate that GD patients bear a genetic feature related to Tie-2 expression that is not found in HT. In this regard, it has been described as a significant association between sickle leg ulcers and a Tie-2 single nucleotide polymorphism (44). Thus, it would be of interest to determine the possible presence of single nucleotide polymorphisms or other factors associated with enhanced expression of Tie-2 in GD.

In summary, altogether our data indicate that the Ang/Tie2 system may participate through different mechanisms in the pathogenesis of AITD. Thus, it is very likely that the synthesis and release of angiopoietins into the thyroid gland by TFC may be important to induce the generation of new blood vessels, a phenomenon that is necessary to sustain the gland hyperplasia seen in GD. More interestingly, we found that the Ang/Tie-2 system may participate in the local inflammatory reaction by regulating monocyte recruitment to the thyroid gland. Therefore, it is possible to speculate that the Ang/Tie-2 system could be a linking factor among the three key elements of AITD and other autoimmune conditions (*e.g.* rheumatoid arthritis or psoriasis), the inflammatory, angiogenic, and hyperplasic phenomena. Therefore, this pleiotropic system could be a potential therapeutic target for the treatment of inflammatory conditions that are accompanied by tissue hyperplasia such as GD.

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Disclosure Summary: N.F.-V., M.A.-P., C.C.-M., F.S.-M., R.M.-O., R.G.-A., and M.M. have nothing to declare.

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