

Structural Changes in the Angiofollicular Units between Active and Hypofunctioning Follicles Align with Differences in the Epithelial Expression of Newly Discovered Proteins Involved in Iodine Transport and Organification

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In animals, as well as in humans, the thyroid gland is made of active follicles, with cuboidal cells and hypofunctioning follicles, with flattened cells. In this study, the functional status of human follicles was dissected out, based on immunohistochemical detection of TSH receptor, Na⁺/I⁻ symporter, pendrin, thyroperoxidase (TPO), thyroid oxidases (ThOXs), and T₄-containing iodinated Tg (Tg-I). To ascertain that angiofollicular units exist in the human, we studied the microvascular bed of each follicle, in correlation with detection of vascular endothelial growth factor (VEGF), of nitric oxide synthase III, and of endothelin in normal and goitrous thyroids. In hypofunctioning follicles, pendrin, TPO, and ThOXs were not detected, and there was no Tg-I in the colloid. At the opposite, in

active follicles, pendrin, TPO, and ThOXs were detected in thyrocytes, and Tg-I was present in the colloid. In normal and goitrous thyroids, the capillary networks surrounding active follicles were larger than those surrounding hypofunctioning follicles. Immunoreactivity for nitric oxide synthase III and endothelin was solely detected in active follicles. Only a few follicles in normal thyroids were immunostained for VEGF, regardless of their functional status. In multinodular goiters, VEGF was detected in contact with the extracellular matrix at the basal pole of the cells. In conclusion, the present study endorses the likelihood of angiofollicular units in the human thyroids. Vascular changes are related to the functional status of thyrocytes. (*J Clin Endocrinol Metab* 87: 1291–1299, 2002)

AS FOR MANY other mammals, the human thyroid gland is characterized by a marked functional and morphological heterogeneity. This intrinsic property has been widely considered to understand the pathogeny of multinodular goiter (MNG) (1). At the anatomical level, MNG consists of clustered functioning (hot) and resting (cold) nodules, as evidenced by iodine-123 and technetium-99m scans. At the microscopical level, the interfollicular heterogeneity is clearly visible, as evidenced by autoradiography after iodine-125 injection in experimental animal models (2–4). Autoradiographic investigations in the human are obviously scarce, although one study showed that some cold follicles cannot trap iodine, whereas others do so but without organification (5).

An alternative approach to characterize the functional status of follicles is to investigate the expression of proteins involved in iodine transport and organification. Thyrocytes are able to actively transport iodine through the basolateral membrane via a Na⁺/I⁻ symporter (NIS) (6–8) and to deliver it to the apical pole of the cell, likely through another

iodide transporter, the product of the pendred syndrome gene (PDS), pendrin (9–14). The Tg iodination and coupling processes take place at the apical pole, on the follicular side of the cell membrane. They require the presence of a thyroperoxidase (TPO), as well as an H₂O₂-generating system, including members of the NADPH oxidase family, thyroid oxidases (ThOXs) (15, 16). By using specific antibodies raised against these different components, combined with the specific detection of T₄-containing iodinated Tg (Tg-I) in the follicular lumen (17), it is possible to get a functional overview of the follicles.

The likely role of the microvascular bed in the pathogeny of the glandular heterogeneity has also been considered. Changes in vascular structures have already been studied in goitrous experimental models (18–21). We previously showed, in the mouse, that a correlation exists among the functional status of the follicles, the extent of the microvascular bed, and the expression of nitric oxide synthase III (NOSIII). These results suggested that the thyroid gland consists of clustered angiofollicular units regulated by local paracrine exchanges between epithelial cells and their neighbor capillary bed (22). This hypothesis remains speculative in the human thyroid gland.

In the present study, we address this specific question using human material. We first characterized the functional

Abbreviations: ET, Endothelin; mAb, monoclonal antibody; MNG, multinodular goiter; NIS, Na⁺/I⁻ symporter; NOS, nitric oxide synthase; PDS, pendred syndrome gene; Tg-I, T₄-containing iodinated Tg; ThOX, thyroid oxidase; TSHr, TSH receptor; TPO, thyroperoxidase; VEGF, vascular endothelial growth factor; Vv, relative volume.

status of follicles through the immunohistochemical detection of the TSH receptor (TSHr), NIS, pendrin, TPO, ThOXs, and Tg-I, in correlation with the morphological aspect of the follicles. To verify the hypothesis about the presence of angiofollicular units in normal and goitrous human thyroids, we analyzed variations in the vascular bed, along with the functional aspect of the follicles, by morphometrical methods. We then correlated them with the expression of angiogenic and vasoactive factors [the vascular endothelial growth factor (VEGF), NOSIII, and endothelin (ET)].

Materials and Methods

Human thyroid tissues

Five human thyroid samples, obtained after autopsy, were used as normal glands. Five MNGs were selected from a tissue bank of the pathology department. Tissues were fixed in Bouin's fluid and embedded in paraffin. Five other MNGs and four normal paranodular thyroids were obtained from surgical cases, after the patients gave their informed consent. Tissue samples were rapidly frozen in liquid nitrogen to perform cryostat sections.

Immunohistochemical analysis of thyroid-specific proteins (TPO, NIS, TSHr, ThOXs, pendrin, Tg-I, and Tg) and of angiogenic and vasoactive peptides (NOS, VEGF, and ET)

Cryostat sections (5- μ m) were used to perform TPO [monoclonal antibody (mAb)47, gift from J. Ruf, U38 INSERM, Marseille, France], NIS [mAb (23)], TSHr (polyclonal antibody; DAKO Corp., Glostrup, Denmark), ThOXs [polyclonal antibody (15)], and pendrin (polyclonal antibody, gift from I. Royaux, NIH, Bethesda, MD) immunostainings. Paraffin sections (5- μ m), laid on superfrost glass slides, were used for Tg-I [mAb (17)], Tg (polyclonal antibody, DAKO Corp.), TPO, NIS, VEGF (polyclonal affinity-purified antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), NOSIII (polyclonal affinity-purified antibody; Transduction Laboratories, Inc., Lexington, KY), and ET (mAb; Biogenesis, Poole, UK) immunostainings. Paraffin sections were dewaxed and rehydrated just before the procedure. Paraffin-embedded sections used for NOSIII and NIS immunostainings were pretreated in a microwave oven in citrate buffer (0.01 M, pH 6) for one cycle of 3 min at 750 W and three cycles of 3.5 min at 350 W.

Immunostainings were performed as previously described (22). Both cryostat- and paraffin-embedded sections were washed with PBS supplemented with 1% BSA (ICN Biochemicals, Inc., Costa Mesa, CA) (PBS-BSA) and thereafter incubated in PBS-BSA containing normal serum from the animal species from which the second antibody was raised (1/50) for 30 min at room temperature. First antibodies were then applied either overnight or for 1 h (Table 1). After two washes in PBS-BSA, the binding of antibodies was detected using a secondary antibody conjugated to a polymer labeled with peroxidase (Envision detection, DAKO Corp.) for 30 min at room temperature. The peroxidase activity was revealed with AEC substrate (3-amino-9-ethylcarbazole, DAKO

Corp.). Sections were counterstained with Mayer's hematoxylin, rinsed, and mounted in Faramount (DAKO Corp.). The binding of anti-ET antibody was detected using the ABC perox kit (Vector Laboratories, Inc., Burlingame, CA), that of anti-Tg-I by using a monoclonal secondary antibody conjugated with peroxidase, and that of polyclonal anti-Tg with a polyclonal antibody conjugated with peroxidase. For Tg-I and Tg, the peroxidase activity was revealed with 3-3'-diaminobenzidine tetrahydrochloride (Aldrich, Bornem, Belgium); sections were dehydrated and mounted in DPX (BDH, Poole, UK).

Several negative controls were performed: absence of the first antibody, immunoneutralization with the peptide when available (anti-VEGF and ET antibodies), or replacement of the first antibody by the preimmune serum. Controls of the Tg-I antibody specificity have been realized by ELISA, showing that this antibody does not recognize non-iodinated Tg but recognizes highly iodinated Tg and that the antibody binding is displaced by T₄ (17). We have performed the same controls by immunohistochemistry, and the results were similar.

For all immunostaining procedures performed on paraffin sections, follicles were ranked as described below, and the proportion of positive *vs.* negative immunostained follicles was recorded. Follicles were considered as positively stained when all their cells were positive; otherwise, they were considered as partly positive or negative. For Tg-I immunostaining, the aspect of the follicular lumen was taken into account.

Ranking of the follicles according to their histological aspect

Ranking was performed on thick sections (5-m) stained with Hemalun-Eosine-Safran. Follicles were ranked into two categories, depending on the aspect of their epithelium. Follicles were considered as hypofunctioning when they were lined with a flat epithelium containing dense nuclei. On the contrary, follicles were considered as active when they were lined with a cuboidal or cylindrical epithelium with rounded nuclei (2–4). Follicles with an intermediary aspect were excluded from the quantitative study. The validation of this qualitative ranking by morphometrical analysis was previously reported in the mouse (22).

Morphometrical analysis of the microvascular bed

After follicles were ranked as hypofunctioning or active, according to their morphological aspect, the morphometrical evaluation of the vascular bed was performed by point counting on 150 follicles per section, according to Weibel *et al.* (24), as previously reported (22). The relative volume (V_v) of capillaries in contact with thyrocytes, the number of sections of capillaries in relation with each follicle, and the mean area of capillary profiles were calculated separately for hypofunctioning and active follicles.

Statistical analysis

Statistical differences between active and hypofunctioning follicles within the same group and between normal thyroid and MNG were tested by one-way ANOVA, followed by Fisher or Dunnett tests. Results

TABLE 1. Experimental conditions for immunohistochemistry

Section	First antibody	Incubation time	Concentration	Secondary antibody	Substrate
Cryostat	TPO	1 h	1/10000	Envision mouse	AEC
Cryostat	TSHr	1 h	1/75	Envision mouse	AEC
Cryostat	ThOXs	1 h	1/75	Envision rabbit	AEC
Cryostat	Pendrin	1 h	1/200	Envision rabbit	AEC
Cryostat	NIS	1 h	1/10	Envision mouse	AEC
Paraffin	Tg-I	Overnight	1/1500	mAb anti-IgG-HRP	DAB
Paraffin	Tg	3 h	1/1500	Polyclonal anti-Ig HRP	DAB
Paraffin	VEGF	Overnight	1/100	Envision rabbit	AEC
Paraffin	NOSIII	1 h	1/75	Envision rabbit	AEC
Paraffin	ET	Overnight	1/100	ABC rat	AEC
Paraffin	NIS	1 h	1/20	Envision mouse	AEC
Paraffin	TPO	1 h	1/3000	Envision mouse	AEC

HRP, Peroxidase.

are expressed as mean \pm SEM for the morphometry, or as mean \pm SD for immunostaining counting.

Results

Characterization of active and hypofunctioning follicles (Fig. 1)

In both normal thyroids and MNGs, a mixture of active and hypofunctioning follicles was observed. No systematic correlation between the size of the follicles and the epithelial thickness was observed. Some large follicles were lined with cuboidal or cylindrical epithelial cells with round nuclei and a well-developed cytoplasm. On the contrary, some large follicles, as well as numerous small ones, were lined with flat epithelial cells with flat and dense nuclei. In some cases, the epithelium was so flat that it was nearly indistinguishable on sections.

Immunohistochemical analysis of thyroid-specific proteins directly involved in the glandular function

In cryostat sections, a positive staining for all markers (TSHr, pendrin, ThOXs, and TPO) was seen in active follicles. In contrast, hypofunctioning follicles were positive only for TSHr. Pendrin, ThOXs, and TPO were either not detected or only observed in very few cells (Fig. 2, A–F). The immunohistochemical pattern varied, though, from one marker to another. Staining for TSHr was almost exclusively located at the basal pole of the cells (Fig. 2A), whereas pendrin was mainly observed at the apical pole (Fig. 2D). In contrast, ThOXs staining was present in the cytoplasm under the apical pole (Fig. 2B), and TPO immunolabeling was detected diffusely throughout the whole cytoplasm (Fig. 2F). The immunohistochemical detection of NIS showed a very heterogeneous pattern. The number of follicles with positive cells varied from one gland to another, without differences between normal and goitrous thyroids. Some glands contained numerous negative follicles regardless of their functional status. Positive cells were stained at the basolateral pole of the cells (Fig. 2, G and H). There was also an intraglandular variation in the number of positive cells among follicles. Thus, NIS immunoreactivity was not indicative about the functional status of the follicles. In both normal thyroids and MNGs, Tg-I immunostaining in the colloid was positive in active follicles, whereas it was negative in hypofunctioning ones (Fig. 2, I and J). Nevertheless, Tg-I-negative follicles contained Tg, given that they were positively stained with a polyclonal antibody against Tg (Fig. 2, K and L).

In paraffin-embedded sections, the immunostaining for Tg-I and TPO was quantified. In normal thyroids, the colloid of 90% of active follicles was positively stained for Tg-I *vs.* 23% of hypofunctioning follicles ($P < 0.001$). In MNGs, a slight decrease in the number of Tg-I-positive follicles was observed (74%, $P < 0.05$), whereas the number of positive hypofunctioning follicles remained constant (22%) (Fig. 3A). In normal thyroids, TPO immunoreactivity was detected in 97% of active follicles. Only 6% of hypofunctioning follicles were positive ($P < 0.001$), 58% of them were totally negative, and 36% were partially labeled. In MNGs, the proportion of positive hypofunctioning follicles was slightly (but not significantly) increased, compared with normal glands (Fig. 3B).

In summary, the morphological aspect of the epithelium correlated with several markers of the functional status of the epithelium. Tg-I-negative follicles correlated with a decreasing iodination capacity of the cells, as evidenced by the absence of staining for the iodination apparatus (ThOXs and TPO), as well as for the apical iodide transport, pendrin.

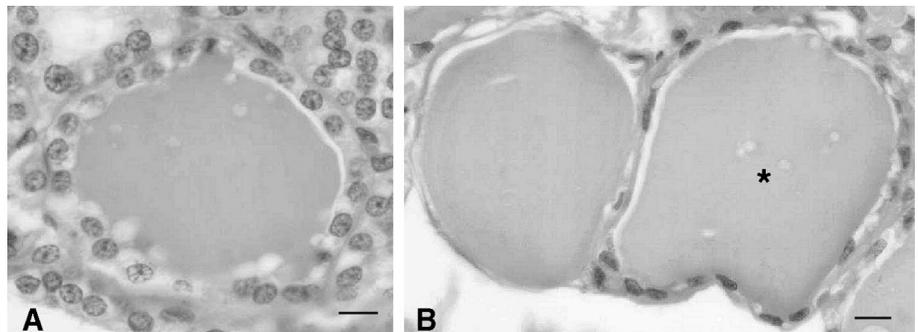
Morphometrical analysis of the microvascular bed

V_v of the capillaries (Fig. 4A). In normal thyroids, as well as in MNGs, *V_v* of capillaries located in close contact with active follicles was 4- to 5-fold larger than *V_v* of capillaries located around hypofunctioning follicles ($P < 0.001$). In addition, *V_v* of the capillaries of active follicles in MNGs was larger than that of active follicles in normal thyroids (0.23 ± 0.05 in MNGs *vs.* 0.125 ± 0.003 in normal thyroids, $P < 0.001$). Similarly, *V_v* of capillaries around hypofunctioning follicles was significantly larger in MNGs (0.06 ± 0.004 in MNGs *vs.* 0.03 ± 0.003 in normal thyroids, $P < 0.001$).

Number of capillaries per follicular area (Fig. 4B). In both normal thyroids and MNGs, the number of capillaries surrounding hypofunctioning follicles was about half of that located around active follicles ($P < 0.001$). The number of capillaries around active follicles in MNGs was greater than that of the corresponding follicles in normal thyroids (0.378 ± 0.01 in MNGs *vs.* 0.254 ± 0.007 in normal thyroids, $P < 0.01$). The same conclusion was drawn for hypofunctioning follicles (0.187 ± 0.008 *vs.* 0.128 ± 0.007 , $P < 0.001$).

Mean capillary area (Fig. 4C). In normal thyroids, the mean area of capillaries surrounding hypofunctioning follicles was twice as small than that of capillaries surrounding active follicles ($P < 0.001$). In MNGs, the difference was of 3-fold ($P < 0.001$). The mean area of capillaries surrounding active

FIG. 1. Typical morphological aspect of active (A) and hypofunctioning (B) follicles in a normal paraffin-embedded human thyroid stained with Hemalun-Eosine-Safran. Active follicles are lined with a cuboidal epithelium with rounded nuclei. Two kinds of hypofunctioning follicles are observed. In the first type (★) the epithelium is flat with visible flat nuclei; whereas in the second type, the epithelium is barely visible. Scale bar, 10 μ m.



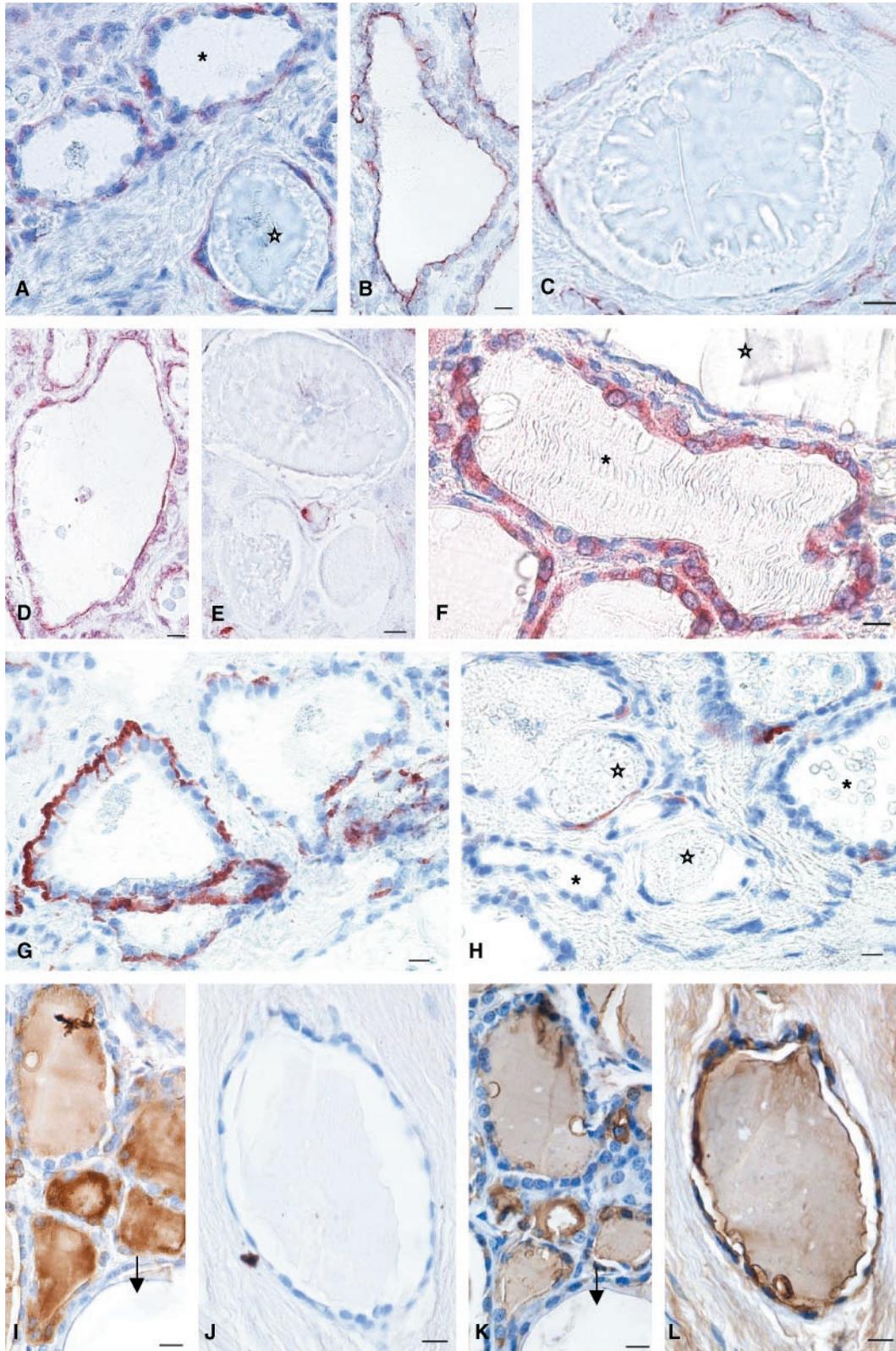


FIG. 2. Immunohistochemical analysis of thyroid-specific proteins. Scale bars, 10 μ m. A, In MNG, TSHr immunoreactivity is detected at the basal pole of the cells (cryostat section). Follicular cells in both active (*) and hypofunctioning (★) follicles are positively stained. B and C, Immunostaining of ThOXs in MNG (cryostat section). Epithelial cells in active follicles (B) are positive, whereas most of the cells of hypofunctioning follicles (C) are negative. D and E, Immunostaining of pendrin in MNG (cryostat section). Active follicles are positive (D). In contrast, hypofunctioning follicles are totally negative (E). F, Immunostaining of TPO in normal thyroid (paraffin-embedded section). Active follicles (*) are positive, whereas hypofunctioning follicles (★) are mostly negative. G and H, Immunostaining of NIS in normal thyroid (cryostat section)

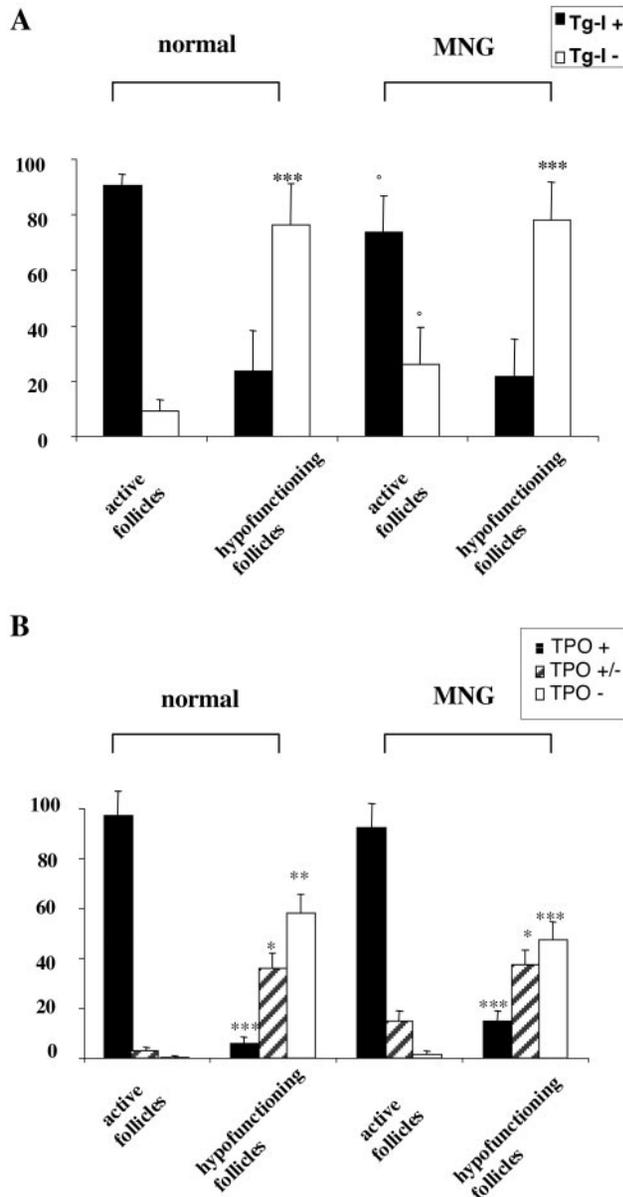


FIG. 3. Percentage of active and hypofunctioning follicles containing positive or negative immunoreactivity for Tg-I (A) or positive, negative, or partially positive immunoreactivity for TPO (B). The results are expressed as mean \pm SD, n = 5. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$ hypofunctioning follicles vs. active follicles in the same group. °, $P < 0.05$, MNG vs. normal thyroids.

follicles in MNGs was larger than that surrounding active follicles in normal thyroids (121.5 ± 2.3 vs. $82 \pm 2 \mu\text{m}^2$, $P < 0.001$). The same observation was made for capillaries surrounding hypofunctioning follicles (43.4 ± 2.5 vs. $32 \pm 2.3 \mu\text{m}^2$, $P < 0.001$).

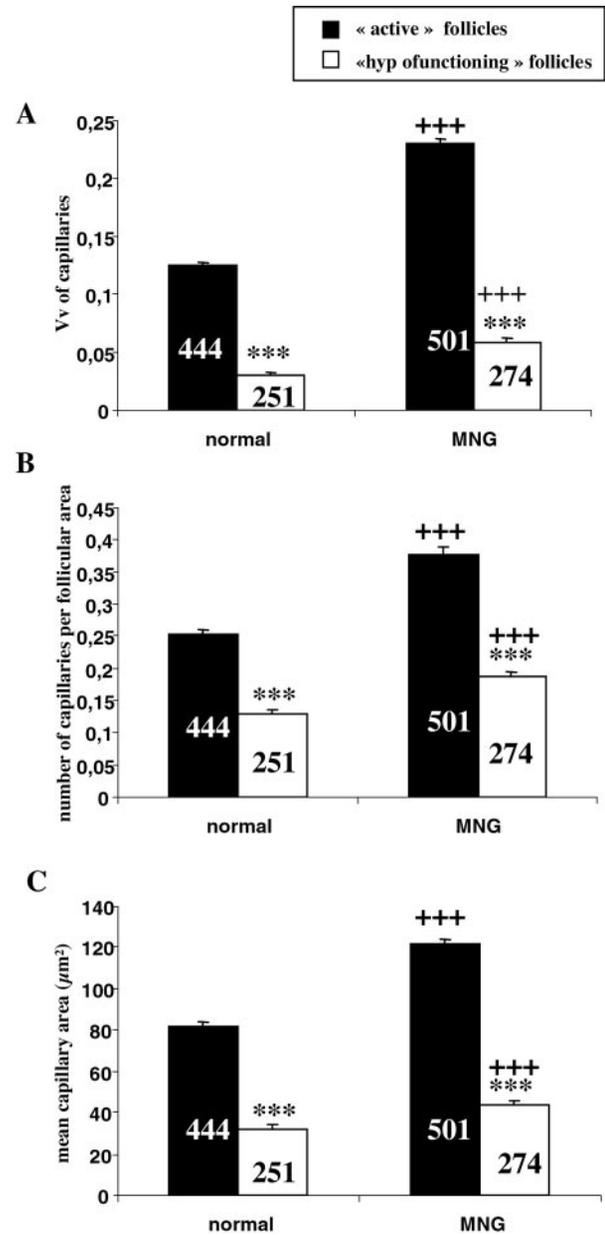


FIG. 4. Quantitative changes of the microvascular vascular bed. A, Vv of capillaries. B, The number of capillaries per follicular area. C, Mean capillary area (μm^2). Results are expressed as means \pm SEM. ***, $P < 0.001$, hypofunctioning follicles vs. active follicles in the same group. + + +, $P < 0.001$, MNG vs. normal thyroids. The number in the column indicates the number of analyzed follicles.

Immunohistochemical analysis of angiogenic and vasoactive factors

VEGF. In normal thyroids, a weak VEGF immunoreactivity was detected in epithelial cells of some follicles, others re-

(G, H). Staining was very heterogeneous. Active follicles (*) are entirely positive, partially positive or totally negative. Similar pattern is observed in hypofunctioning follicles (★). I and J, Immunostaining of Tg-I in normal thyroid (paraffin-embedded section). Colloids of active follicles are positive (I), whereas those of hypofunctioning follicles are negative (J). K and L, Immunostaining of Tg with a polyclonal antibody in normal thyroid (paraffin-embedded section, serial from I and J). Colloids of both active (I) and hypofunctioning (J) follicles are positively stained. Note the empty follicle in I and K (arrows).

maining negative regardless to their functional status (Fig. 5A). In MNGs, an intense immunoreactivity was detected at the basal pole of the cells (Fig. 5B). Nevertheless, the signal was heterogeneous within the same gland. VEGF was also detected around capillaries. In both normal and goitrous thyroids, smooth-muscle cells of arteries were positive, whereas endothelial cells remained negative (data not shown). When VEGF-positive follicles were counted, there was no significant difference between the two kinds of follicles or between normal thyroids and MNGs (Fig. 6A).

NOSIII. In normal thyroids, NOSIII immunoreactivity was detected in follicular cells of active follicles but not in cells of hypofunctioning follicles (Fig. 5C). All endothelial cells were negative. A similar observation was made in MNGs, although the endothelial cells of capillaries were strongly positive (*inset* in Fig. 5C). Counting of follicles showed that 94% of active follicles and 8.5% of hypofunctioning follicles were positive for NOSIII in normal thyroids ($P < 0.001$); 49% of hypofunctioning follicles were totally negative, whereas 42% contained few positive cells. There was no significant difference when compared with MNGs (Fig. 6B).

ET. In normal thyroids and in MNGs, an immunoreactivity for ET was detected only in follicular cells of active follicles (Fig. 5D). When ET-positive follicles were counted, 90% of active follicles and 8% of hypofunctioning follicles were positive in normal thyroids ($P < 0.001$); 51% of hypofunctioning follicles were totally negative, and 41% contained only few positive cells. There was no significant difference when compared with MNGs (Fig. 6C).

Discussion

Hot and cold follicles represent two separate, but coexisting, functional entities within the thyroid gland. They have been well characterized in the mouse, by correlating the aspect of the epithelium with short time autoradiographic studies after iodine-125 administration (2–4). Cold follicles are lined with a flat epithelium, whereas the lumen is filled with a dense colloid. In contrast, hot follicles are lined with a cuboidal epithelium, but the lumen is usually filled with a paler colloid. Based on these morphological features, two categories of follicles, namely active and hypofunctioning follicles can also be identified in the human thyroid. To extend the observations made in experimental models to human thyroids for which autoradiographic studies are obviously lacking, we attempted to better characterize the functional status of follicles, thanks to the immunohistochemical analysis of proteins involved in the iodine transport and organification pathways.

In follicles defined as hypofunctioning, we found that neither TPO, nor pendrin, nor ThOXs are expressed in the epithelial cells. A similar observation has been recently reported (25). Noteworthy, the colloid was also devoid of Tg-I, suggesting that the absence of iodination activity was associated with the absence of proteins involved in this pathway. When an iodination activity was present in the so-called active follicles, as shown by the positive immunostaining for Tg-I in the colloid, the epithelial cells were positively immunostained for TPO, ThOXs, and pendrin. ThOXs were localized at the apical pole of the cells, confirming recent data by *Caillou et al.* (25). From these data, we postulate that each follicle in the human thyroid gland can be categorized as

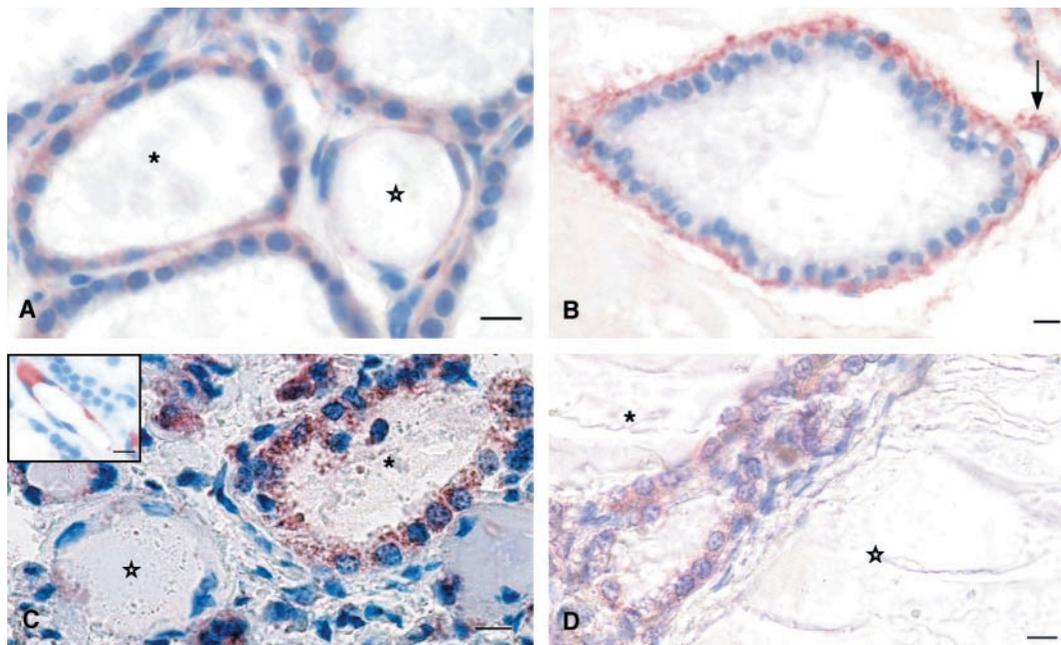


FIG. 5. Immunohistochemical detection of VEGF, NOSIII, and ET (scale bars, 10 μ m). Immunostaining of VEGF. In the normal thyroid follicular cells of both active (*) and hypofunctioning (★) follicles are positive (A). In the MNG, VEGF is detected in contact with extracellular matrix at the basal pole of follicular cells and around capillaries (*arrow*) (B). Immunostaining of NOSIII. In normal thyroid, active follicles (*) are positive and hypofunctioning follicles (★) are negative. In MNG, endothelial cells of capillaries are intensively stained (*inset*) (C). Immunostaining of ET in normal thyroid. Active follicles (*) are positive and hypofunctioning follicles (★) are negative (D).

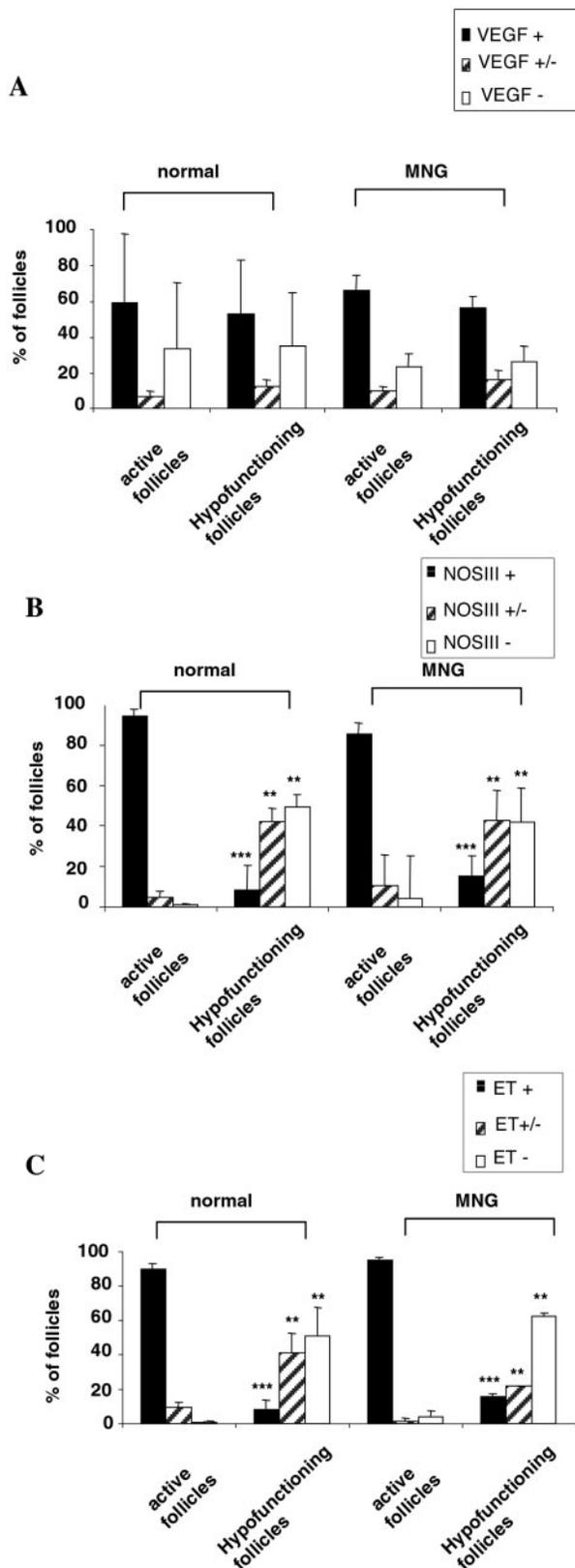


FIG. 6. Percentage of active and hypofunctioning follicles with positive, negative, or partially positive (\pm) immunoreactivity for VEGF (A), NOSIII (B), and ET (C). The results are expressed as mean \pm SD, $n = 5$. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.1$ resting follicles *vs.* active follicles in the same group.

active or hypofunctioning, based on its morphological aspect but also on the detection of proteins involved in iodination (TPO, ThOXs, pendrin), or reflecting this process, as Tg-I.

Irrespective of the functional status, TSHr and Tg were detected in both types of follicles. This suggests that cells of hypofunctioning follicles are not totally inactive, because they still are sensitive to TSH. This is in accordance with previous observations made in mice. Indeed, the proportion of cold follicles decreases progressively when a goiter is experimentally induced, indicating that when TSH increases, cold follicles become active (22, 26). These data are in accordance with those of Maenhaut *et al.* (27) stating that TSHr is always present in thyroid epithelial cells, even if they are dedifferentiated.

The immunostaining for TPO was seen in the whole cytoplasm, although this protein is known to act at the apical pole of the cell. This agrees with previous studies showing that only a small fraction of TPO is associated with the cell surface (28, 29). Most of the TPO is detected by confocal analysis in the perinuclear region, whereas only a small fraction is seen on the apical membrane (30). In fact, intracellular TPO is misfolded and not correctly glycosylated (29). Our data indicate that the antibody used in the present study most likely recognizes a linear epitope that is common for the different molecular TPO configurations (29).

We previously showed, in a model of old goitrous mice, that the thyroid is composed of clustered angiofollicular units having their own local vascular regulatory system. In this model, the microvascular bed was closely related to the functional aspect of the follicles. For instance, Vv of capillaries surrounding hyperfunctioning follicles was larger than those surrounding hypofunctioning follicles (22). Results of the present work in normal and goitrous human thyroids where active and hypofunctioning follicles coexist are in the same line. The capillary network surrounding active follicles was larger than that surrounding hypofunctioning follicles, because of an increased area and number of capillaries. In addition, the vascular bed was always larger in MNGs than in normal thyroids.

As in the mouse, the immunoreactivity for NOSIII in normal and goitrous thyroids was restricted to follicular cells of active follicles, suggesting a paracrine role for NO in regulating the microvascular tone. In MNGs, endothelial cells of capillaries were also intensively stained, suggesting a possible role for NO in the increased vasculature observed in nodular lesions, because NO is known to act as a vasodilator (31). The detection of NOSIII exclusively in active follicles associated with a higher area of capillaries surrounding these structures led us to hypothesize that a deficit in NO production by epithelial cells of hypofunctioning follicles could be responsible for the reduced vasodilation of capillaries surrounding them. There was also a strong correlation between NOSIII and TPO immunostainings. These data strengthen the idea that NO exerts autocrine or paracrine actions in follicular cells, as already suggested by previous reports (32–36).

ET was also exclusively detected in active follicles. ET may act as a vasoconstrictive or vasodilative factor, depending on the activated receptor. Vasoconstrictive effects of ET are me-

diated via the ETA receptor, whereas the presence of the ETB receptor on endothelial cells is associated with vasodilation (37). In normal rat thyroid, the ETB receptor is the most abundant (38). Hence, the deficit in ET production in hypofunctioning follicles could also contribute to the reduced capillary bed observed around these follicles.

A weak VEGF immunoreactivity was observed only in few follicles of the normal human thyroid. This is different from our observations in old mice, where VEGF was detected in all follicles. However, as in the mouse, no difference in this immunoreactivity was observed between active and hypofunctioning follicles in the normal human thyroid. In MNG, VEGF immunostaining was clearly extracellular, suggesting the crinopathy phenomenon, *e.g.* the trapping of growth factors in association with the extracellular matrix, as previously reported (39, 40).

As for ET, VEGF is known to act as an angiogenic factor by altering the mobility and the proliferation of endothelial cells (41–43). Several reports showed that VEGF and ET-1 are unable to induce, separately, angiogenesis *in vivo*. They must be present together to influence the expression of their respective gene (44, 45). In addition, the detection of VEGF in the extracellular matrix was associated with a strong positive signal of NOSIII in endothelial cells. Because Vv of capillaries around both active and hypofunctioning follicles increased as a result of both expanded area and number of capillaries, these data indicate that both vasodilation and angiogenesis are stimulated in goiters. One could suggest that VEGF induces NO production by endothelial cells, allowing angiogenesis (46–48), and thereby increasing the number of capillaries. On the other hand, enhanced NO production could induce vasodilation and therefore expand the capillary area. NO, ET1, and VEGF are all known to induce angiogenesis. Thus, NO modulates the endothelial cell matrix adhesion, thereby activating the first steps for the migration of endothelial cells when induced by VEGF. If NO production is blocked, VEGF is unable to induce endothelial cell migration and angiogenesis (46–48). A similar role for NO in ET-induced migration of endothelial cells has been described *in vitro* (49). During goiter formation in the rat, both NOSIII and ET increase and are associated with the expanded vasculature (50, 38). Local defects in the production of one (or more) of these factors may therefore affect the function of the others, resulting in a local decrease in the number of capillaries, as observed around hypofunctioning follicles. Moreover, other factors known to act on vascularization and detected in thyroid cells (such as the fibroblast growth factor, the angiopoietins, and thrombospondin) could also affect the local microvascularization surrounding thyroid follicles (51–53).

In conclusion, the morphological and microvascular properties of the follicles are correlated with their function, in particular with their iodination capacity, as evidenced by the regulated expression of TPO, pendrin, ThOXs, and Tg-I. In addition, angiofollicular units having their own regulation are present in normal and goitrous human thyroids. They are controlled by vasoactive and angiogenic factors. This regulation involves complex regulatory networks including NO, VEGF, and ET.

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References

- Ramelli F, Studer H, Bruggisser D 1982 Pathogenesis of thyroid nodules in multinodular goiter. *Am J Pathol* 109:215–223
- Studer H, Forster R, Conti A, Kohler H, Haerberli A, Engler H 1978 Transformation of normal follicles into thyrotropin refractory “cold” follicles in the aging mouse thyroid gland. *Endocrinology* 102:1576–1586
- Tamura S, Fujita H 1981 Fine structural aspects on the cold follicles in the aged mouse thyroid. *Arch Histol Jpn* 44:177–188
- Mesdagh C, Many MC, Halpern S, Briancón C, Fragu P, Denef JF 1990 Correlated autoradiographic and ion-microscopic study of the role of iodine in the formation of “cold” follicles in young and old mice. *Cell Tissue Res* 260:449–457
- Schürch M, Peter HJ, Gerber H, Studer H 1990 Cold follicles in a multinodular goiter arise partly from a failing iodide pump and partly from deficient iodine organification. *J Clin Endocrinol Metab* 71:1224–1229
- Dai G, Levy O, Carasco N 1996 Cloning and characterization of the thyroid iodide transporter. *Nature* 379:458–460
- Smanik PA, Liu Q, Furminger TL, Ryu K, Ying S, Mazzaferrri EL, Jhiang SM 1996 Cloning of the human sodium iodide symporter. *Biochem Biophys Res Commun* 226:339–345
- Eskandari S, Loo DF, Dai G, Levy O, Wright EM, Carasco N 1997 Thyroid Na⁺/I⁻ symporter. *J Biol Chem* 272:27230–27238
- Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Bascavanis AD, Sheffield VC, Green ED 1997 Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat Genet* 17:411–422
- Rutishauser J, Kopp P 1998 Surprising news: a putative sulfate transporter is defective in Pendred’s syndrome. *Eur J Endocrinol* 138:623–624
- Kopp P 1999 Pendred’s syndrome: identification of the genetic defect a century after its recognition. *Thyroid* 9:65–69
- Scott DA, Wang R, Kreman TM, Sheffield VC, Karnishki LP 1999 The pendred syndrome gene encodes a chloride-iodide transport protein. *Nat Genet* 21:440–443
- Royaux IE, Suzuki K, Mori A, Katoh R, Everett LA, Kohn LD, Green ED 2000 Pendrin, the protein encoded by the pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. *Endocrinology* 141:839–845
- Bidart JM, Mian C, Lazar, Russo D, Filetti S, Caillou B, Schlumberger M 2000 Expression of pendrin and the Pendred syndrome (PDS) gene in human thyroid tissue. *J Clin Endocrinol Metab* 85:2028–2033
- De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, Miot F 2000 Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem*. 275:23227–23233
- Dupuy C, Pomerance M, Ohayon R, Noel-Hudson MS, Deme D, Chaaraoui M, Francon J, Virion A 2000 Thyroidoxidase (THOX2) gene expression in the rat thyroid cell line FRTL-5. *Biochem Biophys Res Commun* 277:287–292
- den Hartog M, De Boer M, Veenboer JM, De Vijlder J 1990 Generation and characterization of monoclonal antibodies directed against noniodinated and iodinated thyroglobulin, among which are antibodies against hormonogenic sites. *Endocrinology* 127:44–49
- Wollman SH, Herveg JP, Zeligs JD, Ericson LE 1978 Blood capillary enlargement during the development of thyroid hyperplasia in the rat. *Endocrinology* 103:2306–2314
- Denef JF, Haumont S, Beckers C 1980 Morphological changes in mice thyroid induced by iodine deficiency. *Virchows Arch B Cell Pathol* 32:191–199
- Denef JF, Haumont S, Cornette C, Beckers C 1981 Correlated functional and morphometric study of thyroid hyperplasia induced by iodine deficiency. *Endocrinology* 108:2352–2358
- Many MC, Denef JF, Haumont S 1984 Precocity of the endothelial proliferation during a course of rapid goitrogenesis. *Acta Endocrinol (Copenh)* 105:487–491
- Gérard AC, Xhenseval V, Colin IM, Many MC, Denef JF 2000 Evidence for co-ordinated changes between vascular endothelial growth factor and nitric oxide synthase III immunoreactivity, the functional status of the thyroid follicles, and the microvascular bed during chronic stimulation by low iodine and propylthiouracyl in old mice. *Eur J Endocrinol* 142:651–660

23. Pohlenz J, Duprez L, Weiss RE, Vassart G, Refetoff S, Costagliola S 2000 Failure of membrane targeting causes the functional defect of two mutant sodium iodide symporters. *J Clin Endocrinol Metab* 85:2366–2369
24. Weibel ER, Kistler GS, Scherke WR 1966 Practical stereological methods for morphometric cytology. *J Cell Biol* 30:23
25. Caillou B, Dupuy C, Lacroix L, Nocera M, Talbot M, Ohayon R, Deme D, Bidart JM, Schlumberger M 2001 Expression of reduced nicotinamide adenine dinucleotide phosphate oxidase (ThoX, LNOX, Duox) genes and proteins in human thyroid tissues. *J Clin Endocrinol Metab* 86:3351–3358
26. Many MC, Mahmoud I, Deneff JF 1986 Postmitotic alteration of follicular iodine metabolism during cold follicle formation. *Front Thyroidol* 2: 907–911
27. Maenhaut C, Brabant G, Vassart G, Dumont JE 1992 *In vitro* and *in vivo* regulation of thyrotropin receptor mRNA levels in dog and human thyroid cells. *J Biol Chem* 267:3000–3007
28. Kuliawat R, Lisanti MP, Arvan P 1995 Polarized distribution and delivery of plasma membrane protein in thyroid follicular epithelial cells. *J Biol Chem* 270:2478–2482
29. Fayadat L, Niccoli-Sire P, Lanet J, Franc JL 1998 Human thyroperoxidase is largely retained and rapidly degraded in the endoplasmic reticulum. Its N-glycans are required for folding and intracellular trafficking. *Endocrinology* 139:4277–4285
30. Penel C, Gruffat D, Alquier C, Benoliel AM, Chabaud O 1998 Thyrotropin chronically regulates the pool of thyroperoxidase and its intracellular distribution: a quantitative confocal microscopic study. *J Cell Physiol* 174:160–169
31. Moncada S, Higgs A 1993 The L-arginine-nitric oxide pathway. *N Engl J Med* 329:2002–2012
32. Esteves RZ, Van Sande J, Dumont JE 1992 Nitric oxide as a signal in thyroid. *Mol cell Endocrinol* 90:R1–R3
33. Millatt LJ, Jackson R, Williams BC, Whitley GS 1993 Nitric oxide stimulates cyclic GMP in human thyrocytes. *J Mol Endocrinol* 10:163–169
34. Millatt LJ, Johnstone AP, Whitley GSJ 1998 Nitric oxide enhances thyroid peroxidase activity in primary human thyrocytes. *Life Sci* 63:PL373–PL380
35. Bocanera LV, Krawiec L, Silberschmidt D, Pignataro O, Juvenal GJ, Pregliasco LB, Pisarev MA 1997 Role of cyclic 3',5'-guanosine monophosphate and nitric oxide in the regulation of iodide uptake in calf thyroid cells. *J Endocrinol* 155:451–457
36. Costamagna ME, Cabanillas AM, Coleoni AH, Pellizas CG, Masini-Repiso AM 1998 Nitric oxide donors inhibit iodide transport and organification and induce morphological changes in cultured bovine thyroid cells. *Thyroid* 8:1127–1135
37. Fujitani Y, Oda K, Takimoto M, Inui T, Okada T, Uraday Y 1992 Autocrine receptors for endothelins in the primary culture of endothelial cells of human umbilical veins. *FEBS Lett* 298:79–83
38. Colin IM, Nava E, Toussaint D, Maiter DM, vanDenhove MF, Luscher TF, Ketelslegers JM, Deneff JF, Jameson JL 1995 Expression of nitric oxide synthase isoforms in the thyroid gland: evidence for a role of nitric oxide in vascular control during goiter formation. *Endocrinology* 136:5283–5290
39. Feige JJ, Baird A 1995 Crinopexy: extracellular regulation of growth factor action. *Kidney Int Suppl* 49:S15–S18
40. Wang JF, Milosveski V, Schramek C, Fong GH, Becks GP, Hill DJ 1998 Presence and possible role of vascular endothelial growth factor in thyroid cell growth and function. *J Endocrinol* 157:5–12
41. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N 1989 Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306–1309
42. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J 1989 Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 84: 1470–1478
43. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH 1994 Different signal transduction properties of KDR and Flt-1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269:26988–26995
44. Salani D, Taraboletti G, Rosano L 2000 Endothelin-1 induces angiogenic phenotype in cultured endothelial cells and stimulates neovascularization *in vivo*. *Am J Pathol* 157:1703–1711
45. Matsuura A, Yamochi W, Hirata K, Kawashima S, Yokoyama M 1998 Stimulatory interaction between vascular endothelial growth factor and endothelin-1 on each gene expression. *Hypertension* 32:89–95
46. Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bisknell R 1997 Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 99:2625–2634
47. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sesse WC 1997 Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 100:3131–3139
48. Noiri E, Lee E, Testa J, Quigley J, Colflesh D, Keese CR, Giaever I, Goligorsky MS 1998 Podokinesis in endothelial cell migration: role of nitric oxide. *Am J Cell Physiol* 274:C236–C244
49. Noiri E, Hu Y, Bahou WF, Keese CR, Giaever I, Goligorsky M 1997 Permissive role of nitric oxide in endothelin-induced migration of endothelial cells. *J Biol Chem* 272:1747–1752
50. Colin IM, Selvais PL, Rebai T, Maiter DM, Adam E, vanDenhove MF, Ketelslegers JM, Deneff JF 1994 Expression of the endothelin-1 gene in the rat thyroid gland and changes in its peptide and mRNA levels in goiter formation and iodide-induced involution. *J Endocrinol* 143:65–74
51. Becks GP, Logan A, Phillips ID, Wang JF, Smith C, DeSousa D, Hill DJ 1994 Increase of basic fibroblast growth factor (FGF) and FGF receptor messenger RNA during rat thyroid hyperplasia: temporal changes and cellular distribution. *J Endocrinol* 142:325–338
52. Patel VA, Hill DJ, Eggo MC, Sheppard MC, Becks GP, Logan A 1996 Changes in the immunohistochemical localisation of fibroblast growth factor-2, transforming growth factor-beta 1 and thrombospondin-1 are associated with early angiogenic events in the hyperplastic rat thyroid. *J Endocrinol* 148:485–499
53. Ramsden JD, Cocks HC, Shams M, Nijjar S, Watkinson JC, Sheppard MC, Ahmed A, Eggo MC 2001 Tie-2 is expressed on thyroid follicular cells, is increased in goiter, and is regulated by thyrotropin through cyclic adenosine 3', 5'-monophosphate. *J Clin Endocrinol Metab* 86:2709–2716