Metabolic, Endocrine and Genitourinary Pathobiology

Iodine Deficiency Induces a Thyroid Stimulating Hormone-Independent Early Phase of Microvascular Reshaping in the Thyroid

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Expansion of the thyroid microvasculature is the earliest event during goiter formation, always occurring before thyrocyte proliferation; however, the precise mechanisms governing this physiological angiogenesis are not well understood. Using reverse transcriptase-polymerase chain reaction and immunohistochemistry to measure gene expression and laser Doppler to measure blood flow in an animal model of goitrogenesis, we show that thyroid angiogenesis occurred into two successive phases. The first phase lasted a week and involved vascular activation; this process was thyroid-stimulating hormone (TSH)-independent and was directly triggered by expression of vascular endothelial growth factor (VEGF) by thyrocytes as soon as the intracellular iodine content decreased. This early reaction was followed by an increase in thyroid blood flow and endothelial cell proliferation, both of which were mediated by VEGF and inhibited by VEGF-blocking antibodies. The second, angiogenic, phase was TSH-dependent and was activated as TSH levels increased. This phase involved substantial up-regulation of the major proangiogenic factors VEGF-A, fibroblast growth factor-2, angiopoietin 1, and NG2 as well as their receptors Flk-1/VEGFR2, Flt-1/VEGFR1, and Tie-2. In conclusion, goiter-associated angiogenesis promotes thyroid adaptation to iodine deficiency. Specifically, as soon as the iodine supply is limited, thyrocytes produce proangiogenic signals that elicit early TSH-independent microvascular activation; if iodine deficiency persists, TSH plasma levels increase, triggering the second angiogenic phase that supports thy-

rocyte proliferation. (Am J Pathol 2008; 172:748–760; DOI: 10.2353/ajpath.2008.070841)

Vascular supply is an absolute requirement for all organs that cannot solely rely on diffusion of oxygen and metabolites for their survival. In endocrine glands, in addition to allocating nutrient supply, the microvascular network is particularly well adapted to secretory functions. Among all endocrine glands, the thyroid is the most vascularized. The expansion and the shrinkage of the vascular bed are dynamically coupled with its functional status.^{1–3}

Microvascular adaptation is the earliest morphological event occurring during goiter formation, before any alteration in other tissue compartments.^{4,5} The vascular bed may expand up to twofold, along with an increase in microvessel density and blood flow to reach a plateau when a new equilibrium is achieved.^{6,7} Changes in vasculature are closely related to those in intraglandular iodine content. Hence, the thyroid blood flow sharply rises when iodine supply decreases and promptly falls when iodine supply is restored.⁸ Variations in blood flow likely participate in intraglandular autoregulatory mechanisms that keep thyroid hormone release constant.⁹ The maintenance of a steady iodide supply is one of the foremost steps in hormone synthesis. Hence, increasing vascular supply contributes to the optimization of iodide uptake. Of note, iodine may be hazardous for thyrocytes when it is overloaded in

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iodine-deficient glands. To limit this toxicity because of free radicals released in excess, a transient blockade in thyroid hormone synthesis occurs (Wolff-Chaikoff effect).¹⁰ In the meantime, iodide access to thyrocytes becomes strongly hindered, limiting iodine-induced deleterious effects and disables the Wolff-Chaikoff effect. This is achieved, at least in part, by a prompt vasoconstriction^{11–15} and a down-regulation of the sodium-iodide symporter (NIS).¹⁶ Thus, thyroid angioarchitecture is perfectly adapted to bring iodine supply in line with thyrocyte demand and finely tuned to quickly react to adverse environmental conditions.

Previous studies reported that each follicle is surrounded by its own capillary network that remains totally independent from neighboring follicles.¹⁷ The extent of each microvascular bed is closely related to the functional status and proliferation of nearby thyrocytes. Hence, the thyroid is composed of angio-follicular units that are independent from each other but able to react to thyrotropin (thyroid-stimulating hormone or TSH) stimulation on its own. This physiological process entirely relies on a permanent paracrine dialog between endothelial and epithelial cells.^{1–3}

As for changes in the epithelial compartment, recent evidence suggests that changes affecting endothelium are also of utmost importance for the development of goiters. The development of hyperplastic goiter consecutive to iodine deficiency should therefore be considered as an adaptation involving angiogenic processes synchronized with adjustments in thyrocyte function and proliferation, events that evolve throughout the entire adult life. In contrast with the chaotic nature of malignant angiogenesis, thyroid-specific angiogenic processes are both tightly controlled and perfectly reproducible using animal models of goitrogenesis. This makes the thyroid gland an easily workable model to understand how epithelial and endothelial cells cooperate in physiological conditions to keep intact organ homeostasis. Numerous questions remain regarding the role of angiogenesis during goiter development. To answer them, we studied the early stages of angiogenesis in a mouse model of goiter formation. We analyzed the expression of the major proangiogenic factors vascular endothelial growth factor (VEGF)-A, fibroblast growth factor-2 (FGF2), and angiotensin-1 (Ang-1) and their receptors, as well as the possible involvement of pericytes in this process. We found that two successive phases of vascular remodeling take place during goitrogenesis. The early one, which had not previously been described, involves vascular activation and occurs as an immediate response to dropping iodine supply. It is TSH-independent and is directly triggered by thyrocytes. The later angiogenic phase has been previously described, is fully TSH-dependent, and involves blood vessel growth along with thyroid hyperplasia. Our study brings new understanding to the processes that control angiogenesis in regulated growth.

Materials and Methods

Animals and Treatments

Two-month-old NMRI mice received a low-iodine diet (LID) (0.1 μ g iodine/day; Animalabo, Brussels, Belgium) supplemented with perchlorate in the drinking water (ClO_4^- , 1%) for 0, 1, 2, 3, 4, 6, 12, or 24 days where indicated. In addition to LID/ ClO_4^- , animals were injected intraperitoneally with a saline solution of bevacizumab (10 mg/kg/6 days; Roche, Welwyn Garden City, UK) or with a solution of SU5416 (25 mg/kg/day; Sigma, St. Louis, MO) in dimethyl sulfoxide. Control and perchlorate-treated animals were also injected with an equimolar solution of irrelevant human IgGs (bevacizumab control) or dimethyl sulfoxide (SU5416 control, 100 μ l i.p./day). Mice were housed and handled according to Belgian Regulation on Laboratory Animal Welfare.

TSH Radioimmunoassay

Plasma TSH concentrations were measured by radioimmunoassay using the rat thyroid-stimulating hormone (rTSH) Biotrak assay system (Amersham Int., Bucks, UK) according to the manufacturer's protocol.

Preparation of Tissue Samples for Microscopy

In a first set of experiments, five animals from each group (0, 1, 2, 3, 4, or 6 days of goitrogen treatment) were injected with ^3H -thymidine (30 μCi /mouse, Amersham) 1 hour before sacrifice. They were anesthetized with pentothal and perfused as previously described.³ One thyroid lobe was fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 1.5 hours, postfixed in 1% osmium tetroxide for 1 hour, and embedded in LX112 resin (Ladd Research Industries, Burlington, VT). Thin sections (0.5 μm) were stained with toluidine blue and analyzed for morphometric analysis. Autoradiographies were performed on 1- μm -thick sections from Epon-embedded lobes as previously described.^{4,5} Ultrathin sections were prepared and stained with uranyl acetate and lead citrate and examined with an electron microscope Zeiss EM169 (Carl Zeiss, Oberkochen, Germany). The second thyroid lobe was fixed in paraformaldehyde [4% in phosphate-buffered saline (PBS)] for 36 hours and embedded in paraffin. Thick sections (5 μm) were used for immunohistochemistry. In a second set of experiments, animals (0, 6, 12, or 24 days of treatment, five animals per group) were anesthetized with pentothal. The thyroid lobes were dissected without perfusion. One lobe was immersed in paraformaldehyde (4% in PBS) for 36 hours and then embedded in paraffin. Thick sections (5 μm) were used for immunohistochemistry. The second lobe was directly frozen in liquid nitrogen and used for RNA analysis.

Morphometric Analysis

Morphometric measurements were performed using the point-counting method described by Weibel and col-

leagues¹⁸ on sections crossing the center of the lobe and considered to be representative of the entire gland. For each thyroid, 1000 points were counted, and the relative volumes (Vv) of epithelium, follicular lumen, capillaries, and interstitium were measured.

Autoradiographic Measurements

³H-thymidine-labeled nuclei were counted in epithelial and endothelial cells and expressed as percentage of total epithelial or endothelial nuclei.

¹²⁷I Distribution on Semithin Section

The elemental distribution of phosphorus and iodine (³¹P and ¹²⁷I) was obtained by NanoSIMS50 imaging.^{19–22} Maps were acquired under standard analytical conditions, a Cs⁺ ion primary beam with impact energy of 16 keV and a current of 1 pA. The surface analyzed was 30 × 30 μm². Under these conditions, a lateral resolution of 100 nm is expected. The detection of these elements was done in the multicollection mode with parallel detection of the carbon, nitrogen, and sulfur (¹²C, ¹²C¹⁴N, and ³⁴S). All images were acquired in 256 × 256 pixels with a counting time of 20 ms per pixel.

Immunohistochemistry

Tissue sections were washed with phosphate-buffered saline (PBS, pH 7.4) supplemented with 1% bovine serum albumin (PBS-bovine serum albumin) and incubated at room temperature for 30 minutes with normal goat or horse serum (1/50; Vector Laboratories, Burlingame, CA) in PBS-bovine serum albumin. Slides were then incubated at room temperature in the presence of a primary antibody (see Supplemental Table S1 at <http://ajp.amjpathol.org>). The antibody was detected using an EnVision (DakoCytomation, Carpinteria, CA) or ABC Perox kit (Vector Laboratories, see Supplemental Table S1 at <http://ajp.amjpathol.org>). The peroxidase activity was revealed using AEC (DakoCytomation) as substrate. Sections were counterstained with Mayer's hematoxylin. For detection of VEGF and angiopoietin 1, sections were pretreated in a microwave oven in citrate buffer (0.01 mol/L, pH6) for one cycle of 3 minutes at 750 W, followed by four cycles of 3.5 minutes each at 350 W. Negative controls were performed by omitting the primary antibody or by adding

a neutralizing peptide in excess (VEGF and FGF2; Santa Cruz Biotechnology, Santa Cruz, CA).

Western Blotting

Five animals per group (0, 1, 2, 3, 4, or 6 days of treatment) were used for thyroid protein analysis. Animals were anesthetized with pentothal. The thyroid lobes were dissected and frozen in liquid nitrogen. Thyroid homogenates were suspended in Laemmli buffer (50 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, and 10% glycerol) containing a protease inhibitor cocktail (Sigma). Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Proteins (30 μg/lane) were heated at 95°C for 5 minutes in the loading buffer (Laemmli buffer containing 100 mmol/L dithiothreitol and 0.1% bromophenol blue), proteins separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Roosendaal, The Netherlands). Membranes were blocked for 1 hour at room temperature in PBS (pH 7.4), 5% nonfat dry milk, 0.1% Tween, and incubated overnight at 4°C with a polyclonal antibody raised against VEGF-A (1/500). Membranes were washed with PBS/Tween 0.1%, incubated for 1 hour at room temperature with EnVision (1/200, DakoCytomation) peroxidase-labeled secondary antibody, washed, and visualized with enhanced chemiluminescence (SuperSignal West Femto, Pierce) on CL-Xposure TM films (Pierce). The same membranes were also treated with an anti-β-actin antibody (1/8000, Sigma).

RNA Purification and Reverse Transcription

One thyroid lobe of each experimental condition (0, 1, 2, 3, 4, or 6 days of treatment in one set of experiments, and 0, 6, 12, and 24 days in a second set) was homogenized and suspended in TriPure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA was purified according to the manufacturer's protocol and resuspended in 6 μl of H₂O. Reverse transcription was performed by incubating RNA with 200U Moloney murine leukemia virus-reverse transcriptase (M-MLV-RT; Invitrogen, Merelbeke, Belgium) in the recommended buffer containing 4 U of RNasin (Promega, Madison, WI), 0.5 mmol/L dNTP (Promega), 2 μmol/L oligodT (Promega), and 10 mmol/L dithiothreitol (20

Table 1. Forward and Reverse Primers and Annealing Temperatures Used

	Primer forward	Primer reverse	Annealing t°
Actin	5'-CATCCTGCGTCTGGACCT-3'	5'-AGGAGGAGCAATGATCTTGAT-3'	62°C
GAPDH	5'-CTGACGTGCCGCTGGATGAAA-3'	5'-TTGGGGGCGGAGTTGGGATAGG-3'	55°C
TPO	5'-CAGGTTTTGGTGGGAGAA-3'	5'-CTGCACACTCATTAAACATCTT-3'	58°C
VEGF	5'-CACTGCACCCTGGCTTTACTGC-3'	5'-CGCCTCGGCTTGTCACA-3'	57°C
Flk1	5'-GCTCCGCCCCCAATCCT-3'	5'-CTCATAGCCCGCATCCTCCACA-3'	57.5°C
Flt1	5'-AACCCCGGAGTATGCCACACACCTGA-3'	5'-GTCCCGCCTCCTTGTTTTACTCG-3'	59°C
Ang-1	5'-CACGAAGGATGCTGATAACG-3'	5'-AAGTGGCGATTCTGTTGACC-3'	59°C
Tie2	5'-GTGGGGGCCCGACTGTAG-3'	5'-CATCCTTGGCCTGCCTTCTTCT-3'	57.5°C
NG2	5'-GCTCCGGATGGCCAGTCAGTCA-3'	5'-CCAGGGCGAGAGTGAGGTAGAGA-3'	65°C

μ l final volume) overnight at 42°C. H₂O (80 μ l) was then added and products were used for polymerase chain reaction (PCR) amplification analyses.

PCR

cDNAs (2.5 μ l) were mixed with 0.625 U TaKaRa TaqDNA polymerase (TaKaRa Bio, Shiga, Japan), 0.2 mmol/L dNTP, and 0.5 μ mol/L of specific primers (Table 1) in recommended buffer (25 μ l total volume). Amplification cycles were as follows: 94°C for 3 minutes, then cycles of 94°C for 1 minute, annealing temperature for 2 minutes (Table 1), and 72°C for 1 minute, followed by 72°C for 10 minutes. The number of cycles was selected after having verified the linearity of the amplification reaction. PCR products were separated by agarose gel (1%) electrophoresis. Control reactions contained water instead of cDNA products.

Quantitative PCR

cDNAs (2 μ l) were mixed with 500 nmol/L of each selected primers (Table 1), 3 mmol/L MgCl₂, and SYBR Green reaction mix (Roche) in a final volume of 20 μ l. Reactions were performed using a LightCycler apparatus (Roche) as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, annealing temperature for 7 seconds (Table 1), and 72°C for 30 seconds. Amplification levels were normalized to that of GAPDH.

Laser-Doppler Blood Flow Measurement

Anesthetized mice (ketamine/xylazine) underwent surgery during which skin was removed, and submaxillary glands and pretracheal muscles were set aside to allow exposure of intact thyroid lobes. The thyroid blood flow was measured using a Laser Doppler imager (Moor Instruments, Axminster, UK). Thyroid lobes were identified and delineated based on anatomical images and blood flow in area of interest was quantified from colored histogram pixels.²³ During measurements, mice were placed on a heating pad (37°C).

Statistical Analysis

All data are expressed as mean $\pm$ SEM. For TSH radioimmunoassay, morphometric analysis, autoradiographic assay, and thyroid blood flow measurements, statistical analyses were performed using analysis of variance followed by a Tukey-Kramer or Newman-Keul multiple comparisons posthoc test (GraphPad InStat, San Diego, CA). $P < 0.05$ was considered as statistically significant.

Western blots were scanned and quantified by densitometry using pixel densitometry with the NIH Scion Image Analysis Software (National Institutes of Health, Bethesda, MA). Data were normalized with β -actin. For thyroperoxidase (TPO) RT-PCR, digital imaging of ethidium bromide-stained agarose gels served for pixel quantification by densitometry using the Scion Image Analysis

Software. Data were normalized with β -actin. The statistical analysis was performed using unpaired t -test. $P < 0.05$ was considered as statistically significant.

Results

Changes in the Thyroid Function

Plasma TSH levels are a marker of thyroid gland function, and they increase when thyroid hormone synthesis decreases. When mice received a goitrogen treatment from day 0, TSH levels remained at control values for 6 days, followed by a significant increase at days 12 and 24 (Figure 1A). To evaluate thyroid hormone contents, we used a Tg-I antibody (Tg-I) that specifically recognizes thyroglobulin with high T4 content.²⁴ Tg-I staining was positive up to day 6 of goitrogen treatment, indicating that the gland was still containing some hormone reserves during the first week of treatment. At days 12 and 24, Tg-I staining was no longer visible indicating that hormone reserves were empty (Figure 1B). TPO is a key enzyme involved in thyroid hormone synthesis, so we measured its level of expression, known to be strongly regulated by TSH,^{25,26} to assess the degree of thyrocyte stimulation by TSH. TPO mRNA expression remained low during the first 6 days of goitrogen treatment and increased thereafter at days 12 and 24 (Figure 1A). This translated into a similar pattern of protein expression measured using immunohistochemistry: We observed no changes in TPO expression up to 6 days of goitrogen treatment and then an increase at day 12 and day 24 (Figure 1B). Of note, TPO protein expression was detected only in thyrocytes from active follicles in normal thyroids, whereas inactive ones remaining negative, as previously reported.² The profiles of TPO mRNA and protein expression matched perfectly those of serum TSH, thereby indicating that the thyroid function was preserved for the first 6 days, and hypothyroidism started afterward, along with TSH-induced stimulation of thyrocytes.

Because LID/CIO₄⁻ treatment decreases thyroid iodine content, we measured ¹²⁷I thyroid content using multiple isotope mass spectrometry. (NanoSIMS). In the control mice, iodine was detected at high concentration in the colloid (Figure 2A, asterisk), as well as in the interface between apical pole and colloid that appeared as a highlighted edge (Figure 2A, arrowhead). Low levels of ¹²⁷I were also found in the cytoplasm of thyrocytes, in the basal membrane (Figure 2A, arrows), and in the interstitial tissue just underneath. At day 1 of treatment, ¹²⁷I was still present at high concentration in the colloid (Figure 2C, asterisk) and in the apical membrane but was not visible either at the basal pole of thyrocytes (Figure 2C, arrows) or in interstitial tissues. Of note, ¹²⁷I colloid contents somewhat faded in some follicles. These results suggest that less than 1 day after iodine deprivation, iodine supply to the cells and its availability for thyroid hormone synthesis are already compromised. After 24 days of iodine deficiency, the iodine content of the gland was severely reduced because only a faint signal persisted (Figure 2E).

Figure 1. Changes in the thyroid function during goiter formation. **A:** Serum TSH concentration and TPO mRNA expression. Mice were treated with LID + ClO₄⁻ 1% starting from day 0. TSH (red line) and TPO mRNA levels (bars) were followed throughout the goitrogen period, in two separate experiments spanning 0 to 6 days (**left**) and 0 to 24 days (**right**). TSH levels were measured by radioimmunoassay in duplicate, and TPO mRNA levels by RT-PCR. There were no changes in TSH and TPO levels during the first 6 days of treatment. Both parameters increased at 12 and 24 days. Plasma TSH levels are expressed as mean ± SEM, *n* = 15 or 10. Densitometric values of TPO mRNA expression were adjusted to β-actin values and expressed as mean ± SEM, *n* = 5. **P* < 0.05 versus controls. **B:** Tg-I and TPO immunohistochemistry. Thyroid paraffin sections were stained with the Tg-I antibody that identifies thyroid tissues containing high levels of T₄ (**top**) or TPO (**bottom**). A strong Tg-I staining was observed until day 6. Afterward, the signal was virtually absent suggesting that T₄ contents were low. Changes in TPO protein expression were parallel to those of TPO mRNA levels. Scale bars = 10 μm.

B

Tg-I

TPO

Day 0

Day 6

Day 12

Day 24

Goitrogen treatment

Iodine Deficiency Promotes a Biphasic Thyroid Vascular Adaptation

Iodine is normally delivered to thyrocyte from blood vessels, and TSH is a known proangiogenic signal that promotes the extension of the thyroid vascular network.²⁷ Here, we hypothesized that this late adaptive response could be preceded by an early response aimed at preventing a decrease in thyroid hormone synthesis. We therefore focused on the thyroid vascular compartment between day 0 and day 6 of goitrogen treatment.

We first observed that the relative volume of blood vessels was significantly increased by 1.5-fold (*P* < 0.05) (data not shown) after day 6, indicating that the expansion of the microvasculature occurs very early in the course of goiter formation. Interestingly, this response was not associated to gross morphological changes in the tissue structure of the thyroid. We noticed only a moderate decrease in the volume of the colloid after 3 days of treatment, whereas the volume

occupied by epithelial cells remained unchanged. These early vascular changes were confirmed when measuring the thyroid blood flow using a laser Doppler imager (Figure 3A) as a 1.3-fold increase was observed at day 2. At days 4 to 6, values dropped to those of control glands. A second increase ranging from 1.8- to 1.96-fold was then observed at days 12 and 24, respectively, along with elevated TSH plasma levels.

The electron microscopy analysis of the thyroid vasculature on control versus 1 day-treated mice showed a different pattern of endothelial cell/pericyte interaction. Although pericytes were closely bound to endothelial cells (Figure 3B) in controls, they were separated from endothelial cells after 1 day of treatment (Figure 3C), which is typical of angiogenesis initiation.²⁸ To illustrate further the microvascular changes, we assayed epithelial and endothelial cell proliferation in thyroids of 0-, 1-, 2-, 3-, 4-, or 6-day treated mice injected with ³H-thymidine 1 hour before sacrifice. The percentage of labeled epithelial cells remained low

Figure 2. NanoSIMS detection of ^{127}I and ^{31}P . ^{127}I (A, C, E) and ^{31}P (B, D, F) signals were localized using NanoSIMS in the thyroid of mice treated for 0 (A, B), 1 (C, D), or 24 days (E, F) with ClO_4^- (1%). ^{31}P detection gives some information on cell morphology. ^{127}I and ^{31}P concentrations were evaluated by a color scale ranging from black (–) to red (highest concentration). ^{127}I was detected at high concentration in the colloid (asterisk) and in the interface between apical pole (arrowhead) and colloid of control and 1-day-treated mice. It was also present in the basal membrane (arrows), as well as in interstitial tissues underneath. As soon as day 1 after starting iodine deprivation, the signal was still present in the colloid (asterisk) and in the apical pole (arrowhead), but faded away in the basal region suggesting that alterations in the iodine supply to thyrocytes yet occurred. After 24 days of goitrogen treatment, the signal nearly vanished. Scale bars = 6 μm .

during the 6 first day period (Figure 3D). In contrast, the number of labeled endothelial cells significantly increased already at day 2 (fourfold increase versus control, $P < 0.05$) and reached a peak at day 4 (9.4-fold increase versus control, $P < 0.05$), in accordance with previous findings from our laboratory.^{4,5} Altogether, these results demonstrate that a TSH-independent vascular activation occurs during the first week of goiter formation. This first phase is followed by a second angiogenic phase that occurs concomitantly with rising plasma TSH levels.

Iodine Deficiency Induces Biphasic Changes in the Expression of Factors Involved in Angiogenesis

The onset of angiogenesis requires the synthesis and release of proangiogenic factors that stimulate relevant pathways in vascular cells. To analyze the kinetic of molecular events that take place during the early phase of vascular activation, we analyzed the expression of

major factors involved in the onset of angiogenesis: VEGF-A and its receptors flk-1/VEGFR2 and flt-1/VEGFR1, FGF2, Ang-1 and its receptor Tie-2, and NG2, a marker of pericyte activation.

The immunohistological analysis of VEGF-A protein (Figure 4A) showed a weak signal in thyrocytes of control thyroids. VEGF-A signal strongly increased after day 1 and remained high at day 2. Because this signal was detected in thyrocytes, we propose that the angiogenic signal originates from these cells in response to the drop in intracellular iodine contents. VEGF-A staining decreased at day 3, but rose again at day 4, exhibiting the most intense labeling at day 6. The biphasic modulation of VEGF-A protein expression was confirmed using Western blot analysis (Figure 4C). VEGF-A mRNA expression was analyzed by qRT-PCR (Figure 4B) and was found to follow the same trend, with an early rise as soon as day 1 (2.7-fold increase as compared to control, $P < 0.05$), a decrease at day 3 and a second, larger increase (2.3- and 3-fold, respectively; $P < 0.05$) at days 4 and 6, which accurately correlated with the immunohistological observations. On long-term goitrogen treatment, an increase in VEGF-A staining was also noticed (Figure 4D). After 12 days of treatment, VEGF-A protein was not only intracellular, but also found in the interstitium surrounding follicles. VEGF-A protein expression decreased at day 24, but remained higher than in controls. Analysis of mRNA expression (Figure 4B) showed a similar pattern, with a maximum at 12 days of treatment (7.5-fold increase, $P < 0.05$) and a decrease at day 24, whereas remaining higher than in control mice (5.1-fold increase, $P < 0.05$).

We also measured the mRNA levels of VEGF receptors Flk1/VEGFR2 and flt-1/VEGFR1 (see Supplemental Figure 1, A and B, at <http://ajp.amjpathol.org>). In both cases, it followed a single phase increase that became significant at day 4 of treatment (4.0- and 2.9-fold increase, for Flk1/VEGFR2 and flt-1/VEGFR1, respectively) and reached a peak at day 12 as previously reported.^{29,30} FGF2 protein expression followed the two vascular remodeling phases as well. FGF2 was detected at low levels from day 0 to day 2 of treatment (Figure 5A), a strong increase occurred at day 3, followed by a subsequent drop at days 4 and 6. The staining was the strongest during the second phase, especially at day 12 (Figure 5B). In contrast with VEGF-A and FGF2, Ang-1 expression was detected in capillaries surrounding follicles at days 0 and 1 (Figure 6A) and faded from days 2 to 6. The number of positive capillaries was 6.5/10 follicles $\pm$ 1.8 in control and remained stable at day 1 (Figure 6B). This number gradually decreased from day 2 to become very low at day 6. Changes in Ang1 expression were not associated with changes in Tie2 receptor expression in the thyroid, as determined using qRT-PCR (see Supplemental Figure 1C at <http://ajp.amjpathol.org>). We only noticed a sharp increase in thyroid Tie2 mRNA content on day 12 that faded at day 24.

NG2 expression followed that of VEGF-A and FGF2, as well as the two consecutive vascular remodeling phases. Low expression was seen around capillaries of control thyroids (Figure 7A). At day 1 of treatment, NG2 expression strongly increased and then returned to control values between days 2 and 6. In long-term goitrogen treatment NG2

Figure 3. Evidence for two vascular remodeling phases during goiter formation. **A:** Thyroid blood flow was measured *in vivo* using a laser Doppler. Blood perfusion increased between day 0 and day 4, dropped to almost control values between days 4 and 6, and increased again at days 12 and 24. Results are expressed as percentage of control, mean $\pm$ SEM, $n = 6$ (days 0, 6, and 24), 7 (days 1 and 4), and 5 (days 2 and 12). * $P < 0.05$ as compared to control, * $P < 0.05$ as compared to day 2. Electron microscopy analysis of thyroids of mice treated with LID + ClO_4^- 1% for 0 (**B**) and 1 day (**C**). In control mice, endothelial cells (**1**) and pericytes (**3**) were tightly connected through basal laminae (**2**). At day 1 of goitrogen treatment, the pericyte (**3**) was detached from the endothelial cell (**1**). **D:** Mice treated with LID + ClO_4^- 1% for 0, 1, 2, 3, 4, or 6 days were injected with ^3H -thymidine 1 hour before sacrifice. Thyroid lobes were removed, paraffin-embedded, and ^3H -thymidine-labeled epithelial and endothelial cells counted. Results (mean $\pm$ SEM, $n = 5$) are expressed as percentage of total epithelial or endothelial cells. Although epithelial cell proliferation remained quiescent up to day 6 of goitrogen treatment, endothelial cells started to proliferate at day 2 and reached a peak at day 4. Scale bars = 0.5 μm .

expression reached a peak at day 12 (Figure 7B). It is noteworthy that NG2 immunolocalization was quite different since it was mainly detected in the cytoplasm of thyrocytes at this time of treatment, whereas microvascular structures remained negative. Analysis of NG2 mRNA levels by qRT-PCR (Figure 7C) showed no significant changes up to day 6 of treatment, whereas the expression reached a peak at day 12 (6.1-fold increase versus control, $P < 0.05$).

Altogether, our results indicate that the early vascular activation phase takes place during the first 3 days of goitrogen treatment. This early phase is independent of hormone stimulation and is characterized by activation of the endothelium, increase in blood perfusion, and detachment of pericytes, and it correlates with rising expression of two key angiogenic factors VEGF-A and FGF2, whereas the endothelium stabilizing factor Ang-1 is down-regulated.

Bevacizumab- and SU5416-Induced Inhibition of Vascular Remodeling

To put our observations in a dynamic perspective and to test VEGF-A involvement in the vascular events oc-

curing during iodine deprivation, blocking antibody against VEGF (bevacizumab), which was recently shown to bind mouse VEGF-A,³¹ or the VEGF receptor inhibitor SU5416 were administered to animals. The morphometric analysis showed that administration of bevacizumab induced a significant decrease (50%, $P < 0.05$) in the relative volume of vessels, whereas animals receiving control IgGs showed instead a significant increase in this parameter after 12 days (Figure 8A). Similar results were obtained with SU5416 (data not shown). In addition, bevacizumab abolished the rise in blood perfusion observed at day 2 in IgG-treated animals (Figure 8B). This suggests that the first vascular activation that occurs in the early time course of goiter development is indeed VEGF-dependent.

Discussion

Our results show for the first time that an initial phase of vascular activation takes place in the early days of goiter formation. These changes are TSH-independent but are

Figure 4. Goitrogenesis is associated with a biphasic increase in VEGF-A expression. **A:** VEGF-A protein was detected by immunohistochemistry on paraffin thyroid sections of mice treated with LID + ClO_4^- 1% for 0, 1, 2, 3, 4, and 6 days (the experiment was repeated twice). For qRT-PCR, mice were treated with LID + ClO_4^- 1% for 0, 1, 2, 3, 4, and 6 days or for 0, 6, 12, and 24 days in two separate experiments. **B:** Total RNA were extracted and VEGF-A transcripts levels were quantified and normalized to GAPDH transcripts. Values are expressed as mean $\pm$ SEM, $n = 5$. * $P < 0.05$ as compared to control, + $P < 0.05$ as compared to day 6. **C:** Total protein extract of thyroids from mice treated with LID + ClO_4^- for 0, 1, 2, 4, or 6 days were analyzed by Western blot using a VEGF-A antibody. Densitometric values normalized to β -actin were expressed as mean $\pm$ SEM, $n = 5$. * $P < 0.05$ as compared to control. **D:** VEGF-A protein was detected by immunohistochemistry on paraffin thyroid sections of mice treated with LID + ClO_4^- 1% for 0, 6, 12, and 24 days. VEGF-A protein and mRNA expression increased as soon as day 1 of treatment, decreased at day 2 (mRNA) or day 3 (protein), and further increased at day 6 to reach a peak at day 12. Scale bars = 10 μ m.

FGF

A

B

Figure 5. Goitrogenesis is associated with a biphasic increase in FGF2 expression. **A:** FGF2 protein was detected by immunohistochemistry on paraffin thyroid sections of mice treated with LID + ClO_4^- 1% for 0, 1, 2, 3, 4, and 6 days (the experiment was repeated twice). **B:** FGF2 protein was detected by immunohistochemistry on paraffin thyroid sections of mice treated with LID + ClO_4^- 1% for 0, 6, 12, and 24 days. Scale bars = 10 μm .

Ang 1

A

B

Figure 6. Goitrogenesis induces decreased angiopoietin 1 expression. **A:** Ang-1 immunodetection on paraffin thyroid sections of mice treated with LID + ClO_4^- 1% for 0 (ctrl), 1, 2, and 6 days. Ang-1 was detected in vessels surrounding thyroid follicles. **B:** Number of Ang-1-positive vessels. Values are expressed as mean $\pm$ SEM, $n = 5$. From day 2 to day 6, the number of positive vessels gradually declined. * $P < 0.05$ as compared to control. Scale bars = 10 μm .

driven from thyrocytes themselves, likely in response to dropping intracellular iodine contents. Activation of the endothelium during this first phase is associated with increased expression of key factors of angiogenesis and a VEGF-A-dependent mechanism.

Changes in the microvasculature in the late phase of goiter development have been extensively documented in the past in various animal models and in humans.²⁷ They are intimately associated with rising TSH plasma levels. Nevertheless, previous studies showed that changes in the thyroid blood flow can sometimes occur without any changes in TSH levels.^{8,32–35} Our results redefine these apparent discrepancies because they show that variations in the thyroid blood flow in response to goitrogen treatment occur in two distinct phases: an early phase that is completely TSH-independent and a second, later phase that is, in contrast, TSH-dependent. It should be noted that the early TSH-independent endothelial cell proliferation was originally reported more than 25 years ago,^{4,5} without any satisfactory explanation having been given about the underlying mechanisms.

The first phase of vascular activation is accompanied by an increase in the thyroid blood flow and an up-regulation in angiogenic factor expression. VEGF-A, which is detected in normal thyroids, is immediately up-regulated after the start of goitrogen treatment. This is one of the earliest cellular reactions occurring as a response to the fall in intracellular iodine contents. Hence, both events seem to be linked because the immunohistochemical analysis showed that VEGF-A expression increases in thyrocytes as soon as the intracellular iodine content drops, as observed by ionic microscopy. The two VEGF-A receptors flk-1/VEGFR1 and flt-1/VEGFR2 were also up-regulated after iodine deprivation, as was FGF2. As for VEGF-A, thyrocytes appear to be its main source of production. Concomitantly, Ang-1 expression decreased from the second day of goiter formation at a time corresponding to the onset of endothelial cell

proliferation and reached its lowest level when TSH levels were rising up (at 12 days).

Our results provide some insights into the involvement of pericytes in thyroid vascular remodeling. Peri-

NG2

A

B

C

Figure 7. Expression of the pericyte marker NG2. **A:** NG2 protein was detected by immunohistochemistry on thyroid sections from mice treated with LID + ClO_4^- 1% for 0, 1, 2, 3, 4, or 6 days. NG2 protein was detected around endothelia. Its expression was low in control mice and increased at day 1 of treatment. **B:** NG2 protein was detected by immunohistochemistry on thyroid sections from mice treated with LID + ClO_4^- 1% for 0, 6, 12, or 24 days. After 12 and 24 days, NG2 protein was detected in thyrocyte cytoplasm. No labeling was observed in negative controls when first antibody was replaced by PBS/bovine serum albumin, indicating that the labeling was specific and not attributable to endogenous peroxidase activity (*inset*). For qRT-PCR, mice were treated with LID + ClO_4^- 1% for 0, 1, 2, 3, 4, and 6 days and for 0, 6, 12, and 24 days in two separate experiments. **C:** Total RNA was extracted and NG2 transcripts were quantified and normalized to GAPDH transcripts. Values are expressed as mean $\pm$ SEM, $n = 5$. * $P < 0.05$ as compared to controls. NG2 mRNA expression remained unchanged during the 6 first days of treatment and showed a significant increase at day 12. Scale bars = 10 μm .

Figure 8. Bevacizumab-induced inhibition of the early phase of vascular remodeling. **A:** A morphometric analysis by point counting of the relative volume of vessels, epithelium, colloid, and interstitium was performed on semithin sections of mice treated for 12 days with bevacizumab (10 mg/kg/6 days) or human IgGs (10 mg/kg/6 days) in addition to LID/ ClO_4^- . Values are expressed as mean $\pm$ SEM, $n = 5$. * $P < 0.05$ as compared to control. ** $P < 0.05$ as compared to goitrous mice treated with IgGs. The increase in the relative volume of vessels induced by goitrogen treatment was significantly reduced by bevacizumab. **B:** The thyroid blood flow was measured in mice receiving bevacizumab (10 mg/kg) or human IgGs (10 mg/kg) in addition to LID/ ClO_4^- for 0, 1, 2, or 4 days. Values are expressed as mean $\pm$ SEM, $n = 5$. * $P < 0.05$ as compared to 2 days LID/ ClO_4^- . ** $P < 0.05$ as compared to IgGs + LID/ ClO_4^- .

cytes are thought to play a role in the stabilization of vessels. In fact, recent data suggest even more important roles for these cells in the regulation of local blood flow, in vascular permeability, and in endothelial cell proliferation and differentiation. These could occur through paracrine interactions and direct cell-to-cell contact-dependent mechanisms. Although the original hypothesis proposed that pericytes should pull out from endothelial cells for angiogenesis being initiated, more recent data suggest that pericytes may directly play a role in angiogenesis by releasing angiogenic and fibrinolytic factors and, in some cases, by guiding the migration of endothelial cells.^{28,36–39} Vascular stability is regulated by a molecular interaction in which perivascular cells, such as pericytes, produce Ang1, which activates the Tie2 endothelial cell surface receptor. Ang1 activation of Tie2 is constitutive in the normal adult vasculature and is necessary to maintain its quiescence. The initiation of angiogenesis is associated with a decrease in Ang1 production and pericyte detachment from

blood vessels.^{37,40} In this study, electron microscopy analysis clearly shows that pericytes separated from endothelial cells at day 1 of treatment, suggesting that the disconnection of pericytes could favor the proliferation of adjacent endothelial cells, as previously reported.^{41–43} In addition, we show that expression of NG2, a marker of activated pericyte at the onset of angiogenesis,^{44–46} is strongly enhanced at day 1 along with up-regulated VEGF-A expression, in accordance with previous studies showing that VEGF-A induces NG2 up-regulation.⁴⁶ It is therefore likely that pericytes participate in the early phase of vascular activation in the thyroid. The increased VEGF-A expression accompanied by the detachment of pericytes from endothelial cells and the down-regulation of Ang-1 expression may therefore explain the increased blood flow observed after 2 days of treatment. Moreover, the suppression of this response in the presence of bevacizumab strongly suggests that this increased blood flow is VEGF-dependent.

The first phase of vascular activation has not been described previously. However, the second phase involving angiogenesis and thyroid hyperplasia has already been described previously²⁷ and depends on TSH.^{29,47–52} The first phase correlates with the increased expression of several factors involved in angiogenesis and the increased blood flow. Our work provides novel information about a possible involvement of pericytes and NG2 in this TSH-dependent angiogenesis.

Taken together, our data suggest that the first phase of the vascular activation is triggered by signals coming from the thyrocyte as soon as iodine cellular stores drop. One of the best ways for the thyroid to achieve this goal is to massively increase the local blood flow that represents a limiting step for iodide delivery. We propose that this early vascular reaction works as a rescue mechanism triggered by the thyrocyte to safeguard iodide supply, a key element for thyroid hormone synthesis. The blood vessel remodeling is immediate and likely depends on paracrine signals coming directly from the thyrocytes, thereby acting on adjacent endothelial cells and pericytes. Hence, VEGF-A synthesis is turned on, quickly followed by FGF2, and a decrease in Ang-1 expression. Pericytes also take part in this process by optimizing the local conditions to facilitate the migration and proliferation of angio-follicular unit endothelial cells. The blood flow can then increase accordingly. Our data support the idea that the intracellular signal responsible for this early angiogenic reaction is closely associated with intracellular levels of iodine. Thus, a very sensitive intracellular iodine sensor should exist, enjoining thyrocytes to react quickly to iodine deficiency by increasing the release of angiogenic signals as soon as they perceive a decrease, even slight, in iodine supplementation. Further investigations are in progress to verify this hypothesis. Our data indicate that this intrinsic rescue system is quickly activated and that it covers the gap because of delayed increase in TSH plasma levels. The second phase of angiogenesis corresponds to a more classical TSH-dependent adaptation of the gland to iodine deprivation. This angiogenic reaction is more robust and is associ-

ated with thyrocyte hypertrophy and proliferation. It may optimize nutrient and oxygen supplies needed to sustain complex trophic changes. This tissue adaptation results when a new equilibrium is reached between environmental parameters, such as iodine supply and the utmost role of the thyroid gland: safeguarding of hormone synthesis.

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