ORIGINAL RESEARCH

Angiogenic study in Graves' disease treated with thyroid arterial embolization

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Abstract

Purpose: To investigate angiogenesis in the thyroid of Graves' disease (GD) treated with thyroid arterial embolization through analysis of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and microvessel density (MVD).

Materials and methods: Forty-two GD patients were treated with thyroid arterial embolization and followed up for 1-68 months after embolization. Before embolization and at 7 days, 3, 6, 12, 36 and 48 months following embolization, TT3, TT4, FT3, FT4, TSH and thyroid stimulating antibody (TSAb) were tested respectively. Thyroid biopsy was performed under the guidance of computed tomography for immunohistochemical staining of VEGF and bFGF, and MVD within the thyroid gland was marked by CD34.

Results: VEGF and bFGF were mostly expressed in the cytoplasm and on the cell membrane. The expression of VEGF was increased ($P < 0.05$) at ≤ 6 months compared with before embolization and decreased ($P < 0.05$) at ≥ 1 year compared with either at ≤ 6 months or before embolization. The expression of bFGF was not statistically different at ≤ 6 months compared with before embolization but was decreased ($P < 0.05$) at ≥ 1 year compared with either at ≤ 6 months or before embolization. Thyroid MVD marked by CD34 had similar changes to those of the VEGF

expression after embolization. There was a positive correlation between VEGF and bFGF ($P < 0.05$) and between VEGF or bFGF and MVD ($P < 0.05$). Thyroid hormones mostly returned to normal and TSAb was decreased in longer follow-up.

Conclusion: Thyroid arterial embolization can decrease the expression of VEGF, bFGF and MVD. Consequently, angiogenesis within the GD thyroid will be decreased in the long term after embolization and may serve as the basis for reduced thyroid size and function.

Graves disease (GD) is an autoimmune disorder of the thyroid characterized by the production of autoantibodies, especially thyroid-stimulating autoantibody (TSAb) against the thyrotropin receptor leading to receptor activation and subsequent hyperthyroidism.^{1, 2} Treatment strategies for GD hyperthyroidism include medical therapy with antithyroid drugs, thyroid surgery or radioiodine therapy.^{3, 4} However, because of multiple and even severe side-effects caused by medication, surgery and radioiodine such as allergic reactions, agranulocytosis and hepatotoxicity for medication^{5, 6}, and hypothyroidism for surgery and radioio-

dine^{4, 7}, a new method of thyroid arterial embolization has emerged for the treatment of GD due to considerable progress in the endovascular technology.⁸⁻¹³ The mechanism of thyroid arterial embolization to treat GD is to occlude most of the thyroid vessels to reduce thyroid hormone secretion and, hence, restoring the patient to euthyroidism.⁸⁻¹³ To date, clinical experience with this therapy has been minimal. Further research is needed because many questions remain mysterious even though we have studied the clinical¹¹, long-term immunological⁹, pathological¹⁰ and apoptotic¹² effects of thyroid arterial embolization on GD hyperthyroidism. However, angiogenesis in the embolized thyroid of GD patients has not been studied. Angiogenesis is the mechanism of blood vessel formation after the first few days of embryogenesis and is essential for all tissue growth. In adults, angiogenesis occurs in the thyroid during disease processes including goitre, GD, thyroiditis and cancer¹⁴⁻¹⁶, in which increased vascularity takes place in the thyroid gland. This study was conducted to investigate the changes of angiogenesis in GD thyroid gland after thyroid arterial embolization.

Materials and Methods

During the period between November 2001 and August 2007, 42 GD patients (10 male, 32 female) were enrolled in the study. The protocol was approved by the Institutional Review Board and the Ethics Committee of the First Affiliated Hospital of Kunming Medical College. Inclusion criteria were noncompliance with or having serious side effects to antithyroid drugs, and refusing surgical and radioactive iodine therapies. The patients, aged 14 - 51 yr (mean 32.6 yr), had GD confirmed clinically and by laboratory studies. They provided signed informed consent before thyroid arterial embolization and biopsy, except for the one 14-year-old patient whose consent was provided by her father after the risks and benefits of the endovascular thyroid embolization had been thoroughly explained to the patients or their relatives. All

patients had goitre, grade I in 5 cases, II in 16, and III in 21, classified in accordance with World Health Organization recommendations.¹⁷ Twenty two of the patients had vascular murmurs in the thyroid area. None of the patients had a history of other autoimmune, endocrinological and infectious diseases or tumour.

Equipment used in the study included an angiography suite of German SIEMENS BICOR PLUS/TOP model (1250mA), Holland Phillips MX800 4-row spiral CT scanner and US BD FACSCalibur flow cytometer. Reagents used were FT3, FT4 and rT3 solid phase radioimmunoassay kits (Jiuding Bioengineering Co. Ltd, Tianjin, China), TT3 and TT4 radioimmunity kits (Xiehe Medical Technology Co. Ltd., Tianjin, China), radioreceptor assay kits of human blood TSAb autoimmune antibodies, hTSH radioimmune kits (IRLAND Corporation, US), streptavidin-peroxidase immunohistochemistry kits (Fuzhou Manxin, Fuzhou, China), and mouse VEGF, bFGF and CD34 monoclonal antibodies against human (Fuzhou Manxin, Fuzhou, China).

Three days before thyroid arterial embolization, a beta-blocker (propranolol, 10mg, 3 times daily) was administered to bring the patients' heart rate < 100 bpm. Methimazole (20-40 mg, 3 times daily) or propylthiouracil (PTU, 50-100 mg, 3 times daily) was also administered to control hyperthyroidism. Experienced interventional radiologists performed thyroid arterial embolization using similar techniques (Fig.1).

The embolization protocol and efficacy assessment of thyroid embolization had been described previously.¹¹ In brief, after thyroid arterial angiography, bilateral superior arteries were embolized using a mixture of polyvinyl alcohol, papaverine and a non-ionic contrast agent (Omnipaque 300, Amersham Health, Shanghai, China) because these two arteries are usually the major thyroid supplying vessels. In patients with moderate to severe goitre, an inferior thyroid artery was also embolized to enhance the embolization effect. Since the parathyroids are supplied

FIGURE 1. Thyroid arterial embolization. A. Angiography before embolization showing enlarged thyroid arteries, thyroid staining and increased blood flow. B. Six months after embolization, the trunk of the thyroid gland remains patent with decreased thyroid staining and sparse blood supply.

mainly by the bilateral thyroid inferior arteries, one inferior artery was usually left unembolized to avoid parathyroid hypo-function.¹¹ During the procedure, special attention was paid to preventing regurgitation of embolic agent to avoid mis-embolization of other arteries.

Three days before embolization and at 3 and 7 days, and at 3, 6, 12, 24, 36 and 48 months after embolization, 5 ml venous blood were harvested in the morning after fasting to study thyroid function. This included total T4 (TT4), total T3 (TT3), free T4 (FT4), free T3 (FT3), reverse T3 (rT3), thyroid-stimulating hormone (TSH), and thyroid stimulating antibody (TSAAb). Thyroid biopsy was performed in different patients under computed tomography (CT) guidance in 16 cases before embolization, 5 cases at 7 days, 4 at 3 months, 2 at 6 months, 6 at 1 year, 4 at 2 years, and 16 at ≥ 3 years following thyroid embolization. In 1 case at 3 months, 2 at 1 year and 3 at

≥ 3 years after embolization, no thyroid tissue was present in the pathological sections of the biopsied thyroid tissue. Thus, 47 cases had pathological sections for immunohistochemistry examination. After the biopsy, the punctured thyroid was compressed for ten minutes to preventing complications. The biopsy cases were divided into three groups for analysis: 16 cases in group one of pre-embolization, 10 in group two at ≤ 6 months and 21 in group three at ≥ 1 year after embolization. Immunohistochemical staining was performed for vascular endothelial growth factor (VEGF) expression, basic fibroblast growth factor (bFGF) and CD34 to mark microvessel density (MVD). Analysis of the immunohistochemical staining for expression of VEGF and bFGF was based on the semi-quantitative standard proposed by Mattern et al.¹⁸ while the counting of MVD was based on the criteria recommended by Weidner et al.¹⁹. Three independent observers evaluated the results from the immunohistochemical

staining without knowledge of each patient's clinical data and the order of the slides. The few cases of discrepancy among these investigators were reevaluated and then classified according to the most frequent classification. For evaluation of angiogenic factor expression, a semi-quantitative scoring system was adopted based on both the staining intensity (0 or -, negative; 1 or +, weak; 2 or ++, intermediate; 3 or +++, strong) and the percentage of positive cells (0, 0% cells; 1, $\leq 25\%$ positive cells; 2, 26-50% positive cells; 3, $>50\%$ positive cells)¹⁸. The MVD was determined in the area of most intense vascularization (hot spot) of each section. Individual microvessel counts were made on a $\times 250$ field ($\times 25$ objective and $\times 10$ ocular, corresponding to an area of 0.363 mm²) by three independent observers. The average count from the three observers was used as the final score.¹⁹

Statistical analysis

Statistical analysis was performed with SAS application software package, version 6.12 (SAS Institute Inc., North Carolina State University, NC, USA). Continuous data were expressed as mean ($\bar{x} \pm SD$), or as the median and range if not normally distributed. Statistical tests were performed with $P < 0.05$ being considered significant and $P < 0.01$ highly significant.

Results

Complications and Efficacy

No severe complications occurred immediately after the embolization procedure except slight-to-moderate neck pain caused by thyroid arterial embolization. Immediately after embolization, vascular murmurs in the anterior neck region disappeared, the enlarged thyroid gland decreased, and blood flow was reduced as shown by ultrasound and angiographic examination (Fig.1).

Forty-two patients were studied for one year after embolization, among whom 30 were restored to euthyroidism (71.4%), seven improved (16.7%) and

five recurred (11.9%). Thirty-nine patients were followed for one to three years following embolization, with 28 being euthyroid (71.8%), 4 improved (10.3%) and 7 recurred (17.9%). Twenty-two patients were followed up over three years, among whom 17 were euthyroid (77.3 %) and 5 improved (22.7%) with reduced dose of antithyroid drugs for maintenance. Six more patients with long-term follow-up (>3 yr) were found to be recurrent and were re-embolized (for 3 arteries), and thyroid function 6 mos after embolization was euthyroid. No side effects occurred during long-term follow-up.

Changes of thyroid and autoimmune function (Table 1)

Compared with before embolization, serum TT3, TT4, FT3 and FT4 increased at three days, decreased at one month after embolization, and remained normal or showed some inflection (FT4) later. Within 1 month of embolization, TSH index remained very low compared with before embolization. Starting from the third month, TSH suddenly increased and remained within the normal range in the remaining follow-up. The TSAb index was much greater before than at any time after embolization, with a general trend of gradually decreasing over time.

Immunohistochemical

VEGF expression: The positive expression of VEGF showed yellow - dark brown particles mainly located in the cytoplasm and on the cell membrane of thyroid cells, with no expression in the nuclei. VEGF expression was distributed in fragments around the thyroid tissue, with different staining intensity. In regions of active proliferation of follicular epithelial cells and in regions of lymphocyte infiltration, expression of VEGF was stronger. Compared with group 1 before embolization, expression of VEGF was enhanced in group 2 at ≤ 6 mos after embolization but in group 3 at ≥ 1 year following embolization. Compared with group 2 at ≤ 6 months after embolization,

TABLE 1. Changes of thyroid hormones and specific antibody before and after embolization ($\bar{X} \pm S$)

	TT3(nmol/L)	TT4(nmol/L)	FT3(pmol/L)	FT4(pmol/L)	TSH(uIU/ml)	TSAb(u/l)
NR	1.08-3.1	77.2-154.4	2.50-9.82	10.0-25.0	0.25-4	<14
PrE	7.37±3.12	227.32±79.51	18.65±7.66	58.68±18.37	0.11±0.08	173.5±126.38
3d PoE	9.87±5.47*	354.5±108.73**	29.35±16.74**	88.75±14.32**	0.21±0.15	138.65±117.47
7d PoE	7.73±2.52	213.35±85.08	12.62±8.77	25.63±15.75**	0.18±0.04	125.46±110.12
1m PoE	2.93±1.22**	118.11±39.75**	6.15±2.70*	27.78±16.25**	0.09±0.05	111.11±107.61
3m PoE	3.27±1.23**	103.54±37.74**	5.75±2.17*	20.69±12.95**	2.27±1.13**	96.81±92.75
6m PoE	3.22±1.17**	120.75±49.57**	7.25±3.03*	22.24±14.12**	2.49±1.79**	51.17±37.98*
1y PoE	3.18±1.11**	115.54±33.73**	6.74±3.15*	26.15±14.35**	0.51±0.13*	29.65±8.57*
2y PoE	2.31±0.83**	137.74±22.34**	7.47±1.44*	35.86±16.59*	0.85±0.81*	17.29±5.77**
3y PoE	2.43±0.95**	130.73±42.45**	7.74±3.15*	29.24±11.2*	1.18±0.54**	25.65±4.82*
≥3yPoE	2.38±0.82**	124.64±42.41**	8.02±3.21*	36.04±10.7*	1.27±0.81**	27.61±4.67*

TT4: total T4, TT3: total T3, FT4: free T4, FT3: free T3, TSH: thyroid-stimulating hormone, NR: normal range, PrE: pre-embolization, PoE: postembolization, d: day, m: month, y: year,

* $P < 0.05$: compared with pre-embolization values,

** $P < 0.01$ compared with pre-embolization values.

expression of VEGF was highly decreased in group 3 at ≥ 1 year after embolization (Table 2 and Fig. 2).

bFGF expression: Expression of bFGF showed yellow - brown particles located mainly in the cytoplasm of thyroid cells with some expression on the cell membrane but not in the nuclei. Expression of bFGF was heavily enhanced with no difference in groups 1 & 2. However, expression of bFGF was weakened in group 3 at ≥ 1 year following embolization compared with group 1 or 2 (Table 2 and Fig.2).

MVD expression: The CD34 protein was stained as yellow - brown particles located mainly in the cytoplasm of vascular endothelial cells. However, there was also some expression on the cell membrane. In GD thyroid tissue, MVD marked by CD34 was distributed irregularly. In regions of active proliferation

of follicular epithelial cells and in regions of lymphocyte infiltration, expression of MVD was stronger and more intense. Compared with the MVD value of 70.25 ± 15.55 in group 1 before embolization, expression of MVD was enhanced in group 2 (MVD 82.45 ± 12.62) at ≤ 6 months after embolization but reduced in group 3 (MVD 26.56 ± 9.76) at ≥ 1 year after embolization. Compared with group 2 at ≤ 6 months after embolization, expression of MVD was also decreased in group 3 at ≥ 1 year after embolization (Fig. 3).

Correlations among VEGF, bFGF and MVD: Correlation analysis demonstrated a positive correlation between VEGF and bFGF and between VEGF or bFGF and MVD (Table 3).

Discussion

Angiogenesis is the sprouting of new blood vessels from pre-existing capillaries, and requires multiplication of endothelial cells, their migration, remodeling of the extracellular matrix, tube formation and recruitment of surrounding structures to maintain the newly formed vessels.²⁰ Angiogenesis is tightly controlled and occurs rarely in the adult vasculature ex-

TABLE 2. Frequency of VEGF and bFGF expression after thyroid arterial embolization

	Group 1		Group 2		Group 3	
	-/+	++/+++	-/+	++/+++	-/+	++/+++
VEGF	8	8	1	9	17	4
bFGF	7	9	3	7	16	5

Group 1. 16 cases before embolization. Group 2. 10 cases at ≤ 6 months after embolization. Group 3. 21 cases at ≥ 1 year after embolization. (-, negative staining; +, weak staining intensity; ++, intermediate; +++, strong).

FIGURE 2. Expression of VEGF (A-C) and bFGF (D-F), $\times 400$.

A. Before embolization. Expression of VEGF was yellow- brown particles mainly in cytoplasm and on cell membrane.

B. At 3 mos. Expression of VEGF - dark brown particles with similar staining intensity (+++).

C. At 3 yr. Less expression of VEGF (+).

D. Before embolization. Expression of bFGF - yellow-brown particles in cytoplasm and on cell membrane, expression intensity ++.

E. At 1 month. Expression of bFGF was similar to before embolization (++)

F. At 1 yr. A few cells had the amber bFGF expression in the cytoplasm with the staining intensity -.

FIGURE 3. MVD marked by CD34, $\times 400$.

A. Before embolization, CD34 protein - brown particles in cytoplasm, MVD - 55.25. B. At 6 mos. More new vessels marked by CD34. MVD - 90.65.

C. At 3 yr. Fewer new vessels marked by CD34. MVD - 32.58.

TABLE 3. Relationship between VEGF or bFGF expression and MVD after embolization

	VEGF		bFGF	
	Cases	MVD ($\bar{X} \pm S$)	Cases	MVD ($\bar{X} \pm S$)
-	10	22.73 $\pm$ 7.62	5	30.55 $\pm$ 7.18
+	8	41.32 $\pm$ 11.56	14	42.76 $\pm$ 12.07
++	8	67.13 $\pm$ 13.41	7	55.16 $\pm$ 15.91
+++	5	90.67 $\pm$ 10.52	5	74.23 $\pm$ 17.17

Note: Pearson correlation analysis between VEGF and MVD: $r=0.65$, $P<0.05$; between bFGF and MVD: $r=0.46$,

$P<0.05$. (-, negative staining; +, weak staining intensity; ++, intermediate; +++, strong).

cept with wound healing, the menstrual cycle, and in pathological conditions including diabetic retinopathy, tumour formation, hyperplastic goitre and GD in which increased vascularity presents.¹⁴⁻¹⁶ Neovascular growth is regulated through a balance of soluble angiogenic stimulators and inhibitors, with promoters of angiogenesis including VEGF, acidic FGF, bFGF, insulin-like growth factor, transforming growth factor, NO, angiotensin-II.¹⁶ VEGF and bFGF are two major promoters of angiogenesis. Accumulating evidence shows that VEGF and its receptors are important in the thyroid in GD, thyroiditis, goitre and cancer.²¹⁻²⁴ bFGF alone may act as an angiogenic factor in the thyroid, with direct effects on both endothelial and follicular cell growth¹⁶.

In this study, we found that the expression of VEGF was greater in regions of active proliferation of follicular epithelial cells and lymphocyte infiltration. After thyroid arterial embolization, expression of VEGF was enhanced ≤ 6 months but reduced at ≥ 1 year compared with before embolization. Expression of bFGF was heavily enhanced both before and at ≤ 6 months after embolization but weakened at ≥ 1 year following embolization. MVD was strong and intense in regions of active proliferation of follicular epithelial cells and in regions of lymphocyte infiltration. After thyroid arterial embolization, MVD was enhanced at ≤ 6 months but reduced at ≥ 1 year compared with before embolization.

There was a correlation between VEGF and bFGF and between VEGF or bFGF and MVD. The changes of VEGF, bFGF and MVD after thyroid arterial embolization may be caused through many mechanisms. Embolization of the thyroid arteries led to acute occlusion of small arteries and, consequently, acute aseptic inflammation and necrosis of the thyroid tissue. Acute occlusion of arteries caused thyroid hypoxia which could upregulate the expression of VEGF.²⁵⁻²⁷ Moreover, hypoxia could enhance the stability of VEGF and prevent its breakdown^{28, 29}, thus increasing its concentration after embolization. Acute necrosis of thyroid tissue may stimulate expression of VEGF through cytokines and inflammation factors such as insulin-like growth factor, transforming growth factor etc.³⁰⁻³⁴ Consequently, at ≤ 6 months after thyroid arterial embolization, expression of VEGF was increased to enhance production and hyperplasia of MVD within the thyroid gland. However, at ≥ 1 year when the ischemic and necrotic tissues within the thyroid gland had been repaired by hyperplastic tissue and the amount of thyroid follicular cells had been reduced because of ischemia and necrosis⁸⁻¹², the total amount of oxygen needed by the thyroid gland decreased because of decreased secretory function. Subsequently, expression of VEGF was reduced and returned to normal. MVD within the thyroid was also reduced with decreased expression of VEGF.

As for bFGF, its expression was not different at ≤ 6 months after embolization but decreased at ≥ 1 year. This suggests that bFGF may not play a major role in angiogenesis shortly after embolization. In the long run, bFGF expression was also reduced together with decreased expression of VEGF and MVD. The mechanism for the change of bFGF after embolization is not clear but may be related to the repair and decreased function of the thyroid gland. Moreover, interaction with VEGF may also play a role in the changes of the expression of bFGF because there is a complex interdependent relationship between VEGF and bFGF.¹⁶

After thyroid arterial embolization, most of the thyroid hormones showed fluctuating changes but returned to normal in the long run, as did the TSH index. Three days after embolization, serum thyroid hormones were temporarily increased because the embolized thyroid tissue had become necrotic and released quantities of the existing hormones stored within the gland. Later, serum thyroid hormone concentration gradually decreased to normal probably because embolization had decreased the volume of functioning thyroid tissue and the subsequent reduction of the synthesis and secretion of hormones after the stored hormones had been released.^{8, 10, 11} Three years after embolization, some thyroid function tests remained abnormal because of recurrence in some patients whose abnormal thyroid function resulted in the thyroid function increase of the whole group. The TSAb autoantibody concentration gradually decreased with time, but had not returned to normal. These changes demonstrate that the thyroid function had greatly decreased. Hyperthyroidism was restored to euthyroidism even if abnormal immune status remained.

In conclusion, thyroid arterial embolization can decrease the expression of VEGF and bFGF and reduce MVD in GD thyroid in the long term. This decreases abnormal growth of the thyroid tissue and converts hyperthyroidism to euthyroidism.

Acknowledgments

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