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The morphological analysis of vasculature in thyroid tumours: immunoexpression of CD34 antigen

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Abstract: Angiogenesis represents an important process manifested in tumour growth and in development of metastases. Using immunohistochemistry, the authors evaluated number of vessels in various nodular lesions of the thyroid (54 cases). Expression of CD34 antigen and microvessel density were evaluated in sections of archival paraffin blocks originating from the Department of Pathological Anatomy, University Medical School and the Lower Silesia Centre of Oncology in Wrocław, Poland. Microvessel density was assessed in ten different fields per section in "hot spots". Expression of CD34 was quantified using computerised image analysis and, then, mean micrvessel count (MVC) and microvessel area (MVA) were calculated. In thyroid tissue with benign lesions, the MVC (31.7) was higher than in neoplastic lesions (22.3), although no differences in MVA were observed. This observation points to differences in the size of newly formed vessels in individual nodular lesions of the thyroid.

Key words: Angiogenesis - Microvessel density - Computer image analysis - Thyroid tumours

Introduction

Angiogenesis involves formation of new blood vessels from the preexisting vascular bed. It represents a multistage process, related to modelling of the extracellular matrix, proliferation and migration of endothelial cells, differentiation and, then, anastomosing of capillaries [10, 19]. The alterations remain under control of various angiogenic factors. In physiological conditions, angiogenesis can be observed in developmental and regenerative processes [10, 15, 17]. Abnormal and unfavourable angiogenesis can be seen in pathological processes, *e.g.* in the course of neoplastic growth. The latter type of angiogenesis significantly affects proliferation and metastasing potential of tumour cells [16, 17]. In many tumour types, angiogenesis has been noted to be of prognostic significance. Its relation to development of metastases was demonstrated for the first time in malignant melanoma [14, 40]. Subsequently, studies appeared which evaluated angiogenesis in tumours of other organs, including larynx [22], lungs [18, 25, 44], prostate [9, 31, 41], ovary [5], uterine cervix, kidney [39], liver [28, 35] oesophagus, large intestine [3, 11, 13], urinary bladder [7, 21, 33, 36, 43] and haematological disorders [6,10,12]. Neovascularisation in neoplastic lesions, measured by density of microvessels, was found to strictly correlate with metastatising potential in breast cancer [8, 23]. The observations were confirmed in many other solid tumours [8, 23].

Thyroid carcinomas are characterised by extensively variable prognosis [1]: the most frequent papillary carcinoma carries a very good prognosis as to survival of the patient, while the anaplastic carcinoma is burdened by the lowest five-year survival values [20]. In several studies, expression of vascular markers has been found to correlate with tissue vascular supply and we used such markers in our study to quantify microvessel density in selected tumours of the thyroid [7-9].

This study was aimed at comparing density of the vascular network, visualised by the expression of CD34 antigen, in benign and malignant thyroid lesions and in individual types of thyroid tumours.

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Materials and methods

The study was performed on samples of thyroid lesions originating from 54 patients. The material included 18 benign lesions (10 cases of nodular goitre and 8 cases of follicular adenoma). In the other 36 patients, malignant tumours were diagnosed (9 cases of follicular carcinoma, 23 cases of papillary carcinoma and 4 cases of medullary carcinoma). Paraffin blocks originated from the Department of Pa-

Table 1. Analysis of selected variables (number of vessels and vascular surface area) in benign and in malignant lesions of the thyroid.

Variable	Benign lesion, n=18		Malignant lesion, n=36	
	mean	SD	mean	SD
Number of vessels (MVC)	31.7	12.3	22.3*	8.4
Vascular surface area [mm^2] (MVA)	0.307	0.132	0.267	0.117

*$p<0.001$

Table 2. Number of vessels and vascular surface area in individual histological types of studied thyroid lesions

Histological type of lesion	Number of vessels (MVC)		Vascular surface area [mm^2] (MVA)	
	mean	SD	mean	SD
Nodular goitre, n=10	30.3 [2]	12.7	0.278	0.149
Follicular adenoma, n=8	33.5 [2]	12.3	0.344	0.105
Follicular carcinoma, n=9	18.7	5.2	0.266	0.135
Papillary carcinoma, n=23	24.4 [1]	8.9	0.277	0.109
Medullary carcinoma, n=4	18.0	8.9	0.210	0.143

[1]Significantly ($p<0.05$) different from follicular and medullary carcinomas; [2]significantly ($p<0.05$) different from follicular, medullary and papillary carcinomas.

thological Anatomy, University Medical School in Wrocław and from the Department of Pathomorphology, Lower Silesia Centre of Oncology in Wrocław. All the patients were subjected to surgery in the period 1992-2002 due to clinical symptoms of nodular goitre in the absence of hyperthyroidism and on the basis of previous thin needle biopsy or intraoperative histopathological examination. The type of lesions was microscopically diagnosed according to WHO criteria [20, 26, 29]. Before the surgery, the patients were not subjected to therapy.

The expression of CD34-positive cells was determined immunohistochemically in paraffin-embedded specimens fixed in 4% buffered formalin. Histological slides, 4 μm in thickness, were deparaffined in xylol. Slides were heated in 0.01 M citrate buffer target retrieval solution (DAKO, Glostrup, Denmark, cat # N S1699) 3 × 10 min in microvave oven (700 W). After cooling for 20 min and washing in PBS, endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide for 5 min. The samples were incubated with 1:50 dilution of monoclonal mouse anti-CD34 antibody (DAKO, Glostrup, Denmark, cat # N 1632), expressed on vascular endothelium. After washing in PBS, the sections were treated with streptavidin/peroxidase complex standard solution (DAKO, LSAB 2, Denmark, cat # K 3468) for 20 min. Visualisation of signal was achieved by incubation in a substrate solution composed of 250 μL Tris pH 7.6, 2.5 ml diaminobenzidine tetrachloride (DAB) and 1.7 μL 0.3 % H_2O_2 in 5 mL of double distilled H_2O for up to 2 min. Sections were counterstained with hematoxylin and mounted. All procedures were carried out at room temperature.

At first under a light microscope, at a low magnification (× 40), the studied lesions were screened to select regions which manifested the highest density of microvessels (hot spots). The vessels were then counted using × 200 magnification. CD34-positive vessels were quantified using Olympus BX 50 light microscope with visual mode and MultiScan 5.10 software for computer-assisted image analysis. In every analysed case, total number of vessels present within the × 200 magnification field (MVC) and area occupied by the stained vessels in the same field (MVA) were established. In every case, the measurements were performed in 10 representative fields and each field was scored thrice.

Results were subjected to statistical analysis using ANOVA and Levene's tests of variance and the STATISTICA software. For benign and malignant lesions, mean values and standard deviations were calculated for the number and area of vessels. Differences at the level of $p<0.05$ were regarded as statistically significant.

Results

In the histopathological material originating from 54 patients with nodular lesions of the thyroid, expression of CD34 antigen was detected in the stroma of thyroid follicles. Expression of the antigen included an evident cytoplasmic reaction in endothelial cells. Any brown stained endothelial cell or endothelial cluster clearly separated from each other was considered a single countable microvessel (Fig. 1). Evident, statistically significant differences in microvessel count (MVC) were disclosed between benign and malignant lesions (Tab. 1) and, less prominent ones, between malignant tumours of different histological character (Tab. 2). Statistical analysis of MVC in the primary tumours permitted to group the tumours into three categories: lesions with high MVC values, such as nodular goitre and follicular adenoma, lesions with evidently lower MVC values including follicular carcinoma and medullary carcinoma and lesions with intermediate MVC values, *i.e.* papillary carcinoma (Tab. 2). Evaluation and statistical analysis of microvessel area (MVA) demonstrated no significant differences. In turn, when both parameters, *i.e.*, the number of vessels and their area, were analysed in parallel, the quantitative differences between benign and malignant lesions and the differences between various histological types of carcinomas were found to be related mainly to the number of vessels, while their area showed no significant differences. This observation points to

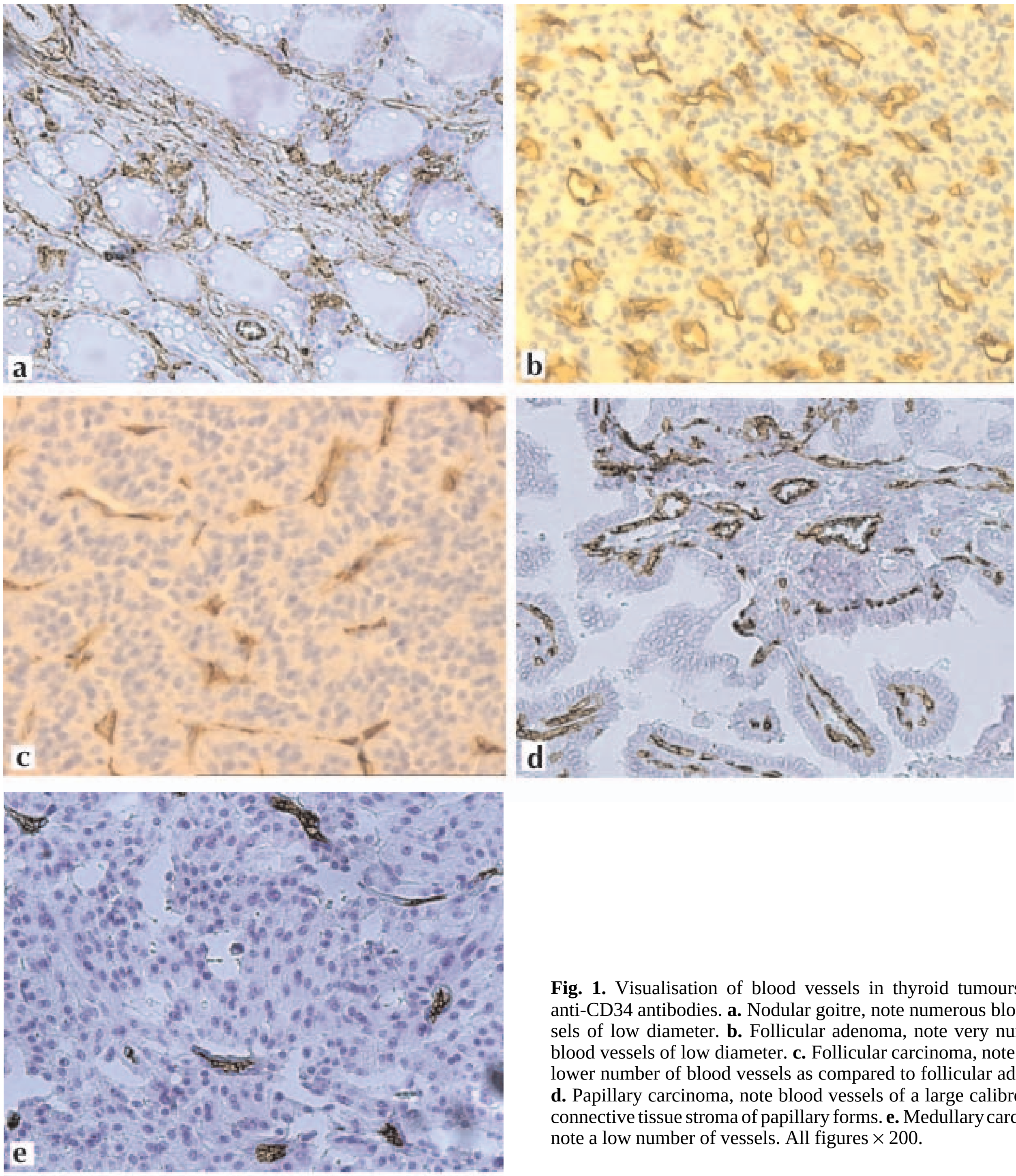

Fig. 1. Visualisation of blood vessels in thyroid tumours using anti-CD34 antibodies. **a.** Nodular goitre, note numerous blood vessels of low diameter. **b.** Follicular adenoma, note very numerous blood vessels of low diameter. **c.** Follicular carcinoma, note clearly lower number of blood vessels as compared to follicular adenoma. **d.** Papillary carcinoma, note blood vessels of a large calibre in the connective tissue stroma of papillary forms. **e.** Medullary carcinoma, note a low number of vessels. All figures × 200.

differences in the size of newly formed vessels in individual nodular lesions of the thyroid.

Discussion

Angiogenic potential of nodular (including neoplastic) lesions of the thyroid has been a topic of studies aimed at, *i .a.*, defining prognosis and at applying appropriate treatment, including anti-angiogenic therapy. In physiological conditions and in normal organs, angiogenesis is insignificant and remains at a stable level, typical for a given tissue. For tumour development, formation of new vessels represents an indispensable process thought to be induced by activation of genes coding for angiogenic factors [4, 10, 15, 17]. The density of vascular network in selected tumours is evaluated by employing immunohistochemical demonstrations of various antigens (VEGF, CD34, CD31 and factor VIII). Their expression visualises vessels in normal tissues, pseudo-neoplastic hypertrophies and in neoplasms [7, 32, 35]. We used anti-CD34 antibody because it requires a simple procedure and is effective in recognizing small-caliber microvessels associated with neovascularisation described as a "vasculogenic mimicry", to emphasize *de novo* generation of blood vessels [9] and is more efficient than other antibodies [7]. A widely employed technique involves scoring of capillaries in the most vascularised fields (hot spots) [7, 32, 34]. Comparability of fields selected for evaluation remains a problem. Thyroid tumours, irrespectively of their histological type, differ also between each other, showing heterogeneity of various components of the neoplastic tissue. For example, the papillary carcinoma of the thyroid may in various sites manifest either papillary or vesicular structure. Heterogeneity of the thyroid neoplasms, application of various antibodies for visualisation of angiogenesis-associated antigens and application of variable parameters of its evaluation may result in divergent results and conclusions [27].

Our studies have shown that number of microvessels demonstrates variability in individual histological types of the lesions. In benign lesions of nodular goitre or follicular adenoma types, the number of vessels exceeded that in malignant tumours (follicular carcinoma, papillary carcinoma, medullary carcinoma). Among malignant tumours, the highest number of microvessels was detected in papillary carcinoma. This might be ascribed to the specific histological structure of the lesion, with a strongly vascularised stalk of the papillary structures, typical for morphology of these tumours. Thyroid papillary carcinomas do not constitute a uniform group in respect to their histological differentiation: low differentiation types can also be distinguished, in which papillary structures are few or absent, which may cause difficulties in evaluation of angiogenesis intensity. In our group of papillary carcinomas, highly mature types have prevailed, with numerous papillary forms and with stalks containing numerous vessels which, in our opinion, has resulted in the observed high vascular density.

We also analysed the area of vascular surface (MVA) which has not shown significant differences between benign and malignant lesions. These data allow to conclude that angiogenesis accompanying benign, hypertrophic lesions involves formation of numerous vessels of a low diameter while in malignant tumours less numerous vessels of a larger diameter dominate. Analysis of the same parameter (MVA) in individual histological types of thyroid lesions has not demonstrated significant differences, either. The area occupied by vessels in individual nodular lesions of the thyroid was similar in spite of differences in the number of the newly formed microvessels. Also in the case of thyroid papillary carcinoma, no significant increase could be demonstrated in the vascular area. The largest and least numerous vessels were observed in follicular and medullary carcinomas.

Reports in which angiogenesis has been studied in thyroid tumours are not numerous. Wong *et al.* [45], employing antibodies against CD34 in 15 tumours of follicular adenoma type and 15 tumours of follicular carcinoma type have noted that the two types cannot be differentiated by comparisons of their vascular surface area. Kumar Dhar *et al.* [29] evaluated angiogenesis in 71 differentiated thyroid carcinomas. They examined 16 cases of follicular carcinoma, 2 cases of papillary carcinoma (follicular variant) and 53 cases of papillary carcinoma. Intensity of angiogenesis was evaluated by reaction with anti-CD34 antibodies. The spread and intensity of the reaction was evaluated by a semi-quantitative approach employing 0 to 3 scale for each of the variables. The obtained results allowed to distinguish two types of thyroid neoplasms: the hypovascular and the hypervascular ones. The latter group included large tumours (over 3 cm in diameter) with infiltration beyond the thyroid. However, the vascular supply to the tumours has not been analysed in relation to the histological type of the tumours. Scarpino *et al.* [38] examined angiogenesis in 31 papillary carcinomas, monitoring expression of von Willebrand factor. They detected a three-fold higher density of vessels in non-neoplastic tissue strictly adjacent to papillary carcinoma. They also stressed a distinct type of vascular supply in papillary carcinomas, resulting from location of the vessels in the form of vascular complexes, resembling glomeruli in connective tissue stroma of papillary structures. The authors suggested that in papillary carcinomas, the vascular supply of the tumour is initiated by extensive vascular spaces with muscle fibres located in the peri-neoplastic fibrous tissue. Similar conclusions were drawn by Akslen *et al.* [2], who studied 128 papillary carcinomas and found that angiogenesis evaluated by intensity of factor VIII expression and by the area of the colour reaction was three-fold higher in papillary carcinomas as compared to the surrounding tissue, not affected by the tumour. They detected also an inverse correlation between the intensity of angiogenesis on one hand and patient age, tumour size, histological grade of the lesion and advancement of the neoplastic process on the other. They found that more pronounced angiogenesis was accompanied by better prognosis. In the Polish literature of the subject we have found just one report related to the density of vascular supply of the thyroid [30]. The authors evaluated angiogenesis in 69 neoplastic and non-neoplastic lesions, examining expression of vascular endothelial growth factor (VEGF). They detected positive immunohistochemical reaction only in a proportion of the lesions. The intensity of the colour reaction, was evaluated semi-quantitatively, and its type: diffuse or focal one was also assessed. The authors detected low intensity of the reaction in small regions of benign nodal lesions (adenoma, nodular goitre) and a strong expression of

VEGF in malignant tumours of the thyroid, except for medullary carcinoma cases.

In our study, similarly to the above discussed experiments with the use of markers permitting evaluation of angiogenesis, application of immunohistochemistry with the use of anti-CD34 antibodies allowed us to demonstrate evident differences in the density of vascular supply between nodular lesions in the thyroid. The density was related to the type of lesion (benign vs. malignant) and to its histological variant.

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