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ARTICLE

Expression of CD34 in Gastric Cancer and its Correlation with Histology, Stage, Proliferation Activity, p53 Expression and Apoptotic Index

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The formation of new blood vessels is essential for tumor growth and progression. Until today there are only few studies of the immunohistochemical assessment of angiogenesis in gastric cancer by the evaluation of the expression of CD34 antigen. The aim of this study was to analyze the relationship between microvessel density (MVD) expressed as the mean count of CD34 immunostained vessels and clinicopathologic features of gastric tumors (the histological type according to the Lauren classification, tumor grade – G; presence of lymph node metastases – N; depth of tumor invasion; stage of disease (UICC-AJCC 1988–1992), p53 expression, tumor cell proliferative activity described as the Ki67 labelling index and apoptotic index of tumor cells – TUNEL method). We assessed formalin-fixed, paraffin-embedded tissue samples obtained during potentially radical gastrectomy from 58 patients with primary gastric adenocarcinoma. The representative tissue blocks from each tumor were used for the immunohistochemical assay and examined by two pathologists independently. MVD was counted in five tumor areas of the most intensive neovascularization (x 200 field by light microscopy) and the mean counts were recorded. The mean MVD (CD34 expression value ± SD) in this study was 43,15 ± 19,8 per x 200 field. The study demonstrated the statistically significant correlation between MVD and two main histological parameters: tumor grading ($p < 0.001$) and tumor histological type according to Lauren's classification ($p<0.05$). In well – and moderately – differentiated tumors (G1/2) MVD was significantly lower in comparison to the group of poorly differentiated cancer – G3 (mean value: 31,62 vs. 49,89). MVD was higher in diffuse type of gastric cancer comparing to intestinal type (50.05 ± 19,03 vs. 39.17 ± 20,09). However, the authors failed to find a significant correlation between MVD and other investigated histopathological features in malignant gastric tumors. The close relationship between CD34 immunostaining, gastric cancer tumor vascularity and main histological parameters was shown in this study. It can be stated that analysis of expression of angiogenesis in gastric cancer may be helpful for better estimation of hematogenous recurrence and the selection of the group of patients for adjuvant antiangiogenic treatment. (Pathology Oncology Research Vol 7, No 2, 129–134, 2001)

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Introduction

Gastric carcinoma is a heterogenous neoplasm, which although diagnosed with high frequency in the world, is still characterized by poor prognosis.[1] Analysis of biological variables can help to understand the cancer and to determine prognosis. Malignant tumor growth and metas-

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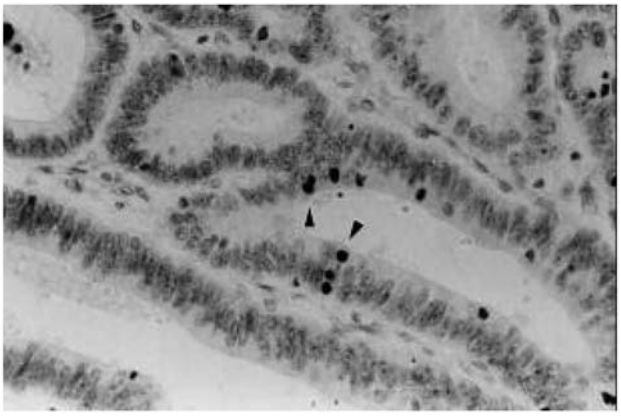

Figure 1. TUNEL staining of apoptotic cells (arrowheads) in intestinal type of gastric cancer (H counterstained, x250).

tasis require persistent new blood vessel growth. Therefore, tumor-associated angiogenesis plays a critical role in development and spread of malignant tumors.[2] It has been demonstrated that intratumoral neovascularization in some types of cancer is a significant prognostic factor e.g. in breast, colorectal or lung neoplasm.[3,4,5]

One of the modern methods of quantitative assessment of angiogenesis is a detection of the expression of CD34 antigen. CD34 is a surface glycoprotein - human hematopoietic progenitor cell antigen, which expression has been also detected in vascular endothelium [6]. The anti-CD34 monoclonal antibody can recognize small-caliber vessels in tumors, so in this study it was used for immunostaining of intratumor endothelial cells.

Only a few inconsistent data exist concerning the association of microvessel density (MVD) as a parameter of tumor angiogenesis and clinicopathological variables in gastric carcinoma. The aim of the study was to estimate the microvascularisation detected with CD34 antibodies in the primary resectable gastric adenocarcinomas and its relationship to clinicopathological and biological features of the tumor. We analyzed the correlation between the MVD and clinicopathologic features of tumors, such as: the histological type of cancer, tumor grade; presence of lymph node metastases; depth of tumor invasion; p53 expression, tumor cell proliferative activity described as the Ki67 immunostaining and apoptosis of tumor cells.

Materials and Methods

Patients and tissue samples

We assessed archival tissue samples obtained during potentially curative surgical treatment from 58 patients with primary gastric cancer, who were underwent operation in Department of Surgical Oncology, Medical University of Lodz, Poland, in the period 1990–1999. There were 15 women and 43 men. The patients ranged in age from 40 to 77 years. All patients had not received preoperative radiotherapy or chemotherapy.

Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin. Histological type was established on haematoxylin/eosin stained sections according to Lauren classification.[7] Then the histological grading (G1-G3 scale), regional lymph nodes status (presence of metastases), depth of tumor invasion (T) and stage of disease (according to UICC-AJCC 1988-1992 classification) was estimated. Among the tumors 22 were diffuse type and 36 intestinal type. The representative tissue blocks from each tumor were used for the immunohistochemical assay.

CD34, Ki-67, p53 and cell apoptosis immunostaining

Immunohistochemical study for CD 34 antigen was performed using the avidin-biotin-peroxidase complex technique on formalin-fixed and paraffin-embedded tumor sections. Then tissue sections after the enzyme digestion were treated with monoclonal anti-CD34 antibody (diluted 1:25; overnight; Novocastra Laboratories, UK).

Immunohistochemical staining for p53 anti-p53 mouse monoclonal antibody (Dako, Copenhagen, Denmark) and monoclonal Ki-67 antibody (Dako, Copenhagen, Denmark) was performed using the avidin-biotin-peroxidase complex method as it has been described previously.[8]

Apoptotic cancer cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method (ApopTaq, Boehringer-Manheim).

For Ki-67, p53 immunostaining and apoptosis analysis all labeled nuclei in tumor specimens were regarded as positive. From each case, 1000 nuclei in areas of the section with the highest labelling rate in high-power view (x400) were examined. Ki-67 labelling index (Ki67 LI) was expressed as a mean percentage of Ki67 positive cells. For p53 immunostaining cases were classified in two groups: positive (+) and negative (–). The apoptotic index (AI) was expressed as average percentage of immunostained tumor cells in five high power fields (x 400; *Figure 1).*

All series included negative controls.

Scoring of MVD

All specimens were examined by two pathologists independently.

For MVD assessment microvessels were counted (x200 field; by light microscopy) in each of the five most vascularized, separately located areas (hot spots) as it was demonstrated by Weidner et al.[9] The hot spots were identified during the scanning the entire section at low magnification (x100) as areas having the highest density of CD-34-positive cells.[10] In ulcerated tumors the luminal areas

with granulation tissue were excluded from analysis by a line drawn arbitrarily across the section.[11] Furthermore the areas of glands in well differentiated tumors were also excluded from analysis based on the criteria giving by Weidner[9] i.e. vessels counted had to be in stroma within the tumor, surrounded by malignant glands. Any stained endothelial cell/cells, which were separated from adjacent microvessels and could not contain the lumen, were identified as a microvessel *(Figure 2)*. MVD was expressed as the mean count of CD34 immunostained vessel in each case (CD34 index).

Statistical analysis

The statistical analysis was performed using Mann-Whitney U test (Statistica; Statsoft). A value of $p<0.05$ was considered statistically significant.

Results

We investigated vascular count in 58 postoperative specimens. In *Table 1* there is shown the detailed data of 58 tumors and 58 patients. The mean microvessel density (CD34 expression value ± SD) in this study was 43,15 ± 19,8 per x200 field.

This study revealed the close relationship between tumor vascularity and two main histological parameters: tumor type according to Lauren's classification ($p<0.05$) and tumor grade ($p<0.001$) (*Table 2*). In well and moderately differentiated tumors MVD was significantly lower in comparison to the group of poorly differentiated cancers G3 (mean value: 31,62 vs. 49,89) ($p<0.001$). MVD was higher in the diffuse type of gastric cancer in comparison with the intestinal type (50.05 ± 19,03 vs. 39.17 ± 20,09) ($p<0.05$).

There was no significant correlation between CD34 value and sex, lymph node metastasis, depth of tumor invasion (T) and stage of disease. We also failed to find a significant correlation between p53 staining and MVD.

To evaluate the relationship between proliferative activity of cancer described as the Ki-67 labeling index and CD 34 tumors were divided into the two groups according to the mean Ki67 LI (29,98%). We did not find any significant differences in MVD between high and low Ki-67 LI groups.

The similar division was made for evaluation of correlation between AI and CD34 index – the cut off point was based on the mean value of AI (5.28%). We did not observe any significant correlation between AIs and MVD.

Table 1. **Patient characteristics**

Male/Female	15/43
Age, years (mean +/–SD)	59,21 +/– 9,9
Histologic type (intestinal/diffuse)	36/22
Lymph nodes involvement (absent/present)	19/39
Tumor depth	
T1,2	20
T3,4	38
Tumor grading	
G1/2	21
G3	37
MVD = CD34 expression (mean +/– SD)	43,15 +/– 19,8
AI (mean% +/– SD)	5,28% +/– 4,34%
Ki67 LI (mean +/– SD)	29,98 +/– 20,17
P53 expression	
absent	32
present	26

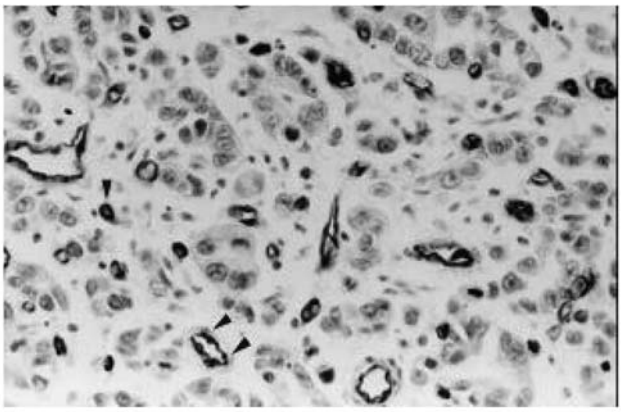

Figure 2. *Detection of endothelial cells by anti-CD 34 antibody in intestinal type of gastric cancer: numerous blood vessels with well defined lumen (double arrowheads) and microvessels (arrowhead) are seen (H counterstained, x 400).*

Discussion

One of the essential factors, on which malignant tumor progression depends, is the induction of a microcirculation from the surrounding environment.

In this study the formation of microvessels in gastric adenocarcinoma was detected by using the CD34 immunostaining method to visualize the endothelial cells. It is documented that CD34 immunostaining is a reproducible, reliable method of assessment of cancer neovascularization and comparable to other techniques as anti-CD31 or anti-Factor VIII-related antigen immunostaining.[3] CD 34 identifies the small caliber microvessels more efficiently than vWF.[12] There is a strong correlation between CD34 expression in gastric cancer and levels of the angiogenic factor IL-8.[13] In esophageal cancer[14] and

gastric cancer[15,16] it has also been shown that expression of another relevant angiogenic-regulating factor, VEGF (vascular endothelial growth factor) is associated with MVD.

In this study there was a significant correlation between MVD analyzed by the CD34 method and histological tumor type. It is well known that patients with gastric cancer of diffuse type are characterized by much worse prognosis, so we can probably conclude that analysis of angiogenesis may be helpful for better estimation of individual survival and selection the group of patients with high risk of recurrence. Our results suggest that more intensive angiogenesis in diffuse type of gastric adenocarcinoma could be important factor for higher metastatic potential of this type of tumors in comparison to intestinal type gastric adenonocarcinomas. It has been proved that MVD is related to clinical outcomes in some types of malignant tumors. Intratumoral vessel density has been reported as a prognostic factor in breast,[3] lung,[4] ovarian cancer[17] and colorectal cancer.[18] These results are consistent with the suggestions about the role of angiogenesis in tumor development process. It is proposed that these small, leaky vessels may allow the tumor cells to reach the blood circulation and increase the probability of metastases.

Table 2. **Microvessel density (CD34 expression value) and clinicopathological parameters in gastric cancer**

Pathological parameter	*CD 34 value +/- SD*	*P statistical significance*	*Number of patients*
Gender			
Male	42,16 +/-18,46	N.S.	43
Female	46,21 +/-23,98		15
Lauren's type			
Intestinal	39,17 ± 20,09	P<0.05	36
Diffuse	50,05 ± 19,03		22
P53 status			
Negative p53	49,74 ± 24,9	N.S.	32
Positive p53	38,51 ± 16,8		26
Depth of tumor invasion			
T1,2	40,21 ± 18,44	N.S.	20
T3,4	44,75 ± 20,92		38
Lymph nodes metastases			
Absent N0 (-)	40,11 ± 20,0	N.S.	19
Present N1/2 (+)	44,55 ± 20,18		39
Stage			
I, II	37,83 ± 15,9	N.S.	24
III, IV	47,03 ± 21,9		34
Histological grading			
G1/2 (well- and moderately-differentiated)	31,62 ± 14,04	P<0.001	21
G3 (poorly differentiated)	49,89 ± 19,71		37
Apoptosis			
Low < 5,28%	45,3 ± 17,55	N.S.	31
High > 5,28%	40,67 ± 25,67		17
Ki 67 LI			
Low	38,92 ± 18,85	N.S.	19
High	47,41 ± 24,45		29

N.S. - not significant

In our study we also observed strong positive correlation between angiogenesis in gastric malignant tumors measured by CD34 antigen expression and histological grade. The prognostic impact of histological grading in gastric cancer is established.[19,20] Differences in vascularization between well and poorly differentiated tumors might reflect the stromal reaction, interaction of the tumor cells with environments (matrix components, enzymes, growth factors), moreover depends upon a balance between positive and negative angiogenesis regulators.[21] The process and interaction between tumor cells, endothelial cells and stroma during tumor progression is very dynamic and determined the tumor growth. At the later stages of tumor progression the balance of angiogenesis stimulators and inhibitors produced by tumors stroma „switched" to stimulation of blood vessels formation and tumor cell growth.

Among various growth factors, vascular endothelial growth factor (VEGF),[22,23] platelet-derived endothelial cell growth factor -identical to thymidine phosphorylase (PD-ECGF),[24] basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and metaloproteinases (MMPS) are considered as the most important.[25] In gastric carcinoma microvessels density was significantly higher in tumors VEGF and PD-ECGF-positive, than in tumors that were both VEGF and PD-ECGF negative.[26] On the other hand, expression of VEGF was more frequently found in well differentiated gastric carcinoma.[15] Tumors with strong VEGF staining had significantly higher vascularity than those with weak VEGF.[15] That was suggested that in poorly differentiated colorectal tumors elevated tissue pressure in the center of tumors causes compression of their lumens and underestimation of the microvessels density.[27]

The vessels-containing stroma is regarded to originate from myofibroblasts, smooth muscle cells,[28] pericriptal fibroblasts and other specialized mesenchymal cells with myoid features. Those cells are CD34 negative and α smooth muscle actin (ASMA) positive.[29] In contrast, the most of the stromal cells (dendritic interstitial cells) in the normal colorectal submucosa, muscularis propria, subserosa, perirectal tissue and colorectal adenomas were positive for CD34 and negative for ASMA, so the lack of CD 34 expression in stromal cells is associated with desmoplastic reaction in tumors.[29] CD34 plays a role in

the maturation and proliferation of adjacent mesenchymal and epithelial stem cells along with immune mediated response.

To evaluate the possible impact of microenvironment in tumor, proliferative activity and apoptosis were investigated in this study. Apoptosis is a programmed cell death which contributes to the homeostasis of organisms and is essential for controlling cell proliferation. The study did not demonstrate a statistically significant correlation between MVD and apoptotic index. This last observation is not consistent with recent studies of Keguchi et al,[30] which have indicated an inverse correlation between the apoptotic index and intratumoral MVD. In the low AI group CD34 index was slightly increased in comparison to the high AI group (45,3 vs 40,67). Recent studies have shown that apoptotic regulation of tumor progression can be enhanced by low neovascuarization.[31] We also failed to find a significant correlation between MVD and tumor proliferative activity measured by Ki-67 labelling index. This may be the consequence of heterogeneity of proliferative activity in gastric neoplasia as was reported previously.[32]

It has been shown that the gene for p53 may act as tumor suppressor gene and play an important role in the regulation of cell-cycle control and tumorigenesis.[33] However the role of expression of p53 protein in gastric cancer needs further investigation. In our study we did not reveal any relationship between MVD and p53 expression.

In addition this study has not demonstrated a statistically significant correlation between lymph node involvement and microvascularization as was shown in breast cancer, for example.[9] The reason for this es be that our study was performed on rather small number of specimens, but also the fact that CD34 expression as an indicator of neovascularization correlates instead with hematogenous metastases.[12,34,35]

Immunohistochemical techniques are reproducible, objective and available in most hospitals. Although an immunohistochemical evaluation of MVD with CD34 antibodies in gastric cancer does not assess the mechanism of angiogenesis, it may help in estimating of probability of hematogenous metastasis. The identification of angiogenesis in gastric carcinoma may also help predict responses to experimental adjuvant antiangiogenic therapy.

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