J. Exp. Clin. Cancer Res. 26,3, 2007

Evaluation of Angiogenesis with the Expression of VEGF and CD34 in Human Non-Small Cell Lung Cancer

A.M. Inda, L.B. Andrini, M.N. García, A.L. García, A. Fernández Blanco, C.C. Furnus, S.M. Galletti, G.D. Prat, and A.L. Errecalde

Cátedra de Citología, Histología y Embriología "A" . Facultad de Ciencias Médicas. UNLP, La Plata - Argentina

Angiogenesis is an essential process in the progression of malignant tumors and the most potent angiogenic factor is the vascular endothelial growth factor (VEGF). On the other hand, the CD34 is an endothelial antigen that has been used to highlight the microvasculature vessel density (MVD) as a direct marker of the degree of neoangiogenesis. In the present study we report the VEGF expression and its relationship with MVD, measured by CD34, in two lineages of non-small cell lung cancer (NSCL): low differentiated adenocarcinomas and epidermoid carcinomas, in order to consider the possibility of using the correlation between both antibodies as a prognostic factor. Tumor sections were stained by immunohistochemistry for CD34 and VEGF. The results showed that the mean value of VEGF for adenocarcinoma was significantly higher than the one for epidermoid carcinoma ($p < 0.001$). However, the mean of MVD did not show significant differences between both types of tumors. The conventional factors taken into consideration (age over 60, sex, and presence of lymph nodes) was not significantly related to the angiogenic factors examined. In conclusion, we could affirm that CD34 is a better prognostic marker of neoangiogenesis in NSCLC, because both types of tumors have the same clinical prognosis, and so we expected the same behaviour from both markers.

Key Words: Lung cancer, VEGF, CD34, Angiogenesis, Epidermoid carcinoma, Adenocarcinoma

Angiogenesis is an essential process in the progression of malignant tumors due to the fact that solid tumors cannot grow beyond 1-2 mm in diameter without neovascularization (1). Various growth factors have proved to stimulate angiogenesis, including the fibroblast growth factor (FGF), the transforming growth factor alpha (TGFα), the plateled-derived growth factor (PDGF) and the vascular endothelial growth factor (VEGF) (2) which is the most potent angiogenic factor, a glycoprotein with angiogenic, mitogenic, and vascular permeability enhancing activity in endothelial cells (3). Moreover several studies have reported that the potential of VEGF signal-pathway inhibitors as anticancer agent in non-small cell lung cancer (NSCLC) treatment disturbs growth and migration of the cancer cells (4,5).

In 1991 Weidner *et al.* (6) showed that assessing tumor microvessel density (MVD) could be useful in evaluating the aggressiveness of breast carcinoma. Since then similar observations have been found for other types of malignancies including NSCLC. However, several studies have failed to show MVD as a significant prognostic factor (7,8).

The CD34 is an endothelial antigen that has been used to highlight the MVD as a direct marker of the degree of neoangiogenesis; however, it can react with not only "newly forming" vessels but also with normal vessels just trapped within a tumor tissues (9)

In the present study we report the VEGF expression and its relationship with MVD in two NSCLC lineages: adenocarcinomas and epidermoid carcinomas with low differentiation, using immunohistochemistry with anti VEGF and anti CD34, in order to consider the possibility of using the correlation between both antibodies as a prognostic factor for patients' survival.

Materials and Methods

Tumor

Tumor specimens from 72 patients with previously untreated epidermoid lung carcinoma and adenocarcinoma with low differentiation in stage I and II surgically treated at the Hospital Interzonal Especializado de Agudos y Crónicos San Juan de Dios, La Plata, were analyzed for VEGF expression and microvessel densi-

ty. The histological classification of tumors was based on the guidelines of the World Health Organization (1981). The mean age of the patients was 56.5 years (range 49-67), 59 male and 13 female.

Determination of VEGF and CD34 Expression

Desparaffinized and rehydrated sections were microwaved for 10 min. in buffer citrate, PH 6. Endogenous peroxidase was blocked for 20 min. The primary antibody against VEGF-C1 (mouse monoclonal antibody; Santa Cruz Biotechnology, California, USA 1:100 dilution) and a mouse monoclonal antibody against CD34 protein (Santa Cruz, Biotechnology, California, USA 1:80 dilution) were incubated for 60 min. Bound primary antibody was detected by Envision Systen (Dako) for 30 min. and the reaction was developed using diaminobenzidine, and counter staining with Mayer hematoxylin. The positive control was a section of lung tumor that had shown to have a high VEGF content previously, by immunohistochemistry. VEGF staining was seen in the tumor cell cytoplasm.

The expression of VEGF was assessed acording to the percentage of immunoreactive cells in a total of 1000 neoplastic cells (quantitative analysis). There was > 95% agreement between the two observers for the VEGF evaluation. A final score was determined by consensus after re-examination. MVD was assessed using the criteria of Weidner *et al.* (6). The areas of highest vascularization were identified as regions of invasive carcinoma with the highest numbers of discrete microvessels stained for CD34. Any brown stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumor cells and other connective tissue elements was considered a single, countable microvessel. Each count was expressed as the highest number of microvessel identified within a 0.3 mm^2 fields at a magnification of X 200. Ten fields of the most intense vascularization (hotspot) were analyzed for each tumor. Two investigators performed all counts simultaneously, both had to agree on what constituted a single microvessel before a vessel was included in the count.

Statistical Analysis

The statistical analysis of VEGF expression was performed using Student Test, because the distribution of samples was normal. The marked tumoral cells were recorded by counting 50 areas, and the total nuclei every 10 areas. The results were expressed as a percentage of marked cells.

The CD34 expression was analyzed by Student Test and was expressed as X ± SE of the total of marked vessels in the selected areas for every tumor.

Results

Table I: summarizes all the clinical data about the patients enrolled in this study.

Table II: shows the immunohistochemical expression of the angiogenic factors evaluated. High expression of VEGF was seen in 28 patients (82%) with adenocarcinoma and in 9 (24%) with epidermoid carcinoma, and a high expression of MVD as assessed by CD34, was seen in 18 patiens (53%) with adenocarcinoma and in 22 (58%) with epidermoid carcinoma.

Table III: shows the mean of immunohistochemical parameters of angiogenic factors and the conventional risk factors taken into account in this study, like age, sex, and the presence of positive lymph nodes. In this table we could also observe that VEGF expression in adenocarcinoma was significantly higher than in epidermoid carcinoma ($p< 0.001$), but the MDV measured by CD34 expression did not show any significant difference between both tumor types.

At the end of our study two patients died, a high expression of CD34 was observed in both, but only one had a high expression of VEGF. Both of them, evidenced the presence of positive lymph nodes, and they were smokers.

Discussion

Angiogenesis is a complex process that involves endothelial cell migration, capillary bredding, neovascular remodelling, in addition to endothelial cell proliferation. The growth of solid tumors like those analized in this study, needs an adequate vascular network for the supply of oxygen and nutrients, and in order to remove waste products, although it has been established that tumor cell proliferation decreases with increasing distances from the blood vessels (10).

Ushijima *et al.* (11) have demonstrated that high MVD is associated with the advancement of NSCLC, and this was particularly evident in conjunction with high VEGF expression. On the contrary, in our study we found a higher VEGF-C expression in adenocarcinomas than in epidermoid lung carcinoma, even though the microvessel density, marked with CD34 expression, did not show differences between tumors. These observations are in agreement with Ghio P. *et al.* (12) but not with Nakashima T. *et al.* who found that

Table I - Study population features divided according to tumoral type

Conventional risk factors	Total (n)		Percentage (%)	
	Adeno	Epid.	Adeno	Epid.
Sex				
- Male	29	30	76.3	88.2
- Female	9	4	23.7	11.8
Smoker				
- No	4	5	10.5	14.7
- Yes	34	29	89.5	85.3
Surgical procedures				
- lobectomy	30	32	78.9	94.1
- pneumonectomy	8	2	21.1	5.9
Grading				
- G1	10	4	26.3	11.8
- G2	28	30	73.7	88.2
Age				
≤55	5	2	13.2	5.9
56 to 65	26	27	68.4	79.4
≥65	7	5	18.4	14.7

(n): Number of patients
Adeno.: Adenocarcinoma
Epid.: Epidermpoid carcinoma

Table II - Number of patients with NSCLC and their inmunohistochemical parameters

	VEGF		CD34	
	Adeno	Epid.	Adeno	Epid.
Strong	28/34	9/38	18/34	22/38
Low	6/34	29/38	16/34	16/38

Adeno.: Adenocarcinoma
Epid.: Epidermpoid carcinoma
The values were expressed as number of patients /total number of patients.

Table III - Immunohistochemical parameters of angiogenic factors and conventional risk factors

	Adenocarcinoma	Epidermoid	p <
(n)	34	38	
VEGF (x)	44 ± 6.1	1.8 ± 0.5	0.001
CD34 (x)	5.1 ± 1.2	6.3 ± 0.8	ns
Age more than 60	12	27	
Male	29	30	
Female	5	8	
Lymph nodes +	18	20	
Lymph nodes -	16	18	

P: probability
(n): number of patients
(x): mean ± SD

VEGF-C expression is one of the significant prognostic factors in patients with squamous cell carcinomas (13). Our results as for low differentiated lung carcinomas may be consistent with other studies (14,15) and carcinoma of oesophagus (16), who found no correlation of microvessel density with tumor cell proliferation.

Interestingly, VEGF-C expression is higher in adenocarcinomas than in epidermoid carcinoma, while the MVD and the positive presence of lymph nodes are similar in both tumors´ lineages. This can be due to the fact that the CD34 is more strictly related to the metastatic process than to neoangiogenesis itself. Yano *et al.* have recently demonstrated a higher incidence of

distant metastases and shorter survival in patients with high grade of MVD as assessed by CD34 (17).

All the patients were operated last year (2006), so we can affirm that the survival was high, because only two patients died, but we assume that more time will be necessary to reach a real conclusion.

The main limitation of our study is the small sample size: we chose only untreated patients with stages I and II of both tumoral lineages with low differentiation. Even though previous works assumed that VEGF and CD34 are excellent markers of neoangiogenesis in NSCLC (2-4,17-19), on the basis of our preliminary results, we could affirm that CD34 is a better prognostic marker of neoangiogenesis in NSCLC, because both types of tumors have the same clinical prognosis, and so we expected the same behaviour from both markers.

Acknowledgements. The Authors wish to thank Maite Roji Gutierrez and Javiera Marini for their technical assistance.

References

1. Folkman J., Watson K., Ingiber D., Hanahan D.: Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature (Lond). 339: 58-61, 1981.
2. Mattern J., Koomägi R., Volm M.: Association of VEGF expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. British Journal of Cancer. 73: 931-4, 1996.
3. Mineo T., Ambrogi V., Baldi A., et al.: Prognostic impact of VEGF, CD31, CD34 and CD105 expression and tumour vessel invasion after radical surgery for IB-II A non-small cell lung cancer. J. Clin. Pathol. 57: 591-7, 2004.
4. Tanno S., Ohsaki Y., Nakanishi K., Toyoshima E., Kikuchi K.: Human small cell lung cancer cells express functional VEGF receptors, VEGFR-2 and VEGFR-3. Lung Cancer. 46: 11-9, 2004.
5. Shepherd F., Sridhar S.: Angiogenesis inhibitors under study for the treatment of lung cancer. Lung Cancer. 41: 63-72, 2003.
6. Weidner N., Semple J., Welch W., Folkman J.: Tumor angiogenesis and metastasis correlation and invasive breast carcinoma. N. Engl. J. Med. 324: 1-8, 1991.
7. Chandrachud L., Pendleton N., Chisholm D., Horan M., Schor A.: Relationship between vascularity, age and survival in non-small cell lung cancer. Br. J. Cancer. 76: 1367-75, 1997.
8. Pastorino V., Andreola S., Tagliabue E., et al.: Immmunocytochemical markers in stage I lung cancer: Relevance to prognosis. J. Clin. Oncol. 15: 2858-65, 1997.
9. Vermeulen P., Gasparini G., Fox P., et al.: Quantification of angiogenesis in solid human tumors: an international consensus on methodology and criteria of evaluation. Eur. J. Cancer. 32 A: 2474-84, 1996.
10. Tannock I.F.: The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumor. Br. J. Cancer. 22: 258-73, 1968.
11. Ushijima C.H., Tsukamoto S., Yamazaki K., Yoshino I., Sugio K., Sugimachi K.: High vascularity in the peripheral region of non-small cell lung cancer tissue is associated with tumor progression. Lung Cancer. 34: 233-41, 2001.
12. Ghio P., Cappia S., Selvaggi G., et al.: Prognostic role of protease-activated receptors 1 and 4 in resected stage IB non-small-cells lung cancer. Clin. Lung Cancer. 7: 395-400, 2006.
13. Nakashima T., Huang C.L., Liu D. et al. : Expression of vascular endothelial growth factor - A and vascular endothelial growth factor - C as prognostic factors for non-small cell lung cancer. Med. Sci. Monit. 10: 157-65, 2004.
14. Fox S., Gatter K., Bicknell R., et al.: Relationship of endothelial cell proliferation of tumor vascularity in human breast cancer. Cancer Res. 53: 4161-63, 1993.
15. Vartanian R.K., Weidner N.: Correlation of intratumoral endothelial cell proliferation with microvessel density tumor angiogenesis and tumor cell proliferation in breast carcinoma. Am. J. Pathol. 144: 1188-94, 1994.
16. Porschen R., Classen S., Pointek M., Borchard F.: Vascularization of carcinomas of the esophagus and its correlation with tumor proliferation. Cancer Res. 54: 587-91, 1994.
17. Yano T., Tanikawa S., Fujie T.: Vascular endothelial growth factor expression and neovascularization in non-small cell lung cancer. Eur. J. Cancer. 36: 601-9, 2000.
18. Mineo T.C., Ambrogi V., Baldi A., et al.: Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB - IIA non-small cell lung cancer. J. Clin. Pathol. 6: 591-7, 2004.
19. Kojima H., Shijubo N., Yamada G., et al.: Clinical significance of vascular endothelial growth factor - C and vascular endotelial growth factor receptor 3 in patients with T1 lung adenocarcinoma. Cancer 8: 1668-77, 2005.

Received: January 19, 2007

Accepted after revision: April 2, 2007

Dra. Ana M. Inda
Cátedra "A" de Citología, Histología y Embriología
Facultad de Ciencias Médicas
Calle 60 y 120 1900) La Plata - Argentina
Telephone (54) 221 483 5524 - Fax (54) 221 425 8989
E-mail: aminda@med.unlp.edu.ar