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Original Paper

Vessel Counts and Vascular Endothelial Growth Factor Expression in Pancreatic Adenocarcinoma

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Angiogenesis is essential for growth and metastasis of solid malignancies. In several tumours, tumour vessel count and expression of vascular endothelial growth factor (VEGF), a potent angiogenic factor, have been associated with prognosis. To determine if vessel count and VEGF expression are prognostic factors in pancreatic cancer, we examined these parameters in resected tumour specimens from 22 patients who did not receive pre-operative therapy. Paraffin-embedded tumour specimens were immunohistochemically stained for factor VIII (surrogate for vessels) and VEGF. Vessel counts and VEGF expression were evaluated without knowledge of patient outcome. The median follow-up for the entire group had not been reached as of 23.1 months (range 10–69 months). The mean vessel count and VEGF expression were no different between those patients who had recurrences and those who did not. By linear regression analysis, the correlation of VEGF expression with vessel count did not reach statistical significance ($P = 0.0685$). Survival and time to recurrence were similar in patients with high and low vessel counts and VEGF expression of 1, 2 or 3. Tumour differentiation or lymph node positivity had no effect on either VEGF expression or vessel count. Our data suggest that, in contrast to findings in other solid malignancies, vessel count and VEGF expression are not predictors of survival or recurrence in patients with resectable adenocarcinoma of the pancreas.

Key words: pancreatic cancer, angiogenesis, metastasis, vascular endothelial growth factor, recurrence

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INTRODUCTION

RECENT ADVANCES in the multimodality management of localised, potentially resectable pancreatic cancers have altered patterns of treatment failure, with a significant decrease in local-regional tumour recurrence [1]. However, this has translated into only a modest improvement in survival duration because the majority of patients develop liver metastases [1, 2].

Angiogenesis is essential for tumour growth and metastasis [3]. Increased vascularity may allow both an increase in tumour growth and a greater chance for haematogenous metastases [4]. Weidner and colleagues [5] showed a correlation between the incidence of metastases and the number of microvessels in invasive breast carcinomas. Similar studies have confirmed this finding in other malignancies, including melanoma and cancer of the lung [6], prostate [7], cervix [8] and colon [9].

Angiogenesis is not a passive process: it is driven by the production of tumour and/or host derived angiogenic factors [3]. One such factor is vascular endothelial growth factor (VEGF), a very potent and selective endothelial cell mitogen. VEGF has been shown to be associated with tumour progression and metastasis in other gastrointestinal malignancies [9, 10]. The following investigation was therefore carried out (1) to determine if vessel density and VEGF expression are useful prognostic markers in human pancreatic cancer; and (2) to determine if an association exists between VEGF expression and vessel counts in pancreatic adenocarcinomas.

PATIENTS AND METHODS

Patients

From 1990 to 1995, 85 patients at our institution underwent pancreaticoduodenectomy for adenocarcinoma of the

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pancreatic head. 22 of these patients underwent pancreatic resection prior to receiving either radiation or chemotherapy. Patients did not receive neoadjuvant therapy if a tissue diagnosis could not be obtained prior to treatment. Surgical specimens from these patients, confirmed as having adenocarcinoma of pancreatic origin, were examined in this report. Patient data, including pathological data (except for VEGF expression and vessel counts), were entered into a database at the time of treatment.

Immunohistochemical staining

Consecutive 4 μm sections were cut from each paraffin-embedded study block. Sections were immunostained for VEGF and factor VIII (specific for endothelial cells). Immunohistochemical staining was performed by the immunoperoxidase technique following predigestion and trypsinisation. Antibodies used were a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.) at a 1:200 dilution for VEGF and a rabbit polyclonal antibody (Dako Co., Carpinteria, California, U.S.A.) at a 1:250 dilution for factor VIII. To determine the specificity of the antibody, we tested the antibody against two proteins known to have significant homology: placenta growth factor (> 50% homology) and platelet derived growth factor (approximately 20% homology). By Western blot analysis, the antibody for VEGF detected only VEGF and there was no cross-reactivity with placenta growth factor or platelet derived growth factor (data not shown) [10].

For positive controls, tissue from a colon cancer known to express VEGF was stained for VEGF, and umbilical vein tissue was stained for factor VIII. Negative controls were performed using non-specific IgG as the primary antibody.

Evaluation of vessel count and VEGF expression

Vessel count and VEGF expression were evaluated by one investigator (YT) without knowledge of patient outcome. Vessel count was assessed by light microscopy in areas of the tumour containing the highest numbers of capillaries and small venules at the invasive edge. The highly vascular areas were identified by scanning tumour sections at low power (40× and 100×). After the area of highest neovascularisation was identified, a vessel count was performed on a 200× field (20× objective and 10× ocular, 0.739 mm^2 per field). As Weidner and colleagues described [5], vessel lumens were not necessary for a structure to be defined as a vessel.

The intensity of staining for VEGF was evaluated in tumour epithelium. The area of the tumour with the highest staining intensity was graded on a scale of 0–3, with 0 representing no detectable stain and 3+ representing the strongest stain. This method of evaluation has previously been validated in other studies in our laboratory [10].

Statistical analysis

Differences in vessel count and in mean intensity of VEGF staining among groups were analysed by Student's *t*-test. Differences in vessel counts between tumours with varying levels of VEGF expression were examined by ANOVA. The correlation between vessel count and VEGF expression was carried out using linear regression analysis. The above statistical analyses were performed using InStat statistical software (GraphPad Software, San Diego, California, U.S.A.). Survival data were evaluated by log-rank test using Statistica software (StatSoft, Tulsa, Oklahoma, U.S.A.). All differences were deemed significant at the 95% confidence interval.

RESULTS

Follow-up

Patient follow-up for all patients ranged from 10–69 months (median not yet reached at 23.1 months). The minimum follow-up for the patients who did not have recurrences was 17 months. 11 of 22 patients had recurrences within the study period. The median survival in the patients who had recurrences was 15 months. 16 patients received postoperative chemotherapy and radiation therapy (9 who had had recurrences and 7 who had not). Prolonged recovery or refusal of therapy was the reason why patients did not receive postoperative therapy.

Vessel count and recurrence

The mean vessel count (per 0.739 mm^2) for the entire group was 44.9 ± 5.6 (SEM). The mean vessel count in the tumour specimens of patients who suffered a recurrence was 40.4 ± 8.8, which was not statistically significantly different from that in patients who remained disease free (49.5 ± 7.0; $P = 0.4284$).

VEGF expression and recurrence

VEGF immunoreactivity was present in all the tumours studied. The mean VEGF expression for the entire group was 2.1 ± 0.2. The mean VEGF expression in the tumour specimens of patients who had recurrences was 2.4 ± 0.2. The mean VEGF expression in the subgroup who developed liver metastasis was 2.5 ± 0.3. The mean VEGF expression in the patients who did not have recurrences was 1.8 ± 0.2, which was not statistically significantly different from that in the group of patients who experienced recurrences (($P = 0.1330$), Mann–Whitney *U* test).

Vessel count, VEGF expression and survival

Figure 1 demonstrates survival of patients with relatively high and low pancreatic cancer vessel counts. The breakpoint of 41 vessels / 0.739 mm^2 was utilised to delineate high and

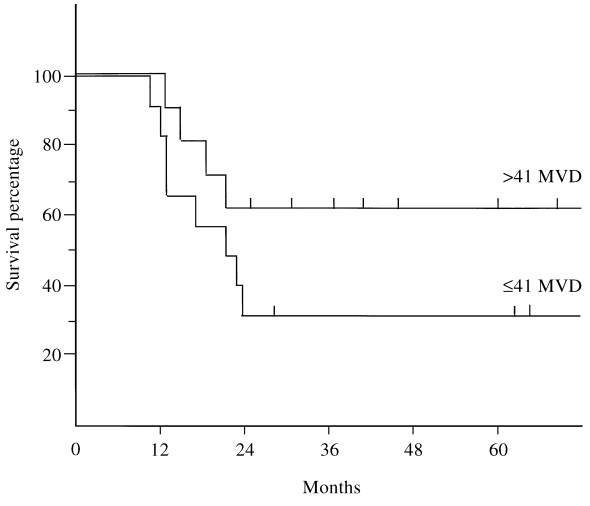

Figure 1. Survival of patients as a function of relatively high and low pancreatic tumour microvessel density (count) (MVD). Patients with a high MVD had a survival similar to that of patients with a low MVD ($P = 0.1296$).

low vessel counts, as this was the median vessel count for the entire group of patients. Patients with a high vessel count had a survival rate and duration similar to that of patients with a low vessel count ($P = 0.1296$). Figure 2 demonstrates survival of patients with pancreatic tumour VEGF expression of 1, 2 or 3. VEGF expression did not impact on survival of patients with pancreatic cancer ($P = 0.5757$).

VEGF expression and vessel counts

The mean tumour vessel counts for the patients with VEGF expression of 1+, 2+ and 3+ were 34.0 ± 6.5, 39.3 ± 6.4 and 58.8 ± 12.2, respectively. By ANOVA, these values were not significantly different ($P = 0.1623$). By linear regression analysis, VEGF did not correlate with vessel count, although this relationship approached statistical significance ($P = 0.1317$) (Figure 3).

VEGF expression, vessel counts and other pathological factors

Tumour differentiation (well, moderate or poor) or lymph node positivity had no effect on either VEGF expression or vessel count ($P > 0.05$). Likewise, there was no correlation between vessel count or VEGF expression and tumour size.

DISCUSSION

Angiogenesis is essential for tumour progression and metastasis. Studies of angiogenic indices in several solid malignancies have clearly demonstrated that vessel count may provide a means of predicting distant recurrence. *In vitro* and *in vivo* experimental studies have also demonstrated that increased angiogenesis secondary to an increase in angiogenic factor expression increases primary tumour growth and metastasis formation. Conversely, mechanisms that down-regulate angiogenic factor expression, and in turn tumour neovascularisation, decrease primary and metastatic tumour growth [11, 12]. The factor most commonly associated with tumour angiogenesis is VEGF. In human colon, lung and breast cancers, the expression of a high level of this factor has been associated with increased vessel counts in resected tumour specimens [9, 13, 14].

Pancreatic adenocarcinoma is an aggressive disease characterised by a high frequency of local recurrence and distant metastasis. Therefore, we examined whether vessel count or VEGF expression could be used to predict tumour recurrence and patient survival. In addition, we sought to determine if an association exists between VEGF expression and vessel count in pancreatic cancer. In the patients studied, we found that neither vessel count nor VEGF expression predicted survival. There was a trend towards an association between VEGF expression and vessel count, but this did not reach statistical significance. However, this lack of correlation could be due to the small size of the patient population. Our findings are generally in agreement with those of Itakura and colleagues [15], who found that VEGF was detectable in all pancreatic carcinoma cell lines studied. VEGF mRNA expression in the cancer tissue was 4.3 times higher than in normal pancreas tissue and 64% of the cancers were immunoreactive for VEGF. However, as in our study, VEGF mRNA expression did not correlate with survival [15]. It is unlikely that any one factor is the causal angiogenic agent for any tumour type. It is more likely that each tumour has a specific angiogenic index, with a unique profile of endogenous angiogenic and anti-angiogenic agents. Thus, VEGF may not be the dominant angiogenic factor in pancreatic cancer as it appears to be in colon cancer [9].

Several recent studies have questioned the previously held belief that vessel count accurately predicts metastasis formation [16, 17]. In pancreatic adenocarcinoma, we did not find vessel count or VEGF expression to be predictive of tumour recurrence or patient survival. At least two possible explanations exist for the failure of neovascularity and VEGF expression to predict tumour recurrence in pancreatic cancer. First, it is possible that the development of metastases is less angiogenesis dependent in some tumour types. For example, we have shown that in intestinal-type gastric cancer (which is often associated with large liver metastases), vessel counts correlate with stage of disease and metastasis formation [10, 18]. In contrast, vessel counts in diffuse-type gastric cancer (which is most commonly associated with multiple

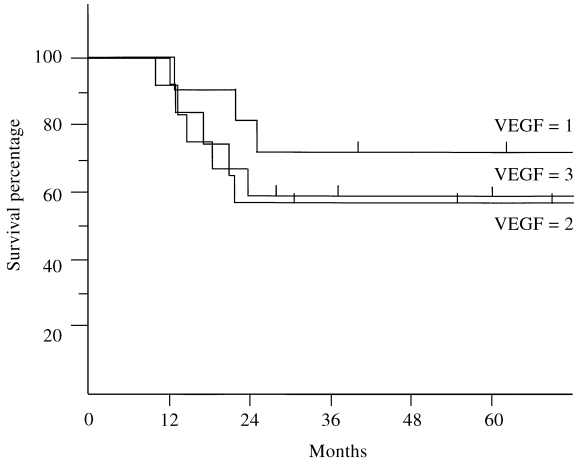

Figure 2. Survival of patients as a function of pancreatic cancer VEGF expression (levels 1, 2 and 3). Expression of VEGF in pancreatic carcinoma had no impact on survival ($P = 0.5757$).

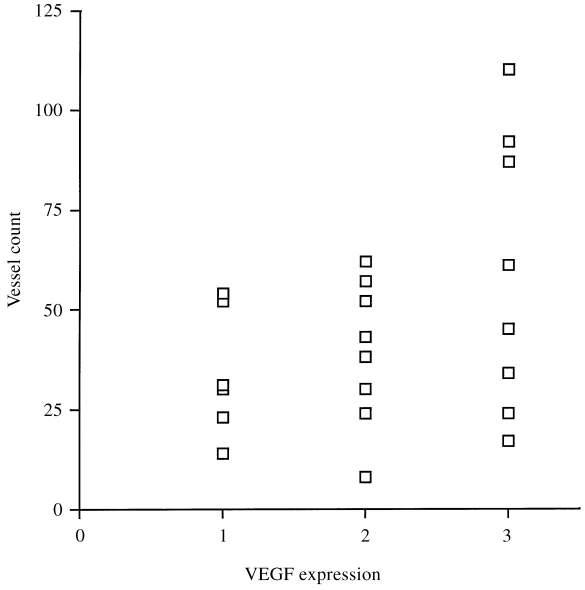

Figure 3. Vessel count as a function of VEGF expression. There was no statistical correlation between vessel count and VEGF expression in pancreatic cancer ($P = 0.1317$, Spearman $R = 0.3315$).

small peritoneal metastases) do not correlate with metastasis formation, and vessel counts in diffuse-type gastric cancer are lower than those in intestinal-type gastric cancer. These findings suggest that diffuse-type gastric adenocarcinoma may not require a high degree of angiogenesis for tumour dissemination. Such may also be the case with pancreatic adenocarcinoma. Pancreatic cancer recurrences may be local-regional, distant or both; and pancreatic cancer metastases are usually multiple and small, possibly because of relatively low angiogenic activity, especially when compared with other metastatic tumours, such as hepatic metastases from colorectal cancer.

A second possible explanation is that angiogenesis is but one step in the multistep process of metastasis formation [19, 20]. Therefore, a high degree of neovascularity and high expression of angiogenic factors may be necessary, but not sufficient to produce metastasis. For example, if a primary tumour has a high angiogenic index and yet does not express other factors necessary for metastasis formation (i.e. adhesion/cohesion molecules, motility factors, growth factor receptors, etc.) then metastasis will not occur [3, 18]. In such a case, a multiparametric study may be necessary to determine which combination of factors must be expressed at high levels to predict early distant metastases [21, 22]. In contrast, certain tumours may possess large populations of cells that express high levels of factors that regulate the other steps in the metastatic cascade and thus the threshold for the angiogenic activity necessary to facilitate metastasis may be lower.

The lack of correlation of vessel count and VEGF expression with prognosis does not abrogate the need to investigate anti-angiogenic therapies in patients with pancreatic cancer. Anti-angiogenic therapies may be most effective when used as adjuvant therapies in patients presumed to have a very small volume of disease to prevent conversion to an angiogenic phenotype. Anti-angiogenic therapies are unlikely to have an impact on patients with large-volume metastatic disease. Inhibiting the growth of micrometastatic disease with long-term anti-angiogenic therapy represents a logical strategy for solid tumours refractory to current systemic therapies. Anti-angiogenic agents are currently being used both before and after surgery in patients with localised pancreatic cancer as part of an investigational pilot study at our institution.

1. Staley CA, Lee JE, Cleary KR, *et al.* Preoperative chemoradiation, pancreaticoduodenectomy, and intraoperative radiation therapy for adenocarcinoma of the pancreatic head. *Am J Surg* 1996, **171**, 118–125.
2. Evans DB, Abbruzzese JL, Rich TA. Cancer of the pancreas. In DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer, Principles and Practice of Oncology*. Philadelphia, J.B. Lippincott, 1997, 1054–1087.
3. Fidler IJ, Ellis LM. The implications of angiogenesis to the biology and therapy of cancer metastasis. *Cell* 1994, **79**, 185–188.
4. Liotta LA, Kleinerman J, Saidel GM. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Res* 1974, **34**, 997–1003.
5. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast cancer. *N Engl J Med* 1991, **324**, 1–8.
6. Macchiarini P, Fontanini G, Hardin M, Squartini F, Angeletti C. Relation of neovascularization to metastasis of non-small-cell lung cancer. *Lancet* 1992, **340**, 145–146.
7. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993, **143**, 401–409.
8. Smith-McCune KK, Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. *Cancer Res* 1994, **54**, 800–804.
9. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995, **55**, 3964–3968.
10. Takahashi Y, Cleary KR, Mai M, Kitadai Y, Bucana CD, Ellis LM. Significance of vessel count and vascular endothelial growth factor and its receptor (*KDR*) in intestinal-type gastric cancer. *Clin Cancer Res* 1996, **2**, 1679–1684.
11. Cheng S-Y, Huang H-JS, Nagane M, *et al.* Supression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. *Proc Natl Acad Sci USA* 1996, **93**, 8502–8507.
12. Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995, **95**, 1789–1797.
13. Mattern J, Koomägi R, Volm M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. *Br J Cancer* 1996, **73**, 931–934.
14. Toi M, Inada K, Hoshina S, Suzuki H, Kondo S, Tominaga T. Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. *Clin Cancer Res* 1995, **1**, 961–964.
15. Itakura J, Ishiwata T, Fujii H, *et al.* Enhanced expression of vascular endothelial growth factor in human pancreatic cancer. *Pancreas* 1996, **13**, 441(A).
16. Goulding H, Rashid NFNA, Robertson JF, *et al.* Assessment of angiogenesis in breast carcinoma: an important factor in prognosis? *Hum Pathol* 1995, **26**, 1196–1200.
17. Ohsawa M, Tomita Y, Kuratsu S, Kanno H, Aozasa K. Angiogenesis in malignant fibrous histiocytoma. *Oncology* 1995, **52**, 51–54.
18. Ellis LM, Fidler IJ. Angiogenesis and metastasis. *Eur J Cancer* 1996, **32A**, 2451–2460.
19. Fidler IJ, Balch CM. The biology of cancer metastasis and implications for therapy. *Curr Probl Surg* 1987, **24**, 137–208.
20. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes Memorial Award Lecture. *Cancer Res* 1990, **50**, 6130–6138.
21. Kitadai Y, Ellis LM, Takahashi Y, *et al.* Multiparametric *in situ* mRNA hybridization analysis to detect metastasis-related genes in surgical specimens of human colon carcinomas. *Clin Cancer Res* 1995, **1**, 1095–1102.
22. Kitadai Y, Ellis LM, Tucker SL, *et al.* Multiparametric *in situ* mRNA hybridization analysis to predict disease recurrence in patients with colon carcinoma. *Am J Pathol* 1996, **149**, 1541–1551.

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