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Prognostic Value of Angiogenesis in Gastro-intestinal Tumours

Y.S. Chung, K. Maeda and M. Sowa

First Department of Surgery, Osaka City University Medical School, 1-5-7 Asahimachi, Abeno-ku, Osaka, 545, Japan

INTRODUCTION

ALTHOUGH ANGIOGENESIS, the physiological process of new vessel formation, is essential in tissue development, reproduction and wound healing, unregulated angiogenesis plays a critical role in neoplasia [1]. New blood vessels are essential for the growth of tumours after reaching the size of a few millimetres [2]. Neovascularisation allows tumour growth by actively exchanging nutrients and metabolites rather than relying solely on simple diffusion. Folkman and associates [3] and Liotta and associates [4] have demonstrated that angiogenesis also contributes to the metastatic process by facilitating shedding of tumour cells into the newly-formed blood vessels. Furthermore, recent evidence suggests that angiogenesis may not only enhance tumour growth by supplying more nutrients to the tumour, but may also work through the paracrine effects of endothelial cells. Endothelial cells are capable of releasing growth factors that evoke tumour cell proliferation [5]. This suggests that there may be an interaction between tumour cells and endothelial cells, where tumour cells release substances that stimulate endothelial cells and in return these vascular cells release substances that promote tumour cell growth.

Quantitative evaluation of neovascularisation may provide information on tumour behaviour and metastatic potential and thus be of prognostic value. Recently, several clinical studies have demonstrated a correlation between the degree of angiogenesis, estimated by immunohistochemical analysis of factor-VIII-related antigen positive cells, and the prognosis of patients with various solid tumours. Thus, patients with higher angiogenesis scores have been found to have decreased survival rates, while the angiogenesis score and grade have been reported to be better predictors of survival than tumour grade, size and site in breast [6–10], lung [11], prostate cancer [12] and head and neck cancer [13].

Thus far, many different prognostic factors other than clinicopathological factors, have been reported in gastrointestinal cancers [14–16]. Among these, both the semiquantitative analysis of angiogenesis (microvessel count) and the immunocytochemical expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), have shown possible clinical application as predictors for survival and recurrence [17–19]. In this article, we have reviewed the prognostic value of tumour angiogenesis in gastro-intestinal cancers, such as gastric and colorectal cancer, and describe our experimental and clinical results.

Correspondence to Y.S. Chung.

EXPERIMENTAL STUDIES

Angiogenic factors in colon cancer cells

Several reports have demonstrated that colorectal cancer cells produce angiogenic growth factors, such as angiogenin [20], basic fibroblast growth factor (bFGF) [21], transforming growth factor-α (TGF-α) and TGF-β [22], which stimulate endothelial cells to proliferate, as well as other factors which induce the formation of capillaries [23]. bFGF and TGF-β may co-operate to enhance endothelial cell proliferation and activity [24]. There is now direct evidence that supports the hypothesis that tumour growth is angiogenesis dependent. Kim and associates [25] reported that a specific antibody against VEGF reduced intratumoral microvessel density (IMD) and caused significant inhibition of tumours that relied on VEGF as their sole mediator of angiogenesis. Similar results, using anti-bFGF monoclonal antibody, were found in a murine tumour by Hori and colleagues [26]. Furthermore, the specific antibody against VEGF and bFGF did not inhibit growth of the tumour cells *in vitro*, which suggested that the inhibition of angiogenesis by antibodies was the direct cause of tumour regression.

Tumour angiogenesis may contribute not only to the enhanced growth of the primary tumour, but also to the establishment of metastatic lesions. Therefore, the ability to produce angiogenic growth factors may influence the metastatic potential of cancer cells. Maeda and associates [18] reported that the VEGF status of gastric cancer was significantly associated with hepatic metastasis. We have also shown that a highly metastatic colon cancer cell line produced higher levels of VEGF than the parental cell line, which only produces a low incidence of liver metastases. We have established a high metastatic variant colon cancer cell line, and shown that the factor produced by these cells, which we have characterised, enhances vascular endothelial cell proliferation, migration and tube formation. These results are described in more detail below.

Highly metastatic variant cell line of colon cancer. Originally, we established a human colon cancer cell line, OCUC-LM (LM; parental cell line), which metastasised to the liver at a low incidence following intrasplenic injection. The highly

metastatic cell line (LM5; daughter cell line) was obtained by applying *in vivo* selection. Thus, liver metastatic lesions, formed after the intrasplenic injection of LM into nude mice, were dissected free and minced into small pieces, and then the cell suspension was recultured and again injected into the spleen of nude mice. This procedure was repeated five times. The incidence of liver metastasis of LM5 was dramatically higher than that of LM. LM and LM5 were used to analyse the production and to characterise the growth factor for vascular endothelial cells.

The angiogenic factor produced by colon cancer cells. The paracrine effects of LM and LM5 on vascular endothelial cell (human umbilical vascular endothelial cells; HUVECs) were studied using a serum-free, conditioned medium (SFCM). Supernatants from LM and LM5 cultures were added to HUVECs plated in dishes, and the proliferative activity of these target cells was determined after 3 days treatment. We found that the proliferation of HUVECs was enhanced significantly by treatment with SFCM from both LM and LM5 cells compared to the control. However, the medium conditioned by LM5 was able to enhance the proliferative activity of endothelial cells to a much greater extent than that of LM cells (Figure 1a). Conversely, the SFCM of LM5 did not influence the proliferation of control cells, such as fibroblasts, even though its effects on vascular endothelial cells was substantial. These findings suggest that the colon cancer cell lines LM and LM5 may produce some growth factor specific for vascular endothelial cells. The effects of SFCM from LM and LM5 cultures was examined on the migration of HUVECs using chemotaxis chambers. It was determined that the number of HUVECs which migrated through the filter was increased in comparison with control medium, and, moreover, that the migration effect of LM5-conditioned material was significantly greater than that of LM (Figure 1b). We also observed the effects of SFCM from LM and LM5 on tube formation by HUVECs. When HUVECs were cultured between two layers of collagen gel, they aggregated together and differentiated into tube-like structures following treatment with SFCM. Quantitative analysis of this tube formation was made by measuring the specific tube lengths using an image analyser. The results showed that the specific tube lengths in culture with SFCM were significantly longer than that of control, and again the contribution of LM5 was significantly greater than that of LM (Figure 1c). From these results, it was concluded that an angiogenic factor appeared to be produced by colon cancer cells and that the effects observed were much stronger when the highly metastatic cells were utilised.

Characterisation of the angiogenic factor. The characteristics of the angiogenic factor produced by LM5 cells were analysed using several techniques, including high power liquid chromatography, SDS polyacrylamide gel electrophoresis and neutralising antibodies against known angiogenic factors, such as VEGF, PDGF (platelet derived growth factor), b-FGF and TGF-β. The data clarified the fact that the angiogenic factor produced by LM5 is VEGF. This conclusion was based on the molecular weight of the active fraction, which at approximately Mr22000 was the same as VEGF, and the finding that the proliferative activity on vascular endothelial cells was best inhibited dose-dependently by anti-VEGF antibody.

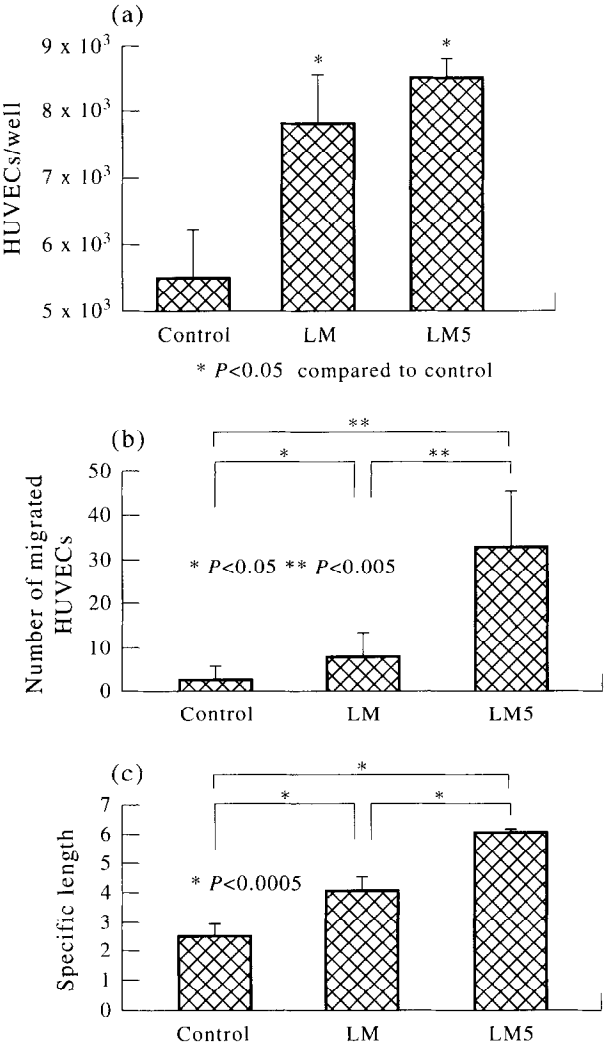

Figure 1. The effects of a secreted factor produced by LH and LH5 cells, on (a) proliferation, (b) migration and (c) tube formation of vascular endothelial cells (HUVEC). Conditioned medium with 10% calf serum was used as the control.

CLINICAL STUDIES

Intratumoral microvessel density (IMD)

After the pioneering report in 1991 by Weidner and associates, [6, 7], many studies have been published correlating IMD and prognosis of patients with primary breast cancer [8–10]. However, there have been few studies on the association of IMD and prognosis of patients with gastro-intestinal tumours (Table 1). Factor-VIII related antigen (F-VIII RAg) was the marker most often used (3 out of 5 studies), but in 2 studies, the antibodies CD-31 or CD-34 were utilised.

We have also used an immunohistochemical technique with an antibody for F-VIII RAg and have investigated the correlation between IMD and prognosis in patients who underwent curative resection for gastric cancer. The mean microvessel count (the mean number of microvessels in the five areas of highest vascular density at 200x magnification) of these patients was 15.9. We therefore classified these patients into two subgroups: a hypervascular group, for which the microvessel count was 16 or greater, and a hypo-

Table 1. Correlation of intratumoral microvessel density with prognosis in gastro-intestinal tumours

[Ref.]	Origin	Marker	Association with metastasis	Recurrence	Survival
[17]	Stomach	F-VIII RAg	SD	SD	SD
[27]	Stomach	CD34	SD	SD	SD
[29]	Rectum	F-VIII RAg	SD		SD
[30]	Colon	F-VIII RAg	SD	SD	
[28]	Colorectum	CD31	NS		NS

F-VIII RA, factor VIII-related antigen; SD, significant difference; NS, not significant.

Table 2. Gastric cancer: recurrent cases after curative resection

Microvessel count	Rate of recurrence		Location of recurrence		
			Liver	43.8 (7/16)	
⩾16 ($n = 38$)	42.1 (16/38)		Peritoneum	37.5 (6/16)	
			Lymph node	6.3 (1/16)	*
		*	Other	12.5 (2/16)	
			Liver	0 (0/10)	
<16 ($n = 54$)	18.5 (10/54)		Peritoneum	80.0 (8/10)	
			Lymph node	10.0 (1/10)	
			Other	10.0 (1/10)	

*$P < 0.05$.

vascular group, for which the count was less than 16. The survival rate of these two groups was calculated using the Kaplan–Meier method. As a result, we found the prognosis of the hypervascular group to be significantly ($P < 0.05$) worse than that of the hypovascular group (Figure 2). These differences in microvessel density appeared to be related to the mode of recurrence. Thus, in the hypervascular group, 7 (43.8%) had hepatic metastases, and 6 (37.5%) had peritoneal dissemination. In contrast, in the hypovascular group, 8 (80%) had peritoneal metastases and none had hepatic metastases (Table 2). The frequency of hepatic metastasis after curative resection was significantly

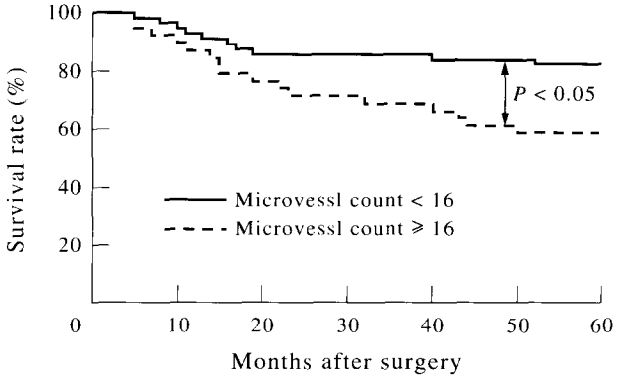

Figure 2. Survival rate in gastric cancer patients, after curative resection, according to microvessel count.

($P < 0.05$) higher in the hypervascular group than in the hypovascular group. Tanigawa and associates [27] also studied the correlation between IMD and prognosis in 110 patients with gastric carcinoma, and reported that IMD is associated with poor survival and haematogenous recurrence.

While Bossi and associates [28] reported that angiogenesis is an early, critical step in colorectal tumorigenesis, it appeared that IMD did not provide any significant prognostic information in patients suffering from colorectal cancer. Conversely, Saclarides and associates [29] and Frank and associates [30] both reported a significant association between high vascularisation of primary tumours and metastasis, recurrence and survival. Thus, Saclarides and colleagues [29] reported that, in patients with rectal cancer, a high incidence of metastasis and shorter survival time was associated with a higher degree of vascularisation in primary tumours. Similar results in 105 patients with node-negative colon cancer were reported by Frank and associates [30]. Here, IMD was significantly higher for patients with recurrent disease, while the 5-year survival rate was significantly higher in the patients who had low microvessel density relative to those with a high microvessel density [30]. These authors concluded that IMD is an important predictor of disease recurrence and could help to identify patients with a high risk of recurrence and shorter survival times.

The angiogenic factor

Tumour angiogenesis may be regulated by angiogenic factors that are produced and secreted by tumour cells. Recently, several angiogenic factors have been identified

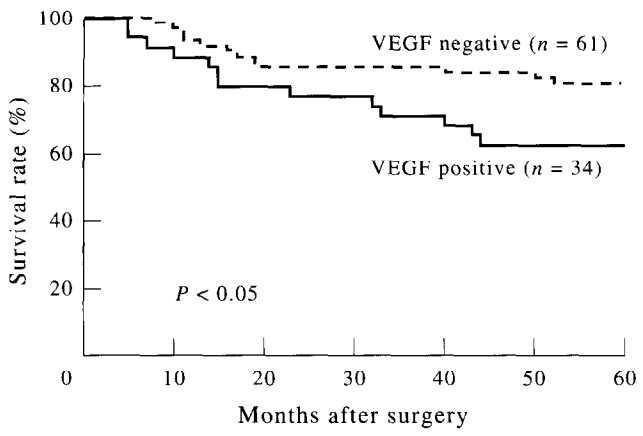

Figure 3. Survival rate in gastric cancer patients, after curative resection, according to VEGF expression.

[28] and VEGF is thought to be one such molecule [31–33]. VEGF is a selective mitogen for endothelial cells and may directly stimulate the growth of new blood vessels [34, 35]. Toi and associates [36] have reported that the expression of VEGF is closely associated with the promotion of angiogenesis and poor prognosis in breast cancer. We investigated the correlation between expression of VEGF and prognosis of patients with gastric or colorectal cancer.

The prognosis of 95 gastric cancer patients who underwent curative resection was studied. As shown in Figure 3, we found the prognosis of the patients with VEGF-positive tumours to be significantly worse than that of those with VEGF-negative tumours. The relationship between VEGF expression and recurrence is shown in Table 3 , with the location of recurrence varying between VEGF-negative or -positive tumours. Thus, 14 (66.7%) of the patients with VEGF-positive tumours had hepatic recurrence, while only 1 (16.7%) of those with VEGF-negative tumours had hepatic recurrence. The frequency of hepatic recurrence following curative resection was significantly higher ($P < 0.05$) in the patients with VEGF-positive tumours than in those with VEGF-negative tumours. The same tendency was seen in the patients with colorectal cancer so that VEGF expression was significantly associated with prognosis and haematogenous metastasis in colorectal carcinoma (Table 3).

Table 4. Correlation between VEGF expression and microvessel density in gastro-intestinal tumours

VEGF expression	Microvessel count Mean ± S.D.	Median (range)
Gastric carcinoma		
Positive ($n = 26$)	22.0 ± 9.2*	17.8 (9.0–50.0)
Negative ($n = 14$)	13.0 ± 9.6	12.5 (4.0–44.5)
Colorectal carcinoma		
Positive ($n = 26$)	22.4 ± 16.8*	18.3 (9.0–90.4)
Negative ($n = 14$)	13.8 ± 5.1	12.0 (8.8–23.6)

*$P < 0.05$.

We also found that the microvessel count in VEGF-positive tumours was significantly higher than in VEGF-negative tumours in both gastric and colorectal cancer (Table 4).

The finding that neovascularisation is more pronounced in VEGF-positive tumours is consistent with the possibility that an enhanced vascular supply reflects an increased risk of metastasis. Tumour cells rarely shed into the circulation before the primary tumour is vascularised [1]. It has been shown that greater numbers of tumour vessels increase the opportunity for tumour cells to enter the circulation. Moreover, newly formed capillaries have fragmented basement membranes and are leaky, making them more penetrable by tumour cells than mature vessels [4]. Therefore, in the hypervascular tumours induced by VEGF, the metastatic process may be enhanced by the 'leaky' nature of newly formed blood vessels which facilitates vascular invasion [37].

CONCLUSION

In conclusion, both microvessel density and VEGF expression may be good prognostic indicators and may be useful as predictors for the mode of recurrence in patients with gastric or colorectal carcinoma. However, more studies, particularly clinical trials, using standardised metrology for

Table 3. Involvement of VEGF expression in recurrence of gastro-intestinal tumours

	VEGF expression	Number of recurrences		Location of recurrence		
Gastric carcinoma	Positive ($n = 59$)	21 (35.6%)		Liver	14 (66.7%)	
				Lung	2 (9.5%)	
			*	Lymph node	5 (23.8%)	*
	Negative ($n = 73$)	6 (8.2%)		Liver	1 (16.7%)	
				Lymph node	2 (33.3%)	
				Others	3 (50.0%)	
Colorectal carcinoma	Positive ($n = 34$)	14 (41.2%)		Liver	7 (50.0%)	
				Lymph node	1 (7.1%)	
			*	Others	6 (42.9%)	*
	Negative ($n = 61$)	12 (19.7%)		Liver	0 (0%)	
				Lymph node	1 (8.3%)	
				Others	11 (91.7%)	

*$P < 0.05$.

the quantification of angiogenic activity are required to confirm the results reviewed here.

1. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992, **267**, 10931–10934.
2. Folkman J, Long D, Becker F. Growth and metastasis of tumour in organ culture. *Cancer* 1963, **16**, 453–467.
3. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition of hyperplasia to neoplasia. *Nature* 1989, **39**, 58–61.
4. Liotta L, Kleinerman J, Saldel G. Quantitative relationships of intravascular tumour cells, tumour vessel, and pulmonary metastasis following tumour implantation. *Cancer Res* 1974, **34**, 997–1004.
5. Folkman J. Angiogenesis and breast cancer. *J Clin Oncol* 1994, **12**, 441–443.
6. Weidner N, Semple SF, Welch WR, Folkman J, *et al.* Tumour angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991, **324**, 1–8.
7. Weidner N, Folkman J, Pozza F, *et al.* Tumour angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992, **84**, 1875–1887.
8. Toi M, Kashitani J, Tominaga T. Tumour angiogenesis is an independent prognostic indicator in primary breast carcinoma. *Int J Cancer* 1993, **55**, 371–374.
9. Gasparini G, Weidner N, Bevilacqua P, *et al.* Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. *J Clin Oncol* 1994, **12**, 454–466.
10. Ogawa Y, Chung YS, Nakata B, *et al.* Microvessel quantitation in invasive breast cancer by staining for factor VIII-related antigen. *Br J Cancer* 1995, **71**, 1297–1301.
11. Macchiarini P, Fontanini G, Hardin MJ, *et al.* Relation of neovasculature to metastasis of non-small-cell lung cancer. *Lancet* 1992, **340**, 145–146.
12. Weidner N, Carrol PR, Flax J, Blumenfeld W, Folkman J. Tumour angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993, **143**, 401–409.
13. Gasparini G, Weidner N, Maluta S, *et al.* Intratumoral microvessel density and p53 protein: correlation with metastasis in head-and-neck squamous-cell carcinoma. *Int J Cancer* 1993, **55**, 739–744.
14. Sowa M, Yoshino H, Kato Y, Nishimura H, Kamino K, Umeyama K. An analysis of the DNA ploidy pattern of gastric cancer. *Cancer* 1988, **62**, 1325–1330.
15. Maeda K, Chung YS, Sowa H, *et al.* Proliferating cell nuclear antigen labeling index of preoperative biopsy specimens in gastric carcinoma with special reference to prognosis. *Cancer* 1994, **73**, 528–533.
16. Chung YS, Yamashita Y, Kato Y, Nakata B, Sawada T, Sowa H. Prognostic significance of T antigen expression in patients with gastric carcinoma. *Cancer* 1996, 77, 768–773.
17. Maeda K, Chung YS, takatsuka S, *et al.* Tumour angiogenesis as a predictor of recurrence in gastric carcinoma. *J Clin Oncol* 1995, **13**, 477–481.
18. Maeda K, Chung YS, Ogawa Y, *et al.* Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996, 77, 858–863.
19. Maeda K, Chung YS, Takatsuka S, *et al.* Tumour angiogenesis and tumour cell proliferation as prognostic indicators in gastric carcinoma. *Br J Cancer* 1995, **72**, 319–323.
20. Shapiro R, Riordan JF, Vallee BL. Characteristic ribonucleolytic activity of human angiogenin. *Biochemistry* 1986, **25**, 3527–3532.
21. New BA, Yeaman LC. Identification of basic fibroblast growth factor sensitivity and receptor and ligand expression in human colon tumour cell lines. *J Cell Physiol* 1992, **150**, 320–326.
22. Anzano HA, Rieman D, Lassalle P, *et al.* Growth factor production by human colon carcinoma cell lines. *Cancer Res* 1989, **49**, 2898–2904.
23. Wernert N, Raes MB, Lassalle P, *et al.* c-ets 1 protooncogene is a transcription factor expressed in endothelial cells during tumour vascularization and other forms of angiogenesis in humans. *Am J Pathol* 1992, **140**, 119–127.
24. Presta H, Maier JA, Rusnati H, *et al.* Modulaton of plasminogen activator activity in human endothelial adenocarcinoma cells by basic fibroblast growth factor and transforming growth factor beta. *Cancer Res* 1988, **48**, 6384–6389.
25. Kim KJ, Li B, Winer J, *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993, **362**, 841–844.
26. Hori A, Sasada R, Hatsutani E, *et al.* Suppression of solid tumour growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Res* 1991, **51**, 6180–6184.
27. Tanigawa N, Amaya H, Hatsumura H, *et al.* Extent of tumour vascularization correlates with prognosis and hematogenous metastasis in gastric carcinoma. *Cancer Res* 1996, **56**, 2671–2676.
28. Bossi P, Viale G, Lee AKC, Alfano RH, Coggi G, Bosari S. Angiogenesis in colorectal tumours: microvessel quantitation in adenomas and carcinomas with clinicopathological correlations. *Cancer Res* 1995, **55**, 5049–5053.
29. Saclarides TJ, Speziale NJ, Drab E, *et al.* Tumour angiogenesis and rectal carcinoma. *Dis Colon Rectum* 1994, **37**, 921–926.
30. Frank RE, Saclarides TJ, Leurgans S, *et al.* Tumour angiogenesis as a predictor of recurrence and survival in patients with node-negative colon cancer. *Ann Surg* 1995, **222**, 695–699.
31. Dvorak HF, Orenstein NS, Carvalho AC, *et al.* Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. *J Immunol* 1979, **122**, 166–174.
32. Senger DR, Connolly DT, Van De Water L, Feder J, Dvorak HF. Purification and NH_2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. *Cancer Res* 1990, **50**, 1774–1778.
33. Brown R, Lanir N, HcDonagh J, *et al.* Fibroblast migration in fibrin gel matrices. *Am J Pathol* 1993, **142**, 273–283.
34. Leung DW, Cachianes G, Kuang WJ, Goedel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989, **246**, 1306.
35. Connolly DT, Heuvelman DH, Nelson R, *et al.* Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989, **84**, 1470–1478.
36. Toi H, Hoshina S, Takayanagi T, Tomonaga T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994, **85**, 1045–1049.
37. Nagy JA, Brown LF, Senger DR, *et al.* Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochim Biophys Acta* 1989, **948**, 305–326.