Original article

Effect of neoadjuvant chemoradiotherapy on angiogenesis in oesophageal cancer

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Background: Vascular endothelial growth factor (VEGF) levels are raised in the serum of patients with oesophageal carcinoma. The aim of this study was to evaluate the tumour microvasculature and the role of tumour-associated macrophages in VEGF production after neoadjuvant chemoradiotherapy and surgery for oesophageal cancer.
Methods: Sections from 92 consecutively resected oesophageal tumours were stained for VEGF, Von Willebrand factor and CD68. Twenty-seven patients received preoperative chemoradiation and 65 underwent surgical excision alone. The cellular source of VEGF was determined by parallel-section staining. Microvessel density and macrophage count were determined for each tumour by means of image analysis software.
Results: There were no significant differences between the two groups in age, sex or tumour type. Local downstaging of disease was evident in most specimens of tumours that had received preoperative chemoradiation. All tumours stained positive for VEGF, including those demonstrating a complete pathological response. Staining of parallel sections confirmed macrophages as the principal source of VEGF. Mean microvessel density was 6·4 per high-power field (h.p.f.) in tumours that received preoperative chemoradiation compared with 5·3 per h.p.f. in those treated by surgery alone ($P = 0{\cdot}13$•). Q2
A significant increase in tumour-associated macrophage infiltration was noted in tumours treated with neoadjuvant chemoradiation (22• per h.p.f.) compared with those treated by surgery alone (14 per h.p.f.) ($P = 0{\cdot}042$). Q3
Conclusion: Preoperative chemoradiation had little effect on the local angiogenic profile of the tumour in patients with oesophageal cancer. Tumour-infiltrating macrophages seem to be the source of persistent VEGF production after chemoradiotherapy and might explain the raised serum levels. Addition of an antiangiogenic agent to this •regimen may be worthwhile in patients with oesophageal carcinoma. Q4

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Introduction

The prognosis for oesophageal cancer is poor, with fewer than 10 per cent of patients surviving 5 years[1]. Although surgery is undertaken with curative intent, most patients succumb to residual or recurrent disease. Preoperative chemoradiotherapy confers a survival advantage over surgery alone in patients with adenocarcinoma[2] and squamous cell carcinoma[3]. However, the benefit is modest and additional strategies are required to further enhance survival rates. Antiangiogenic agents offer one such alternative therapeutic approach[4].

Measurement of the angiogenic index of a primary tumour by assessing microvessel density is a reliable independent prognostic factor in breast[5], non-small-cell lung[6], prostate[7], and head and neck squamous cell[8] carcinomas. Expression of the proangiogenic cytokine vascular endothelial growth factor (VEGF) and tumour microvessel density are useful prognostic indicators in oesophageal

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squamous cell carcinoma[9,10]. VEGF plays a vital role in tumour biology. It is a potent endothelial cell mitogen, promoting tumour angiogenesis and inhibiting tumour cell apoptosis[11], making it an attractive target for novel therapeutic approaches. VEGF levels are raised in the serum of patients with oesophageal cancer[12]. Preoperative chemoradiotherapy does not significantly alter these levels, even in patients who have a complete pathological response[12]. Serum VEGF levels fall after resection of the primary tumour, implying that the source lies in the tumour bed[12]. The source of VEGF in these patients remains unclear and the effect of preoperative chemoradiotherapy on the angiogenic profile of oesophageal carcinoma is unknown.

At tissue level both inflammation and fibrosis occur after chemoradiotherapy[13–15]. The cellular changes mimic those of a granulating wound, with activated macrophages and fibroblasts replacing the malignant cells as they are eradicated[16]. Macrophages may account for a large proportion of a solid tumour mass, comprising as much as 50 per cent of the total cellular content of some breast carcinomas[17]. They are a major source of angiogenic factors in both the healing wound[18] and in solid malignancies[19,20]. Previous work has identified the macrophage as a potent source of VEGF in breast cancer[21]. Similarly, fibroblasts play a significant role in VEGF production in breast carcinoma[22] and in the healing wound[23,24]. The reactive inflammatory changes that occur in a tumour following chemotherapy and radiotherapy may lead to an increase in the macrophage and fibroblast population. These cells may replace the eradicated malignant cells as the principal source of proangiogenic cytokines, explaining the persistently high serum levels of VEGF[12].

It was hypothesized that treatment of oesophageal cancer with chemoradiation might induce inflammatory and fibrotic changes in the tumour resulting in increased macrophage infiltration, a persistence of VEGF production and little alteration in the tumour angiogenic profile. The present study was designed to test this hypothesis, by measuring microvessel density as a marker of angiogenesis and using macrophage immunohistochemistry to determine the source of VEGF production.

Patients and methods

Patients

Following ethics committee approval, paraffin-embedded tumour blocks from 92 consecutive patients who had undergone resection of oesophageal carcinoma between October 1991 and June 2000 were retrieved. Before November 1998, patients with carcinoma of the oesophagus were treated by surgery only. After this date, all patients underwent chemoradiotherapy before surgery. Patients who had preoperative chemoradiotherapy were compared with those managed by surgical resection alone. A subgroup of patients who received chemoradiation and demonstrated a complete pathological response to treatment was also identified.

Preoperative chemoradiotherapy

Patients were treated with preoperative chemoradiotherapy as described previously[2]. Briefly, this consisted of a 5-day course of 5-fluorouracil 15 mg per kg per day followed by cisplatin 75 mg per m^2 body surface area given on day 7. Radiotherapy to a total dose of 40 Gy delivered in 15 fractions was commenced in week 1 and continued in weeks 2 and 3. The course of chemotherapy was repeated on week 6 and patients underwent oesophagectomy on or after week 8.

Pathological stage

Tumour stage was defined according to the American Joint Committee on Cancer classification[25]. A complete pathological response was defined by the absence of residual tumour in the resected specimen and in the lymph nodes.

Immunohistochemistry

Tumour blocks were cut into 4-μm sections, which were mounted on poly-L-lysine-coated slides and stained using immunohistochemical techniques for vascular endothelial growth factor (VEGF), CD68 (a marker of human macrophages) and Von Willebrand factor (endothelial cell marker). Human tonsil sections were used as positive controls and negative controls were obtained by repeating the staining process with the specific antibody omitted. Parallel 4-μm sections from each tumour were stained alternatively for VEGF and CD-68 to determine the cellular source of VEGF production.

Microvessel and macrophage counts

Five areas of high concentration of immunohistochemical staining were identified by scanning the tumour sections under low power (× 4 magnification) with a Eclipse E600 •microscope (Nikon, USA). Microvessel counts were then performed in each of these five areas under high-power magnification (× 40) and the mean• count obtained. Microvessel and macrophage counts were performed using

Lucia Screen Measurement™ Version 4.21 image analysis software (Nikon). Any brown-stained vessel or endothelial cell that was clearly separate from the microvessels was considered a vessel and included.

Statistical analysis

Data were analysed using GB-STAT for Windows Statistical Package (Dynamic Microsystems, •USA). χ^2 and unpaired *t* tests were used to compare data between the two groups. $P \leq 0{\cdot}050$ was considered significant.

Results

Paraffin-embedded tumour blocks from 92 patients with carcinoma of the oesophagus were studied. There were 47 patients (51·1 per cent) with adenocarcinoma and 42 (45·7 per cent) with squamous cell carcinoma. Three patients (3·3 per cent) had a poorly differentiated carcinoma. Twenty-seven patients (29·3 per cent) were treated with neoadjuvant chemoradiotherapy before surgery and 65 (70·7 per cent) had surgical resection alone. There was a predominance of men (55 men *versus* 37 •women). There were no significant differences between treatment groups in terms of age, sex and tumour type (*Table 1*).

Significantly more patients who had undergone neoadjuvant chemoradiotherapy had an early- stage tumour (*Table 1*), consistent with disease downstaging, as has been reported previously[2]. Seven patients (7·6 per cent) had stage 0 tumours at the time of resection, 25 (27·2 per cent) had stage IIa, 28 (30·4 per cent) had stage IIb and 32 (34·8 per cent) had stage III disease. Sixteen of those treated with neoadjuvant therapy had lymph node-negative disease at the time of surgical resection compared with 16 of 65 patients managed with oesophagectomy alone ($P = 0{\cdot}013$) (*Table 1*).

There was no significant difference in microvessel count between the two groups. The mean(s.e.m.) microvessel count in patients who had undergone preoperative treatment was 6·4(1·0) (95 per cent confidence interval (c.i.) 5·2 to 7·3) vessels per high-power field (h.p.f) compared with 5·3(0·7) (95 per cent c.i. 4·8 to 6·7) per h.p.f. for those who had surgery only ($P = 0{\cdot}13$) (*Fig. 1*). Microvessel counts were similar in adenocarcinomas and squamous cell carcinomas. Mean(s.e.m.) microvessel count in specimens demonstrating a complete pathological response was 5·5(0·9) (95 per cent c.i. 5·0 to 6·2) microvessels per h.p.f. (*Fig. 1*). This was not significantly different to that of tumours with a partial response or those treated with surgery alone.

Immunohistochemistry confirmed the presence of VEGF staining in the primary tumour in patients with a complete pathological response to preoperative chemoradiation (stage 0); all four adenocarcinomas and three squamous cell tumours response stained positive for VEGF.

Parallel sections of tumour stained for VEGF and CD68, as a marker of macrophages, demonstrated that areas of high concentration of CD68 staining mirrored high concentrations of VEGF staining, suggesting that tumour-associated macrophages were a potent source of VEGF (*Fig. 2a,2b*). This applied to both squamous cell and adenocarcinomas.

There were significantly more tumour-associated macrophages in sections of tumours treated with chemoradiation than in those from patients who underwent excision alone: mean(s.e.m.) number of CD-68 positive cells 22(1·7) (95 per cent c.i. 18·3 to 26·9) *versus* 14(0·8) (95 per cent c.i. 12·7 to 16·3) per h.p.f. respectively ($P = 0{\cdot}042$). There was no significant difference in the tumour-associated macrophage count between adenocarcinomas and squamous cell carcinomas in either treatment group.

Table 1 Patient characteristics

	NeoAdjuvant ($n = 27$)	Surgery ($n = 65$)	*P*
Sex ratio (M : F)	17 : 10	44 : 21	0·711
Mean age (years)	66	67	0·3
Squamous Cell Carcinoma	11 (40·7)	31 (47·8)	0·147
Adenocarcinoma	15 (55·6)	32 (49·2)	0·22
Stage 0	7 (26·0)	0 (0)	0·0011
Node negative	16 (59·3)	16 (24·6)	0·013

Values in parentheses are percentages.

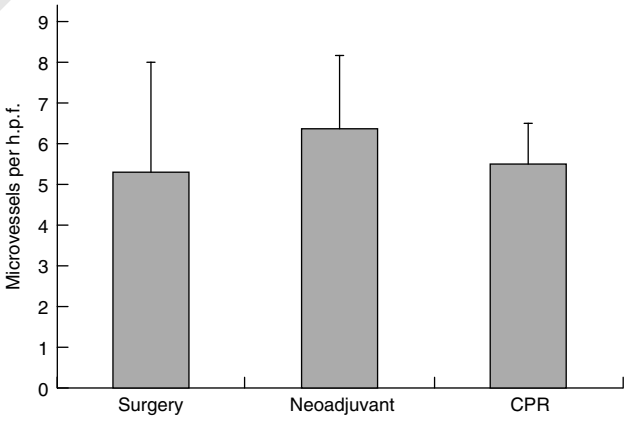

Fig. 1 Effect of neoadjuvant chemoradiotherapy on microvessel density in the primary tumour. h.p.f, High-power field; CPR, complete •response

a VEGF-positive cells

b CD68-positive cells

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Fig. 2 **a** Tumour section stained for vascular endothelial growth factor (VEGF) (brown staining). **b** Parallel section of tumour stained for CD68 (brown staining). The staining pattern corresponds to the areas of most intense VEGF •staining

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Discussion

Preoperative chemoradiation downstages tumours and improves survival of patients with oesophageal cancer, but does not change the serum levels of the proangiogenic cytokine VEGF during or after treatment[12]. It had been expected that treatment with chemoradiation would result in eradication of malignant cells and reduce VEGF levels; however, despite tumour reduction or eradication, VEGF production continued unabated. Immunohistochemical staining of the resected specimens suggested that the tumour cells were replaced with macrophages and fibroblasts, which took over as the principal source of VEGF production.

High microvessel density has previously been associated with a poorer outcome in a number of tumours, including oesophageal squamous cell carcinoma[10]. Tumours with a high microvessel density are reported to be more sensitive to chemotherapy[26], suggesting that microvessel count or tissue VEGF expression might help identify patients who might benefit from adjuvant treatment.

In this series of 92 oesophagectomy specimens, microvessel density in tumours from patients who received neoadjuvant chemoradiotherapy was similar to that in lesions from patients managed with excision alone. Even patients who demonstrated a complete pathological response showed no reduction in tumour vasculature, suggesting that pretreatment with chemoradiotherapy has a minimal effect on the angiogenic component of tumour growth.

Perez-Atayde *et al.*[27] reported that the involution of microvessels in the bone marrow following chemotherapy lagged behind the killing of malignant cells in children with acute lymphoblastic leukaemia. This may explain the persistently high microvessel count seen in tumours from patients who received preoperative chemoradiotherapy, even with a complete pathological response. The timing of surgery after the preoperative regimen may be crucial, at least with respect to the expression of these markers. All patients included in this study underwent oesophagectomy within 2 weeks after the completion of preoperative chemoradiotherapy. Examination of tumours with a longer interval between completion of neoadjuvant treatment and oesophagectomy might help determine whether microvessel regression occurs.

An alternative explanation is that tumour endothelial cells are resistant to the effects of the neoadjuvant regimen and that these cells remain as a potential source, facilitating local tumour recurrence. It seems reasonable to speculate that the addition of an antiangiogenic agent to the existing chemoradiotherapy regimen might be beneficial.

Chemoradiotherapy results in tumour cell apoptosis and necrosis, with subsequent inflammation and fibrosis. This results in an increase in the tumour-associated macrophage count. These cells, together with fibroblasts, are a potent source of VEGF[28–30] and, as demonstrated, the excised mass in patients who display a complete pathological response still stains positive for VEGF. Treatment with chemoradiation may induce an 'angiogenic switch', promoting the growth of new blood vessels within the tumour, with possible detrimental effects. The development of telangiectasia is a long recognized side-effect of radiation treatment.

In addition to its the growth-promoting effects on endothelial cells, VEGF may promote growth of residual primary tumour cells. VEGF is a potent antiapoptotic factor for tumour cells[11] and raised serum levels in patients with oesophageal carcinoma might facilitate the survival

of micrometastatic tumour deposits, giving rise to later disease. As VEGF has been shown to inhibit tumour cell apoptosis[11], and both chemotherapy and radiotherapy exert their effects by means of an induction of apoptotic cell death, VEGF expression within a tumour may enhance its ability to resist the cytotoxic effects of chemoradiation. The combination of persistently raised serum VEGF levels in patients treated with preoperative chemoradiation[12], together with the finding of VEGF-positive cells, suggests that a specific anti-VEGF therapy, rather than a general antiangiogenic strategy, might enhance the efficacy of chemoradiation. Specifically targeting the macrophage might reduce VEGF levels and potentially improve the response to treatment[31].

No differences were noted between adenocarcinomas and squamous cell lesions in terms of the number of patients who received neoadjuvant treatment, the incidence of complete response to treatment, microvessel density, VEGF production or tumour-associated macrophage infiltration. This is in contrast to the findings of Torres *et al.*[32], who reported a significantly higher microvessel density in adenocarcinomas than squamous cell lesions in a series of 67 oesophageal cancers. Several differences between in the two series might explain this disparity. First, in the present series just over half of the samples included were adenocarcinomas, whereas in Torres' series adenocarcinomas accounted for approximately two-thirds of the specimens. In addition, all of the adenocarcinomas included in the latter series were associated with Barrett's oesophagus and the inflammatory nature of this lesion might account for the increase in microvessel density reported. Not all the adenocarcinomas included in the present series were associated with Barrett's change.

Dvorak[33] has described tumours as 'wounds that do not heal' because of the presence of a highly cellular, highly vascularized stroma that resembles the granulation tissue of healing wounds. It is possible that neoadjuvant treatment enhances this granulation-like response within the tumour, accounting for the persistently high serum VEGF levels, microvessel counts and an increase in macrophage infiltration. Abrogation of this proangiogenic inflammatory-type response may be a useful additional therapeutic approach in patients receiving preoperative chemoradiotherapy.

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