

## Relationship of Endothelial Cell Proliferation to Tumor Vascularity in Human Breast Cancer

Stephen B. Fox, Kevin C. Gatter, Roy Bicknell, James J. Going, Peter Stanton, Tim G. Cooke, and Adrian L. Harris

*Nuffield Department of Pathology [S. B. F., K. C. G.] and Imperial Cancer Research Fund, Institute of Molecular Medicine [R. B., A. L. H.], University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, and Department of Surgery, University of Glasgow, Royal Infirmary, Queen Elizabeth Building, Glasgow G31 2ER [P. S., T. G. C.], and Department of Pathology, University of Glasgow, Royal Infirmary, Castle Street, Glasgow G4 0SF [J. J. G.], United Kingdom*

### Abstract

Current studies of tumor angiogenesis rely on the concept that endothelium proliferates 30–40 times faster in tumors than in normal tissues. This evidence is based on histological autoradiographic data largely from animal studies. To assess endothelial cell proliferation in human cancer we used the more sensitive and specific technique of immunohistochemistry. We measured the frequency and distribution of endothelial cell proliferation and examined their relationship to tumor cell proliferation. For the first time, we also correlated endothelial and tumor cell proliferation with tumor vascularity. Twenty breast carcinomas from patients exposed to bromodeoxyuridine 3–8 h prior to surgery were double immunostained using antibodies to CD31 (as a marker of endothelium) and bromodeoxyuridine (as a marker of proliferation). The labeling index (LI) for both tumor and endothelial cells was determined and tumor vascularity was assessed by counting the number of CD31 positive vessels. Endothelial cell proliferation was predominantly at the tumor periphery while tumor cell proliferation occurred throughout the lesion. The mean LIs for endothelium and tumor were 2.2% (range, 0.8–5.3) and 7.3% (range, 1.3–17.1), respectively. There was no correlation between tumor and endothelial cell LI ( $P = 0.414$ ) or between the tumor LI or endothelial cell LI and tumor vascularity ( $P = 0.08$  and  $P = 0.39$ , respectively). These findings suggest that previous studies in animal tumors have significantly overestimated endothelial cell proliferation and that its importance in tumor angiogenesis may be related more to continual remodeling and migration of vessels than to proliferation alone.

### Introduction

Angiogenesis is essential for tumor growth and metastasis (1). Endothelial cell matrix remodeling, migration, and proliferation are central to the angiogenic process (2). In marked contrast to the numerous tumor cell kinetic studies there have been few examining endothelial cell proliferations during tumor angiogenesis (3–7). However, based on this small number of reports it has become generally accepted that endothelial cells proliferate 30–40-fold faster in tumor blood vessels than in the vasculature of normal tissue, irrespective of tumor type, growth rate, or size.

However, this oft quoted figure was obtained from endothelial labeling indices derived from histological autoradiographs of animal tissues exposed to tritiated thymidine. To the best of our knowledge in humans only gliomas have been examined in detail (8). The identification of proliferating endothelium by tinctorial stains used in all of these studies permitted recognition only of larger caliber capillaries (a small proportion of the tumor vessels). This difficulty is likely to make the reported endothelial labeling indices inaccurate and also obscures patterns of endothelial cell proliferation and its relationship with the tumor vascularity.

The weakness of previous studies and the paucity of human data warrant reevaluation of this question with more precise techniques. Using immunohistochemistry and antibodies to endothelium and

BrdUrd<sup>1</sup> we analyzed endothelial cell proliferation in a series of human breast adenocarcinomas. We report on the frequency and pattern of endothelial cell proliferation and their relationship with tumor cell proliferation and, for the first time, tumor vascularity.

### Materials and Methods

**Tissue Specimens.** Twenty invasive breast carcinomas resected between 1989 and 1991 were taken from the archival files of the Glasgow Royal Infirmary, Scotland. The tumors were derived from patients who had been given i.v. injections of 200 mg bromodeoxyuridine 3–8 h prior to mastectomy. Patients' ages range from 40 to 77 years (mean, 61.2 years); 17 were ductal carcinomas of no special type, 1 was a lobular carcinoma, 1 was a medullary carcinoma and 1 was an atypical medullary carcinoma. Of the ductal carcinomas 2 were grade I, 7 were grade II, and 8 were grade III (Nottingham modification of system of Bloom and Richardson) (9). Tumors ranged in diameter from 12 to 120 mm. Nine had histologically confirmed lymph node involvement by tumor.

**Immunohistochemistry.** Four- $\mu$ m sections were cut onto silane coated slides and dewaxed. Double immunostaining was performed on sections using streptavidin-biotin-peroxidase and alkaline phosphatase anti-alkaline phosphatase techniques and the antibodies JC70 (Dako) (10) and Bu20a (Dako) (11), respectively (12). For optimal double staining it was necessary to pretreat sections with 12.5 mg protease type XXIV (Sigma)/100 ml phosphate buffered saline for 20 min at 37°C for JC70 and 2 N HCl for 5 min at 60°C for Bu20a. After immunostaining a light hematoxylin counterstain was applied before mounting in aqueous medium. In 10 cases multiple tissue sections were stained. Single BrdUrd immunohistochemistry was performed in parallel sections for all cases in another laboratory.

**Morphometry.** Labeling indices for both endothelial and tumor cells were determined by scanning the entire tumor section at  $\times 400$ . In 14 of 20 cases the entire cross-section of the tumor was examined and in 5 of 20, due to their size, at least one-half of the tumor diameter was studied. In one tumor, due to its size of 120 mm, a representative section had to be selected which included the invading tumor margin. A positive endothelial cell was identified on the basis of JC70 positive cytoplasm and/or cell membrane and Bu20a positive nucleus. An endothelial cell was considered negative when an immunonegative nucleus was surrounded by JC70 positive cytoplasm and/or cell membrane. Occasional JC70 immunopositive macrophages and plasma cells were excluded on morphological grounds. The LI of tumor cells was scored by selecting the maximally immunostained area. An average of 1860 endothelial cells and 1398 tumor cells (ranges, 689–2728 and 543–2742, respectively) were counted for each tumor. The vascularity of the tumors was assessed by averaging the number of JC70 positive vessels per mm<sup>2</sup> in the three most vascular areas (13).

**Statistical Analysis.** Since the data skew and hence cannot be considered normal, a nonparametric Kendall's rank correlation was performed which makes no such assumptions, to assess the relationship between the variables.

### Results

Endothelial cell BrdUrd labeling was almost restricted to the infiltrating margin of the tumor with only occasional endothelial cell double immunoreactivity of microvessels within the tumor body. Microvessels were often seen in the body of the tumor without an accompanying endothelial cell nucleus. Proliferating endothelium was

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<sup>1</sup> The abbreviations used are: BrdUrd, bromodeoxyuridine; LI, labeling index.

observed in all types of vessel (*i.e.*, arteriole, venule, narrow and dilated capillaries) (Fig. 1, A and B). Labeled tumor BrdUrd nuclei were easily distinguishable from labeled endothelial cell BrdUrd nuclei (Fig. 1C). Tumor cell labeling showed no similar restricted pattern of proliferation with labeling observed throughout the tumors. The endothelial cell LI ranged from 0.8 to 5.3% (mean, 2.2%) and tumor LI ranged from 1.3 to 17.1% (mean, 7.3%). This tumor cell LI did not differ from the tumor LI established by single BrdUrd immunostaining in another laboratory [correlation score, 138 ( $n = 20$ );  $P < 0.0001$  (data not shown)]. There was no correlation between endothelial cell and tumor cell LIs ( $P = 0.414$ ) (Fig. 2) or between the endothelial cell LI and tumor vascularity ( $P = 0.39$ ) (Fig. 3). There was no association between tumor cell LI and tumor vascularity ( $P = 0.08$ ) (Fig. 4).

**Discussion**

In accord with prior studies we observed no relationship between the endothelial cell LI, tumor cell LI, or vascularity (6, 7). However, our findings of a mean endothelial cell LI of 2.2% is in contrast to

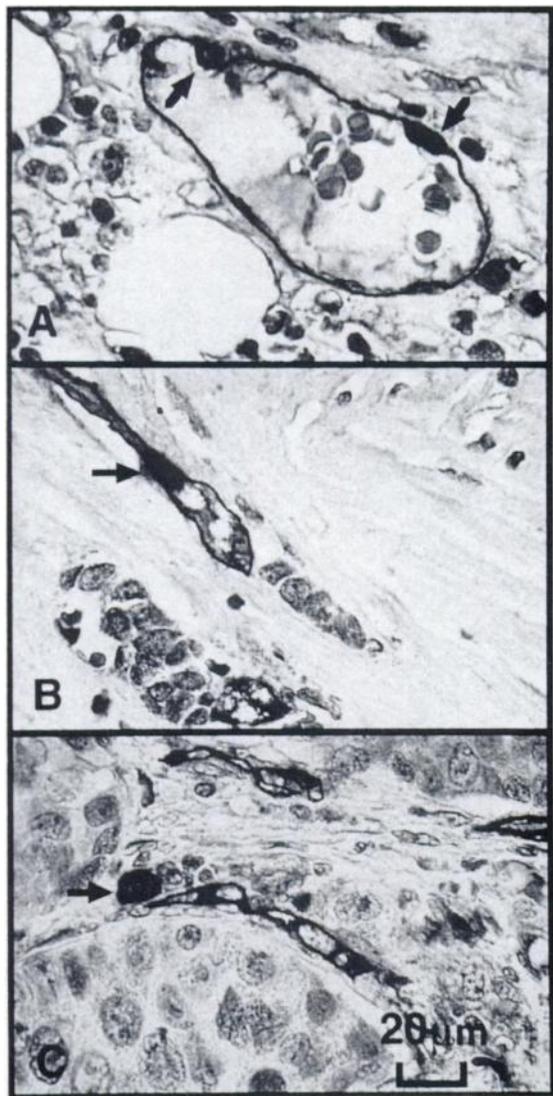


Fig. 1. A, dilated positively stained CD31 capillary containing erythrocytes with two labeled BrdUrd endothelial cell nuclei (arrows) admixed with neoplastic and inflammatory cells. B, CD31 positive microvessel with one labeled BrdUrd endothelial cell nucleus (arrow) adjacent to the tumor with negative nuclei. C, tumor nests with an immunopositive tumor cell nucleus (arrow) and adjacent CD31 positive microvessels with immunonegative nuclei. CD31-diaminobenzidine-immunoperoxidase; BrdUrd-alkaline phosphatase anti-alkaline phosphatase.

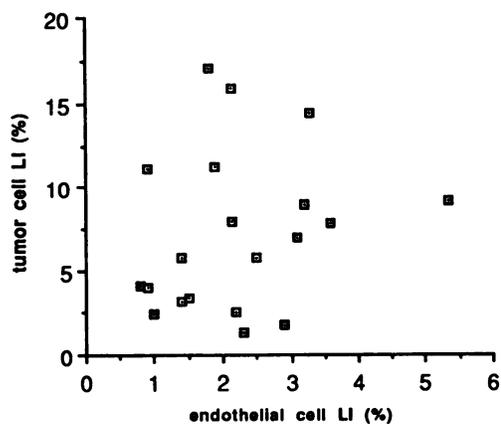


Fig. 2. Tumor cell LI plotted as a function of endothelial cell LI. Score = 25;  $P = 0.414$ .

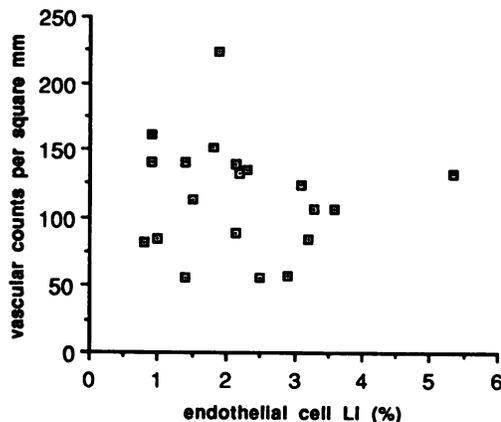


Fig. 3. Vascular counts plotted as a function of endothelial cell LI. Score = 0.084;  $P = 0.39$ .

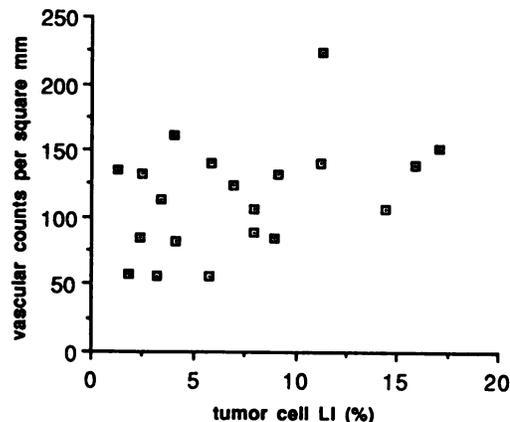


Fig. 4. Vascular counts plotted as a function of tumor cell LI. Score = 53;  $P = 0.08$ .

those reported previously (9%) (3–8). This large discrepancy can be partially explained by the type of vessels examined. Strict morphological definitions based on classical histochemical staining were used to identify vascular endothelium in these previous studies. This necessarily identified endothelial cells only in the larger caliber vessels and therefore the small capillaries, making up most of the tumor vascular network, were excluded. At the time this limitation was recognized but it was assumed that these small budding capillaries, which could not be morphologically identified, would be the vessels likely to proliferate and that the true endothelial cell LI would be still higher (6).

In this study immunohistochemistry with an antibody to CD31, the most sensitive vascular endothelial marker to date (14, 15), was used to highlight endothelium. We selected this endothelial marker rather than an antibody to Factor VIII related antigen which is at least as specific but not as sensitive since we wished to identify the complete tumor vasculature. We chose BrdUrd as a marker of proliferation since we could examine large sections of formalin fixed paraffin embedded tumor in contrast to other markers which rely on frozen material. Reliable BrdUrd immunostaining confirmed by comparing double with single BrdUrd immunostaining performed in a different laboratory. We observed that most endothelial cell proliferation occurred at the tumor periphery and that microvessels in the body of the tumor do not proliferate. This pattern of endothelial cell proliferation has been described in the rabbit brain tumor model but not in other animal or human tumors (16) and is not unexpected since endothelial cell proliferation during angiogenesis occurs adjacent to the "parent vessel" and not at the tips of growing capillary sprouts (17). Indeed, this study might have overestimated the true endothelial cell LI since it was common to observe immunoreactive capillaries within the tumor without being able to identify the accompanying endothelial cell nucleus. Because these are liable to be noncycling endothelial cells, inclusion in the LI denominator might further have reduced the endothelial cell LI. One criticism of these findings might be that BrdUrd did not penetrate into the center of the tumor. This possibility is eliminated by the fact that tumor cells were labeled as intensely in the center of the tumor as at its periphery. The BrdUrd in these labeled tumor cells must have passed through the tumor vasculature, the endothelium of which must have necessarily had a prior exposure to BrdUrd.

One of the other major limitations of other studies has been their reliance on animal material. Marked differences between spontaneously arising human and transplanted animal tumors are recognized. Moreover, most of the experimental animal tumors studied were small and fast growing (3–8) with rapid tumor volume doubling times as compared to the human tumors examined in this series. It has been demonstrated that increasing tumor size retards tumor proliferation (18). Furthermore, in several previous studies (7, 8, 19) continuous labeling was performed to optimize LIs, in contrast to the flash labeling carried out in this study. We were able to find limited data on endothelial cell proliferation in nine assorted human tumors (7) and a series of gliomas (8); endothelial LIs ranged from 2 to 37%. However, as far as can be ascertained from published work, only two tumor types had labeling indices exceeding 5%; gliomas which are known for their prominent vascular pattern and a parotid tumor, the precise type not specified. The remaining tumors were assorted lymphomas and carcinomas (the precise histological types appear not to have been published) which had labeling indices from 2.8 to 4.6% within the range but generally higher than those observed in this study.

It is possible that endothelial cell proliferation might be focal or vary diurnally. Furthermore, it is also possible that since we are detecting cells only in S phase, we are missing a substantial number of cycling endothelial cells. However, we have now examined multiple cross-sections of tumors from 20 cases and any significant variation in location or time of endothelial cell proliferation should have emerged.

These findings show that endothelial cell proliferation occurs at the tumor periphery, which suggests that although endothelial cell proliferation must be required for angiogenesis, after endothelial cell division, remodeling and migration of the existing tissue vascular supply establish the functional vasculature within the body of the tumor. Moreover, the association between tumor cell and endothelial cell proliferation only at the invading edge also suggests that growth factors controlling tumor growth are not the same as those involved in

endothelial cell growth. This is further supported by the lack of correlation between tumor and endothelial cell LIs. We also observed no correlation between tumor cell LI and tumor vascularity ( $P = 0.08$ ). This is supported by other findings using a similar method of assessing microvessel density and Ki-67 and S-phase fractions as indices of tumor proliferation (20).

This study also challenges the proposal of attacking proliferating tumor endothelium as a unique and universal strategy of tumor treatment on the basis of differential endothelial cell proliferation rates in tumors and normal tissues (21). To target proliferating endothelium much more information will be needed about patterns of proliferation and its importance for tumor angiogenesis. Nevertheless, even a 2- or 3-fold proliferation difference (7, 22) between normal and tumor endothelium might still permit effective targeting of cytotoxic treatment to tumors since although many drugs do not adequately penetrate tumors, drug delivery to the tumor periphery would not compromise cytotoxic efficacy.

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