Angiogenesis in Ductal Carcinoma *in situ* of the Breast

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Contents

Abstract

Background

Up to 50% of recurrences of ductal carcinoma in situ (DCIS) of the breast are associated with invasive carcinoma but no pathological or molecular features have yet been found to predict for the development of invasive disease. For a tumour to invade, it requires the formation of new blood vessels. Previous studies have described a vascular rim around ducts involved by DCIS raising the possibility that the characteristics of periductal vascularisation may be important in determining transformation from in situ to invasive disease as well as risk of recurrence.

Hypothesis

I hypothesized that the risk of malignant tranformation (from normal breast to DCIS) and of invasive recurrence, following initial diagnosis and treatment of DCIS, is associated with the extent and pattern of periductal vascularity. It is likely that the periductal vessels are most important in this respect as incipient invasion is most likely to be associated with changes in vessels in the immediate vicinity of the tumour cells.

Methodology

I investigated to see whether changes in vasculature are related to the progression of in situ to invasive carcinoma and if so, which factors may be important in this change. I initially studied vascular density surrounding DCIS with and without invasive cancer and its relationship to specific angiogenic factors secreted by the tumour. Periductal vascular density and phenotype were determined using morphometry and a panel of anti-endothelial

antibodies (von Willebrand factor [vWF], CD31, CD141 and CD34). These were related to the presence of invasive carcinoma at initial diagnosis, the histological features of DCIS and the risk of recurrence. For each histological section, the number of stained vessels and endothelial cells within 100 micrometers of foci of DCIS were counted. Up to 50 foci of DCIS on a single slide were scored and the microvessel density (MVD) and endothelial cell density (ED) were calculated for each focus. Normal lobules at least 2 mm away were used as controls. These studies suggested a change in phenotype and dual fluorescence immunostaining for CD34+ and vWF+ vessels was carried out to confirm the change. The relationship between Thymidine Phophorylase (TP) expression on sections of pure DCIS and periductal vascularity was assessed as it had been previously shown to correlate with stromal vascular density. The relationship between stromal (hotspots) and periductal vascularity was also evaluated.

Results

Compared to normal lobules, pure DCIS exhibited a greater density of CD34+ and CD31+ vessels but a decrease in those that were immunopositive for von Willebrand factor (vWF). DCIS associated with invasive carcinoma showed a profile of vascular immunostaining similar to that of pure DCIS but there were statistically significantly greater numbers of CD34+ and CD141+ vessels and fewer staining for vWF. There was a significant negative correlation between vascular density and both the cross-sectional areas of the ducts involved and the extent of the necrosis of the tumour they contained. A correlation between vascular density and nuclear grade was also noted, being highest in the intermediate grade. On dual staining, the number of vessels stained only by CD34 was

significantly higher around DCIS compared to adjacent normal lobules. For TP, although a relationship was seen between the H-score and MVD, a relationship with recurrence was not identified. Vascularity stained by anti-CD34 around DCIS which recurred was statistically significantly higher than those which did not. There was a significant and positive correlation between the two types of vascularity ($p<0.001$) in sections stained with anti-vWF antibody, but not anti-CD34.

Conclusions

Blood vessels surrounding DCIS appear to have a different immunophenotype when compared with blood vessels surrounding normal breast lobules. Increases in vascular density, as detected with the CD34 antibody, correlates with recurrence and the development of invasive carcinoma. Recurrent disease does not appear to be related to TP expression by DCIS. Periductal MVD appears to be more important than stromal MVD in predicting for recurrence in DCIS.

Abbreviations

Abbreviation	Full term
5'-DFUR	5'-deoxy-5'-fluorouridine
5-FU	5-fluorouracil
Anti-CD141	Anti-Thrombomodulin
Anti-vWF/F8/FVIII	Anti-human von Willebrand factor
BPV	Bovine papillomavirus
BSA	Bovine Serum Albumen
CAM	Chorioallantoic membrane
CIAS	Computerised image analysis systems
CIN	Cervical intraepithelial neoplasia
D/CD34+%	Proportion/percentage of CD34+ vessels also stained with vWF
D/vWF+%	Proportion/percentage of vWF+ vessels also stained with CD34
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DFS	Disease-free survival
DNA	Deoxyribonucleic Acid
EA	Endothelial area
EBSG	European Breast Screening Group
ECM	Extracellular matrix
ED	Endothelial density

EDTA	Ethylenediamine-Tetraacetic Acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EM	Excision margin
EORTC	European organization for research and treatment of cancer
EPWG	European Pathologists Working Group
ER	Estrogen receptor
FGF	Fibroblast growth factor
H&E	Haematoxylin and eosin
HGF	Hepatocyte growth factor
HPV	Human papillomavirus
HUVECs	Human umbilical vein endothelial cells
IGF	Insulin-like growth factor
IL	Interleukin
KD	Ki-67 density
KDR/flk-1	Endothelial cell specific tyrosine kinase receptors
KP	Ki-67 fraction/percentage
M(3)VD	3 highest microvessel densities
M(5)VD	5 highest microvessel densities
MKD	Mean Ki-67 density
MMP	Matrix metalloproteinase
MR(I)	Magnetic resonance (imaging)

MVC	Microvessel count
MVD	Microvessel density
NHS BSP	National Health Service Breast Screening Programme
OS	Overall survival
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
PCNA	Proliferating cell nuclear antigen
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E-2
PR	Progesteron receptor
PSA	Prostate-specific antigen
Rag	Related antigen
RFS	Relapse-free survival
RLUH	Royal Liverpool University Hospital
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SD	Standard deviation
SF	Scatter factor
TAM	Tumour-associated macrophages
TBS	Tris Buffered Saline
TDLU	Terminal duct lobular units
TGF	Transforming growth factor

TM	Thrombomodulin
TNF	Tumour necrosis factor
TP	Thymidine Phosphorylase
TRITC	Tetramethylrhodamine isothiocynate
TSP	Thrombospondin
UEA-1	Ulex europeus agglutinin I
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VNPI	Van Nuys Prognostic index
VPF	Vascular permeability factor

Legends

Declaration

I hereby declare that this thesis has been composed by myself, that it has not been submitted in any previous application for a degree and that the general matter of this thesis is my own general composition.

Preliminary results from this project were read at the national and international scientific conferences between mid-1999 and mid-2001.

No benefit has been received by the author from any commercial party towards this thesis.

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Dedication

I would like to dedicate this work to late Professor J.P. Sloane who sadly passed away on 10th of May, 2000 four months prior to the completion of this project. He had provided me with support and encouragement throughout the whole project.

I would also like to dedicate it to my father Mr. Sian Peng Teo, mother Madam Soo Fun Kok, brother Mr. Nee Chuan Teo, sister Miss Kar Hui Teo. They have all provided me with tremendous love and hope in my moments of deep despair and given me strength to achieve my goals. For this I love and thank them unreservedly.

Foreword

It is with great happiness that I am writing this foreword which marks the end of a period in which I have devoted all my energy to a single project. The idea of this project arose from the description of cancer made by a Persian physician in the seventh century A.D.

"*Cancer is an uneven swelling, rough, unseemly, darkish, painful, and sometimes without ulceration. .... It has its* ***veins*** *stretched on all sides as the animal, the crab has its feet, whence it derives its name.*"

Paul of Aegina

The reference to the tumour vascularity is very clear. Angiogenesis (the development of new blood vessels from an existing vascular network) is an essential requirement for tumour growth and progression. This multi-step process is regulated by a complex network of cytokines (including growth factors), enzymes and adhesion molecules. Angiogenesis has been shown to have a role in invasive breast cancer. Its importance in pre-invasive disease remains to be elucidated.

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Professor J.P. Sloane (late)	Professor in Histopathology, University of Liverpool.
Mr. Chris Holcombe	Consultant Breast Surgeon, Royal Liverpool University Hospital.
Dr. Balvinder Shoker	Consultant Histopathologist, Royal Hampshire County Hospital, Winchester.

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Mr. Allan William	Senior Officer, Medical Illustrations, Department of Pathology, University of Liverpool.
Mr. Lee Martin	Consultant Breast Surgeon, Aintree University Hospital, Liverpool.
Ms. Liz Twelves	Quality Assurance Coordinator, North West Breast Screening Quality Assurance, Department of Public Health Medicine, West Pennine Health Authority, Oldham.

Overview of thesis

The introduction of this thesis reviews the histopathology of DCIS and the importance of angiogenesis in carcinogenesis. The first part entitled ***Histopathology of DCIS (Chapter 1)*** discusses the definition, incidence, local recurrence rates and risk factors for recurrence. Different classifications with their respective ability to predict recurrence or reproducibility are also discussed. The molecular biology of DCIS is covered briefly.

In the mid-1960 that the contribution of angiogenesis in tumorigenesis was appreciated. Most research has focused on invasive cancers or advanced cancers. Angiogenesis-related findings in these groups of cancers is reviewed in ***Chapter 2*** entitled ***Angiogenesis in Cancers***. The basic principles of the mechanisms of angiogenesis in cancer (including angiogenic balance and switch), its relationship with proliferation and malignant transformation and the therapeutic implications are also covered.

Recently, there has been a lot of interest in the intensity of angiogenesis as measured by microvessel density (MVD). Various types of endothelial markers and different methods of assessment of MVD have been used, and these are reviewed in third part of Introduction entitled ***Assessment of Vascularity (Chapter 3)***. Complimentary methods of angiogenesis assessment are also covered.

With the introduction of screening mammography the frequency with which DCIS is detected has increased and the best treatment of DCIS is still controversial. In the UK, the Van Nuys classification is not commonly applied to screen-detected DCIS cases which have breast conservative treatment. ***Chapter 4 (pilot study)*** addresses different treatments and the application of the Van Nuys classification in these patients. In this study, I also reviewed all cases of DCIS cases detected in Merseyside.

From the literature reviews and clinical data above, I concluded that the present assessment of prognosis for DCIS is inadequate. No pathological or molecular features have been found to predict for the development of invasive disease or the risk of recurrence. There is a lot of work on angiogenesis in advanced or invasive cancers but little is known about the role of angiogenesis in DCIS especially the vascularity around the affected ducts.

Chapter 5 highlights my hypothesis that the risk of malignant tranformation and invasive recurrence in DCIS is associated with the extent and pattern of periductal vascularity. It is likely that the periductal vessels are most important in this respect as incipient invasion is most likely to be associated with changes in vessels in the immediate vicinity of the tumour cells. I also hypothesized that there is relationship between stromal and periductal vascularity in DCIS.

Section 3 ***(Chapter 6-8)*** describes the way I investigated whether changes in vasculature are related to the progression of in situ to invasive carcinoma and if so, which factors may be important in this change. The features studied included vascular density surrounding DCIS with and without invasive cancer and how this relates to the demonstration of specific angiogenic factors secreted by the tumour.

The role of angiogenesis in DCIS is investigated in ***chapters 9*** to ***12***. In the ***chapter 9***, the periductal vascularity was compared in pure DCIS and in DCIS associated with invasive carcinoma. Rigorous morphometric methodology was used to compare vascularity in DCIS with normal breast, and a panel of anti-endothelial antibodies were used to determine changes in phenotype as well as numerical changes in vessel density.

Proliferation, differentiation and migration of endothelial cells all play an important role in tumour angiogenesis. The relationship between MVD and endothelial cell density (ED) has not been evaluated to date. ***Chapter 9*** addresses this and examines changes in ED in relation to the progression of in-situ to invasive carcinoma and the correlation between MVD and ED.

MVD increased from low to intermediate grade DCIS but decreased in high grade DCIS. There was a significant and negative correlation between MVD and degree of necrosis in high grade DCIS. This suggested that periductal angiogenesis increases with tumour growth but is unable to cope with most rapidly growing lesions. This suggestion is tested in ***chapter 10*** by investigating angiogenesis in pure DCIS in relation to its proliferation measured with the anti-Ki67 antibody.

As well as a change in number of vessels with grade, a difference in vessel type was noted. There was a significant increase in CD34 positive periductal vessels in DCIS but a reduction in those staining for vWF compared with adjacent normal lobules. These findings suggest a change in phenotype as well as vascular density. In order to confirm the change of phenotype, I examined the relationship between vWF positive and CD34 positive periductal vessels in pure DCIS as well as adjacent normal lobules by means of dual immunostaining for flourescence ***(chapter 11)***.

Having determined that both the type and number of vessels are different in normal breast, in-situ cancer and invasive cancer, this raised the possibility that changes in vascularity may predict the likelihood of recurrence. I therefore compared changes in periductal vessels and their characteristics in pure DCIS in patients who did not recur with those who developed recurrence either as in-situ or invasive carcinoma. This study is also documented in ***chapter 11***.

The emphasis of my investigations has been on periductal vascularity as it is hypothesized that the risk of malignant transformation and recurrence are likely to be associated with vascular changes in the immediate vicinity of the tumour foci. However, most of the studies in angiogenesis in breast cancer to date have concentrated on stromal vascularity and the relationship between these two types of vascular pattern in DCIS has not hitherto been investigated. I therefore examined the relationship between stromal and periductal vascularity to determine which vascular pattern has a more important bearing on the risk of invasive recurrence or development of invasive disease ***(chapter 12)***.

The expression of Thymidine Phosphorylase has also been investigated as this has previously been shown to correlate with stromal vascular density, and I wished to determine whether its expression in DCIS correlates with periductal vascular density. This study is outlined in ***chapter 12***.

The results were summarised and conclusions were suggested in ***chapter 13***. Before pondering into the future (***chapter 15***), I reviewed the values of current markers of angiogenesis as a prognotic indicator from recent literature in ***chapter 14***.

Section 1

Chapter 1

Histopathology of DCIS

Definition

DCIS is defined as a 'proliferation of epithelial cells with cytological features of malignancy within parenchymal structures of the breast .... Distinguished from invasive carcinoma by the absence of stromal invasion across the basement membrane' [National Co-ordinationg Committee on Breast Screening Pathology 1995]. It was first described by Bloodgood et al [1934]. Lewis and Geschickter et al [1938] described comedo DCIS although they failed to distinguish invasive carcinoma from *in situ* carcinoma. Lobular cancerization in DCIS was highlighted by Azzopardi et al [1979]. Page and Dupont et al [1982] identified cribriform, micropapillary and other types of non-comedo DCIS as high risk factors for the development of ipsilateral invasive breast cancer.

DCIS is no longer thought of as a single disease and is instead recognized as a heterogenous group of lesions with a diverse malignant potential. Several sub-types have been described to date, which include neuroendocrine, apocrine, clear cell, secretory, cystic hypersecretory, mucinous and signet ring sub-types. These subgroups are rare and very little is known about their natural history.

Incidence

Until recently, DCIS was diagnosed relatively infrequently, representing only about 1% of all new cases of breast cancer [Nemoto et al 1980]. It was regarded as a single disease with a single treatment, which was mastectomy. Most patients presenting with DCIS had symptoms – a palpable mass or nipple discharge. The frequency of detected DCIS has increased dramatically since the introduction of mammographic screening, accounting for at least 20% of screen-detected breast cancers, compared with about 5% of those in symptomatic women in the UK [Badve et al 1998, Fryberg et al 1994].

Natural history

DCIS has been shown to arise within the terminal duct lobular units (TDLU) of the breast [Wellings et al 1975, Holland et al 1990a&b, Faverly et al 1994], although the size of some of the involved ducts suggest that extralobular ducts are also involved [Rosen et al 1993]. The current belief is that DCIS originates in the terminal ducts and acini of the breast, it grows and expands these ducts until at a certain stage in its natural history, it develops invasive properties and penetrates the basement membrane.

Autopsy based studies have demonstrated that 15% of young asymptomatic women may have DCIS [Nielsen et al 1984 & 87]. It might be argued that DCIS could almost be 'normal' finding, similar in disease frequency to the detection of benign breast disease. Critical review of these studies suggests that at least some cases of post-mortem DCIS detected in these studies might not meet modern diagnostic criteria. However, the

incidence of invasive breast cancer in western Caucasian populations is high. The lifetime risk of development of invasive breast cancer of 1 in 8 to 1 in 12 in North America or Europe and this incidence may indeed be consistent with these autopsy findings.

DCIS is nearly always unicentric and recurrences occur almost invariably at the site of previous excision [Rosen et al 1980, Baird et al 1990, Schwartz et al 1992, Sneige et al 1995]. However, DCIS is usually poorly-delineated because of extensive microscopic spread along the duct system which makes it difficult to define the precise limits of the disease.

The view that DCIS is a single clonal process has been further supported by the observation that in 12 cases of DCIS although apparently multifocal, all showed loss of heterozygosity at the same chromosome locus implying that the foci of DCIS were derived from the same clone [Stratton et al 1995].

The data available on the risk of progression from DCIS to invasive breast cancer is limited. This is mainly due to the fact that once DCIS is detected it is excised. Studies that provide insight about the natural history of DCIS are those in which patients were initially diagnosed as having benign lesions that on subsequent histological review were classified as DCIS. These patients received no further treatment after diagnostic biopsy [Farrow et al 1970, Page et al 1982 & 95, Rosen et al 1980, Betsill et al 1978, Eusebi et al 1994]. The number of patients in these studies was relatively small (range, 8-25) and most of these cases were low-grade non-comedo DCIS with uncertain margins. During the follow-

up period, ipsilateral invasive breast cancer developed in a significant percentage of these women, usually within 10 years of biopsy [Page et al 1982, Rosen et al 1980]. In a more recent study [Page et al 1995a], about 40% of low grade DCIS cases treated by diagnostic biopsy alone become invasive over a time span of 25-30 years.
These studies show that inadequately treated DCIS is associated with a significant risk for the development of subsequent invasive cancer, up to 11-fold higher than the risk expected in the general population. These data support the concept of DCIS as an anatomical 'forerunner' to invasive breast cancer.

There is evidence that not all DCIS progresses to invasive breast cancer. The results of autopsy studies suggest that latent DCIS is relatively common, ranging from 0.2 to 18% in random autopsy series or series confined to women who died of causes other than breast cancer or who were not known to have had breast cancer at the time of death [Bartow et al 1987, Nielsen et al 1984 & 87, Alpers et al 1985, Bhathal et al 1985]. It was estimated that no more than one third of all intraductal carcinomas will progress to invasive breast cancer [Nielsen et al 1984].

Histological classifications

Histological classifications have been developed in an attempt to predict the likelihood of local recurrence and invasive tranformation after initial treatment. DCIS is a heterogeneous group of lesions and no single approach will be appropriate for all forms of the disease. Therefore, better methods for stratifying DCIS according to risk of recurrence have been developed. This in fact has led to a large numbers of new histological

classifications in recent years. To achieve these objectives, 2 basic requirements have to be fulfilled by any prospective histological classification : 1) it should be able to predict clinical outcome such as local recurrence and possibly progression to invasive carcinoma, and 2) it should be able to be used reproducibly. Some of the classifications are listed below:

Architectural pattern

DCIS is divided solely according to the architectural pattern such as comedo, solid, cribriform, micropapillary and papillary. Two categories (comedo and non-comedo) can be produced by combining some of the groups. The cytological grade of the cells is not taken into account.

European Pathologists Working Group

DCIS is divided into three categories according to cytonuclear differentiation and the presence or absence of cell polarization. The cytonuclear DCIS classification system used by the NHS breast cancer screening programme is derived from this.

Van Nuys

DCIS is divided into three categories according to the presence or absence of comedo necrosis and of high nuclear grade cytonuclear differentiation.

Lagios

DCIS is divided into four categories according to nuclear grade, cytoarchitecture and necrosis (I: comedo, II: cribriform/with necrosis, III: cribriform with anaplasia, IV: micropapillary). This has subsequently been simplified into three categories by combining groups I and II.

Nottingham

DCIS divided into three categories according to the architecture and the presence and degree of necrosis.

Ottesen

DCIS is divided into three types according to growth pattern (microfocal, diffuse and tumour-forming). Architectural pattern, comedo necrosis and nuclear size are also evaluated.

Classifications based on architecture

A number of different morphological patterns are recognized. In the solid type the ductules are completely filled by a disorderly proliferation of large epithelial cells with abundant cytoplasm, variable nuclear pleomorphism and increased mitoses. A similar appearance is seen in the comedo type but in addition there is central necrosis in the lumen so that lipid-rich yellow debris is present. The cribriform type is characterized by a proliferation of relatively small and regular cells which form a geometric network of bridges and trabeculae. The micropapillary type also consists of a proliferation of small

epithelial cells, which form small papillary projections into the lumen. In some cases a mixture of one or more patterns may be present.

There are numerous studies which show that there are relationships between DCIS growth patterns and clinical characteristics and outcome. DCIS of comedo type is noted to have a larger microscopic size, is more likely to be associated with microinvasion and most important of all, is more likely to recur after local excision alone [Baird et al 1990, Schwartz et al 1992, Fisher ER et al 1995, Patchefsky et al 1989, Poller et al 1994, Ottesen et al 1992]. Micropapillary types are more extensive [Bellamy et al 1993, Patchefsky et al 1989, Lennington et al 1994] and are significantly more likely than other patterns to involve multiple quadrants of breast, irrespective of nuclear grade or necrosis [Bellamy et al 1993]. Solid DCIS is significantly more often completely excised compared to other types of DCIS [Bellamy et al 1993].

It is important to stress that architecture is not independent of cytology. Approximately 90% of cases of comedo carcinoma are of high nuclear grade which itself shows a correlation with microscopic size of tumour, presence of microinvasion, frequency of incomplete excision and local recurrence rates after local excision in comparison with growth pattern [Bellamy et al 1993, Patchefsky et al 1989]. Although architectural classification of DCIS is widely used, two problems arise: First of all, Badve et al [1998] failed to establish a relationship between growth patterns and the risk of local recurrence after local excision. Secondly, in the presence of heterogenous histological architecture, it is difficult for various groups of pathologists to use the classification reproducibly

[Ottesen et al 1992, Harrison et al 1996]. A relatively low level of consistency was achieved by a number of pathologists participating in the UK National scheme when they classified DCIS by growth pattern with a kappa statistic value of only 0.23 [Sloane et al 1994].

Classifications based on nuclear grade (Table 1.1)

In view of the inadequacies of DCIS classification based on growth pattern alone a number of newer classifications have been introduced that are primarily based on nuclear grade.

Classifications based on nuclear grade: ability to predict recurrence

Lagios et al [1995] assessed the local recurrence rates of patients a mean of 10 years after local excision. The patients with high grade DCIS had the highest risk with a recurrence rate of 32% compared with 10% for intermediate grade. None of those with low grade DCIS recurred. Similar results were achieved by Silverstein et al [1998b]. Bellamy et al [1993] reviewed 130 cases of non-invasive DCIS in women without previous breast carcinoma, they found that local recurrence only followed high grade DCIS. Silverstein et al [1996] divided 238 patients with DCIS treated with conservation surgery into 3 groups : 1– · non-high grade DCIS without comedo-type necrosis, 2 – non-high grade DCIS with comedo-type necrosis, 3 – high-grade DCIS with comedo-type necrosis. There were 31 local recurrences : 3/80 (3.8%) in group 1, 10/90 (11.1%) in group 2, and 18/68 (26.5%) in group 3.

In a study of 141 patients with follow-up of 2 decades, Badve et al [1998] compared the ability of 5 different classifications in predicting recurrence after local excision. No significant correlation was found between recurrence and growth pattern when a traditional architectural-based system was used. A significant correlation was observed between histology and recurrence using the Van Nuys classification. A significant correlation was also found between nuclear grade as defined by the EPWG and recurrence when cell polarization was disregarded, using the classification currently employed by the UK National Health Service and European Commission-funded Breast Screening programs. This was attributed to a small number of recurring cases being downgraded as a

consequence of the presence of cell polarization. These findings strongly support the use of nuclear grade to identify cases of DCIS at high risk of recurrence after local excision.

Classifications based on nuclear grade: reproducibility

23 pathologists from 12 different countries examined 33 cases of DCIS using 5 different classifications : 1) European Pathologists' Working Group, EPWG; 2) European Breast Screening Group, EBSG; 3) a two tiered classification in which the categories are high nuclear grade and other; 4) Van Nuys; 5) a two-category classification based entirely on the presence of comedo-necrosis [Sloane et al 1998].The Van Nuys classification gave the highest overall Kappa statistic value of 0.42, followed by EPWG (0.37) and EBSG (0.35). These values were not particularly high but were better than the 0.23 achieved by up to 250 pathologists over a 3-year period in the first 6 rounds of the UK National External Quality Assessment Scheme using a classification based on architecture alone [Sloane et al 1994]. The likely explanation for high Kappa value obtained by the Van Nuys system was that the three categories do not form a continuous spectrum but are derived from two dichotomous decisions : 1) high grade versus other and 2) necrosis versus no necrosis.

In the study of Douglas-Jones et al [1996], a lower percentage of disagreement was found using the Van Nuys classification than the EPWG and EBSG systems, where the level of agreement was similar. They found the Van Nuys classification the simplest and easiest to use and had greatest difficulty in agreeing on growth pattern. Bethwaite et al [1998] reported higher levels of consistency using nuclear grade and the Van Nuys system than architecture.

	Grade		
	Low	**Intermediate**	**High**
Breast Screening Group	DCIS composed of cells with pleomorphic, irregularly-shaped and usually large nuclei exhibiting marked variation in size, irregular nuclear contour, course chromatin and prominent nucleoli. Mitoses are frequent and abnormal forms may be seen. May have several different growth patterns but is often solid with central comedo-type necrosis. Polarisation of cells is rare.	The nuclei show mild to moderate pleomorphism which is less than that seen in high grade DCIS but they lack the monotony of the small cell type. The nucleo-cytoplasmic ratio is often high and one or two nucleoli may be identified. The growth pattern may be solid, cribriform or micropapillary and some degree of polarization is present.	Composed of monomorphic, evenly-spaced cells with roughly spherical centrally-placed nucleoli. Mitoses are few and there is rarely individual cell necrosis. The architecture is often micropapillary and cribriform with polarization of cells.
European Pathologists Working Group	The nuclei are very pleomorphic with an irregular outline and spacing. The chromatin is coarse, clumped with prominent nucleoli being present. Mitoses are often seen and polarisation of cells is absent or minimal. Central necrosis and individual cell necrosis are usually present often with associated amorphous calcification.	The nuclei show mild to moderate pleomorphism with some variation in size, outline and spacing. The chromatin is fine to coarse and nucleoli are evident. Mitoses are occasionally present and polarisation of cells is seen. Central necrosis is variable but individual cell necrosis may be focally present. Calcifications are amorphous or laminated.	The nuclei are monomorphic with uniform size, regular outline and spacing. The chromatin is uniform and fine, nucleoli are insignificant and mitoses are rare. Polarisation of cells is marked and central necrosis is absent or minimal. Individual cell necrosis is absent and calcifications are laminated and rarely amorphous.
Van Nuys	Nuclei with a diameter of greater than two red blood cells, with vesicular chromatin and one or more nucleoli. Comedo necrosis (DCIS with central lumina containing necrotic debris surrounded by large pleomorphic cells or necrotic neoplastic cells within ducts with other architectural patterns eg cribriform or micropapillary) is usually present but not essential.	Nuclei with low nuclear grade (1-1.5 red blood cells in diameter with diffuse chromatin and inapparent nucleoli) or intermediate nuclear grade (1-2 red blood cells in diameter with coarse chromatin and infrequent nucleoli) and comedo type necrosis. There is no minimum requirement for comedo necrosis although individual necrotic cells are not scored.	Nuclei with low or intermediate nuclear grade and no comedo necrosis.
Lagios	Large nuclei (2 red blood cells in diameter) with vesicular chromatin, 1 or more nucleoli and a high mitotic index (2+ mitoses/10 hpf). Extensive linear coagulative necrosis of comedo type is present.	Intermediate nuclei (1-2 red blood cells in diameter) with coarse chromatin, infrequent nucleoli and an intermediate mitotic index (1-2 mitoses/10 hpf). Punctate necrosis may be present.	Small nuclei (1-1.5 red blood cells in diameter) with diffuse chromatin, inapparent nucleoli and a low mitotic index (<1 mitoses/ 10 hpf). Necrosis is absent.
Nottingham	Pure comedo subtype showing central lumina containing necrotic debris surrounded by large pleomorphic viable cells in solid masses	DCIS with necrosis (non-pure comedo) are tumours with necrotic neoplastic cells within duct lumina but lacking a pure comedo pattern and often showing a cribriform or micropapillary architecture.	DCIS without necrosis show no evidence of necrosis or no more than one or a few necrotic or desquamted cells within intraductal lumina. This includes the majority of classifical cribriform, papillary and micropapillary subtypes.

Table 1.1. Comparison of DCIS grading classifications.

Microinvasion in DCIS

Diagnosis of microinvasion in DCIS is problematic. Histological features resembling invasion can be simulated by *in situ* carcinoma distorted by inflammatory changes and fibrosis. Microinvasion has been increasingly diagnosed in the advent of screening mammography and is commonly associated with extensive comedo-type DCIS [Silver et al 1998, Silverstein et al 1997d, Rosen et al 1997].

According to the UK NHSBSP Guidelines on Pathology Reporting in Breast Cancer Screening [National Co-ordinating Group for Breast Screening Pathology 1995]: "A microinvasive carcinoma is defined as a tumour in which the dominant lesion is DCIS but in which there are one or more clearly separate foci of infiltration of nonspecialized interlobular or interductal fibrous or adipose tissue, none measuring more than 1 mm (about 2 high-power fields) in maximum diameter". This definition is very restrictive and tumours fulfilling the criteria are consequently very rare. If there is sufficient doubt about the presence of invasion, the case should be classified as DCIS. Microinvasion is largely restricted to high nuclear grade types of DCIS, mainly of comedo type. "Cases of apparently pure comedo DCIS should thus be extensively sampled to exclude invasion. Microinvasive carcinomas should likewise be extensively sampled in order to exclude the possibility of larger invasive foci. Where such foci are found, the lesion should be classified as an invasive tumour. Small invasive carcinomas without an in-situ component are classified as invasive."

The incidence of microinvasion varies according to the size and extent of the DCIS. Lagios et al [1982] reported a 2% incidence of microinvasion in patients with DCIS measuring less than 25 mm, compared with a 29% incidence of microinvasion in index lesions larger than 26 mm.

In an attempt to resolve the uncertainty, Prasad et al [1999] recommends the utility of double-immunolabelling of cytokeratin and smooth muscle actin. From the clinical point of view, microinvasion is seldom associated with axillary nodal metastasis and it has a slight poorer prognosis than DCIS [Silver et al 1998, Silverstein et al 1997d, Rosen et al 1997].

Molecular biology of DCIS

Oestrogen receptor (ER): Generally, it is accepted that ER expression is more common in low grade and non-comedo DCIS than high grade comedo DCIS. However, the exact frequency of ER expression varies with different studies. Poller et al [1993b] reported ER expression in 32% of cases of DCIS. They also established relationships between positive expression and non-comedo variants, small cell size, higher proliferation fraction (S-phase fraction) and lack of c-erbB 2 staining. However, higher proportions of positive expression have been reported by other studies [Giri et al 1989, Ronay et al 1990, Pallis et al 1992, Bur et al 1992]. Bur et al [1992] found ER expression in 91% of non-comedo and 57% of comedo DCIS respectively.

Progesterone receptor (PR): Progesterone receptor expression has been found 31% to 73% of cases.[Bobrow et al 1994, Zafrani et al 1994, Pallis et al 1992]. Like ER it correlates with low nuclear grade DCIS.

Proliferation fraction: Higher cellular proliferation fractions are related to the high nuclear grade DCIS with comedo type necrosis. Cellular proliferation has been assessed by Ki 67/MIBI staining of tumour nuclei, S phase estimation using flow cytometric analysis of DNA, and by thymidine labelling [Locker et al 1990, Poller et al 1991, Barnes et al 1991, Kileen et al 1991, Meyer et al 1986].

c-erbB-2 or HER2/neu: c-erbB-2 gene amplification and c-erbB-2 gene protein expression is more frequent in high and intermediate nuclear grades of DCIS and those of comedo type necrosis [Ramachandra et al 1990, Bobrow et al 1994, Zafrani et al 1994].

Other markers: The p53 protein is more frequently expressed in high nuclear grade DCIS with comedo type necrosis [Davidoff et al 1991, Poller et al 1993a, Thorf et al 1992, Walker et al 1991]. Loss of heterozygosity was identified at one locus on Chr 17q in the vicinity of BRCA 1 in 10-20% of cases of DCIS examined [Futreal et al 1994]. One study showed greater expression of the nm23 gene product in comedo DCIS as compared to non-comedo variants [Simpson et al 1994].

Factors predicting clinical outcome

Nuclear grade

Numerous studies have shown that high grade DCIS is associated with high recurrence rates after local excision alone [Baird et al 1990, Schwartz et al 1992, Solin et al 1993, Bellamy et al 1993, Poller et al 1994, Ottesen et al 1992]. High nuclear grade in DCIS is also closely correlated with frequency of incomplete excision [Bellamy et al 1993].

Necrosis

The presence comedo type necrosis is correlated with intermediate and high grade DCIS which often radiologically contains coarse cast-like calcification. Comedo DCIS tends to be have a larger microscopic size, is more likely to be associated with microinvasion and has a higher risk of recurrence after local excision alone [Baird e al 1990, Schwartz et al 1992, Fisher ER et al 1995, Patchefsky et al 1989, Poller et al 1994, Ottesen et al 1992].

Microscopic size

The presence of residual tumour has been shown to be related to DCIS size [Cheng et al 1997]. Residual DCIS was found in 69% of tumours greater than 25mm compared with only 15% of tumours less than 10mm. Tumour size is also predictor of initial margin involvement and residual DCIS [Silverstein et al 1994].

DCIS presenting as a mass (> 1 cm) is associated with a significantly higher incidence of occult invasion, multicentricity, axillary lymph node metastatsis, higher local recurrence rates, and worse overall and disease-free survival than DCIS incidentally diagnosed on screening mammography [Robinson et al 1999, Fonseca et al 1997, Ashikari et al 1971]. Unfortunately, accurate determination of the size or extent of the lesion is not always easy.

Microcalcifications on mammography often underestimate the size of the tumour, particularly in cases of low-grade (well-differentiated) DCIS, in which substantial areas of tumour may not contain microcalcification [Farrow et al 1970, Holland R et al 1990a & 1994]. Holland et al found that in 44% of micropapillary tumours, the lesions were more than 2 cm larger by histological examination than by mammographic estimate, compared with only 12% of the pure comedo subtype [Holland R et al 1990a].

Determination of the lesion size may also be difficult for the pathologist. Macroscopic examination of a specimen containing DCIS rarely reveals a grossly evident tumour that can be measured. Therefore, the assessment of size of the lesion often must be performed on the histological sections. When the lesion is present in a single slide, the greatest dimension can be measured and reported. However, in many cases of DCIS, the lesion is present on more than one slide. In such cases, accurate determination of the size or extent of the lesions is not possible, unless the specimen has been examined in a sequential manner [Silverstein et al 1996].

Excision margin

Margin width – the distance between DCIS and the closest inked margin – confirms the completeness of excision. In a series where mastectomy or re-excision was carried out after local excision, residual tumour found in 76% of cases with an excision margin of less than 1mm, 43% of cases with an excision margin greater than 1mm and in only 5.6% of cases with a margin of 10mm or more [Silverstein et al 1997a]. The value of margin width has also been confirmed by the group from Nottingham [Sibbering et al 1997], where they reported a local recurrence rate of 6% in a group a 48 patients treated with local excision alone and with margins greater than 10mm. Fisher [Fisher ER et al 1995] reported an adjusted relative risk for local recurrence of 2.3 when margins were involved. Among patients with margin widths of 1 to <10mm, there was no statistically significant benefit from adjuvant radiotherapy which was only beneficial to those with an excision margin of less than 1mm [Silverstein et al 1999].

There are limitations associated with pathological assessment of excision margins. Residual tumour may not be present when the excision margins appear involved as tissue around the biopsy is destroyed during the operation. Secondly, the technical difficulties of assessing margins may lead to false impression of margin involvement due to problems such as seepage of ink though the specimen. Furthermore, the exclusion of the involved part of the specimen for histological examination can lead to a false impression of complete excision, this is more common with ill-defined lesions.

Van Nuys Prognostic Index

Nuclear grade, the presence of comedo-type necrosis, tumour size, and the excision margin are important factors which are able to predict local recurrence in patients with DCIS [Silverstein et al 1995a&b, Lagios et al 1989, Silverstein et al 1996, Solin et al 1993, Bellamy et al 1993]. By using a combination of these factors it enables the clinician to select subgroups of patients who do not require radiotherapy after local excision or whose recurrence rate is potentially so high, even with breast irradiation, that mastectomy is preferable. In the Van Nuys Prognostic Index, scores from 1 to 3 were given for each of the three different categories (size of tumour, excision margin and pathological classification) [*Table 1.2*]. The prognostic index was applied on 333 patients who had breast conservation treatment with and without adjuvant irradiation. Patients with low scores (3 or 4), showed no difference in local recurrence regardless of whether they had had radiotherapy. Patients with intermediate scores (5 or 6 or 7), showed a significant decrease in local recurrence rates with radiotherapy. However, the high scores group (8 or 9), the local recurrence rates was very high even with irradiation and therefore they should be considered for mastectomy. According to the formulators, it is a numerical algorithm based on tumour features which can not be controlled by the patient or clinician. It allows clinician to quantify prognostic factors that are measured and to place patients into one of three clearly defined categories.

Score	1	2	3
Size (mm)	< or =15	16 to 40	> or =41
Margin width (mm)	> or =10	1 to 9	<1
Pathologic classification	Non high grade without necrosis	Non high grade with necrosis	High grade with or without necrosis

Table 1.2. Van Nuys Prognostic Index (using microscopic size, excision margin, degree of necrosis and nuclear grade).

Treatment & local recurrence

Local recurrence is one of the most commonly studied clinical outcomes in DCIS as death is rare. The traditional treatment for DCIS was mastectomy with nearly 100% cure rates [Sunshine et al 1986, Morrow et al 1997, Silverstein et al 1995a&b]. In the early 1980s around the same time as breast conservation treatment was popularized for invasive breast cancer by Veronesi, breast conservation was also introduced for DCIS. Breast conservation treatment for DCIS has now assumed a much greater importance with the implementation of the breast screening programmes in the UK as there has been an explosion in the number of newly diagnosed cases of DCIS.

Recurrences after mastectomy are almost all invasive carcinoma, and may present initially as local failure or distant metastases. Recurrence is primarily due to unrecognized invasive carcinoma present at the time of the initial diagnosis, although incomplete removal of breast tissue could allow a new carcinoma to manifest as a local recurrence.

The local recurrence rate for DCIS is between 10-40%, depending on the length of follow-up and type of treatment. [Silverstein et al 1995a&b, Silverstein et al 1996, Silverstein et al 1998a, Solin et al 1993, Fisher ER et al 1995]. Silverstein et al [1992] looked at the local recurrence rate of patients with DCIS 8 years after treatment. The recurrence rates for patients treated by mastectomy, excision with adjuvant radiotherapy or excision alone were 0.5%, 16% and 21% respectively. However, the survival rates were similar. The major difference between mastectomy and conservative breast surgery is thus the effect of treatment on local recurrence rates and not patient survival. This has been confirmed in several other studies [Sneige et al 1995, Silverstein et al 1992 & 98a].

Local recurrence after conservative treatment is demoralizing and it causes marked patient morbidity. Local excision alone is associated with a high recurrence rate and up to half of the recurrences are associated with invasive carcinoma [Silverstein et al 1995a&b and 96, Solin et al 1993].

Numerous studies have shown radiotherapy to be beneficial after breast conserving treatment, reducing the recurrence rate from 8-45% for excision alone to 4-27% for local excision and radiotherapy [Sneige et al 1995]. In the National Surgical Adjuvant Breast Project Protocol B-17 [Fisher B et al 1993], the recurrence rate was lower in patients treated with local excision with adjuvant radiotherapy as compared with local excision alone (15.6% vs 26.2%). A later analysis [Fisher et al 1999] demonstrated that after lumpectomy alone the lowest rates of breast recurrence were observed in patients with

absent or slight comedo necrosis and margins free of tumour (3 per 100 patient-year); when both unfavorable features were present recurrence rates increased to 8.2 per 100 patient-years. The use of radiotherapy reduced the risk of recurrence in both groups, with a 7% absolute decrease in breast recurrences in the favorable group at 8 years. However, this trial has been criticized for lacking analysis of different pathological subsets and the lack of size measurements in more than 40% of cases [Lagios et al 1993, Page et al 1995b]. Other problems are the lack of requirement for mammographic-pathological correlation or specimen radiography; no uniform guidelines for tissue processing or size estimation; and the inappropriate definition of clear excision margin. The margin is defined to be clear if no tumour is transected which implies that a few fat cells or collagen fibres between the tumour and the inked margin are all that is required to call the margin clear. Nevertheless, the study was not designed to find out which subgroups might benefit from the adjuvant treatment and by how much and furthermore its findings have been confirmed by other studies. Preliminary results from EORTC randomized phase III trial 10853 [Julien et al 2000] and UK DCIS Trial [George et al 2000] show that radiotherapy after local excision for DCIS, as compared with local excision alone, reduces the overall number of both invasive and non-invasive recurrences in the ipsilateral breast.

The long-term effect of radiotherapy in reducing local recurrence rates is disputed by some [Lagios et al 1993]. Radiotherapy is accompanied by considerable side effects such as cardiac toxicity, pulmonary fibrosis and radiation fibrosis which changes the texture of the breast, makes mammographic follow-up more difficult which can result in delayed diagnosis of local recurrence. Nevertheless, approximately half of the recurrences after excision and radiation for DCIS are as invasive breast cancer [Solin et al 1994].

The role of tamoxifen in the treatment of DCIS remains undefined. The NSABP B-24 randomized trial [Fisher B et al 1999] compared the clinical outcome among 1800 women with DCIS that were treated with local excision, radiotherapy and either Tamoxifen or placebo. The patient characteristics (age, ethnic origin, tumour size, tumour type, methods of detection, margin status, presence of comedo necrosis) were similar in two groups of women. After a median of 5 years' follow-up, the incidence of breast tumours was 13.4% among the controls and 8.2% in the treatment group (p=0.0009), a relative reduction of nearly 40%. Furthermore, recurrences of DCIS and invasive ductal carcinoma were reduced by 30% and >40% respectively. At present, tamoxifen is usually used as adjuvant therapy for patients with DCIS in the context of a clinical trial.

Conclusions

Present classifications are inadequate to predict likelihood of local recurrence and malignant transformation because DCIS is a heterogenous group of lesions. The prognostic values of current classifications are also inevitably affected by their low reproducibility (kappa values). There are also limitations associated with pathological assessment of excision margin which is one of the most important factors to predict local recurrence. To date, no pathological or molecular features have been found to predict the development of invasive disease. Consequently, the best treatment of DCIS still remains controversial. If further prognostic markers can be defined they would be important. One such potential prognostic marker is angiogenesis. I will review the literature on angiogenesis in carcinogenesis in the next section of the introduction.

Chapter 2

Angiogenesis in Cancer

Introduction

"Cancer is an uneven swelling, rough, unseemly, darkish, painful, and sometimes without ulceration. .... It has its veins stretched on all sides as the animal, the crab has its feet, whence it derives its name."

Paul of Aegina

The above description of cancer was made by Paul of Aegina, a Persian physician in the seventh century A.D. Although the reference to the tumour vascularity is clear, the contribution of angiogenesis in tumorigenesis was not appreciated until the 1960s [Harmey et al 1998]. Since the 1980s, there has been a significant escalation in scientific research secondary to the discovery of endothelial growth factors, endogenous inhibitors and other molecules of the extracellular matrix (ECM). More recently, natural angiogenic inhibitors and pharmacological drugs capable of suppressing specific stages of neovascularisation have been reported. Measurement of the intra-tumoural microvessel density (MVD) has also been found to be an independent prognostic marker.

Angiogenesis in cancer

For a tumour to grow beyond 1- 2 mm^3, it needs to develop its own blood supply for the supply of oxygen and nutrients as well as excretion of catabolites, by inducing the growth of new blood vessels [Folkman et al 1971, Gimbrone et al 1972]. Early tumours are only a few cell layers thick [Folkman et al 1992c] and metabolize by the diffusion of oxygen and nutrients across the cell layers. This limits tumour size as a tumour cell needing to be approximately 100 micrometer from a capillary, this being the upper limit of diffusability of macromolecular substances [Tannock et al 1969]. Tumour vascularisation promotes survival, growth and metastatic spread of cancer [Folkman et al 1971, Gimbrone et al 1972, Brem et al 1976, Brooks et al 1994a, Folkman et al 1992a, Srivastava et al 1988, Weinstat-Saslow D et al 1994] and the quantification of tumour vascularity by measuring the MVD has been reported to be an independent prognostic indicator in carcinomas of breast, prostate, lung adenocarcinoma, melanoma, head and neck cancer [Fregene et al 1993, Gasparini et al 1992 & 93, Hayes et al 1994, Visscher et al 1994, Weidner et al 1991 &92 & 93, Yamazaki et al 1994, Graham et al 1994].

Mechanisms of neovascularisation

Neovascularisation is a complex biological process which consists of three stages [Varner et al 1996] [*Fig. 2.1*]:

1. Initiation : Angiogenic factors are released by the tumour stimulating endothelial cells by interacting with specific cell surface receptors.
2. Invasion and Proliferation : The endothelial cells proliferate and migrate into the tumour. This is facilitated by the degradation of the extracellular matrix by proteolytic enzymes as well as the interaction between cell surface adhesion molecules and adhesion proteins in the matrix.
3. Maturation : Vessel lumens are formed by differentiation of the endothelial cells, facilitated by cell-cell adhesive contacts. The new vessels nourish the tumour and serve as conduits for metastasis, the new vessels being immature and 'leaky', increasing the likelihood of tumour cells entering the general circulation.

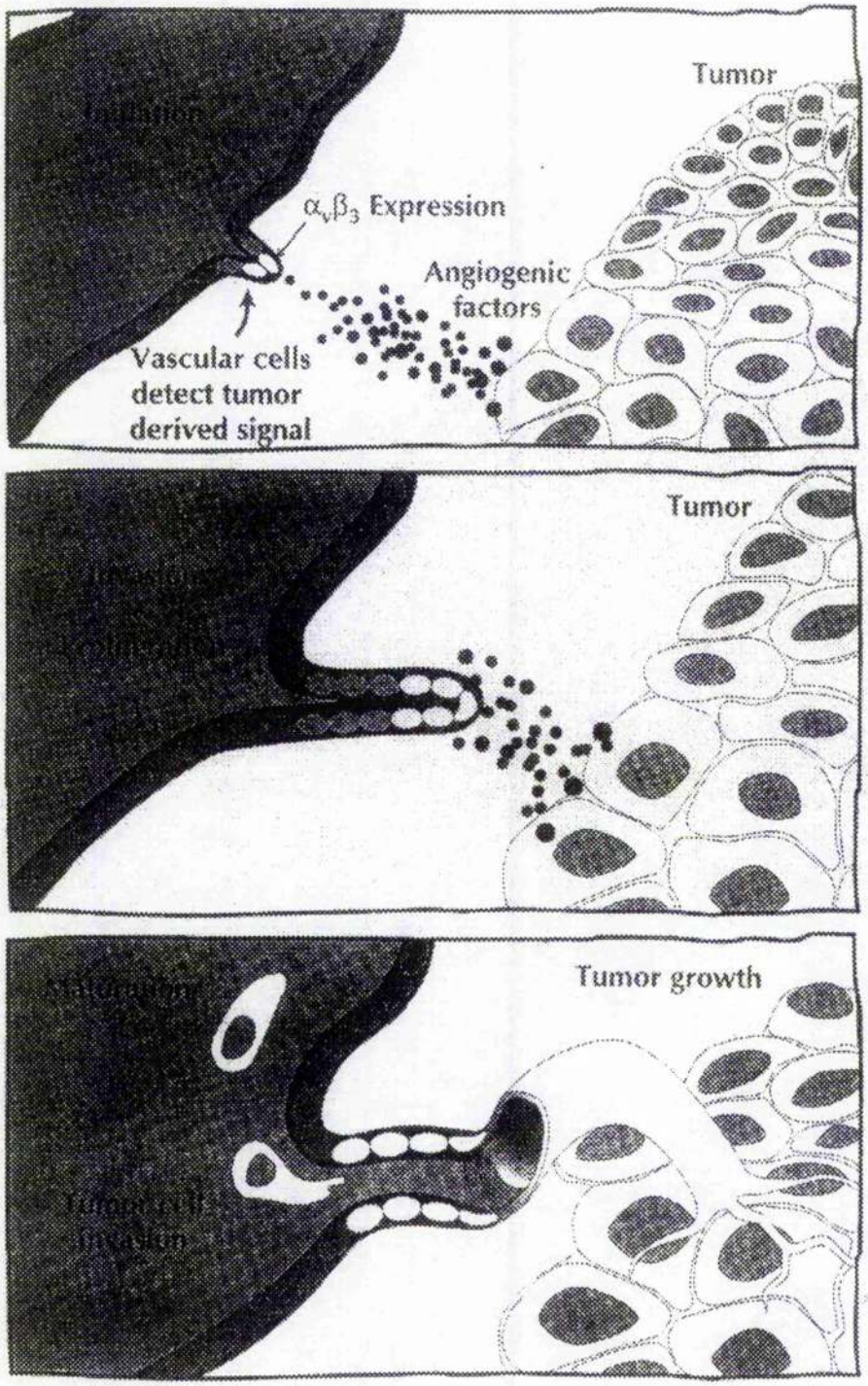


Figure 2.1. Steps in tumour-induced angiogenesis [Varner & Cheresh et al 1996].

Regulation of angiogenesis

There are complex interactions between tumour cells, endothelial cells and inflammatory cells. Secreted pro- and anti- angiogenic factors from the these cells act in an autocrine and paracrine manner [de Jong et al 1998a&b, Folkman et al 1996a]. Physiological control depends on the balance between these pro- and anti-angiogenic factors as well as a response to both oncogene activation and tumour suppressor gene loss [Volpert et al 1997]. Increased production of inducing factors is necessary for neovascularisation but not sufficient unless the inhibiting factors are reduced [Folkman et al 1995a&b, Dameron et al 1994, Hanahan et al 1996].

The angiogenic balance

In terms of the "balance" in normal physiological control, it is the cell phenotype that plays the major role, systemic activation of the VEGF receptor KDR/flk-1 selectively triggering endothelial cells which express an angiogenic phenotype [Ortega et al 1997].

(a) Role of oncogenes

The role of oncogenes, such as ras and myc, in tumour angiogenesis was first reported by Thompson et al [1989]. The oncogenes were able to induce phenotypic changes when introduced into 0.1% of the cells used to reconstitute the mouse prostate gland. Myc induced hyperplasia while ras induced dysplasia and angiogenesis. Ras and myc together induced primarily carcinomas.

Since, further studies have confirmed this role by examining mutant ras oncogene induction or up-regulation of VEGF/VPF in different cells types or systems [Volpert et al 1997, Rak et al 1995, Grugel et al 1995, Larcher et al 1996, Mazure et al 1996, Arbiser et al 1997, Lingen et al 1997, Enholm et al 1997, White et al 1997]. Inactivation of tumour suppressor genes, such as the p53 gene, have also been shown to downregulate anti-angiogenic factors, such as thrombospondin-1, resulting in increased neovascularisation [Dameron et al 1994]. Likewise, levels of thrombospondin-1 are also suppressed in the ras oncogene-transformed fibroblast [Zabrenetzky et al 1994, Sheibani et al 1996]. Oncogenes also interact under physiological conditions, the expression of VEGF/VPF of mutant neu oncogene-transformed NIH 3T3 fibroblasts being upregulated by hypoxia [Viloria-Petit et al 1997].

(b) Role of cytokines and inflammatory cells

During tumour progression, the interactions between tumour cells and endothelial cells are not static, but in constant flux. In pre-malignant cells, growth is inhibited by endothelial-derived cytokines (eg. IL-6, IL-1, TGF-beta). With disease progression, the sensitivity of tumour cells to these inhibitors is reduced, lost or "switched" to growth stimulation. At a later stage, the "balance" favors angiogenesis and tumorigenesis. These reciprocal interactions are mainly evident in melanoma and colorectal carcinoma [Rak et al 1994 & 96].

Inflammatory cells (eg. macrophages and neutrophils) influence certain stages of angiogenesis especially in hypoxic conditions [Harmey et al 1998, Takahashi et al 1996, Sunderkotter et al 1994, Harmey et al 1996, Knighton et al 1983]. It was shown that macrophage density is directly proportional to rate of tumour growth [Van Netten et al 1992]. The inflammatory cells produce substances which induce endothelial cell proliferation, migration and differentiation [Polverini et al 1977]. They produce monokines and proteases which affect the matrix and alter basement membrane composition respectively [Bouck et al 1996]. However, macrophages also produce thrombospondin-1 (TSP-1) which itself is anti-angiogenic [Bornstein et al 1995]. Angiogenic factors are produced and released by activated macrophages in the presence of hypoxia as mentioned earlier, altered pH, lactate, pyruvate as well as a variety of cytokines [Sunderkotter et al 1994].

In breast cancer, tumour-associated macrophages (TAM) and neutrophils enhance tumour angiogenesis and are associated with a poorer prognosis [Leek et al 1996, Polverini et al 1989, Mantovani et al 1994, Welch et al 1989]. Leek et al [1996] examined macrophage infiltration in invasive breast carcinoma using the Chalkley counting method. They reported a significant positive correlation between high vascular grade and increased macrophage index. There was a strong relationship between increased macrophage counts and reduced relapse-free survival and reduced overall survival. Furthermore, the macrophage count was an independent prognostic indicator. Breast cancer cell responsiveness to TNF-alpha produced by TAMs play a role in the regulation of TP expression by tumour cells and in their metastatic behaviour [Leek et al 1998]. Matrix

metalloprotease-9 produced by macrophages as well as by other cell types such as fibroblasts causes extracellular matrix degradation during tumour angiogenesis [Kurizaki et al 1998, Nielsen et al 1997].

In DCIS, two distinct types of inflammatory patterns have been described, type I showing perivascular clusters of B and T cells; and type II, diffuse clusters of macrophages and T cells [Lee AHS et al 1997]. The perivascular pattern is associated with poorer differentiation of DCIS. Different patterns of inflammation are associated with different patterns of vessels. Inflammatory mast cells have been reported to be involved in the regulation of angiogenesis during squamous epithelial carcinogenesis [Coussens et al 1999]. In hyperplasia and dysplasias, mast cells are conscripted to reorganize stromal architecture and hyperactivate angiogenesis, the infiltration of mast cells and activation of the matrix metalloproteinase MMP-9/gelatinase B coinciding with the angiogenic switch in premalignant skin lesions.

Recently, Leek et al [2000] carried out a study to localize VEGF protein in primary breast carcinomas and to determine its relationship to focal macrophages infiltration (macrophage index). They showed a positive correlation between neoplastic cell VEGF expression and macrophage infiltration (index), but not with microvessel density. VEGF expression was also inversely associated with EGFR. The author suggested VEGF expression may be an important factor in the recruitment of tumour-associated macrophages into breast carcinomas and may thus have an additional as well as indirect pathway of angiogenic stimulation.

The angiogenic switch

The theory of "The Angiogenesis Switch" during tumorigenesis was first hypothesized by Hanahan and Folkman et al [1996] [*Fig. 2.2*]. They showed that the "switch" occurs before tumour formation in three transgenic mouse models [*Fig. 2.3*]:

1. Expression of the Tag oncogene in the pancreatic islets and the development of islet cell carcinomas. An intermediate stage called the 'angiogenic islet' was then identified during the malignant transformation of carcinoma in-situ (CIS) into islet cell carcinoma. Histologically, there were capillary sprouting and endothelial cell proliferation.
2. Expression of the BPV-1 oncogenes in the dermis and the development of fibrosarcoma. Angiogenesis was evident in a late preneoplastic stage (aggressive fibromatosis) as well as end –stage fibrosarcomas.
3. Expression of the HPV-16 oncogenes in the epidermis and the development of squamous cell carcinoma. In the dysplastic stage, there were abundant capillaries converging on to the basement membrane separating stroma from dysplastic cells.

The "angiogenic switch" has also been reported in pre-malignant stages of two human cancers, breast carcinoma in-situ and cervical intraepithelial neoplasia [Hanahan et al 1996] [*Fig. 2.4*].

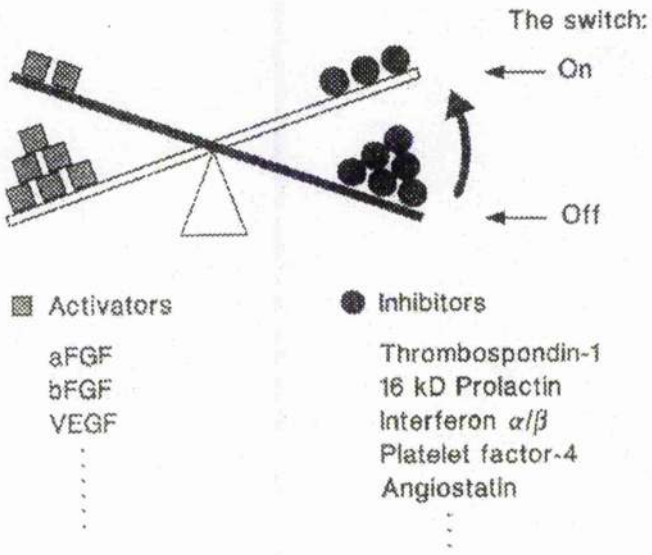


Figure 2.2. The balance hypothesis for the angiogenic switch [Hanahan & Folkman et al 1996].

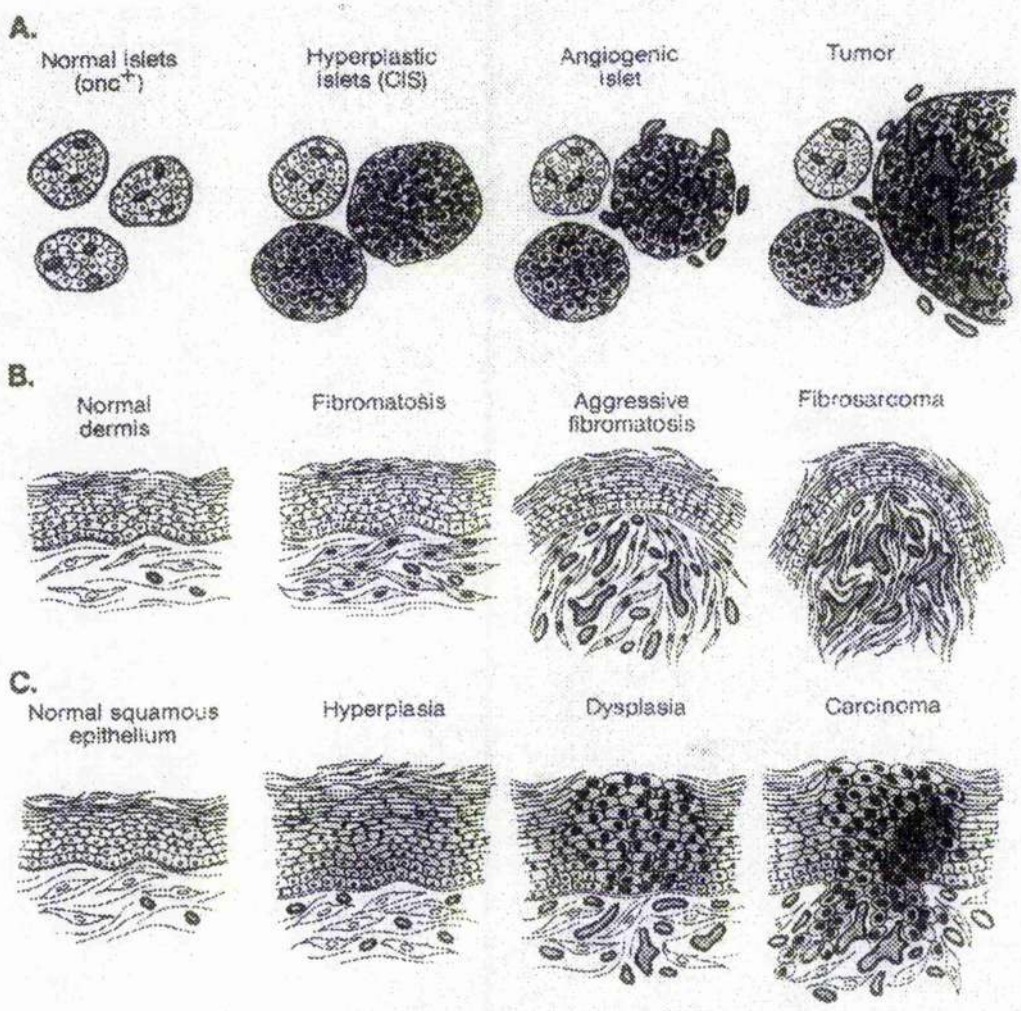


Figure 2.3. The angiogenic switch prior to tumour formation in three transgenic mouse models of tumorigenesis [Hanahan & Folkman et al 1996].

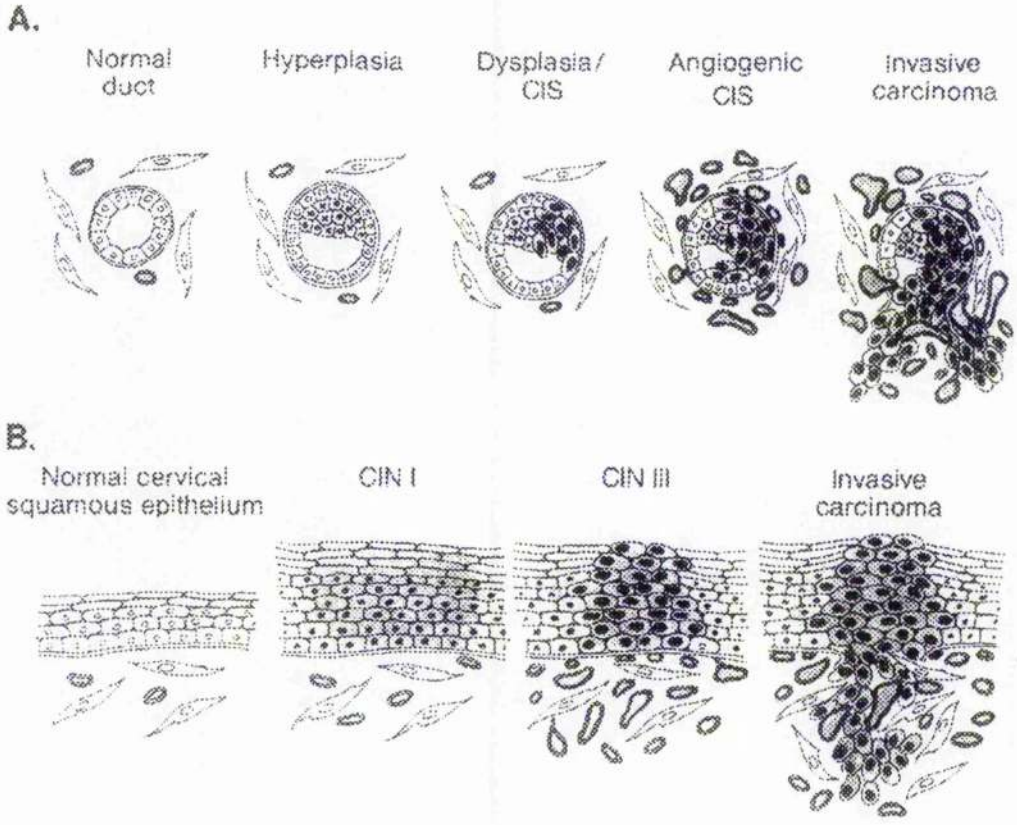


Figure 2.4. The angiogenic switch can be visualised in neoplastic lesions inferred to be progenitor stages to human breast (A) and cervical cancer (B) [Hanahan & Folkman et al 1996].

Interaction of endothelial cells with the environment

There are changes in the interaction of endothelial cells with the environment during angiogenesis in cancer [*Fig. 2.5*].

The expression of membrane receptors (tyrosine kinases) is increased. There is a different pattern of expressed receptors on stimulation by angiogenic factors, for example, receptors for integrins are altered in accordance with the changes that occur to the structure and composition of the matrix during angiogenesis [Brooks et al 1996].

The pattern of the ECM is altered by the proteolytic activity of the endothelial cells . At the early stage of angiogenesis, there is deposition of type I collagen and fibronectin in the ECM, which is followed by deposition of type IV collagen and laminin at the end of the process [Brooks et al 1996]. These activities are due to increased levels of MMP/PA and decreased levels of TIMP/PAI [Werb et al 1997, DeClerk et al 1997]. Degradation of the matrix promotes endothelial cells migration and releases angiogenic factors that are stored in the matrix. At the end of the process, the formation of a network of tubular structures is dependent on a balance of traction forces, created by the alteration of an elastic matrix by the endothelial cells [Sage et al 1994].

At an early stage of angiogenesis, cell junctions between endothelial cells are weakened and broken so allowing cell proliferation and migration. During formation of the blood vessels, the cell junctions are reformed.

There are modifications in the interactions with surrounding cells such as pericytes, smooth muscle cells and accessory cells. At an early stage of angiogenesis, there is no interaction between endothelial cells with these surrounding cells. At a later stage, the interaction is mediated by angiopoitin-2 [Lauren et al 1998, Hanahan et al 1997].

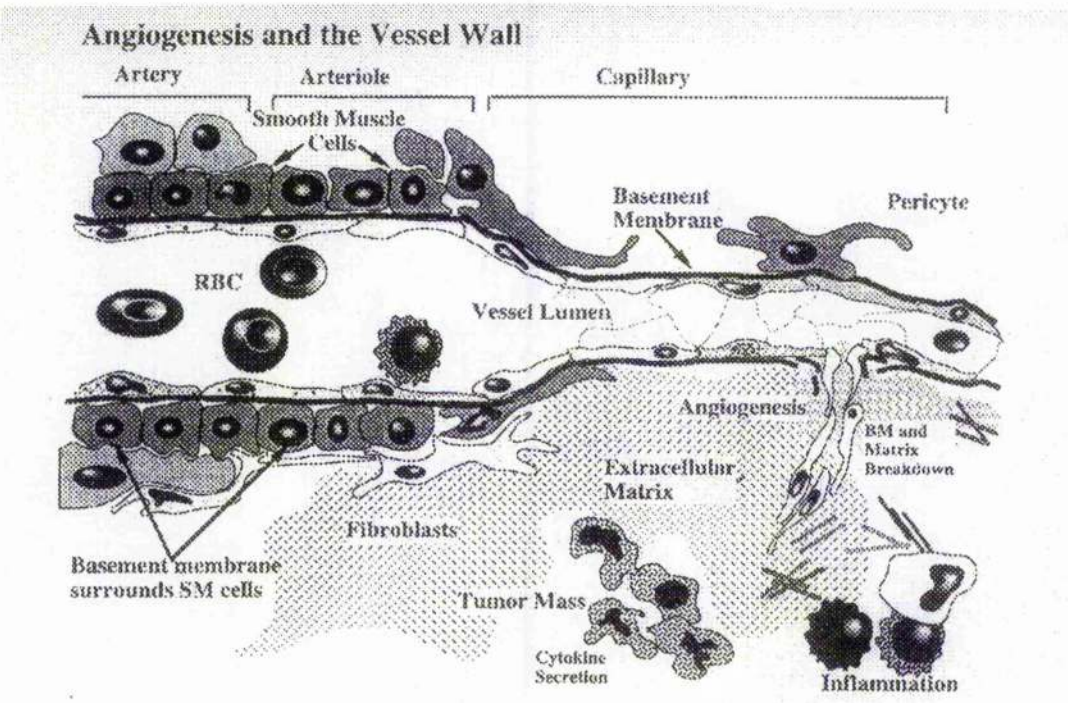


Figure 2.5. Angiogenesis occurs mainly in the capillaries. In response to cytokines stimulation, endothelial cells break down the basement membrane, migrates into the extravascular space, proliferate, and reorganize to form a new vessel [Goldberg & Rosen et al 1997].

Pro-angiogenic factors

Vascular endothelial growth factor (VEGF)

VEGF is expressed on a variety of normal cells and is secreted by a number of human tumour cell lines [Senger et al 1986]. It increases vascular permeability and is an endothelial cell- specific mitogen and angiogenic factor [Ferrara et al 1995, Marme et al 1996]. It binds exclusively to endothelial cell specific tyrosine kinase receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2) [De Veries et al 1992, Terman et al 1992], and it plays a role in the paracrine stimulation of angiogenesis.

Production of VEGF can be induced by PDGF [Colville-Nash et al 1997], TGF-beta [Pertovaara et al 1994], v-sarc oncogene [Mulkhopadhyay et al 1995], mutated p53 tumour suppressor gene [Mulkhopadhyay et al 1995], mutated ras oncogene [Rak et al 1995] and hypoxia [Namiki et al 1995]. Production is inhibited by wild-type p53 [Mulkhopadhyay et al 1995]. VEGF works synergistically with FGF-2 [Pepper et al 1992]. The activity is potentiated by hypoxic conditions [Minchenkoi et al 1994, Levy et al 1995, Grone et al 1995] through the up-regulation of the KDR receptor [Brogi et al 1996].

Structurally, VEGF is homologous with vascular permeability factor (VPF), which itself promotes protein extravasation [Dvorak et al 1995]. This process releases fibrin in the ECM [Senger et al 1996], which in turn promotes angiogenesis.

Study by Ferrara et al [1996] emphasize the key role played by VEGF in embryonic development. Heterozygous mutations inactivating the VEGF gene in mice result in embryonic lethality between day 11.5 and 12.5. Mutant embryos revealed dramatic deficits in angiogenesis and hematopoiesis as well as in the development of the cardiovascular and other systems. These findings indicate that VEGF is a most critical factor in vasculogenesis and angiogenesis, as other factors cannot compensate for even reduced levels of VEGF.

Acidic fibroblast growth factor (aFGF or FGF-1)

FGF-1 is found mainly in neural tissues [Relf et al 1997] and acts via both autocrine and paracrine pathways. It is essential in embryonic development and wound healing and is mitogenic for endothelial cells which in turn promotes angiogenesis [Folkman et al 1987, Thompson et al 1988, Klagsburn et al 1989, Thomas et al 1987]. FGF-1 is expressed in a variety of tumours [Relf et al 1997] but is believed to be suppressed by the angiogenesis suppressor gene which itself is downregulated during carcinogenesis [Folkman et al 1995b]. It has to be released into the ECM to bind with receptors to be angiogenic, however, it is usually confined to the cell of origin as it lacks the signal sequence for secretion [Mulkhopadhyay et al 1995].

Basic fibroblast growth factor (bFGF or FGF-2)

FGF-2 , an endothelial mitogen [Schweigerer et al 1987], is produced and released by capillary endothelial cells. It has been isolated in a variey of normal and tumour cells [Asahara et al 1995]. It acts in an autocrine and paracrine manner. It also acts synergistically with VEGF [Mulkhopadhyay et al 1995]. Like FGF-1, it has to be released to be angiogenic and yet is usually confined to the cell of origin in the absence of the secretory signal peptide [Mulkhopadhyay et al 1995]. It has been shown that angiogenesis is associated with a switch in the ability to secrete FGF-2 from cells during carcinogenesis in a fibrosarcoma model [Kandel et al 1991].

The administration of FGF-2 increases vascularity and promotes tumour growth [Gross et al 1993]. The binding of FGF-2 can be blocked by suramin [Danesi et al 1993], and the blockade of its receptor can suppress tumour growth [Hori et al 1991].

Platelet-derived endothelial cell growth factor (PD-ECGF)

PDECGF is structurally homologous to thymidine phosphorylase [Furukawa et al 1987]. It is produced by platelets [Miyazono et al 1987] and vascular smooth muscle cells [Usuki et al 1989], and acts as an endothelial mitogen. It has a role in endothelial cell migration [Risau et al 1992] and differentiation [Klagsburn et al 1991] as well as during the blood clotting cascade.

Its expression in human breast cancer cells has been shown to correlate with microvessel density [Fajardo et al 1992, Folkman et al 1996, Fox et al 1996]. TP expression is 260-fold higher in invasive bladder cancer [O'Brien et al 1995] and 27-fold higher in invasive breast carcinoma than normal tissue [Patterson et al 1995]. In ovarian carcinoma, areas of increased expression of TP have been associated with high blood velocity as measured by colour Doppler imaging [Reynolds et al 1994].

In most normal organs, TP is most highly expressed in resident tissue macrophages, and may be part of a mechanism controlling angiogenesis in response to injury [Fox et al 1995]. TP is not a classic type of pro-angiogenic factor in tumours as it is thought to exert its angiogenic effects via the metabolites of its enzymatic activity [Moghaddam and Bicknell et al 1992]. DNA released from dying cells and engulfed in apoptotic nuclei may be degraded to thymidine, which can freely enter cells, including tumour cells and TAMs, which then metabolize thymidine via TP to angiogenically active metabolites such as deoxyribose-1-phosphate. TP also catalyses the phosphorolytic cleavage of the chemotherapeutic pro-drug 5'-deoxy-5'-fluorouridine (5'-DFUR) to its therapeutically active form 5-fluorouracil (5-FU) [Patterson et al 1995], and it is thought that resistance to 5'-DFUR therapy may be due to low TP activity in some tumours.

Expression of TP in tumour cells can be modulated by hypoxia [Shweiki et al 1992] and up-regulated by tumour necrosis factor-alpha, interleukin-1 and interferon-gama, and basic fibroblast growth factor [Ho et al 1990, Eda et al 1993]. It is suggested that tumours might directly regulate TP expression through autocrine and paracrine pathways and that

cytokines might also recruit macrophages rich in TP that may themselves, also, through paracrine loops, augment tumour cell TP.

On immunohistochemical examination, the location of TP expression varies. In bladder [Mitutani et al 1997] and lung cancers [Giatromanoraki et al 1997, Koukourakis et al 1997], TP expression was observed mainly in cancer cells. By contrast, however, it was observed mainly in the infiltrating cells around the stroma of gastric [Takebayashi et al 1996] and colorectal cancer [Takahashi et al 1996]. Nagaoka et al [1998] showed both breast cancer cells and normal or benign breast epithethelial cells expressed TP, and that expression tended to be more intense in the former [*Fig. 2.6*]. TP has been shown to be prognostic indicator in colorectal, gastric and breast cancers [Takebayashi et al 1996, Takahashi et al 1996, Nagaoka et al 1998].

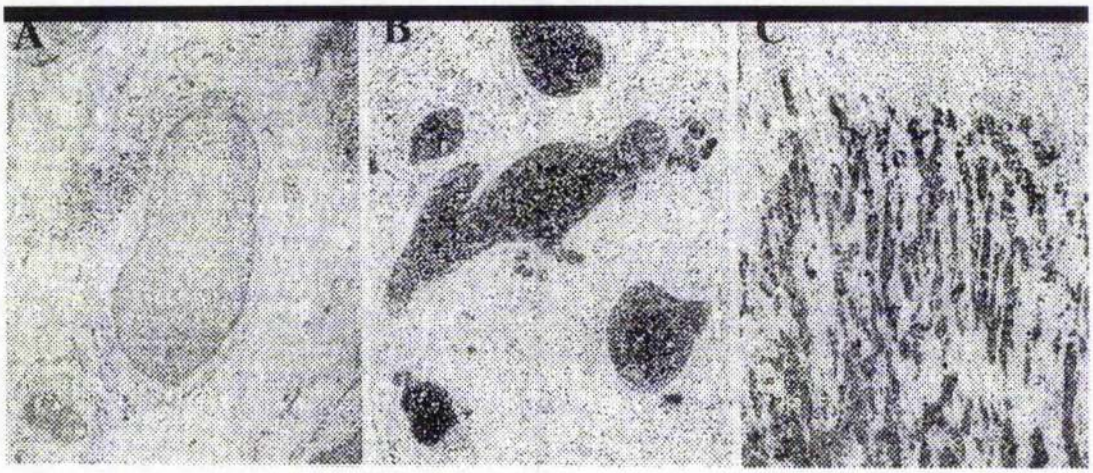


Figure 2.6. TP expression in breast carcinomas. DCIS lesions negative (A), and positive (B) for TP. (C) Invasive ductal carcinoma with higher TP expression at the invading border [Goldberg & Rosen et al 1997].

Transforming growth factor beta (TGF-beta)

TGF-beta is produced by macrophages and platelets. It is chemotactic to macrophages, angiogenic in vivo and promotes wound healing [Thompson et al 1991, Roberts et al 1986, Sporn et al 1987, Shah et al 1995, Pierce et al 1992]. It also upregulates the production of matrix and integrin during angiogenesis, acting in a biphasic manner, low levels having a stimulatory effect and high levels an inhibitory effect. [Pepper et al 1993].

Epidermal growth factor (EGF)

EGF is a mitogenic and angiogenic growth factor. It is produced by tumour-associated macrophages in breast carcinoma [O'Sullivan et al 1993].

Integrins

The integrin family consists of 15 alpha and 8 beta subunits that are expressed in over 20 different alpha/beta heterodimeric combinations on cell surfaces. Integrins bind to extracellular matrix proteins or cell-surface immunoglobin family molecules through short peptide sequences in the ligands. Combinations of different integrins on cell surfaces allow cells to recognize and respond to different extracellular matrix proteins. Integrins facilitate cellular adhesion to and migration on the ECM proteins [Cheresh et al 1993, Hynes et al 1992] as well as regulate cellular entry into and withdrawal from the cell cycle [Dike et al 1988, Guadagnow et al 1993, Varner et al 1995]. Prevention of integrin-ligand interaction suppresses cellular growth and induces apoptotic cell death [Varner et al 1995, Meredith et al 1993, Montgomery et al 1994].

The migration of endothelial cells into a tumour requires adhesion to proteins of the extracellular matrix, including thrombospondin, vitronectin, fibronectin, laminin and collagen [Montgomery et al 1994, Brooks et al 1994b, Good et al 1990]. Laminin is implicated in angiogenesis as the deposition of matrix by newly forming capillaries appears to be critical in angiogenesis. Adhesion molecules, of the selectin family and their ligands sialyl Lewis X/A have been implicated in the differentiation phase of angiogenic lumen formation [Bischoff et al 1995, Nguyen et al 1993] and inhibition of the molecules by monoclonal antibodies has been shown to decrease angiogenesis [Folkman et al 1995b].

Integrin alpha5beta3 is a receptor for a wide variety of extracellular matrix proteins. It is the most 'promiscuous' member of the integrin family, mediating cellular adhesion to vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willebrand factor, osteopontin, and other molecules [Cheresh et al 1987 & 93, Leavesley et al 1992]. It is strongly expressed on cytokine-activated endothelial or smooth muscle cells explaining its intense expression on blood vessels in granulation tissue and tumours [Montgomery et al 1994, Brooks et al 1994b, Enenstein et al 1994, Sepp et al 1994]. The expression is upregulated by bFGF, TNF-alpha and human tumours in vitro and during wound healing in vivo [Montgomery et al 1994, Brooks et al 1994b]. Alpha5beta3 has angiogenic and anti-apoptotic effects by suppressing wild-type p53 activity and increasing the bcl2/bax ratio [Naylor et al 1993]. Antagonists of integrin alpha5beta3 not only suppress angiogenesis in vitro without affecting adjacent blood vessels, but also induce tumour regression up to five fold [Brooks et al 1994b].

Tumour necrosis factor alpha (TNF-alpha)

TNF-alpha is produced by tumour-associated macrophages, especially in breast and ovarian carcinoma [Pusztai et al 1994, Scannell et al 1993] and its expression is up-regulated by hypoxia [Niedbala et al 1991]. It activates endothelial proteases [Fajardo et al 1992], induces granulocyte-macrophage colony stimulating factor production, increases adherence of endothelial cells, and has a role in the blood clotting cascade. It also acts in a dose-dependant manner being angiogenic at low concentrations and vice versa [Leek et al 1994].

Interleukin-8 (IL-8)

IL-8 was first detected in lung tumour from which extracts were shown to be angiogenic in the rabbit cornea angiogenesis assays [Smith et al 1994] and inhibited by anti-IL-8 antibodies [Smith et al 1994]. Recombinant human IL-8 has been reported to stimulate new blood vessel formation in a rabbit cornea assay [Strieter et al 1992].

Endotoxin

Endotoxin, an angiogenic factor, is found in the cell wall of most Gram-negative as well as some Gram-positive bacteria. It up-regulates the production of VEGF, bFGF, TGF-beta, TNF, IL-1, IL-6 and other molecules [Mattsby-Baltzer et al 1994, Watson et al 1994, Li WW et al 1991].

Anti-angiogenic factors

Thrombospondin (TSP)

Thrombospondin, a matrix-bound glycoprotein, is found in endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, monocytes, macrophages and other cell populations [Raugi et al 1982]. It is a potent anti-angiogenic factor, controlling the motility, adhesiveness and proteolytic activity of endothelial cells [Bussolino et al 1997]. During carcinogenesis, the levels of TSP are downregulated [Folkman et al 1992b, Rastinejad et al 1989, Bouck et al 1990]. Angiogenesis is regulated by wild-type p53 through upregulation of TSP, inactivation of the gene resulting in decreased levels of TSP [Rastinejad et al 1989]. In inflammation, TSP potentiates the angiogenic effect of bFGF and endotoxin [BenEzra et al 1993]. Recently, it was shown that in vivo transfection of human breast cancer cells with DNA encoding TSP-1 resulted in a decreased microvessel density [Weinstat-Saslow DL et al 1994].

Angiostatin

Angiostatin is a fragment of plasminogen, which itself is not anti-angiogenic [O'Reilly et al 1994] which suppresses endothelial cell proliferation and tumour growth. It is produced during the development of solid tumours and has been found to suppress growth of metastases [O'Reilly et al 1994]. Systemic administration of angiostatin has been shown to cause tumour regression in a variety of animal models [O'Reilly et al 1996].

Endostatin

Endostatin is a fragment of collagen XVII and acts in a similar way to angiostatin. It blocks endothelial cell proliferation, matrix remodeling and the activity of collagenase. Recombinant endostatin has been shown to inhibit angiogenesis, suppressing growth of primary and secondary tumours [O'Reilly et al 1997]. In treated tumours, angiostatin appears to increase apoptosis but not the proliferation of the lesions [O'Reilly et al 1994].

Angiogenesis in breast carcinoma

To date, there is evidence that breast carcinoma is angiogenesis-dependent:

Indirect evidence (experimental studies)

Brem et al [1977] assessed the angiogenic response induced in the rabbit iris by the transplantation of xenografts of normal, hyperplastic and chemically-induced neoplastic mammary tissues. They showed that angiogenesis preceded the transforamation of mammary hyperplasia to carcinoma. Using a similar experimental model, Jensen et al [1982] showed that angiogenesis is involved in human breast cancer transformation and progression. The angiogenic activity of normal breast tissues from cancerous breasts was twice that of non-cancerous ones. Studies by McLesky et al [1993] and Costa et al [1994] demonstrated that fibroblastic growth factors (FGF) 3 and 4 induce angiogenesis followed by rapid tumour growth and metastasis in human breast cancer cell lines. Zhang et al [1995] reported that human breast carcinoma cells transfected with vascular endothelial growth factor (VEGF) show high vascularity and growth.

Overall, the above studies demonstrate that different peptides induce angiogenesis and facilitate progression and metastasis in vivo.

Indirect evidence (clinical studies)

Using a rabbit iris assay, Brem et al [1978] demonstrated that angiogenesis is a potentially useful marker of preneoplastic lesions and that it is associated with most human breast cancers. An iris angiogenic response was recorded in 30% of tissue fragments from biopsies of human hyperplastic breast lobules compared with 65% of those from invasive breast carcinoma. They also found that the frequency of transformation was higher in the subgroup of hyperplastic lesions with angiogenic activity. By examining the histological sections of breast cancer stained with a specific endothelial marker, Weidner et al [1991] showed that it is possible to observe in situ carcinomas in the prevascular and vascular phase.

In another study, Guinerbretiere et al [1994] suggested that the risk of women with fibrocystic disease developing invasive breast carcinoma increases with the degree of vascularisation within the lesion. Hildenbrand et al [1995] found a significant association between urokinase plasminogen activator (uPA) levels and intratumoural vascularisation in breast cancers. McCulloch et al [1995] demonstrated that the extent of tumour cells shedding into effluent venous blood during breast cancer surgery are associated with the microvessel count of primary tumour, confirming preclinical studies indicating that angiogenesis facilitates metastases [1994].

Direct evidence (experimental studies)

Studies have shown that inhibition of angiogenesis can cause regression of human breast cancer in animal models. Weinstat-Saslow et al [1994] transfected TSP-1 complementary DNA into a metastatic human breast cancer cell line. The genetic modulation inhibited angiogenesis in vivo along with a reduction of tumour growth and metastasis compared with untreated controls. Brooks et al [1995] reported that systemic treatment with an antibody (LM609) against the alphaVbeta3 integrin reduced angiogenesis and tumour growth in a severe combined immunodeficient (SCID) mouse/human chimera bearing subcutaneous transplant of human breast cancer. Folkman et al [1996] administered angiostatin to nude mice bearing diverse solid human tumours. They demonstrated an induced tumour regression through inhibition of angiogenesis and prolonged tumour dormancy.

In conclusion, the current evidence suggests that angiogenesis is necessary, but not sufficient alone, for breast cancer growth, and that inhibition of angiogenesis is a potential therapeutic modality along with conventional anticancer therapies.

Angiogenesis and DCIS

With the advent of screening mammography, the reported incidence of DCIS has increased significantly. However, despite the importance of various pathological features, the prognosis for individual patients cannot be accurately predicted and the optimal treatment for DCIS remains unresolved. Breast conservation with or without adjuvant radiotherapy is associated with a significant risk of recurrence, of which 50% will be invasive carcinoma [Silverstein et al 1995a&b & 96, Solin et al 1993 & 94 & 96]. Several studies have studied neovascularisation and in-situ breast cancer.

Guidi et al [1994] reported two types of vascular pattern, a diffuse pattern (Pattern I) and a dense vascular rim around the involved ducts (pattern II) [*Fig. 2.7*]. This finding was confirmed by Engels et al [1997a], who proposed two angiogenic pathways to account for these morphological findings: Pattern II is thought to result from angiogenic factors being secreted by tumour cells (autocrine pathway) and Pattern I to be mediated via the recruitment of accessory cells, releasing proangiogenic factors themselves (paracrine pathway). Engels et al [1997b] in another study, showed a significant correlation between Thymidine Phosphroylase (TP) and the presence of the type II vascular pattern, although there was no correlation between the vascular patterns or expression of TP with relapse-free survival. Sales et al [1999] reported that there was a significant association between MVD, histological features and nuclear area in both DCIS and ductal hyperplastic epithelial lesions. The two types of angiogenic patterns were again identified, a type II vascular pattern also being seen in hyperplasia.

The association between vascularity and inflammation in DCIS has been studied by Lee AHS et al [1997]. They found the Type I pattern was associated with perivascular clusters of inflammatory cells while pattern II was associated with diffuse inflammation, supporting the hypothesis that vascular patterns result from paracrine or autocrine factors released by the DCIS/inflammatory cells. More recently, they have also reported that the vascular pattern I in DCIS is associated with TP expression in the perivascular inflammatory cells and tumour cells [Lee AHS et al 1999].

Valtola et al [1999] examined the role of VEGFR-3 and its ligand VEGF-C in DCIS. They noted that VEGFR-3 was prominent in vessels adjacent to the basal lamina of affected ducts and its ligand was localized in the cytoplasm of the tumour cells. These results suggest that the role of VEGF-C involves modifying the permeabilities of both blood and lymphatic vessels.

High levels of expression of VEGF mRNA and its receptor mRNA have been found in both tumour cells and microvessel endothelial cells [Brown et al 1995]. Guidi et al [1994] showed that there was a significant correlation between a high Ki-S1 proliferation index and high microvessel counts [Solin et al 1994] as well as VEGF expression [Morales et al 1995]. Parham et al [1992] also reported increased levels of TGF-alpha expression in DCIS.

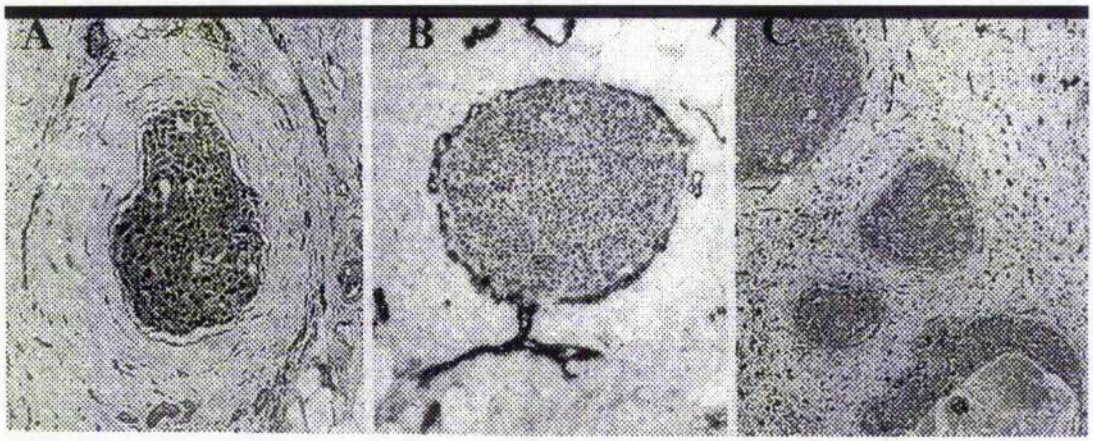


Figure 2.7. Different vessel patterns in DCIS. (A) Distant rim and (B) dense rim of microvessels around a DCIS duct close to the basement membrane. (C) High vascular density between DCIS lesions. All vessels are stained with anti-vWF antibodies [Goldberg & Rosen et al 1997].

Angiogenesis and hormonal control

Oestrogen added to human umbilical vein endothelial cells (HUVECs) grown in an oestrogen-free medium culture cause an increase in endothelial cell proliferation, cell attachment to laminin and enhances the ability of HUVECs to organize into tubular networks, these are all features that are important in angiogenesis [Morales et al 1995]. Oestrogen may also have a role in preservation of endothelial cells by inhibiting apoptosis [Sypridopoulos et al 1997], the apoptosis caused by TNF-alpha on cultures of HUVECs being reversed by the addition of oestrogen.

Partial oestrogen antagonists including Tamoxifen, and pure antagonists ICI 164, 384 and ICI 182, 780 have been shown to inhibit angiogenesis in the chick egg chorio-allantoic membrane [Gagliardi et al 1993]. It has also been shown that the growth of porcine pulmonary artery endothelial cells is stimulated by bFGF and VEGF, and inhibited by Tamoxifen [Gagliardi et al 1995], suggesting that the anti-angiogenic effect of anti-oestrogens is mediated through direct inhibition of growth factor stimulated endothelial cell growth. Male rats treated with Tamoxifen injected with VEGF-impregnated matrigel show a reduction in gel MVD, suggesting that tamoxifen affects VEGF-mediated angiogenesis [McNamara et al 1998]. Angiogenesis is also decreased in ovariectomized mice in the presence of bFGF, suggesting that the angiogenic effects of bFGF are promoted by oestrogen [Morales et al 1995]. Therefore, as well as enhancing the action of angiogenic factors, oestrogen receptors may mediate the control of angiogenesis [Sypridopoulos et al 1997].

Angiogenesis and therapeutic implications

The understanding that tumour growth is angiogenesis dependent has led to the development of agents directed towards the tumour vasculature [*Table 2.1*]. These have yielded striking successes in experimental models, which if translated into the clinical setting may have a substantial effect on patient survival.

Neutralising angiogenesis promoters

This treatment interferes with pro-angiogenic factors produced by tumours. Reported models include:- targeting vascular endothelial growth factor with a monoclonal antibody [Kim et al 1993], viral delivery of dominant negative receptors to VEGF and preventing the release of active fibroblast growth factor 2 from the extracellular matrix by eradicating a binding protein necessary for its release [Czubayko et al 1997].

Endogenous angiogenesis inhibitors

The angiogenic stimulus produced by tumours can be countered by means of endogenous inhibitory proteins. This can be carried out either by direct supply of angiogenic inhibitors such as angiostatin and endostatin [O'Reilly et al 1994 & 96 & 97] or by gene transfer of DNA coding for angiogenic inhibitors such as angiostatin and platelet factor 4.

Endothelial cell targets

Specific markers for tumour endothelial cells can be used to direct a toxin or antibody to the tumour vasculature causing tumour infarction by inducing coagulation. The possible targets are VEGF-receptor complexes, Endoglin and intergrin alpha5beta3. The latter is expressed only on proliferating vessels in healing wounds and in tumours. The antibody promotes tumour regression by inducing endothelial cell apoptosis [Brooks et al 1994a].

Synthetic angiogenic inhibitors

This approach inhibits tumour angiogenesis with drugs that specifically prevent endothelial division. These medications can act synergistically with conventional cytotoxic treatment and with "hypoxia activated" cytotoxic drugs.

Mechanisms	Target	Therapy
Block of proteolytic pathways in extracellular matrix	Metalloproteinases	Metalloproteinase inhibitors
Block of adhesion molecules	Beta1, alphaVbeta3, alphaVbeta5 integrins	Neutralising antibodies
Block of endothelial cell migration	*In vitro* tests for cell migration	Linomide
Block of proliferating endothelial cells	Activated endothelium	Specific growth inhibitors
Block of angiogenic peptides	VEGF, bFGF, etc.	Growth factor inhibitors, neutralising antibodies
Gene therapy	TSP-1, angiostatin & platelet-factor 4	Transfection of tumour cells with gene encoding angiogenic inhibitors

Table 2.1. Anti-angiogenic therapy: possible targets and mechanisms of action [Gasparini et al 1996].

Conclusions

There have been many studies of the process of angiogenesis in carcinoma but most, not surprisingly, have focused on invasive disease. Some, however, have investigated DCIS of the breast or other precursor breast lesions [Lee AHS et al 1997&99, Guidi et al 1994&97, Engles et al 1997a&b, Sales et al 1999, Valtola et al 1999, Brown et al 1995] where 2 patterns have been described: diffuse stromal vascularity and a vascular rim around the involved ducts. Several studies have shown that various pre-malignant lesions of the breast can induce angiogenesis in animal experimental systems and in the human breast [Lichtenbeld et al 1998, Gimbrone et al 1976, Brem et al 1977, Maiorana et al 1978, Strum et al 1983, Heffelfinger et al 1996]. Little work has been done in angiogenesis in DCIS of the breast but there are tantalizing glimpses suggesting that this may be important. The mechanisms involved in the angiogenesis in invasive disease may be applicable to this pre-invasive breast pathology.

Chapter 3

Assessment of Vascularity

Microvessel density/ hotspots

Weidner et al [1991] introduced a method of identifying hotspots (areas with high vascular density) in breast carcinoma. The rationale is that there is an active relationship between the tumour and the endothelial cells in these hotspots. The functionality of endothelial cell in terms of proliferation, differentiation and migration plays an important role in tumour angiogenesis. MVD is believed to reflect the intensity of tumour angiogenesis as well as carrying useful insights on the functionality of the endothelial cells.

The method first involved selecting the appropriate blocks for further study by scanning sections stained with haemotoxylin and eosin. The blocks selected were then immunostained with an endothelial marker (eg. anti-factor VIII antibody) and the hotspots were identified by analyzing the section on low magnification (10-100x) [*Fig. 3.1*]. The hotspots were usually localized around the peripheral tumour margin. The counting was carried out at a higher magnification with a proposed field size of 0.74 mm2. A countable microvessel included any stained endothelial cell or cell cluster clearly separated from adjacent microvessels, tumour cells and other connective tissue components. A lumen or the presence of red blood cells was not required. Single cell sprouts and larger vessels were included. In cases where clusters give the impression of being part of one vessel these were counted as separate microvessels. The following studies have all used hotspot counting to assess MVD.

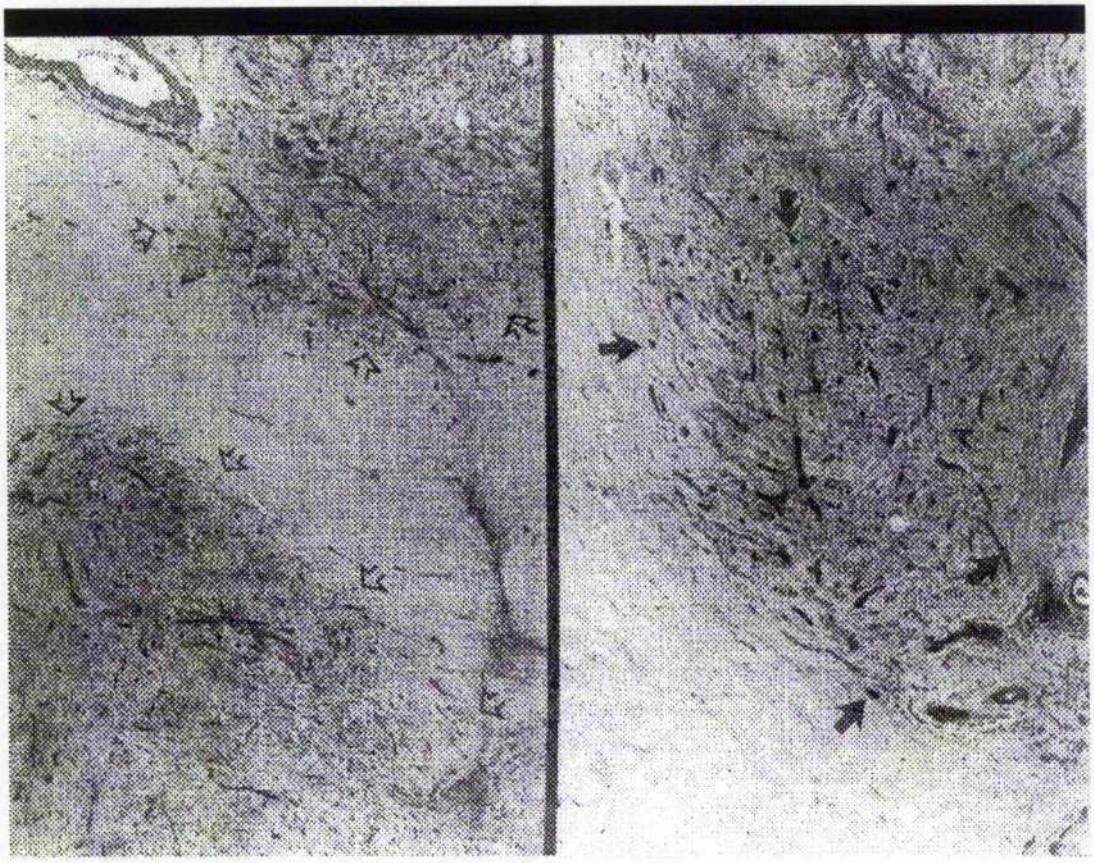


Figure 3.1. Breast carcinoma with microvessels highlighted with anti-vWF antibodies. (Left) Two foci of invasive carcinoma are shown (open arrows). Microvessels are represented by dark clusters, which stand out from carcinoma cells and connective tissue. (Right) Carcinoma with high angiogenesis. Solid arrows indicate representative microvessels [Weidner & Folkman et al 1996].

MVD and its relationship to angiogenic factors

Li et al [1994] measured bFGF in the cerebrospinal fluids of children with various brain tumours and correlated increasing fluid levels with greater MVD and increased risk of recurrence. In gliomas and meningiomas, there was an association between VEGF mRNA expression and MVD [Samoto et al 1995]. Similar findings have been seen with renal and cervical tumours. In human renal cell carcinomas grafted into a murine model, Berger et al

[1995] reported a correlation between MVD and VEGF mRNA expression. Dobbs et al [1997] showed a correlation between MVD and VEGF expression in CIN and SCC of the cervix. Furthermore, there were significant increases in MVD and VEGF expression from the progression from normal cervix through CIN I to CIN III to invasive SCC.

The degree of VEGF mRNA and Thymidine Phosphorylase expression in perivascular inflammation were significantly associated with MVD in DCIS [Guidi et al 1997, Lee et al 1999]. Toi et al [1995a] reported a significant correlation between MVD and VEGF as well as PdECGF expression in invasive ductal carcinoma. A similar association between MVD and VEGF expression was also shown in early stage breast cancers [Toi et al 1995b]. Multivariate analysis confirmed that the former had an independent predictive value for relapse-free survival (RFS), whereas VEGF expression had a similar predictive value when univariate analysis was performed. These studies suggest that MVD provides important information on the degree and functionality of the tumour vasculature, as well as quantifying the vascular morphology.

MVD and uPA/PAI-1 & MMP levels

Urokinase plasminogen activator (uPA) is an extracellular proteolytic enzyme which is produced by endothelial, tumour and inflammatory cells. The migration of endothelial cells is facilitated by the secretion of collagenases, urokinases, and plasminogen activator [Pepper et al 1987, Fox et al 1993, Moscatelli et al 1981]. The degradative enzymes facilitate the spread of tumour cells into and through the adjacent fibrin-gel matrix and connective tissue stroma. Both uPA and plasminogen activator inhibitor-1 (PAI-1) have

independent prognostic values in breast cancer [Janicke et al 1993]. There was a correlation between MVD and the uPA/PAI-1 levels and pro MMP-9 in human breast cancer tissues [Hildebrand et al 1995, Kurizaki et al 1998]. These extracellular proteolytic enzymes activity may contribute to tumour cell invasion and metastases to distant sites. Since activated endothelial cells produce uPA, this represents part of the reciprocal paracrine interaction between tumour and host cells. A higher MVD might represent a more extensive intercellular interaction at the interface between tumour and host.

MVD and tumour cell shedding

McCulloch et al [1995] investigated the relationship between the risk of tumour cell shedding and MVD of operable breast cancer in 16 patients. Before, during and one day after the operation, blood samples obtained via a central venous catheter which was fixed in the ipsilateral proximal subclavian vein. The samples were cytospun and then stained with appropriate cytokeratin antibodies that would detect any epithelial cells. Circulating cells were found in 1 patient before, 6 during, and none after operation. The results suggested that the risk of tumour cell shedding was related to MVD.

MVD and intratumoral tissue plasma volume

The tumour plasma volume can be assessed by means of magnetic resonance imaging (MRI) with the injection of contrast medium [Van Dijke et al 1996]. It was noted that the estimated plasma volume had an exponential relationship with the MVD. This study, along with other related literature mentioned earlier, strongly support the idea that MVD is a reliable measure of tumour angiogenesis.

MVD and prognostic values

Even though Brem et al [1972] were among the first to put forward the idea that there might be a correlation between MVD and the tumour grade and invasive characteristics, it was Srivastava et al [1988] who provided experimental evidence that there was a relationship between the tumour vascularity and the risk of metastasis in human solid tumours by studying cutaneous melanoma. The latter studied the vascularity of 20 intermediate-thickness skin melanomas. Microvessels were stained with *Ulex europaeus-1* agglutinin conjugated with peroxidase, and the stained histology sections analysed with a semiautomatic image analysis system. The 10 patients who developed metastases had a vascular area at the tumour base that was more than twice that seen in the 10 cases without metastases. Patient characteristics, Breslow's tumour thickness and Clark's level of invasion were similar in the two groups.

A number of studies have shown that MVD is associated with tumour aggressiveness (including the risk of metastasis) and patient survival. The associations have been shown in patients with melanoma [Srivastava et al 1986&88, Smolle et al 1989, Fallowfield et al 1991, Barnhill et al 1993, Vacca et al 1993, Graham et al 1993], central nervous system tumours [Li et al 1994], multiple myeloma [Vacca et al 1994], head and neck squamous-cell carcinomas [Mikami et al 1993, Gasparini et al 1993, Albo et al 1994, Williams et al 1994], non-small-cell lung carcinomas [Macchiarini et al 1992&94, Yamazaki et al 1994, Yuan et al 1995], breast carcinomas [Weidner et al 1991&92, Bosari et al 1992, Horak et al 1992, Gasparini et al 1994a&b, Gasparini et al 1995a&b, Obermair et al 1994a&b],

gastrointestinal carcinomas [Saclarides et al 1994, Maeda et al 1995], transitional-cell carcinoma of the bladder [Jaeger et al 1995, Bochner et al 1995], testicular germ-cell malignancies [Olivarez et al 1994], prostate carcinoma [Weidner et al 1993, Fregene et al 1993, Brawer et al 1994, Vesalainen et al 1994], cervical squamous cell carcinoma [Wiggins et al 1995] and ovarian carcinoma [Hollingsworth et al 1995]. In some of the above studies, MVD was shown to have independent prognostic value, confirmed by multivariate analysis.

MVD also has a place in predicting the response or resistance to treatment and to outcome or survival after adjuvant treatment. Kohno et al [1993] reported that cervical carcinomas with high vascularity were more responsive to hypertensive intra-arterial chemotherapy. Zatterstrom et al [1995] noticed that head and neck squamous-cell carcinomas with a higher vascular count were more responsive to radiotherapy. Jacquemier et al [1998] investigated the prognostic value of tumour vascularity in patients with breast carcinoma undergoing conventional adjuvant chemotherapy. The study showed that the chemotherapy did not reduce the high prognostic value of microvessel count (MVC) and suggested that MVC may predict the degree of resistance to chemotherapy. Gasparini et al [1996] showed that MVD added significant prognostic information to ER status in predicting the outcome after adjuvant Tamoxifen treatment in node positive breast cancer patients. The authors found that the outcome for those patients with high MVD was poor after therapy, irrespective of the ER status.

Endothelial markers

Vascular endothelial cells have an important role in coagulation, inflammation, immunity, regulation of vascular tone and in a wide variety of synthetic and metabolic functions. Endothelial cells also have a pivotal role in immunological diseases, angiogenesis in tumours, transplantation rejection, atherogenesis and placentation [Fajardo et al 1989].

The endothelial cells of microvessels can be identified using immunohistochemical methods. There are two major categories of endothelial markers: pan-endothelial cell markers or those that only stain proliferating endothelium [*Table 3.1 &3. 2*]. Both might also stain pericytes and other constituents of the basal lamina. In comparison with the activated endothelial cell-specific antibodies, the pan-endothelial cell-specific antibodies might not represent the dynamic angiogenic capacity of a tumour. The corresponding antigens of the former endothelial markers are usually lost during the tissue fixation or paraffin embedding, at the expense of the sensitivity of the antibodies.

Antibody	Sensitivity	Specificity	Lymphatic
Anti-CD31	Small/large vessels, equal staining in normal & tumour tissue	Non-specific in cryostat section	No
UEA	As above	Low	Yes
Anti-CD34	As above	High	Proportion
PAL-E	As above	High	Proportion
BMA120-BW200	As above	High	?
EN-4	Small/large vessels, restricted distribution pattern	Stromal staining, infiltrate cell staining, occasional tumour staining	?
Anti-8-Rag	Large vessels: sensitive, capillaries: variable & focal, P Ab more sensitive	M Ab: High P Ab: Stromal staining	Proportion
Anti-CD36	Intense staining of small vessels – variable/weak	Monocyte and platelet staining	?

Table 3.1. Endothelial cell-specific antibodies [Vermeulen et al 1996a].

Antibody	Sensitivity	Specificity	Lymphatic
TEC-11	>80% of tumour vessels	Weak staining : Normal endothelial cells, stromal cell	?
E9	Small vessels: positive Large vessels: negative	Weak staining: Normal tissue vessels in skin, mucosa, tonsils	?
EN 7/44	Small vessels in tumour, inflammation, immune reaction	Staining in normal tissues in colon, placenta capillaries	?
4A11	Tissue vessels in tumour, inflammation	Staining in normal tissues in lymph nodes, tonsils, vessels in synovial tissue	?
H4/18	Delayed hypersensitivity in Hodgkin's lymphoma lymph node vessels	Negative in normal tissue	?
FB5	Most of the small tumour vessels	Negative in normal tissue	?

Table 3.2. Activated endothelial cell-specific antibodies [Vermeulen et al 1996a].

Common endothelial markers

vWF

vWF antigen is involved in platelet aggregation and adhesion [Vermeulen et al 1996a]. vWF is found in endothelial cells of arterioles, capillaries, and venules of several tissues, it is also found in high endothelial venules of lymphoid tissue and liver sinusoids, but it is absent in glomerular capillaries, and splenic sinusoids express it only weakly [Turner et al 1987]. F8-Rag is also found in platelets, megakaryocytes, mast cells and the mesangial region of renal glomeruli [Parums et al 1990].

F8-Rag has been established as one of the markers of endothelial cells [Throgeirsson et al 1978], along with the Weibel-Palade body, associated with secretion of vWF [Gimbrone et al 1974, Jaffe et al 1973]. Antibodies against the antigen are highly specific, providing very high contrast between microvessels and other tissue components, and are also highly-reproducible. They stain the large vessels strongly and small vessels weakly [Vermeulen et al 1996a].

It has been shown that their staining is absent on part of the capillary endothelium in tumour tissues [Vermeulen et al 1995a] such as proliferating spindle cells lining the slits in the lesions of Kaposi's sarcoma and angiosarcoma [Vermeulen et al 1995a]. It was suggested that in some pathological conditions, the endothelial cells do not contain sufficient F8-Rag to be demonstrable [Burgdorf et al 1981]. This could also be due to the tumour dedifferentiation, leakage through a defective cell membrane, or increased

turnover rate [Burgdorf et al 1981]. Furthermore, the marker also stains a proportion of lymphatic vessels [Burgdorf et al 1981].

Siitonen et al [1995] reported that this marker is the most sensitive of the commonly used antibodies. However, it has been reported to show lack of specificity, sensitivity, and reproducibility by others, particularly when using polyclonal preparations [Little et al 1986, Millard et al 1987]. It is also claimed that F8-Rag is a less reliable marker of malignant endothelial cells and less specific for endothelial cells of cardiovascular origin [Parums et al 1990].

CD31

CD31 has a role in platelet adhesion, in inflammation and wound healing [Parums et al 1990]. Its antibody is a sensitive pan-endothelial cell markers and doesn't stain lymphatic vessels. It stains large and small vessels in either normal or tumour tissue equally [Vermeulen et al 1996a]. The marker also stains occasional plasma cells and megakaryocytes in formalin fixed sections. This can be due to the fact that endothelial cells contain several proteins, mainly glyproteins, which are antigenically related to proteins isolated from platelet membranes [Borsum et al 1987].

Horak et al [1992], Toi et al [1993] and Giatromanolaki et al [1997] showed that anti-CD31 antibody is more sensitive than anti-F8-Rag. The latter also reported that anti-CD31 is more sensitive for highlighting small, immature microvessels or single endothelial cells. A recent study showed that it has a weaker endothelial staining than anti-F8-Rag antibody

[Martin et al 1997a]. Horak et al [1992] reported that anti-CD31 is more sensitive than anti-CD34. In angiosarcoma, Parums et al [1990] showed that CD31 gave clear, strong staining compared with F8 or UEA-1 , particularly in areas of necrosis and haemorrhage.

Despite this fact the antibody has been recommended in the proposed standard method for intratumoral microvessel density assessment [Vermeulen et al 1996a], there are two major drawbacks to this marker. Firstly, it strongly cross-reacts with plasma cells [De Young et al 1993, Longacre et al 1994] which can significantly obscure the microvessels in tumours in the presence of inflammation. Secondly, the antigen is frequently lost in fixation in the presence of acetic acid. The latter drawback can be solved by means of microwave antigen retrieval [Vermeulen et al 1996a].

CD34

CD34 is a cell surface glycoprotein (cell adhesion molecule) that is selectively expressed within the human hematopoietic system on stem and progenitor cells, and in early blood vessels. It has a role in leukocyte adhesion and endothelial cell migration in neovascularisation [Fina et al 1990].

The antigen is a 105-120kDa single chain transmembrane protein which is expressed on most haematopoietic colony-forming cells from human bone marrow, including unipotent and multipotent progenitors [Civin et al 1984&89&90]. CD34 appears to be expressed at highest levels on the earliest progenitors, and to decrease progressively with maturation [Civin et al 1989]. It is a stage-specific leukocyte differentiation antigen, which is present

on B lymphoid progenitor cells [Civin et al 1989, Loken et al 1989]. The antigen is encoded by a gene located on chromosome 1 [Molgaard et al 1989].

Anti-CD34 antibody is an IgG1 antibody and can be raised by injecting mice with endothelial cell membranes obtained as vesicles from human placenta by injecting a chemical vesicant N-methyl maleimide [Watt et al 1987]. The antibody detects the formalin resistant epitope on the endothelial cells lining all types of vessels. The antibody (clone QBEnd/10) is a class II [defined from the sensitivity of the corresponding CD34 epitopes to degradation to enzymes] monoclonal antibody that recognizes a CD34 epitope which is resistant to neuraminidase, and sensitive to glycoprotease and chymopapain [Sutherland et al 1992]. In addition, the data accumulated by Fina et al [1990], indicates that the binding of the CD34 antibody to vascular endothelium is to the CD34 gene product, and not to cross-reactive epitopes.

The anti-CD34 antibody is a sensitive endothelial marker which stains small and large vessels in normal and tumour tissue equally [Vermeulen et al 1996a]. This marker also highlights perivascular stromal cells and a proportion of lymphatic vessels [Vermeulen et al 1996a]. According to a recent study by Martin et al [1997a], the marker is more sensitive, more reproducible and gives consistently higher vessel counts than anti-F8-Rag or anti-CD31 antibodies.

Other endothelial markers

Thrombomodulin (TM)

Thrombomodulin (TM) is an endothelial cell-associated protein (cell-surface receptor) that functions as a potent natural anticoagulant through its cofactor role in the thrombin-catalyzed activation of human protein C [Bourin et al 1988, Preissner et al 1990]. It converts thrombin from a pro-coagulant protease to an anticoagulant and inhibits the activity of thrombin [Bourin et al 1988, Preissner et al 1990]. It is widely distributed in normal human tissues, such as endothelial cells of blood vessels, lymph vessels and syncytiotrophoblasts in placenta [Maruyama et al 1985]. It was recently reported by Calneck et al [1998] that the TM-dependent anticoagulant activity is regulated by VEGF, a pro-angiogenic factor.

TM has been shown to be a specific and a highly sensitive marker for angiosarcoma in comparison with F8-Rag or UEA-I [Yonezawa et al 1987]. It had been reported that the decreased TM expression could be associated with metastases of tumour cells [Tesuka et al 1995, Suehiro et al 1995, Collins et al 1992]. In a study by Kim et al [1997], low expression of TM in invasive breast carcinoma was significantly associated with a high relapse rate. The authors proposed that TM might have a role in cancer invasion and metastasis as well as could have prognostic values.

Ulex europeus agglutinin I (UEA-I)

Ulex europeus agglutinin I (UEA-I) is a lectin that reacts with alpha-L-fucose in a variety of cell types [Holthofer et al 1982]. It is a pan-endothelial marker which stains small and large vessels in normal as well as tumour tissue [Vermeulen et al 1996a]. It also stains lymphatic vessels. It is limited by its low specificity and reproducibility [Ordonez et al 1984]. UEA-1 shows variable staining of angiosarcoma cells [Miettenen et al 1983] and is not specific for endothelial cells, binding to squamous cell carcinomas and synovial sarcomas [Alles et al 1988] as well as to neoplasm arising from colonic epithelium [Yonezawa et al 1983]. It is a less reliable marker for malignant endothelial cells and may be less specific for endothelial cells of cardiovascular origin [Parums et al 1990].

It has been claimed that it is a more sensitive marker than F8-Rag and works well in paraffin wax sections [Miettenen et al 1983]. Stephenson et al [1986] reported that it is a more reliable endothelial marker of vascular endothelium in follicular carcinoma of the thyroid than F8. Furthermore, according to Yonezawa et al [1987], UEA-I is more sensitive for benign vascular tumours than thrombomodulin or F8.

Mab E9

This monoclonal antibody was developed by Wang et al [1993&94]. It was raised against proliferating endothelial cells of human umbilical vein origin. The antibody is very sensitive to small intratumoral vessels of all tumours, vessels in regenerating or inflamed tissues, and vessels in fetal organs [Vermeulen et al 1996a]. It is highly specific with only weak staining in normal tissue vessels [Vermeulen et al 1996a]. The drawback for this new

marker is that most of the corresponding antigen is lost during tissue fixation and paraffin embedding [Vermeulen et al 1996a]. E9 and another new marker, TEC-II, are both directed at endoglin (CD105) which resembles the TGFbeta type III receptor, and antibodies directed at members of the cell adhesion molecule family integrins [Weidner et al 1991], which might react more specifically to the fraction of endothelial cells affected by the angiogenic drive of the tumour/host.

Kumar et al [1999] using anti-CD105 and anti-CD34 antibodies in the assessment of microvessel density in breast cancers found that vessel counts using anti-CD105 antibodies, but not anti-CD34 antibodies, showed a significant correlation with overall or disease-free survival. Multivariate analysis also confirmed that microvessel density using anti-CD105 antibodies was an independent prognostic factor.

Tie2

Tie2 is a monoclonal antibody against the endothelial Tie receptor tyrosine kinase [Salven et al 1996, Peters et al 1998]. So far, it has been used in the assessment of breast cancer angiogenesis with favorable results. Several authors proposed that it is an antigenic marker for evaluation of tumour angiogenesis [Salven et al 1996, Peters et al 1998].

Potential pitfalls in assessment of microvessel density (Weidner's method)

MVD has been shown to be an independent prognostic factor for disease-free or overall survival in a variety of solid tumours. However, conflicting results have been produced by a number of studies. The discrepancies can be due to performance of different endothelial markers (antibodies) for immunostaining and the lack of consistency in the methodology for vessel counting, selecting sections of representative tumour block and selecting 'hot-spots'. Furthermore, conflicting results can be secondary to inaccurate staging, inadequate patient follow-up, biased case selection and inappropriate statistical data analysis.

Selection of a representative block is very precarious in the presence of heterogeneity between different blocks from the same tumour. De Jong et al [1995] showed a high average coefficient of variation of 24% if more than one tissue block was assessed as compared to 15% when only counts within one section of one block were made. They proposed that single sections should be taken from multiple blocks and each should be scanned to localize the 'hot-spots'. However, Martin [Martin et al 1997b] assessed MVD in sections from each end and one from the centre of the tumour. They reported that vessel counts among blocks varied by less than 20% in 14 out of 20 cases (70%). The results were similar to those shown by Van Hoef et al [1993], De Jong et al [1995] and Bosari et al [1992]. Martin [Martin et al 1997b] suggested that a tumours' angiogenic potential can be assessed on one histological section making this technique suitable for clinical practice.

Ideally, proper scanning at low magnification for 'hot-spots' requires low background staining and a highly specific and intense labeling of endothelial cells. Most of the current antibodies (endothelial markers) are not able to fulfill this requirement. This makes the training and experience of the investigator extremely important. Barbareschi et al [1995] reported that only the counts of experienced investigators were significantly associated with relapse-free survival in both univariate and multivariate analysis. In a case-control study by Vermeulen et al [1995b], the prognostic value of MVD in a sample set of node-negative breast cancer patients was shown. For experienced investigators, a cut off point could be identified for which a significantly different distribution of MVD for an unfavorable versus a favorable outcome group was present. This was not observed for inexperienced investigators. When the counts of two experienced investigators were compared according to their median, agreement was reached in 76% of cases. Axelsson et al [1995] reported a agreement ratio of 73%.

In early studies of MVD as a prognostic indicator, the histological sections were scanned at low magnification (x40 and x100) for 'hot-spots'. These were more likely to be found at the margins of the tumour ('leading edge'), only one x200 microscopic field was then assessed for microvessel density. In subsequent studies, between 1 and 5 'hot-spots' were counted and MVD recorded either as the highest count [Weidner et al 1991, Horak et al 1992, Gasparini et al 1994a] or as the mean of three, four or five fields [Bosari et al 1992, Toi et al 1993, Van Hoef et al 1993, Hall et al 1992, Miliaras et al 1995, Ogawa et al 1990]. The rationale is that the intratumoral vessel density is heterogeneous and the blood vessel invasion (systemic dissemination) of tumour cells is more likely to occur in the areas

of highest vessel density [Horak et al 1992]. The question is : What is the optimum number of 'hot-spots' considered to be desirable? Martin et al [1997a] assessed the current methodology by scanning for ten hot-spots at low magnification followed by point-counting stained vessels at x200 magnification. The apparent 'hot-spot' selected at low magnification often did not contain the highest MVD at high magnification. The first count was the highest in only 20% of cases. As for the apparent highest five fields, the highest count was only found in 65% of cases. The authors suggested that counting ten fields reduced the chance of missing the most vascular area as well as shortening the training period for prospective investigators.

Weidner et al [1991] suggested an optimum field size of 0.74mm2 at high magnification in order to count the labeled microvessels. However, a wide range of magnification and related field sizes have been applied with conflicting results in the assessment of MVD. These range from 0.12 to 1.00 mm2. A small area at higher magnification tends to allow the identification of more single endothelial cell sprouts. Vermeulen et al [1996b] reported a 2-fold higher MVD at magnification x400 than at magnification x200 in breast cancer sections. An area larger or smaller than the 'hot-spot' will result in loss of information. Weidner et al [1991] showed that as counting area decreased from an optimal size of 0.74 mm2, statistical power for predicting outcome diminished. The deviation from the Weidner procedure is likely to be one of the contributing factors why Hall et al [1992], Van Hoeff et al [1993], Axelsson et al [1995] and others failed to show the prognostic value of MVD in solid tumours.

Regarding the definition of microvessel at high magnification, strict application of objective criteria is likely to result in low inter-observer variability when analyzing 'hot-spots' [Vermeulen et al 1996b]. Hall et al [1992] believed that a lumen was necessary for definition of a vessel and that single cells were frequently not of vascular origin, which may partly explain why they failed to relate the MVD to metastasis in breast carcinoma.

Other methods of MVD assessment

MVD grading

This is a semi-quantitative grading system which is rather subjective and requires a long period of training to achieve comparable results between different observers. The method is time-efficient but unlikely to be suitable for multi-centre trials involving MVD assessment. It was reported that there is a relationship between MVD grading and quantitative MVD [Guidi et al 1994]. Weidner et al [1992] showed that both quantitative and semi-quantitative MVD scores were significantly related to the RFS and OS in breast cancer patients.

By means of anti-CD34 antibodies on 836 patients, Hansen et al [2000a] showed vascular grade was significantly associated with axillary node involvement, tumour size, malignancy grade, oestrogen receptor status and histological type. In univariate analysis, vascular grade significantly predicted recurrence free survival and overall survival for all patients, node-negative patients and node-positive patients. Cox multivariate regression analysis showed that vascular grading contributed independent prognostic value in all patients.

Chalkley counting

This method was developed by Fox et al [1995]. It involves scanning the histological section at low magnification for 'hot-spots'. At high magnification (x200-250), an eyepiece graticule containing 25 randomly-positioned dots is rotated so that the maximum number of dots are on or within the vessels of the 'hot-spots'. Then, the dots are counted. Fox et al [1995] showed that there is a significant correlation between MVD assessment according to the Weidner method and Chalkley point counting. It was also reported that this method had independent prognostic value in breast [Hansen et al 2000a] and transitional bladder carcinoma [Dickinson et al 1994]. Gasparini et al [1996] found the Chalkey score was the strongest significant independent predictor of outcome as well as being useful for studies of angiogenesis between multicentres. Even though the selection for 'hot-spots' remains subjective, no decision is needed on whether adjacent stained structures are separate microvessels or otherwise.

Hansen et al [1998] showed that the Chalkey method has less observer variation in comparison with other MVD methods and suggested that the former may be superior from a methodological point of view. The same author [Hansen et al 2000b] also showed a significant correlation between high Chalkey counts and axillary lymph node metastasis, large tumour size, high histological malignancy grade, and histological type. A high Chalkey count showed a lower probability of recurrence-free survival and overall survival.

Multiparametric computerised image analysis systems (CIAS)

The advantage of this system is that it is able to measure the number of vessels within a certain dimension range, the vessel lumen area, the vessel lumen perimeter and the percentage of immunostained area per microscopic field. The main drawback is the heterogeneity of microvessel morphology such as size, length and anastomoses; and the locoregional differences in immunostaining intensity.

In a study involving node-negative ductal carcinoma patients, Barbareschi et al [1995] showed that MVD measured by experienced investigators and the microvessel area – consisting of the endothelial cells plus the vessel lumen, measured by CIAS, were independently associated with RFS. This suggested that the prognostic value of microvessel area is less dependent on the experience of identifying 'hot-spots'. In breast carcinoma, Dickinson & Fox et al [1994] found a significant correlation between manual Chalkey counting and microvessel area, microvessel perimeter and microvessel number measured by CIAS.

The apparent disadvantage of interactive computer-aided IMD measurements is the time-consuming nature of the method. Automated hot-spot selection would solve both this problem and the problem related to the subjectivity associated with manual IMD assessment. Fox et al [1995] were unsuccessful in their attempt to perform completely automatic quantification, due to the extremely low level of interference of non-microvessel structures allowed by CIAS. Highly specific endothelial cell markers are needed and variations in staining intensity have to be eliminated or compensated for, for example, by

automatic background subtraction. The use of reference slides and adjustment of fixation techniques in multicentre studies have to be considered.

In conclusion, CIAS seems to provide additional information on the morphology of the tumour vasculature. Most studies seem to indicate that MVA, EA, and MVP are a quantitative reflection of the degree of pathophysiological involvement of tumour angiogenesis in tumour progression. Integration of IMD, MVA, EA, and MVP with information of the activity status of the tumour-related endothelium might provide a more dynamic picture of the vascular component of a tumour. CIAS might be introduced as a more objective method of microvessel quantification and eventually to perform automated hotspot selection. The high signal-to-noise ratio of the immunostaining required for CIAS IMD assessment might result in a considerable number of tumour sections unsuitable for evaluation. Artificial intelligence-based pattern recognition systems with a learning capability might have to be developed to counter the inherent degree of irreproducibility related to immunohistochemical techniques.

Complementary methods of assessing angiogenesis

These methods are complementary to the histological quantification of angiogenesis. Generally, they provide non-invasive analysis of the whole tumour as well as allowing repetitive sampling throughout the treatment of the tumours, including during the follow-up sessions.

Doppler ultrasound scan

It has been proposed that colour Doppler ultrasound may have a role in the non-invasive differential diagnosis of breast lesions [Cosgrove et al 1990]; where malignant lesions to be colour Doppler positive (moderate to high flow signals) and benign lesions negative (no flow signals). A study showed that although the colour Doppler mapping is useful in distinguishing benign from malignant breast lesions, the intensity of signal and velocity of flow had no correlation with the extent of angiogenesis of breast cancer [Lee et al 1995]. They did, however, find that the presence of a high-flow tumour signal in early breast cancer is significantly associated with the presence of axillary lymph node metastases.

Serum and tissue sampling of angiogenic peptides

In colorectal carcinomas, more than 80% of pre-operative patients were found to have elevated serum levels of bFGF and /or VEGF. This was reduced to 33% post-operatively. Yamamoto et al [1996] reported significant elevated serum VEGF in patients with advanced primary breast cancer compared with those at an early stage. The pre-operative VEGF serum levels were elevated in association with an increase in MVD. The levels dropped in the majority of the patients after surgery.

Increased serum levels of bFGF and VEGF were observed in patients with metastatic colorectal, breast, ovarian and renal carcinoma [Dirix et al 1997]. Furthermore, levels of bFGF have been reported to be elevated in the urine of patients with a variety of cancers [Nguyen et al 1994].

Contrast-enhanced MRI scan

Hulka et al [1995] and Esserman et al [1996] reported that the kinetic profiles of contrast uptake and washout in contrast enhanced MRI appeared to correlate with tumour grade and MVD in breast cancer. In an animal model, MRI derived tumour tissue plasma volume increased exponentially with increasing histological vascular density [Van Dijke et al 1996].

In DCIS, it was suggested that angiogenesis might play a role in the contrast enhancement on MR angiogram of this pre-invasive tumour [Giles et al 1995]. Contrast-enhanced MRI is considered to be inferior to mammography in the detection of DCIS because the induced angiogenesis is relatively faint and difficult to be detected by the scan [Friedrich et al 1998]. Bone et al [1998] showed that there was a significant correlation between contrast enhancement at MR scan of breast cancer and both tumour angiogenesis and proliferative cellular activity as shown by PCNA immunoreactivity. There was also a correlation between contrast enhancement and both tumour grade and invasiveness. The authors suggested that MR scan could turn out to be a valuable prognostic tool in breast cancers.

Parameters measured by both techniques (serum and tissue sampling of angiogenic peptides/contrast-enhanced MRI scan) seem to be related to tumour progression or grade rather than simply to tumour volume. This aspect offers promising perspectives for their use in monitoring tumour response during treatment or in predicting tumour relapse. However, it will be necessary to determine the net balance between angiogenic growth factors and natural angiogenesis inhibitors.

Assay Quality Control

It would seem to be a reasonable objective that results produced by a given laboratory for each assay are consistent over time and that results produced by different laboratories from the same samples are comparable. At least one published study has investigated within- and between-laboratory variation in Elisa results in the USA (Kreider 1991b). Results varied significantly and substantially among different laboratories (the greater source of variation) and among different days in the same laboratory. This suggests that single determinations on individual serum samples are not likely to give a reliable estimate of antibody titre. The large variability within laboratories further indicates the need for standard reference pools of positive serum to be included in assays in order to substantiate assay results.

Murray et al [1993] have discussed in some detail the sources of variability of assay results. While these authors concentrated on biochemical assays the same basic principles apply equally well to serology. Each assay must be validated to identify and quantify sources of variation in results. We must also keep in mind that there are non-assay sources of variation. These can be grouped into those factors which precede the assay (how and when a sample is taken, how it is manipulated, stored, transported, and identified) and those which come after (for example transcription errors in report generation). In validating an assay the following areas need to be addressed :

1. ***Specificity*** - A highly specific assay will have a low tendency to show "false positive" reactions in animals exposed to a closely related pathogen. This can be tested by obtaining mono-specific sera raised against a range of other pathogens and including them in the test.
2. ***Sensitivity*** - This is a measure of the ability to detect clinically important but very low levels of antibody. There will sometimes be a trade-off in that the higher the sensitivity of an assay the lower its specificity. In poultry production it is common practice to apply a highly sensitive technique as a screening test in order to identify problem flocks or individuals. then apply more specific tests only to these or on repeat sampling of the flock. The sensitivity of a test can be evaluated by diluting known positive samples sequentially and determining the dilution at which the reaction is lost.
3. ***Accuracy*** - This is a measure of the ability of the test to measure purified amounts of the substance sought when it is added in measured amounts to a typical test sample. Rarely will we have purified antibody available for this type of study but neither is this

required in that we will not be reporting results in "milligrams of antibody". If known positive field sera are available then they can be used as a pool in repeated assays. This will be most valuable if this pool is also submitted to a reference laboratory for testing using a recognized and already validated procedure. The alternative is to take purified mono-specific antiserum and use this to "spike" sero-negative field serum at different concentrations then use the spiked samples to establish a measure of the variability of the assay results within a given sample.

4. ***Precision*** - This is the ability of the assay to consistently reproduce a result when sub-samples are taken from the same specimen. Within-assay and inter-assay precision are two distinct measures of this can be made as part of the validation procedure. The formulae used for the calculation of CV% are slightly different from the conventional formula (Standard Deviation divided by the mean and multiplied by 100).

Within-assay precision

Assay 10 duplicated samples on the same plate (a total of 20 assays) and calculate an intra-assay coefficient of variation as follows:

$$\frac{\textit{Mean of the Standard Deviations of the Duplicates}}{\textit{Grand mean of the duplicates}} \times 100$$

A figure of 10% or less is considered satisfactory [Murray et al 1993]

Inter-assay Precision

In this case the 10 runs on duplicate samples are run on different days. For each run the mean, the deviation, and the % C.E. are calculated. The interassay coefficient of variation is calculated from the formula:

$$\frac{\textit{Standard Deviation of the means of the duplicates}}{\textit{Grand Mean of the Duplicates}} \times 100$$

Much of the validation work described here will, in the case of commercially available antigens and test kits, be carried out by the manufacturer. A good first step in setting up a serological quality assurance system will be to request the manufacturer to provide data on with-run and between-assay precision. The laboratory can then quickly validate the assay using known positive and negative sera. Once an assay has been validated the continued satisfactory performance should be monitored by the use of a Quality control system.

This system should include a definition of criteria for assay acceptability, along with a means for identifying sources of variation and implementing corrective procedures. One component of such a system might be to repeat an Inter-assay Precision test. Alternatively, simply calculate the %CV for all assays of each standard control serum each month and plot the results over time. A reasonable target for %CV in routine testing is 10-15%.

The prime aim of in-house quality assessment is to confirm that the results generated are consistent over time. Laboratories may also participate in inter-laboratory quality assurance schemes with a view to ensuring uniformity of results between different laboratories. Accreditation schemes take this a step further by independently checking procedures and standards, often with a periodic inspection. The only scheme currently operated in the UK which has a component specific to avian serology is the accreditation scheme for *Mycoplasma gallisepticum* and *M.meleagridis* testing operated by the Ministry for Agriculture Fisheries and Food. For a detailed discussion of the concepts behind quality assessment and accreditation schemes the reader is referred to Manser et al [1994]. At every point in the validation and QA of assays it is important that staff be educated and motivated. It is equally important for those interpreting the results to understand the inherent variability of the results and to be able to express an opinion by comparing them with "population means". This may lead to a decision which will take into account information from a variety of sources.

Section 3

Chapter 4

Merseyside DCIS Review

Introduction

I carried out this study to demonstrate that recurrence in DCIS and transformation from in-situ to invasive ductal carcinoma is important clinically. In other words, angiogenesis in DCIS is a useful question to study and my hypothesis has clinical relevance. Another reason for this study was to recruit DCIS cases detected in Merseyside for my subsequent laboratory work in angiogenesis in DCIS.

The NHS Breast Screening Programme is set up to save lives by detecting breast cancer early. DCIS is a non fatal disease, however, if it progresses to the development of invasion, a significant morbidity and mortality are associated. Furthermore, the majority of DCIS cases detected are those of high nuclear grade and if progression occurs then these patients are more likely to develop high grade invasive carcinomas. The majority of studies that have been published to date on DCIS and its recurrence rate have not been in the screened population. It is therefore difficult to know whether these results can be extrapolated to the NHS breast screening population.

DCIS is a heterogeneous disorder varying in cytology and architecture. Some histological features, particularly nuclear grade and necrosis, are associated with an increased risk of local recurrence [Badve et al 1998, Lagios et al 1995, Silverstein et al 1995a]. Lesion size and excision margin status are also important histological features that predict for recurrence. These histological parameters have been combined into the Van Nuys Prognostic index (size, excision margin, necrosis and nuclear grade) [*Table 4.1*], and have been shown to predict for the risk of recurrence in DCIS that has been treated with breast conservation in North America better than the individual parameters [Silverstein et al 1996]. However, the pathological classification of DCIS used in this index is not standardly used in the National Health Service Breast Screening Programme (NHS BSP). It is therefore important to determine if the VNPI is valid in DCIS detected in the NHS BSP, and if VNPI can be modified to use the pathological data routinely detected in the National Screening Programme.

Score	1	2	3
Size (mm)	< or =15	16 to 40	> or =41
Margin width (mm)	> or =10	1 to 9	<1
Pathologic classification	Non high grade without necrosis	Non high grade with necrosis	High grade with or without necrosis

Table 4.1. Van Nuys Prognostic Index

Patients and methods

With assistance from North-west Breast Screening Quality Assurance Co-ordinators, I obtained a list of DCIS patients in Mersey region. At the same time, I scanned the Pathology database in RLUH for symptomatic patients. I reviewed the case sheets of these 355 patients and recorded relevant details as follows:

- Family history, menstrual status
- Detection: date, method, presentation, side affected, examination findings, X-ray findings
- Treatment: date, operation, adjuvant therapy
- Histology data
- Most recent follow-up: date, alive?, recurrence(s)?

  Cases of DCIS with microinvasion were excluded. I also applied the VNPI and modified VNPI (using only grade) to those with pure DCIS treated by breast conservation with or without adjuvant therapy.

Results

Data from 355 patients with DCIS was assessed. The patients had a mean age of 57 years (range 35-83) and mean follow up of 51 months (range 1-252). 80% of the cases were screen-detected. 97% of patients were still alive and only one patient had died of breast cancer. The patients had undergone a number of different treatment modalities and these included mastectomy or wide local excision with and without adjuvant hormonal treatment or radiotherapy. The recurrence rates and types of recurrence following individual treatment combinations is summarised in *Table 4.2* and *Figs.4.1 &4. 2*. The majority of patients underwent a wide local excision rather than a mastectomy and most of the patients received adjuvant tamoxifen but no radiotherapy. The overall five-year recurrence rate was 13%. The recurrence rate was highest (28%) in those patients treated with wide local excision alone and lowest in those patients that had adjuvant radiotherapy or had a mastectomy with adjuvant tamoxifen.

	WLE	WLE/T	WLE/Rx	WLE/Tam/Rx	Mx	Mx/Tam	Total
Number	57	115	8	26	68	82	355
In-situ recurrence: number (%)	10 (17.5)	10 (8.7)	--	--	0 (0)	--	20
Invasive recurrence: number (%)	6 (10.5)	4 (3.3)	--	--	2 (3)	--	12
Total recurrence: number (%)	16 (28)	14 (12)	0 (0)	0 (0)	2 (3)	0 (0)	32

Table 4.2. Comparison of outcome among different treatment modalities in DCIS.
Abbreviation: wide local excision (WLE), tamoxifen (Tam), radiation (Rx), mastectomy (Mx)

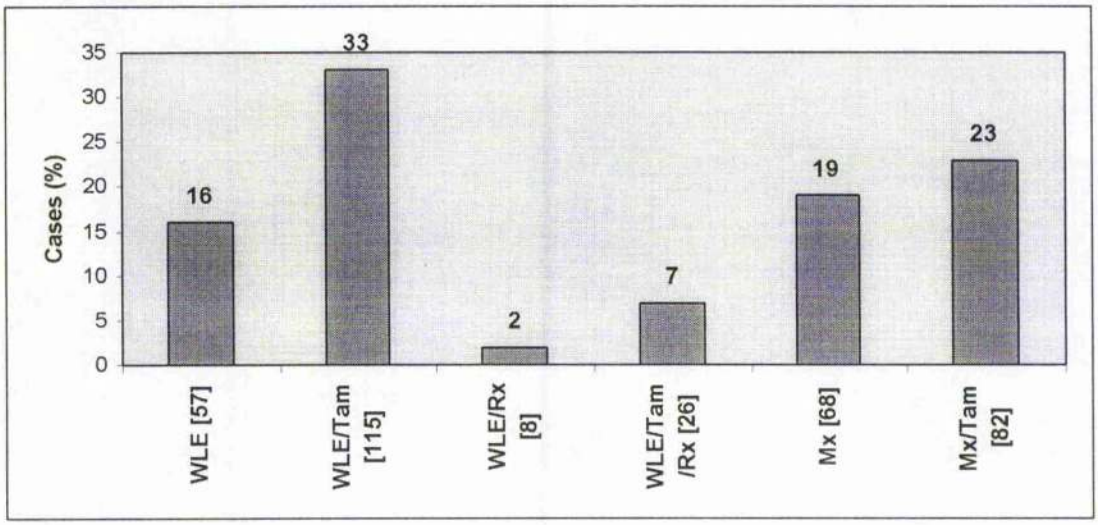


Figure 4.1. Treatment: number on top of each column shows percentage of patients in each type of treatment, number in bracket under the column shows number of patients in that treatment.
Abbreviation: wide local excision (WLE), Tamoxifen (Tam), radiation (Rx), mastectomy (Mx)

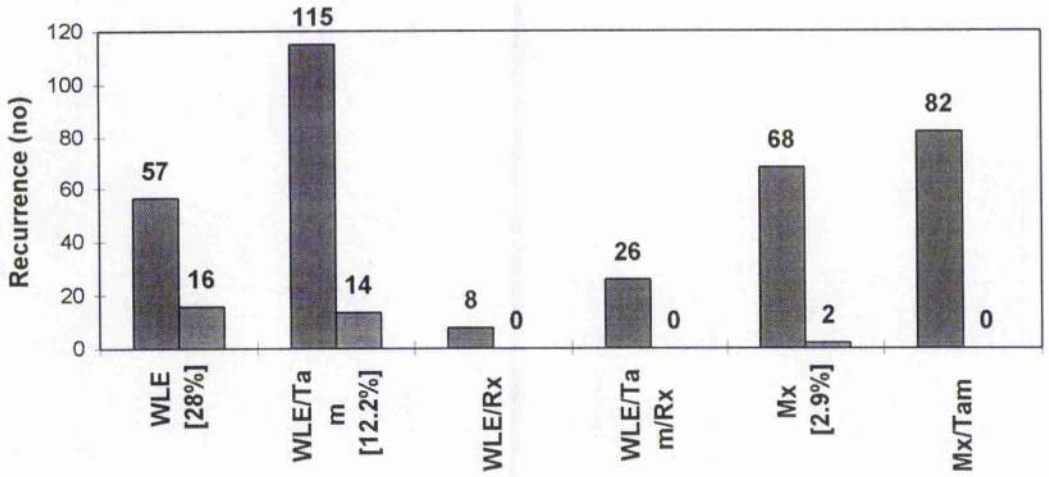


Figure 4.2. Treatment and outcome: number on top of first column shows number of patients in each type of treatment, number on top of second column shows number of recurrence, number in bracket under both columns shows percentage of recurrence in each type of treatment.
Abbreviation: wide local excision (WLE), Tamoxifen (Tam), radiation (Rx), mastectomy (Mx)

Of the 32 recurrences, 12 (37.5%) were invasive of which 3 had regional nodal involvement and 1 had bone marrow involvement. The patient with bone marrow metastasis eventually died of breast cancer. The break-down of recurrences are shown in *Table 4.3.*

Treatment	*DCIS*	*Invasive*	Local Invasive	Regional Nodes	Metastatic
WLE	10	6	5	1	0
WLE/Tam	10	4	3	1	0
Mx	0	2	0	1	1

Table 4.3. Break down of recurrences in the study.

In addition, there were 8 cases of contralateral breast cancers of which half were invasive. None of those patients given adjuvant Tamoxifen treatment developed contralateral [*Fig. 4.3*].

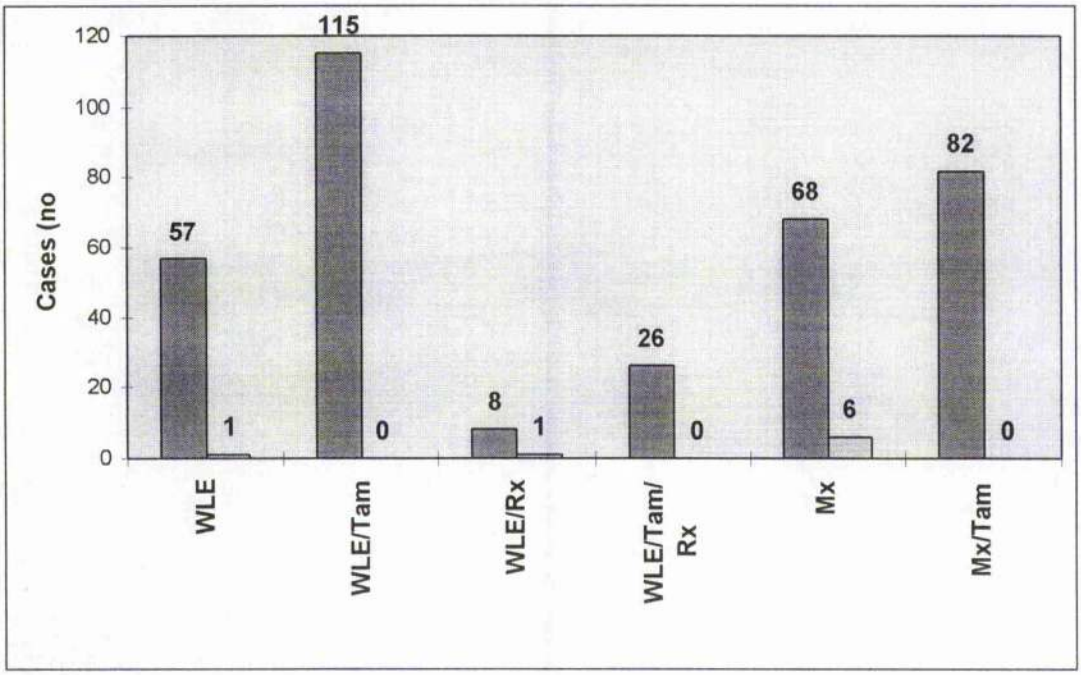


Figure 4.3. Treatment and contralateral event: number on top of first column shows number of patients in each type of treatment, number on top of second column shows number of contralateral event.
Abbreviation: wide local excision (WLE), Tamoxifen (Tam), radiation (Rx), mastectomy (Mx)

The VNPI and modified VNPI (NHS breast screening program nuclear grade substituted for the Van Nuys histopathological grade) were applied to 104 patients with pure DCIS treated by breast conservation with or without adjuvant therapy that had undergone histopathological review. The whole series could not be assessed as a size measurement was not available for all. The findings are shown in *Table 4.4*.

	VNPI				**Modified VNPI**		
	High	**Intermediate**	**Low**		**High**	**Intermediate**	**Low**
Number	9	76	19		9	75	20
Recurrence: number (%)	2 (22.2)	4 (5.2)	2 (10.5)		2 (22.2)	4 (5.3)	2 (10)

Table 4.4. Comparison between conventional and modified VNPI on patients with breast conservation treatment.

The results with both the VNPI and the modified VNPI were very similar. Both a high VNPI and high modified VNPI predicted for an increased likelihood of recurrence. Taking treatment into account, the highest recurrence rate was in those with a high VNPI and wide local excision only 1/3 (33.3%), this recurrence rate was reduced by Tamoxifen 1/5 (20%) and abolished by radiation 0/1, however, the numbers in each group are very small [*Fig. 4.4*].

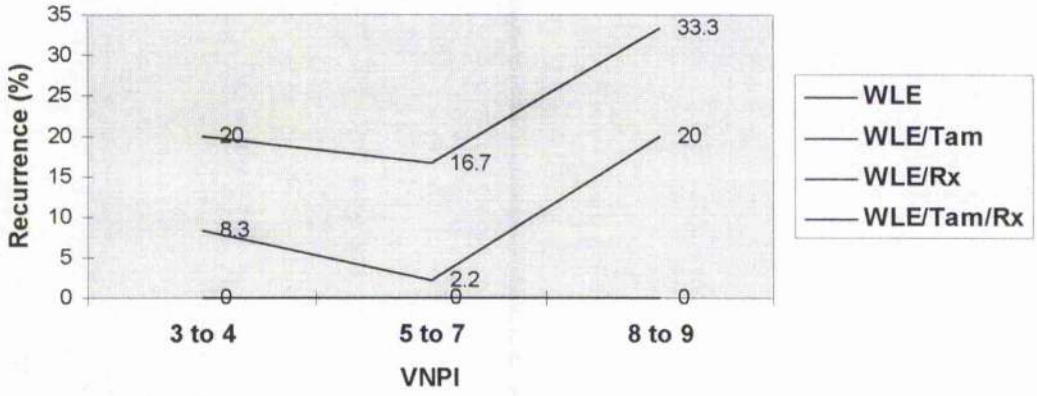


Figure 4.4. Breast conservation treatment in patients with different VNPI and outcome. None of those patients who had adjuvant radiotherapy recurred.
Abbreviation: wide local excision (Ex), Tamoxifen (Tam), radiation (Rx)

Discussion

The local recurrence rate for DCIS ranges from 10-40%, and is dependant on tumour histology, type of treatment, and length of follow-up [Silverstein et al 1995a&b, Silverstein et al 1996, Solin et al 1993, Bellamy et al 1993, Fisher ER et al 1995, Fisher B et al 1998]. Silverstein [Silverstein et al 1992] showed that the local recurrence rates 8 years after treatment for DCIS patients who had mastectomy, excision with adjuvant radiotherapy and excision alone were 0.5%, 16% and 21% respectively. The NSABP-06 was a randomised trial to compare local excision alone, breast-conservation treatment, and mastectomy in patients with early invasive breast cancer [Fisher et al 1989]. A subset of 76 patients was found to have DCIS on subsequent pathologic review [Fisher et al 1991]. 7% (2/27) of these patients who had wide local excision with adjuvant radiotherapy and 43% (9/21) who had wide local excision alone developed ipsilateral recurrence. No tumour recurrence was reported in the mastectomy group (n=28). In this series, wide local excision alone was associated with a high rate of local recurrence (28%) as compared with those of wide local excision with radiotherapy (0%) or mastectomy alone (3%).

Local recurrence after conservative treatment is demoralising and it causes marked patient morbidity. It is associated with invasive carcinoma in up to 50% of cases [Silverstein et al 1995a&b and 96, Solin et al 1993&94&96]. In this study, 37.5% (12/32) of the recurrences were invasive. An invasive recurrence in a patient previously treated for DCIS upstages that patient from stage 0 disease to at least stage I breast cancer. This does have

profound implications as invasive recurrence is a potential threat to life. One patient in this series died of breast cancer which recurred as invasive carcinoma with distant metastases.

Nuclear grade, the presence of comedo-type necrosis, tumour size, and the excision margin are important factors which are able to predict local recurrence in patients with DCIS [Silverstein et al 1992&95a&95b&96&97c, Fisher B et al 1998, Lagios et al 1989]. However, the index uses the Van Nuys pathological classification and this is not standardly used in the NHS Breast Screening Programme. The screening programme instead stratifies DCIS according to nuclear grade. I therefore substituted nuclear grade for the Van Nuys pathological classification and found that stratification of patients into the Van Nuys Prognostic Index were similar and correlated with the risk of recurrence. It therefore appears that the index is still a useful tool when data from the NHS Breast Screening Programme is used.

To sum up this clinical data, the treatment for DCIS varies widely and so do the recurrence rates. These recurrences have huge impact on the patients as well as resource implications. However, the patients' concern at diagnosis is always for long term survival and therefore to avoid the risk of invasive recurrence. As shown in my previous literature review, present methods of histological assessment are inadequate to predict the clinical outcome and to provide the patients with the best possible, and most reliably informed treatment choice. This clinical data shows the importance of studying angiogenesis in DCIS.

Chapter 5

Literature review & pilot study conclusions

After the literature review and pilot study, I conclude that:

- current histological classifications are inadequate to predict the development of invasive disease,
- no pathological or molecular features have been identified to predict the risk of recurrence or malignant transformation in DCIS,
- the best treatment of DCIS remains controversial,
- little is known of angiogenesis in DCIS; especially those with increased periductal vascularity,
- there is a need to study angiogenesis in DCIS and its prognostic value.
- assessments of angiogenesis in breast carcinoma have been mainly of stromal vascularity, and little work has been done on periductal vascularity
- highest microvessel counts (hotspots) have been used to assess stromal vascularity, which has been associated with inconsistent findings due to selection bias of hotspots at low power field
- most studies have employed a single anti-endothelial marker, and therefore have overlooked potential phenotypic changes of the microvessel
- no work has been done to correlate the stromal and periductal vascularity in DCIS

Hypothesis

I hypothesize that:

- changes in vasculature are related to the progression of in situ to invasive carcinoma,
- the pattern and extent of periductal vascularity is related to the risk or potential for in situ or invasive recurrence in DCIS,
- the two vascular patterns in DCIS are related to one and another.

Aim of the study

The aim of my study is to determine whether changes in vasculature (vascularity and phenotype) are related to the progression of in situ to invasive carcinoma and those factors that may predict for recurrences. The feature studied included periductal and stromal vascular density and phenotype in DCIS with and without invasive cancer and how this relates to the histological features, proliferative activity, and Thymidine Phosphorylase expression of the tumour. The relationship between the two vascular patterns is also examined.

Clinical relevance

While the majority of patients are cured by the treatment of their DCIS a small proportion go on to develop invasive breast cancer, and therefore have their life threatened by what, at initial presentation, was a curable disease. It may be that measurable changes in microvessel density and angiogenesis at diagnosis may predict these who will subsequently develop invasive cancer or recurrence, and therefore need more aggressive treatment following initial diagnosis.

Section 2

Chapter 6

Methodology

If angiogenesis is important in malignant transformation from in situ to invasive cancer, it is likely that MVD or vessel phenotype will be different around DCIS with invasive carcinoma compared with pure DCIS. Periductal vessels are likely to be most important in transformation from in-situ to invasive carcinoma as incipient invasion is most likely to be associated with changes in vessels in the immediate vicinity of the tumour cells.

Chapter 9

Periductal angiogenesis in pure DCIS and DCIS associated with invasive cancer

My first experiment in ***chapter 9*** was to compare the periductal angiogenesis in pure DCIS and DCIS associated with invasive cancer. Studies of breast cancer angiogenesis in the past have been associated with inconsistent findings, I therefore used rigorous morphometric methodology to compare vascularity in DCIS with normal breast and employed a panel of anti-endothelial antibodies to take account of potential phenotypic as well as numerical changes. As part of this study I also determined if vascular density and phenotype have any relationship to the histological features, of nuclear grade, necrosis and duct size. The correlation between MVD and endothelial density was also examined. 20 consecutive cases of DCIS were examined in each group.

Chapter 10

Proliferation in DCIS

MVD increased from low intermediate nuclear grade but decreased in high grade lesions, I therefore went on to measure proliferation in DCIS using Ki-67 and correlate this with MVD in ***chapter 10***.

Chapter 11

Microvessel density and recurrence & dual staining immunofluorescene of periductal blood vessels

MVD would be expected to be increased at diagnosis in those who subsequently developed a recurrence compared to those that did not. Periductal vessel density from a group of women with pure DCIS (no known development of recurrent disease) was compared with a group that subsequently developed recurrence either as *in situ* or invasive carcinoma. Out of 355 DCIS cases recruited in Merseyside with mean follow-up of 51months, there were 20 cases of in situ and 12 cases of invasive recurrences respectively, and the MVD at diagnosis was measured in these cases and compared to those with no recurrence.

The results in ***chapter 9*** suggest a phenotypic changes in the periductal vessels from normal lobules to DCIS, to DCIS with invasion. To confirm the above findings, I carried out dual staining immunofluoresence to determine the phenotype of individual periductal blood vessels.

Chapter 12

Correlation between periductal and stromal vascularity & Thymidine Phosphorylase, periductal vascularity and recurrence

Having demonstrated that changes in periductal vascularity are associated with the presence of an invasive carcinoma and recurrence, I investigated the relationship between periductal and stromal vascular patterns and investigated which as a better prognostic marker in DCIS.

In addition, I investigated the association of Thymidine phosphorylase with the increased periductal vascularity previously identified and with disease recurrence. Thymidine phosphorylase is thought to have a role in endothelial cell migration [Risau et al 1992] and has been shown to be present within DCIS [Engels et al 1997b, Lee et al 1999].

Chapter 7

Materials

Sections

- Original haematoxylin and eosin (H&E) sections
- Formalin-fixed paraffin-embedded sections
- Slides
- Coverslips

Basic hardware and software

- Pressure cooker
- Microwave
- Microscope
- Image analysis (Zeis Axiohome with software version 3.0, Germany)
- Computer with SPSS package (Version 10.0 for Window 97/NT)

Primary antibodies

- Monoclonal anti-CD31 (JC/70A, DAKO, Denmark, Code No. M823)
- Anti-CD34 (Qbend/10, DAKO, Denmark, Code No. M7165)
- Anti-CD141 (Anti-Thrombomodulin, DAKO, USA, Code No. MO617)
- Polyclonal anti-human von Willebrand factor, vWF (DAKO, Denmark, Code No. A082).

- Polyclonal anti-Ki 67 antibodies (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK)
- Monoclonal Thymidine Phosphorylase (Clone P-GF.44C, NeoMarkers, USA)

Secondary antibodies

- Biotinylated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Essex UK)
- Swine anti-rabbit TRITC Conjugated antibody (DAKO, Cambridge, UK)
- Swine α Anti-Rabbit Immunoglobulin TRITC

Chemical solutions

- Xylene
- Industrial methylated spirits
- Mixture of H_2O_2/methanol [12ml H_2O_2 (30%) in 400 ml methanol]
- 0.2g of trypsin
- 0.4g of calcium chloride
- Tris Buffered Saline, TBS [50mM Tris-HCL, 150mM NaCl, pH 7.4]
- Mixture of 5% BSA/TBS [1g Bovine Serum Albumen in 20 ml TBS]
- Ethylenediamine-Tetraacetic Acid (ED2SS, Sigma Chemical, St Louis USA)
- Phosphate buffered solution

Chromogen

- EnVision Labeled Polymer (mouse or rabbit as appropriate)
- 33' diaminobenzidine, DAB (chromogen)
- Commercial kit [DAKO EnVisionTM + System, Peroxidase (DAB), USA, Code No. K4010 (rabbit) and K4006 (mouse)]
- Haematoxylin solution
- Flourescein Avidin DCS (A-2011; Vector Labs, Peterborough UK)
- 4',6-diamidino-2-phenylindole (Sigma)

Mountant

- Resinous mountant (DPX, BDH Laboratory supplies, England)
- Anti-fading medium (Vectashield, Vector Laboratories, Peterborough UK)

Chapter 8

Methods

Patients and tumours

Formalin-fixed paraffin-embedded breast samples of patients with ductal carcinoma *in situ* were collected from the archives of the Pathology Department of the Royal Liverpool University Hospital and peripheral hospitals.

The original haematoxylin and eosin (H&E) stained sections from the primary tumour were reviewed by 2 pathologists (BS & JPS) for classification by architecture and nuclear grade following the guidelines of the European Commission and UK National Breast Screening Programme [National coordinating group for breast screening pathology 1997, The consensus conference committee 1997]. A representative block for each patient was selected for subsequent immunostaining.

Immunohistochemistry

Sections were stained using monoclonal anti-CD31 (JC/70A, DAKO, Denmark, Code No. M823), anti-CD34 (Qbend/10, DAKO, Denmark, Code No. M7165), anti-CD141 (Anti-Thrombomodulin, DAKO, USA, Code No. MO617), polyclonal anti-human von Willebrand factor, vWF (DAKO, Denmark, Code No. A082), polyclonal anti-Ki 67 antibodies (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and monoclonal Thymidine Phosphorylase (Clone P-GF.44C, NeoMarkers, USA).

Sections were dewaxed through two changes of xylene and industrial methylated spirits. Endogenous peroxidase activity was blocked with a mixture of H_2O_2/methanol [12ml H_2O_2 (30%) in 400 ml methanol] for 12 minutes. Antigen retrieval was performed by treating the sections with 0.2g of trypsin and 0.4g of calcium chloride in 440 ml Tris Buffered Saline, TBS [50mM Tris-HCL, 150mM NaCl, pH 7.4] at 37°C for 20 minutes. No pre-treatment was needed for the staining with Thymidine Phosphorylase antibodies. For staining with anti-Ki 67 antibodies, the sections were treated with 2000ml of Ethylenediamine-Tetraacetic Acid (ED2SS; Sigma Chemical, St Louis USA) in a pressure cooker for 8 minutes.

Prior to staining with the polyclonal antiserum, sections were treated with a mixture of 5% BSA/TBS [1g Bovine Serum Albumen in 20 ml TBS] for 10 minutes. The antibodies were diluted 1:5 for anti-CD31, 1:10 for anti-CD141, 1:20 for anti-CD34, 1:1000 for anti-human vWF, 1:1000 for anti-Ki 67 and 1:100 for Thymidine Phosphorylase antibodies in 5% BSA/TBS.

The sections were incubated with primary antibodies at room temperature for 40 minutes. Secondary antibodies were incubated for 40 minutes using EnVision Labeled Polymer (mouse or rabbit as appropriate). Sections were washed with TBS between incubation steps. 3,3' diaminobenzidine, DAB was used as a chromogen. The last two steps were carried out using a commercial kit [DAKO EnVisionTM + System, Peroxidase (DAB), USA, Code No. K4010 (rabbit) and K4006 (mouse)].

The cellular nuclei were counter-stained blue with Haematoxylin solution. The sections were dehydrated through four changes of IMS and three changes of xylene before being mounted in resinous mountant (DPX, BDH Laboratory supplies, England). Omission of the primary antibody was used as a negative control and the microvessels of the normal adjacent breast tissue served as internal positive controls.

Assessment of tumour periductal vascularity

Vascular density was determined without knowing how the architecture or nuclear grade had been classified. No scanning of stained microvessels or endothelial cells to identify 'hot-spots' at low magnification was undertaken. The assessment of completely transected involved ducts started from the upper right of all stained sections, moving downwards and to the left. All or the first 50 foci (duct cross sections) encountered were assessed on each section, thus eliminating selection bias. Up to three representative histological sections for each patient were chosen by two pathologists. These sections on average have 30 foci of DCIS for each patient.

In the assessment of DCIS accompanied by invasive carcinoma, only those foci that were at least 2 mm away from the invasive component were assessed to minimize the detection of direct local effects of the invasive disease.

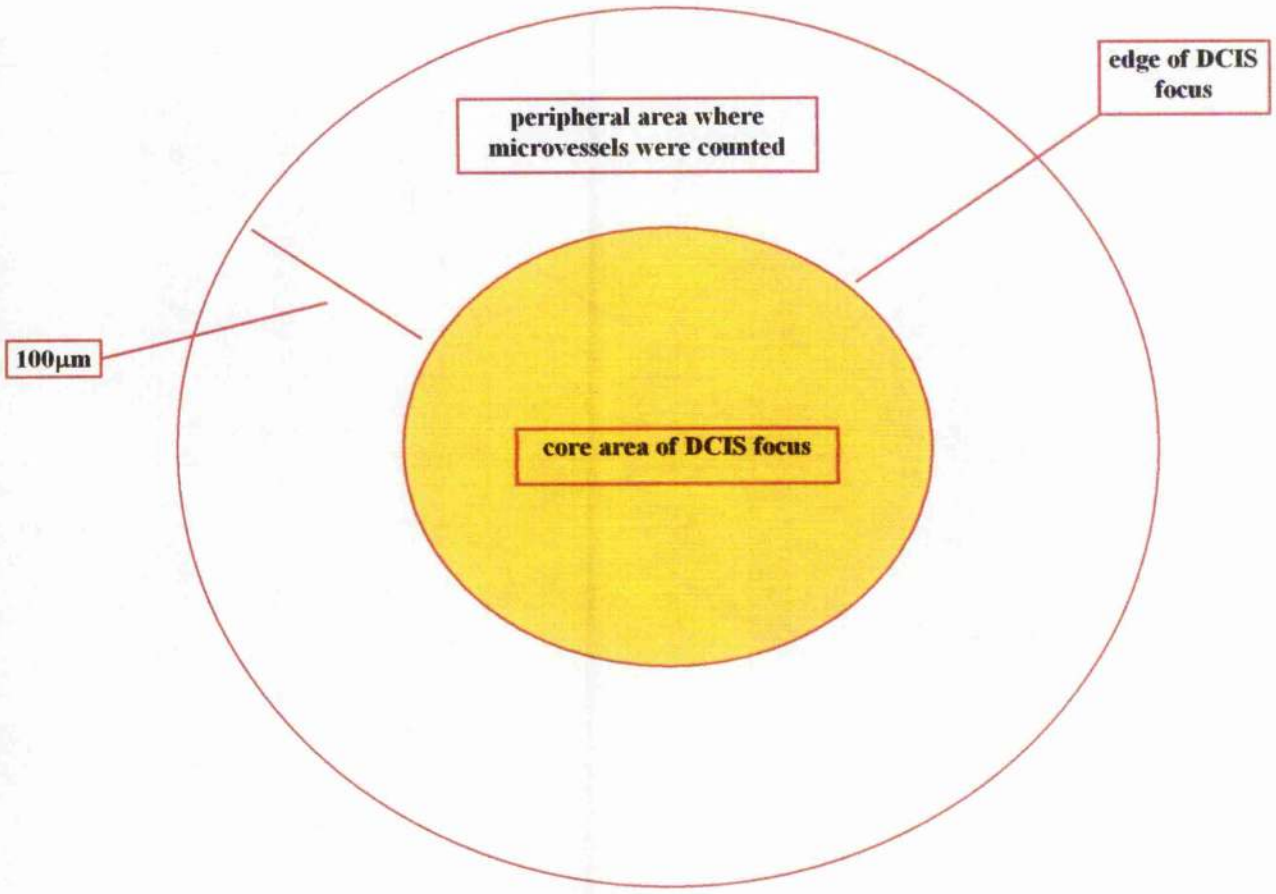
Evaluation the area of DCIS

I measured the area rather the circumference because I intended to evaluate the vascular density using the formula under the heading of '*Counting periductal microvessels and endothelial cells*' below. In addition, I planned to assess the relationship between periductal vascular density and the core size of DCIS.

Two areas were measured: a) the area of each DCIS focus (core area) and, b) the core area plus the area around the DCIS focus to a width of 100 micrometer from the edge of the individual focus of DCIS (total area). I chose to assess those vessels lying within 100 micrometers from the edge of the tumour (each focus) because I believe these vessels are least likely to be affected by factors secreted by adjacent tumour (either invasive or DCIS). These areas were measured at x200 magnification using image analysis (Zeis Axiohome with software version 3.0, Germany). The peripheral area, where the microvessels or endothelial cells were counted, was obtained by subtraction of core area from total area [*Fig.8.1*].

$$Peripheral\ area = Total\ area - Core\ area$$

Figure 8.1. Simplified diagram of an individual focus of ductal carcinoma in situ (DCIS) and peripheral area where the periductal microvessels were counted.



Counting periductal microvessels and endothelial cells

The microvessels and endothelial cells within the peripheral area were counted at high magnification (x400) for each focus. Eligible microvessels included any immunostained endothelial cell or cluster of cells around a visible lumen clearly separated from adjacent microvessels, tumour cells and other connective tissue components [*Fig. 8.2-8.5*]. The presence of red blood cells was not required. It was not possible to distinguish blood and lymphatic vessels. Where vessels were in clusters, each was counted as separate if it met the above criteria. Endothelial cells were counted at the same time. Microvessel and endothelial density for each individual focus was calculated using the following formula:

$$\textit{Microvessel or endothelial density} = \frac{\textit{Number of microvessels or endothelial cells}}{\textit{Peripheral area}}$$

The assessment of microvessels and endothelial cells were carried solely by myself. I underwent three-month-training with two pathologists. The study only began once we reached an agreement ratio of at least 70%.

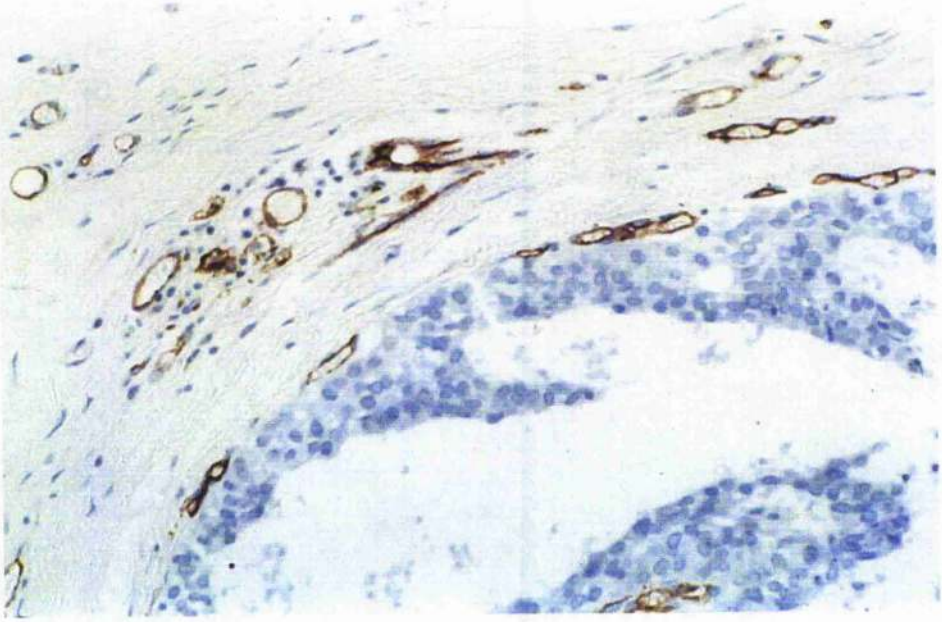


Figure 8.2. Periductal microvessels stained with anti-vWF antibody (high power field).

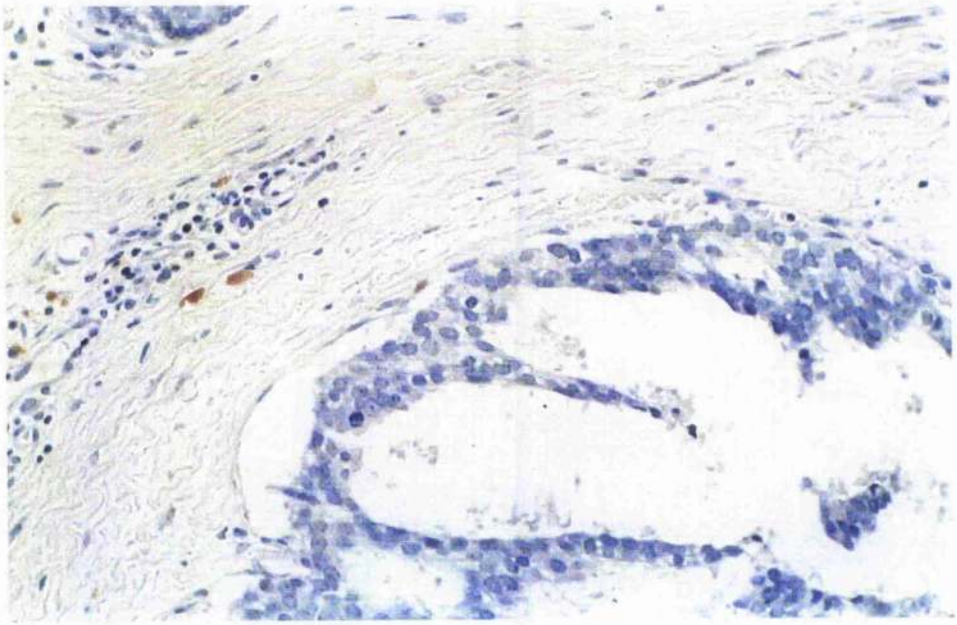


Figure 8.3. Periductal microvessels stained with anti-CD31 antibody (high power field).

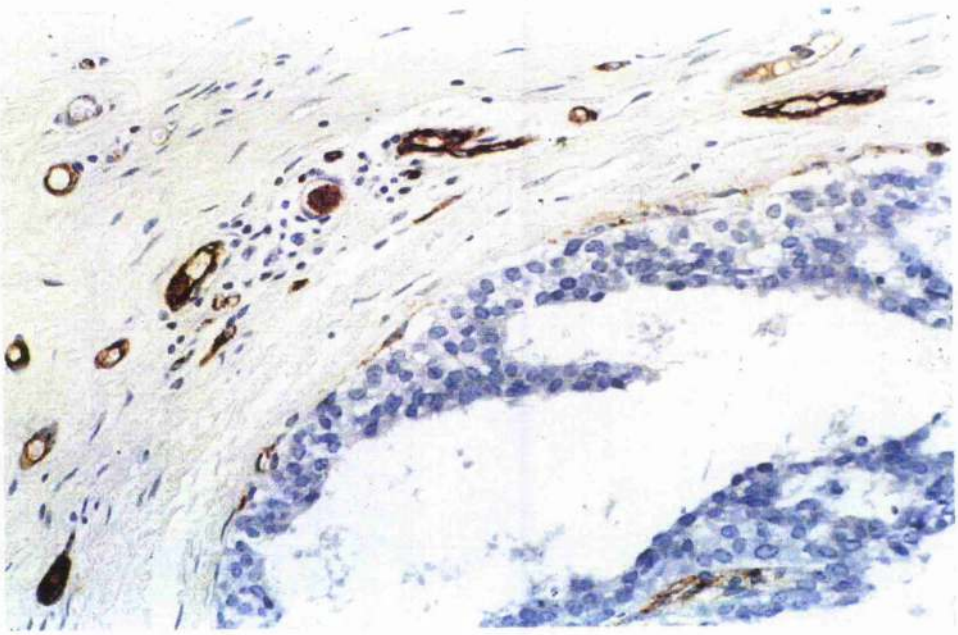


Figure 8.4. Periductal microvessels stained with anti-CD141 antibody (high power field).

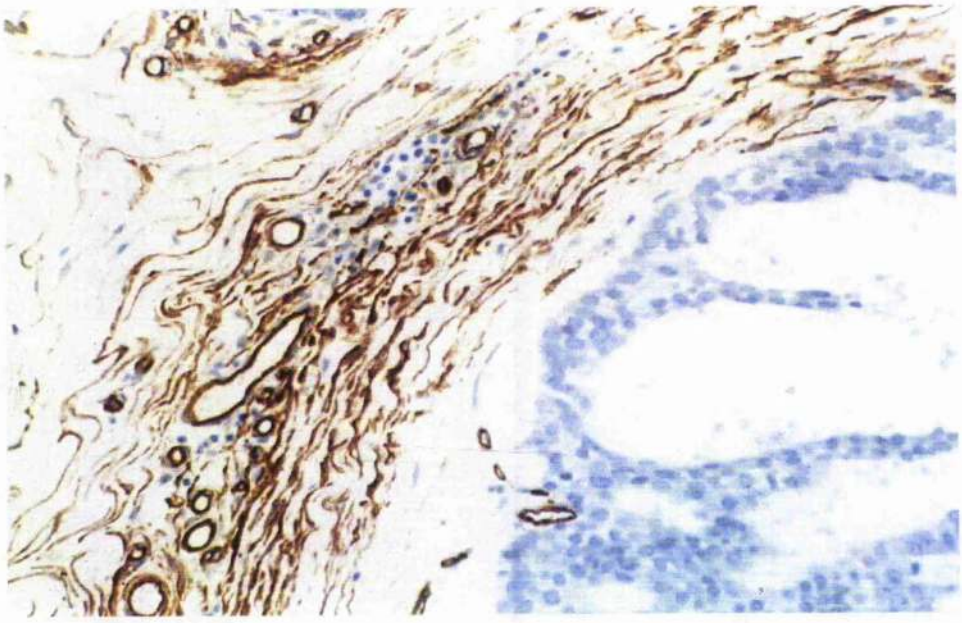


Figure 8.5. Periductal microvessels stained with anti-CD34 antibody (high power field).

Assessment of stromal tumour vascularity

Microvessel count was determined as described by Weidner et al [Weidner N et al 1991]. Microvessels were assessed in the areas of stroma containing the most capillaries (hotspots). Areas with high vascularisation were identified by scanning the sections at low power fields (x40 and x100) [*Fig. 8.6*]. Only those stromal areas at least 2 mm from the edge of the nearest tumour were assessed. Up to 30 individual highly vascularised areas were then examined at high power field (magnification: x200; field size: 0.32 mm^2). The 5 highest counts were recorded to give the data for hotspots (highest microvessel density), mean stromal M(5)VD (average of 5 highest microvessel densities) and mean stromal M(3)VD (average of 3 highest microvessel densities).

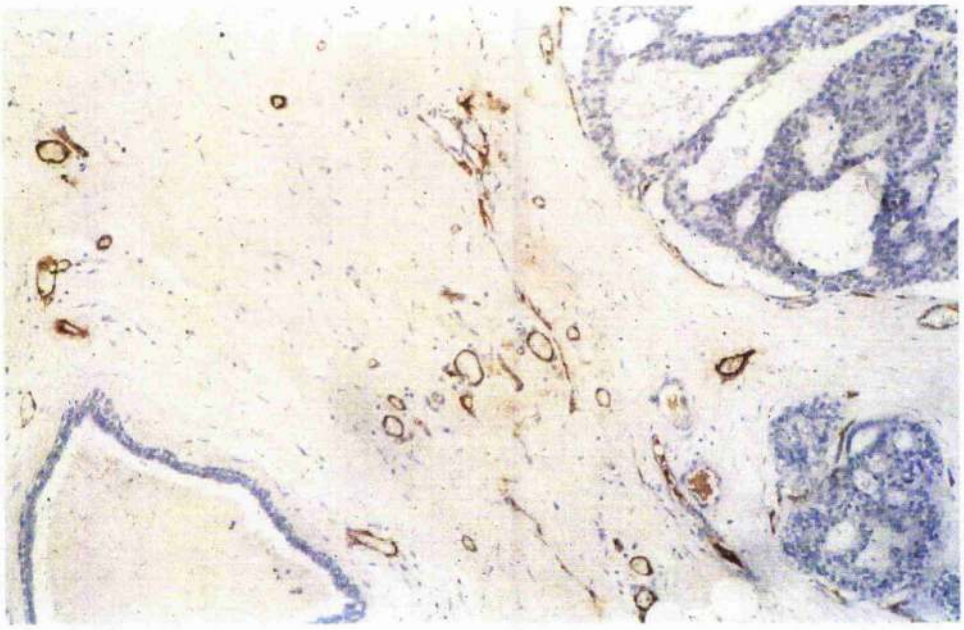


Figure 8.6. Stromal microvessels stained with anti-CD34 antibody (low power field).

Controls

Five normal lobules in the surrounding breast were used for internal controls and were assessed for microvessel and endothelial density in the same manner. The normal lobules were assessed only if they were situated more than 2 mm from the nearest tumour.

Evaluation of degree of necrosis

As part of the histological assessment, the degree of necrosis was semi-quantitatively assessed in sections of high grade DCIS from patients with and without invasive disease. There were three categories:

Area of necrosis	Score
None	1
1- 50%	2
> 50%	3

Quantification of Ki-67 staining

a) Ki-67 density (KD)

All or the first 50 foci of DCIS encountered were assessed on each section, thus eliminating selection bias. The core areas of DCIS foci were measured at the x200 magnification using image analysis (Zeis Axiohome with software version 3.0, Germany). The Ki-67density for each individual focus was calculated by dividing the number of Ki-67 positive cells in the focus with the corresponding core area of the tumour [*Fig. 8.7 & 8.8*]. The mean Ki-67 density (MKD) was also calculated for each section.

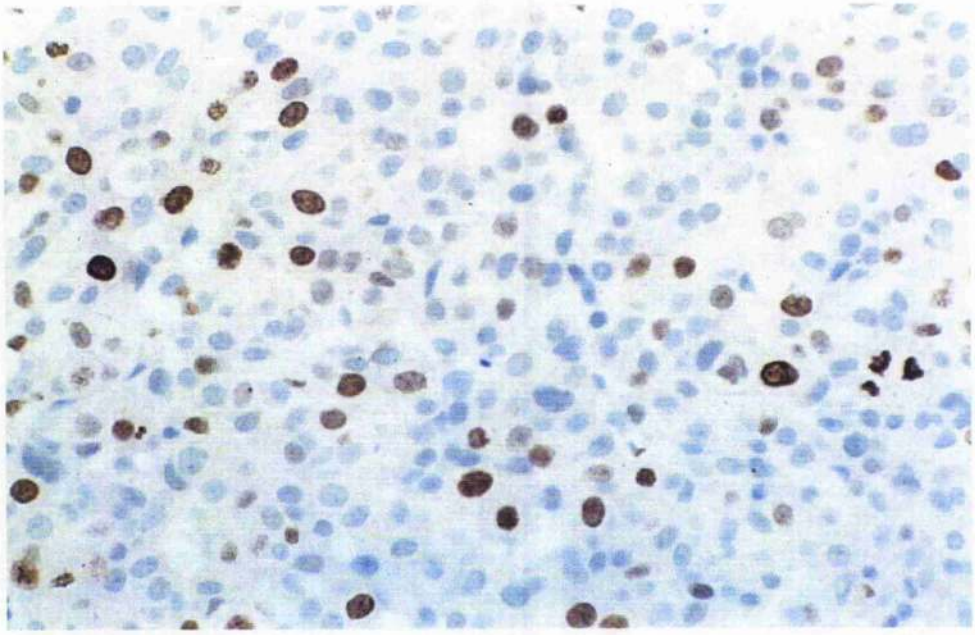


Figure 8.7. High density of parenchymal cells (DCIS) positively-stained with anti-Ki67 antibody (high power field).

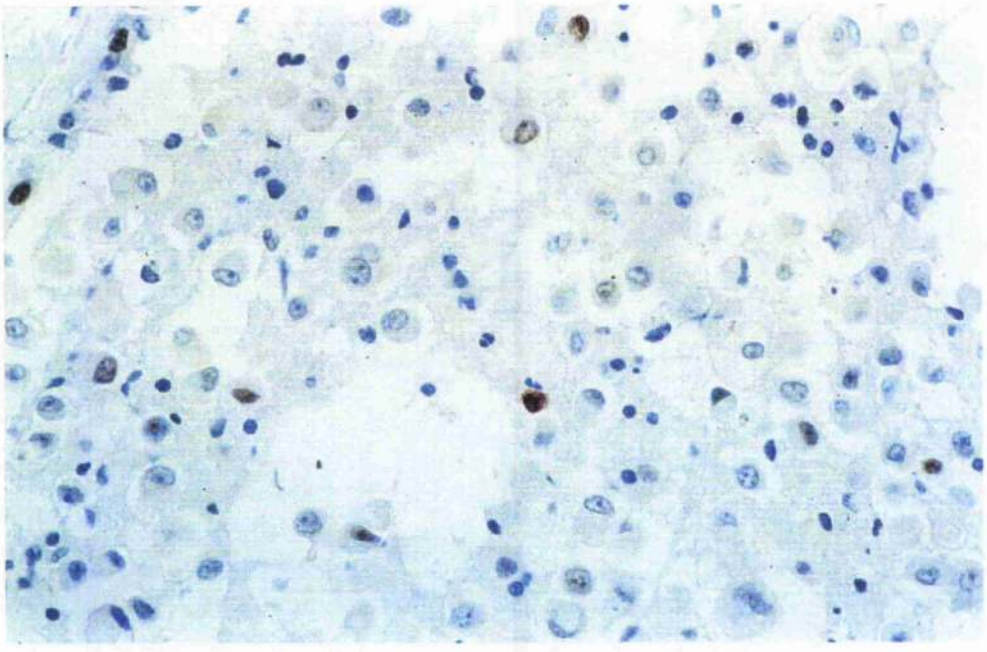


Figure 8.8. Low density of parenchymal cells (DCIS) positively-stained with anti-Ki67 antibody (high power field).
b) Ki-67 fraction/percentage (KP)

On each section, the first 1000 tumour cells were counted randomly. The number of Ki-67 positive cells was expressed in percentage.

Assessment of Thymidine Phosphorylase expression

Sections were examined at low power field (x40 and x100) [*Fig. 8.9*]. TP expression in carcinoma cells was both cytoplasmic and nuclear, with the former predominating. The nuclear and cytoplasmic staining was not separated, scoring was given to overall staining. The expression in cancer cells was evaluated both for intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and percentage of cell staining [*Fig. 8.10 & 8.11*]. The product of multiplication of intensity and percentage of cell staining were then added to produce a 'H-score' with a range from 0 to 300.

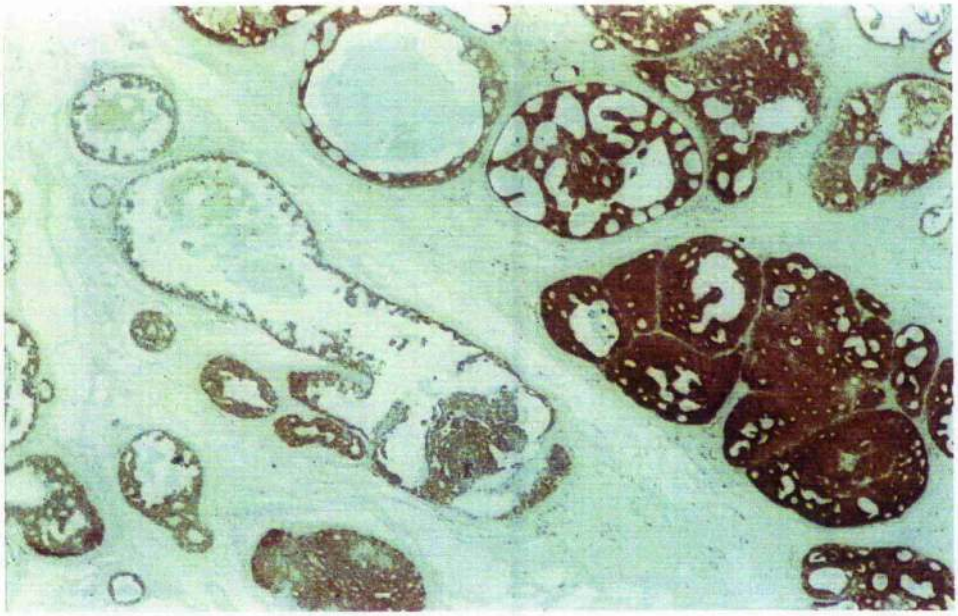


Figure 8.9. Thymidine Phosphorylase expression in DCIS parenchymal cells (low power field).

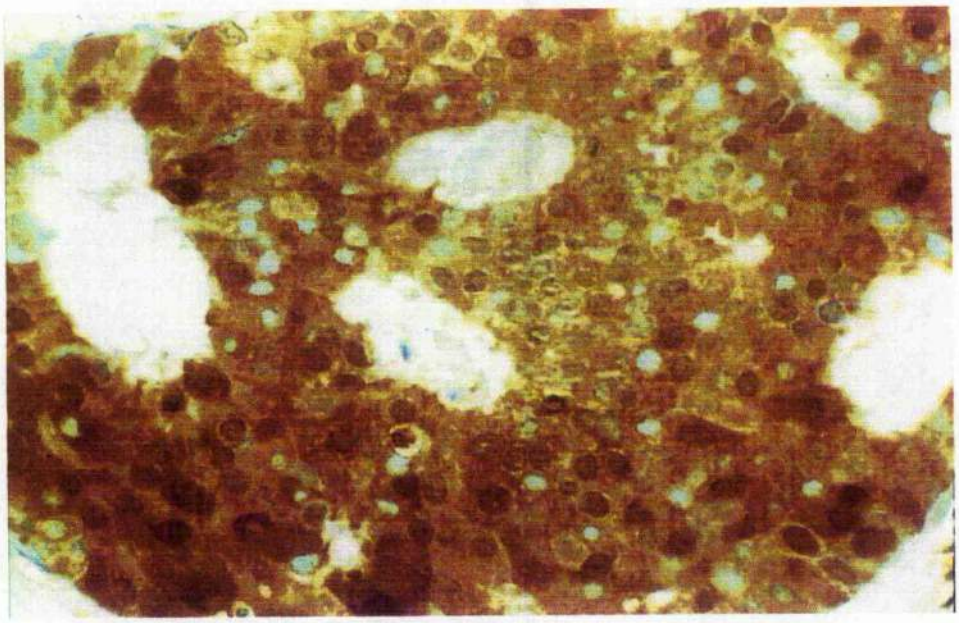


Figure 8.10. High intensity of Thymidine Phosphorylase expression in DCIS parenchymal cells (high power field).

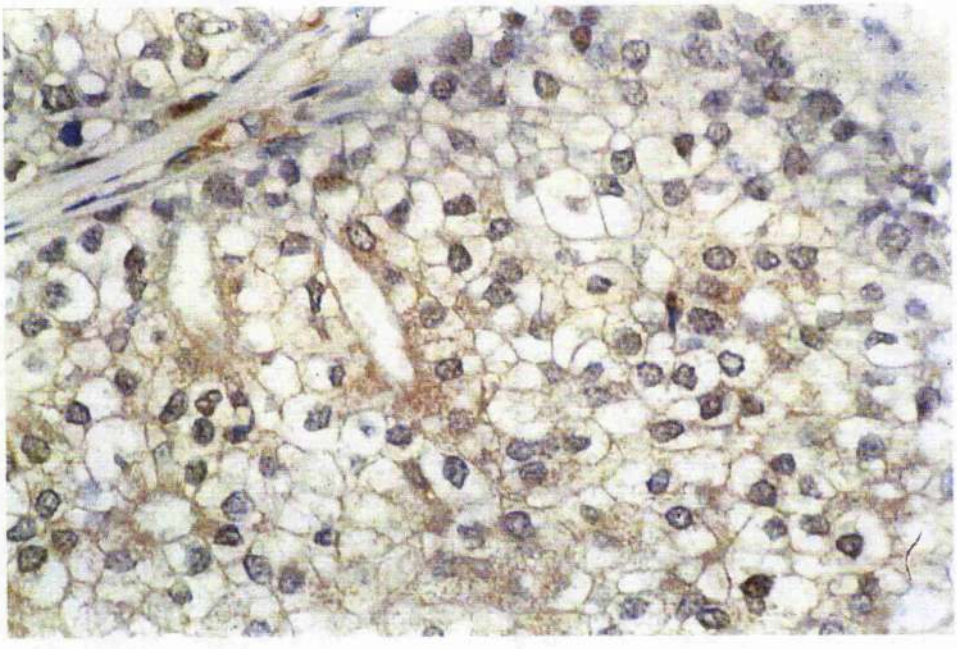


Figure 8.11. Low intensity of Thymidine Phosphorylase expression in DCIS parenchymal cells (high power field).

Immunocytochemistry for dual immunostaining (vWF and CD34)

Sections were dewaxed through two changes of xylene and IMS respectively. The sections were treated with 500 ml of Ethylenediamine-Tetraacetic Acid (ED2SS; Sigma Chemical, St Louis USA) in the microwave for 20 minutes. Prior to incubation with primary antibodies, sections were treated with a mixture of 5% BSA/TBS for 10 minutes. The antibodies were diluted 1:10 for anti-CD34 and 1:1000 for anti-human vWF in 5% BSA/TBS. The sections were incubated with primary antibodies at room temperature for 40 minutes. Secondary antibodies were applied for 30 minutes using a biotinylated sheep anti-mouse antibody (Amersham Pharmacia Biotech, Essex UK) diluted 1:100 and a swine anti-rabbit TRITC Conjugated antibody (DAKO, Cambridge, UK) diluted 1:50. Sections were then incubated for 30 minutes using Flourescein Avidin DCS (A-2011; Vector Labs, Peterborough UK) diluted 1:100 and Swine α Anti-Rabbit Immunoglobulin TRITC diluted 1:50. Sections were washed with TBS between incubation steps.

DNA was stained by immersion in a solution of 4',6-diamidino-2-phenylindole (Sigma) at a concentration of 250 ng/ml in phosphate buffered saline for 10 min and coverslips were mounted onto the tissue sections in an antifading medium (Vectashield, Vector Laboratories, Peterborough UK). Control slides were included in each analysis by performing the same procedures and substituting non-immune serum for primary antibodies and secondary antibodies individually.

Assessment of slides in dual immunostaining

Quantification of the fluorochrome-labeled vessels was performed as above. Each vessel was examined under a high power lens for the red (TRITC), green (fluorescein) and blue (4',6-diamidino-2-phenylindole) fluorochromes using the appropriate filters in succession to assess the presence or absence of double-labeled vessels. A triple band filter in which all three fluorochromes could be seen simultaneously was used for confirmation of dual staining. In my study, CD34- and vWF-positive endothelial cells stained green and red in colour respectively [*Fig. 8.12 & 8.13*].

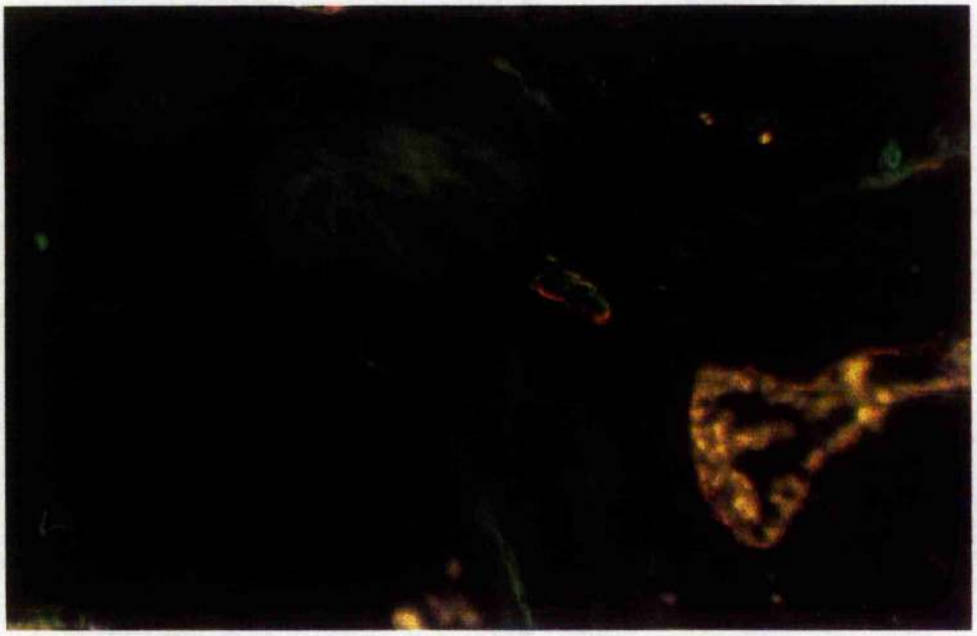


Figure 8.12. High power field: CD34-, vWF-, and dual positive (in the middle) microvessels were present.

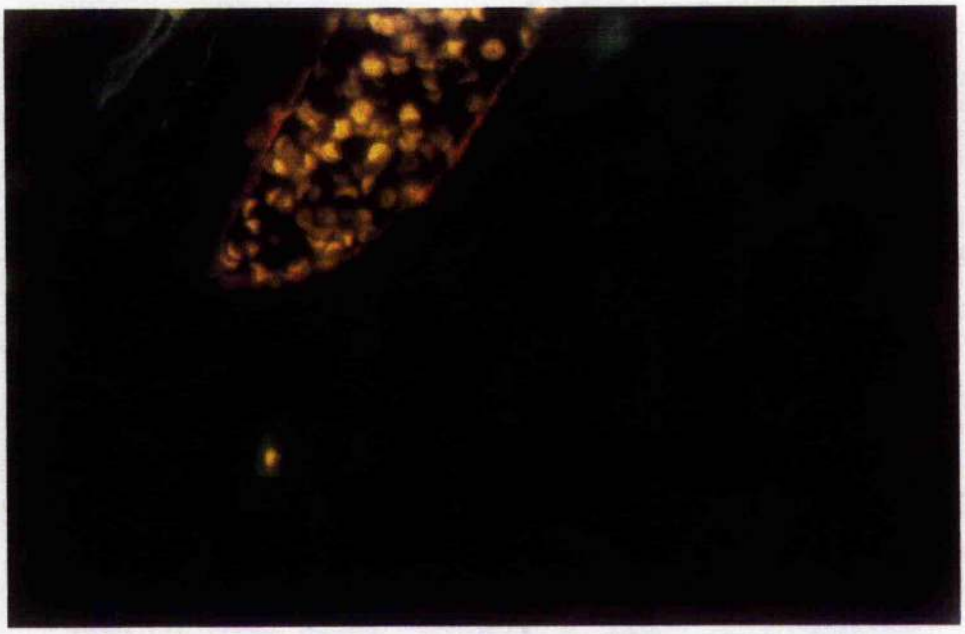


Figure 8.13. High power field: only CD34- and vWF-positive microvessels were present.

Statistics

The differences of parameters were analysed using the Kruskal-Wallis method and Mann-Whitney U-test. The relationship between microvessels or endothelial density and a variety of histological features was assessed by means of 2-tailed Spearman's or Pearson's Correlation test as indicated. A p-value below 0.05 was considered statistically significant. All analyses were undertaken using the SPSS package (Version 10.0 for Window 97/NT).

Section 4

Chapter 9

Periductal Vascularity in Ductal Carcinoma *in situ* of the Breast

Results

Nuclear grade of DCIS

In each group of 20 patients with and without invasive breast cancer, 7 lesions were low, 6 intermediate and 7 high nuclear grade.

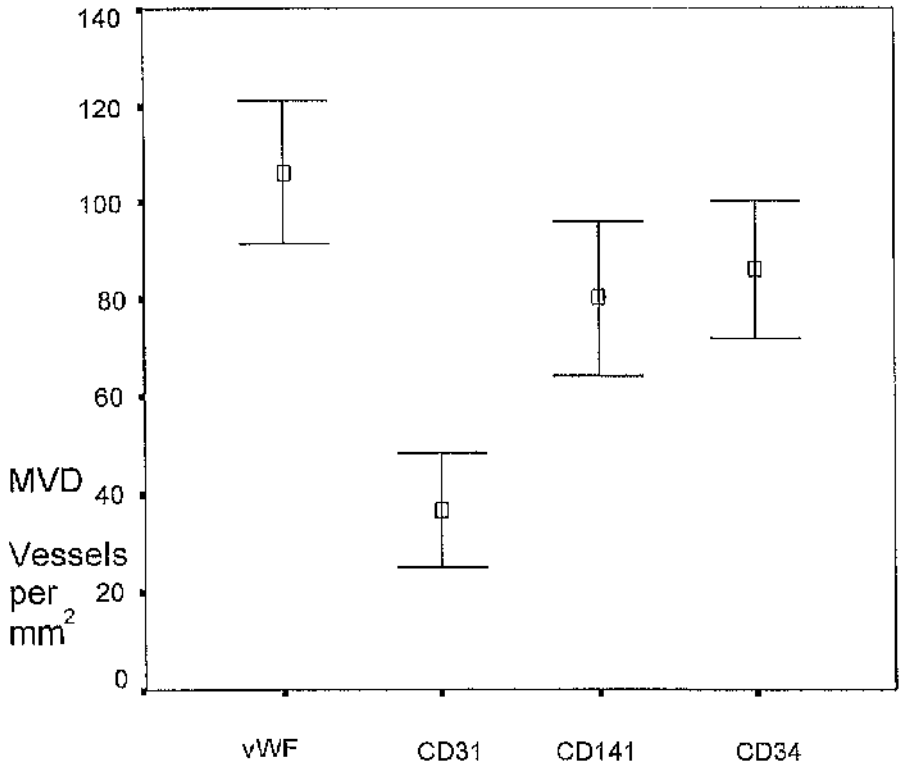
Microvessel density in DCIS without invasive carcinoma

Normal lobules

The highest values of periductal MVD were obtained using the anti-vWF antiserum, followed by monoclonal antibodies CD34, CD141 and CD31. These differences were significant apart from those between CD141 and CD34. [*Fig.9.1*].

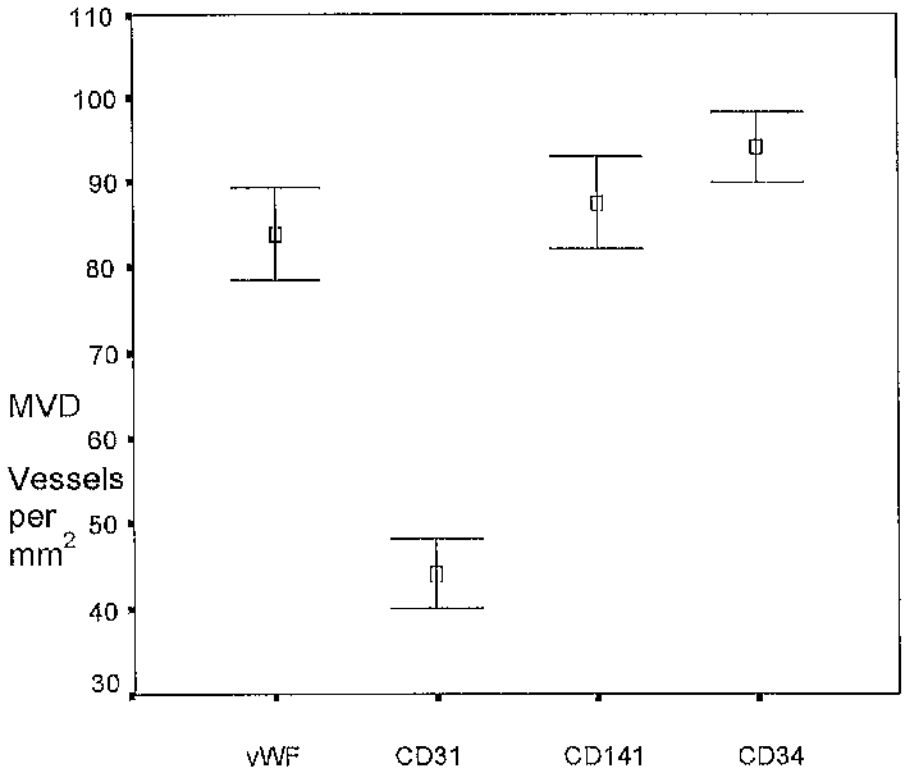
DCIS

The highest periductal MVD was obtained using the antibody to CD34, followed by CD141, vWF and CD31. This reflected increases in CD34, 141 and 31 positive vessels but a decrease in those immunopositive for vWF. Changes in periductal MVD detected by the CD34, CD31 and vWF antibodies were statistically significant compared to those around normal lobules [*Fig.9.2*]. Periductal MVD around DCIS using CD34 was less than that around normal lobules using anti-vWF. However, the difference was not statistically significant.



Comparison	p Value	Comparison	p Value	Comparison	p Value
vWF vs CD31	<0.001	vWF vs CD34	0.010	CD31 vs CD34	<0.001
vWF vs CD141	0.003	CD31 vs CD141	<0.001	CD141 vs CD34	0.279

Figure 9.1. MVD around normal lobules in cases of **DCIS** *without invasive carcinoma.*



Comparison	p Value	Comparison	p Value	Comparison	p Value
vWF vs CD31	<0.001	vWF vs CD34	<0.001	CD31 vs CD34	<0.001
vWF vs CD141	0.244	CD31 vs CD141	<0.001	CD141 vs CD34	<0.001

Figure 9.2. MVD around **DCIS** *without invasive carcinoma.*

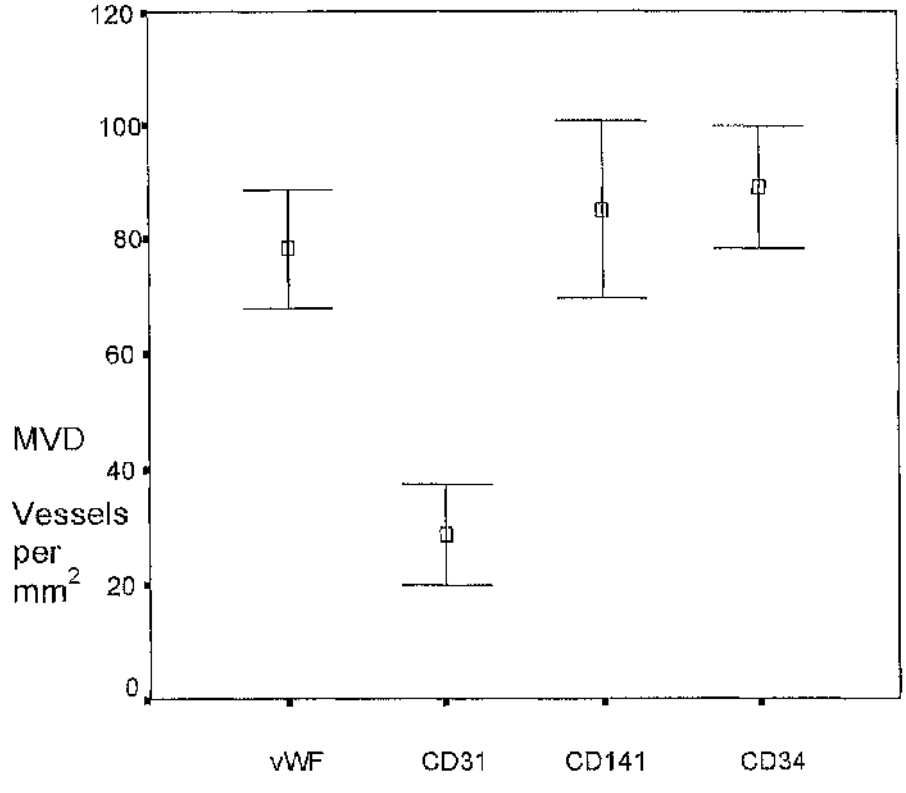
Comparison of values between Fig. 1 and Fig. 2			
Comparison	**p Value**	**Comparison**	**p Value**
vWF	0.001	CD141	0.054
CD31	0.015	CD34	0.004

Table 9.1. Comparison between MVD around normal lobules and **DCIS** *without invasive carcinoma.*

Microvessel density in DCIS with invasive carcinoma

Normal lobules

In contrast to cases of pure DCIS, normal lobules associated with DCIS with invasive carcinoma exhibited the greatest periductal MVD using the CD34 antibody, followed by CD141, vWF and CD31. CD31 gave significantly different values to the other 3 antibodies. There was no significant difference between the periductal MVD as measured by CD34, CD141, and vWF [*Fig.9.3*]. The periductal MVD obtained with vWF was significantly lower than in normal lobules from cases of pure DCIS whereas those obtained with CD31, CD34 and CD141 showed no significant difference.



Comparison	p Value	Comparison	p Value	Comparison	p Value
vWF vs CD31	<0.001	vWF vs CD34	0.348	CD31 vs CD34	<0.001
vWF vs CD141	0.813	CD31 vs CD141	<0.001	CD141 vs CD34	0.093

Figure 9.3. MVD around normal lobules in cases of* DCIS *with invasive carcinoma.

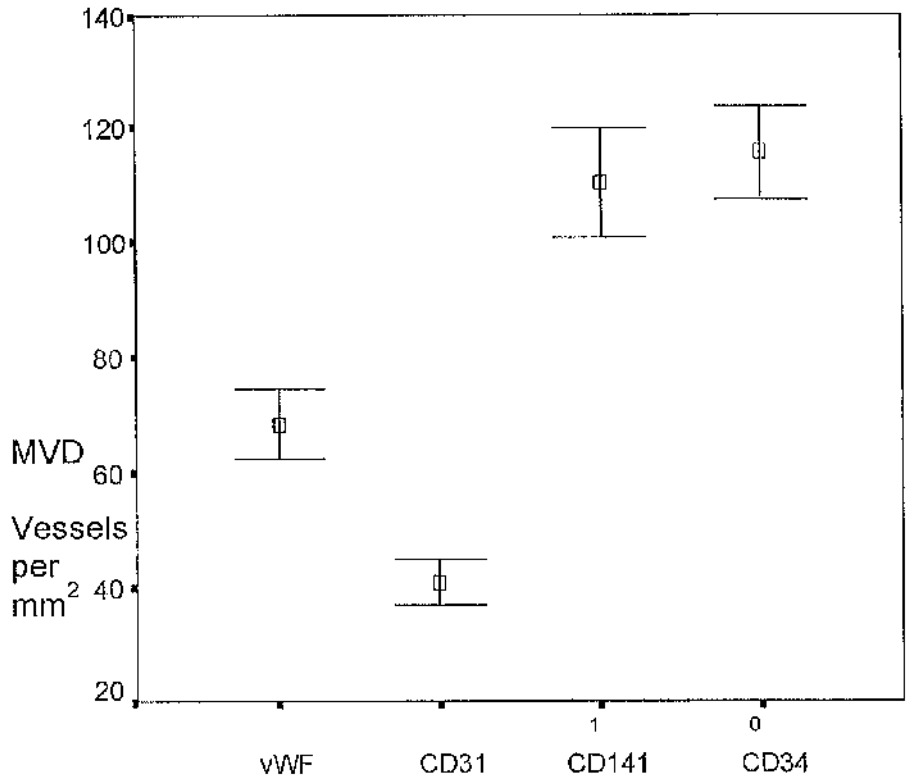
Comparison of values between Fig. 1 and Fig. 3			
Comparison	**p Value**	**Comparison**	**p Value**
vWF	0.014	CD141	0.363
CD31	0.513	CD34	0.209

Table 9.2. Comparison between MVD around normal lobules in cases of* DCIS *with and without invasive carcinoma.

DCIS

The highest periductal MVD was obtained using the CD34 antibody, followed by CD141, vWF and CD31. There were significant differences between all 4 antibodies apart from between CD34 and CD141 *[Fig.9.4]*. In common with cases of pure DCIS, there were increases above normal in CD31, CD34 and CD141 but a decrease in vWF immunopositive vessels. All these changes were statistically significant.

When compared with *pure* DCIS, there were increases in periductal MVD as determined by CD34 and 141 and decreases as determined by CD31 and vWF. Except for CD31, these findings were statistically significant.



Comparison	p Value	Comparison	p Value	Comparison	p Value
vWF vs CD31	<0.001	vWF vs CD34	<0.001	CD31 vs CD34	<0.001
vWF vs CD141	<0.001	CD31 vs CD141	<0.001	CD141 vs CD34	0.034

Figure 9.4. MVD around* DCIS *with invasive carcinoma.

Comparison of values between Fig. 3 and Fig. 4			
Comparison	**p Value**	**Comparison**	**p Value**
vWF	0.030	CD141	0.004
CD31	<0.001	CD34	0.003

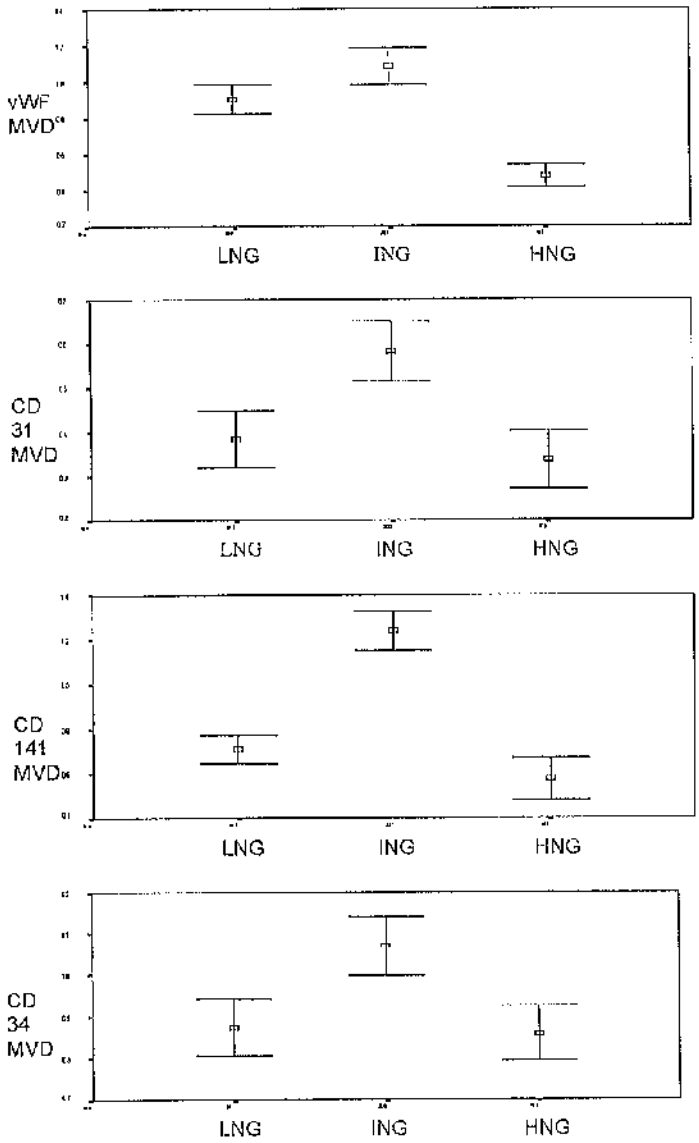
Table 9.3. Comparison between MVD around normal lobules and* DCIS *with invasive carcinoma.

Comparison of values between Fig. 2 and Fig. 4			
Comparison	**p Value**	**Comparison**	**p Value**
vWF	<0.001	CD141	0.001
CD31	0.305	CD34	0.001

Table 9.4. Comparison between MVD around* DCIS *with and without invasive carcinoma.

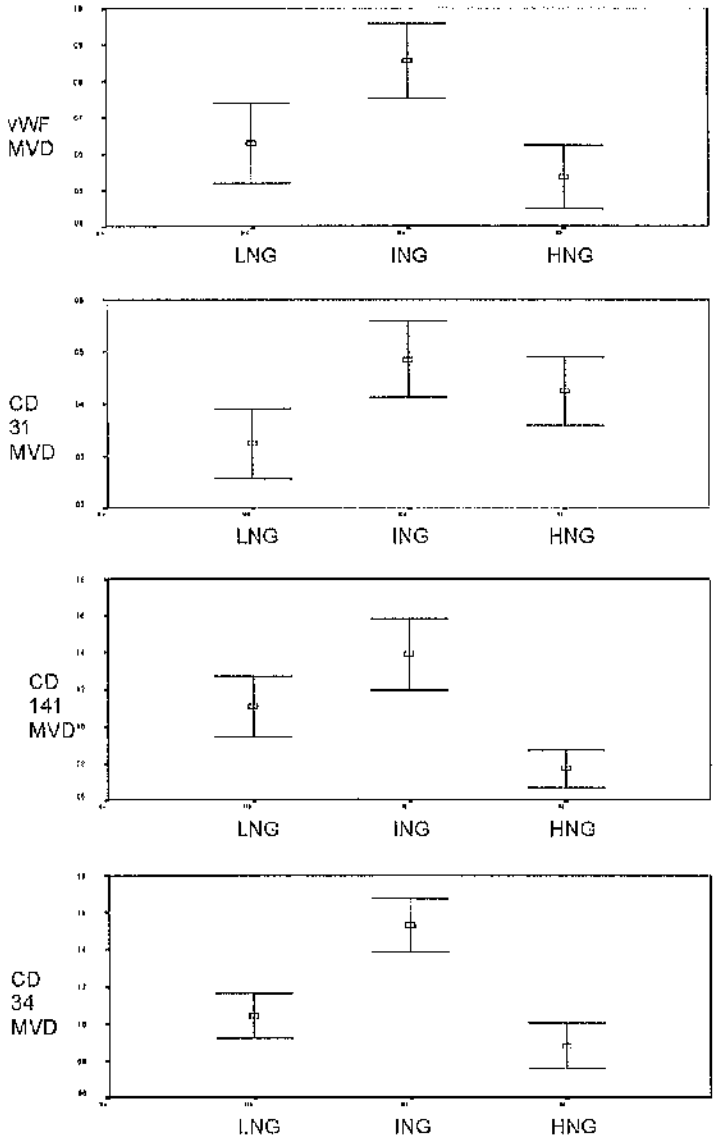
Relationship between MVD and nuclear grade

Periductal MVD increased from low to intermediate nuclear grade but decreased in high grade DCIS usually to a level below that of low grade. The difference between low and intermediate grade DCIS was statistically significant for pure DCIS and those associated with invasive carcinoma using all antibodies (except for pure DCIS using vWF). The difference between intermediate and high grade was significant for both pure DCIS and those associated with invasive carcinoma with all antibodies (except for invasive cases using CD31). The difference between low and high grade, however, was significant only for pure DCIS using CD141, CD31 and vWF antibodies and for invasive cases using CD31 [*Figs. 9.5 & 9.6*].



vWF		CD31		CD141		CD34	
Comparison	**p Value**	**Comparison**	**p Value**	**Comparison**	**p Value**	**Comparison**	**p Value**
LNG vs ING	0.071	LNG vs ING	<0.001	LNG vs ING	<0.001	LNG vs ING	<0.001
LNG vs HNG	<0.001	LNG vs HNG	0.047	LNG vs HNG	<0.001	LNG vs HNG	0.843
ING vs HNG	<0.001	ING vs HNG	<0.001	ING vs HNG	<0.001	ING vs HNG	<0.001

Figure 9.5. Relationship between periductal MVD and nuclear grade in* DCIS *without invasive carcinoma. (LNG = low nuclear grade, ING = intermediate nuclear grade, HNG = high nuclear grade)

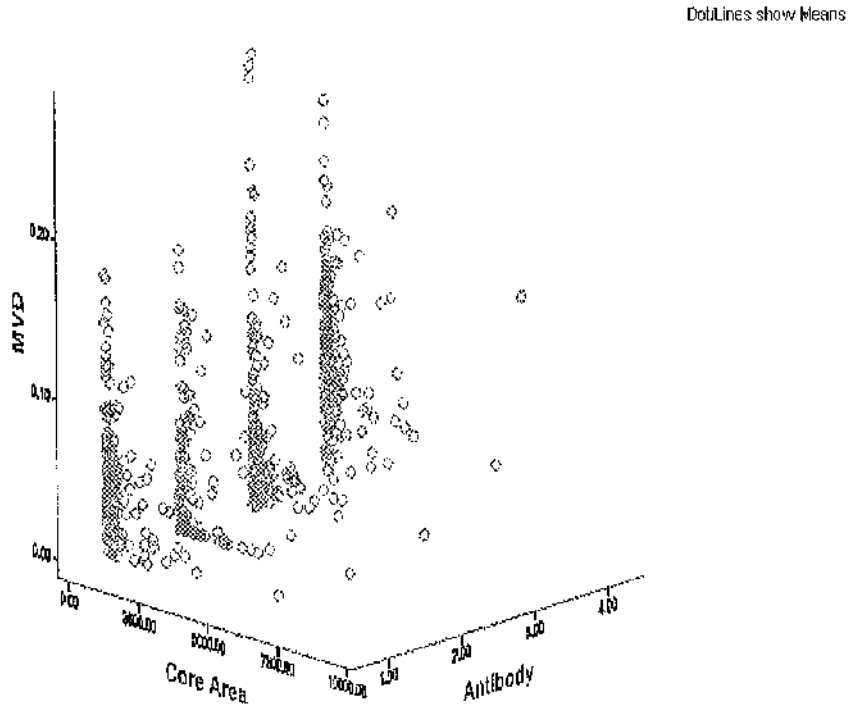


vWF		CD31		CD141		CD34	
Comparison	**p Value**	**Comparison**	**p Value**	**Comparison**	**p Value**	**Comparison**	**p Value**
LNG vs ING	<0.001	LNG vs ING	<0.001	LNG vs ING	0.007	LNG vs ING	<0.001
LNG vs HNG	0.800	LNG vs HNG	0.001	LNG vs HNG	0.064	LNG vs HNG	0.136
ING vs HNG	<0.001	ING vs HNG	0.473	ING vs HNG	<0.001	ING vs HNG	<0.001

Figure 9.6. Relationship between periductal MVD and nuclear grade in* DCIS *with invasive carcinoma. (LNG = low nuclear grade, ING = intermediate nuclear grade, HNG = high nuclear grade)

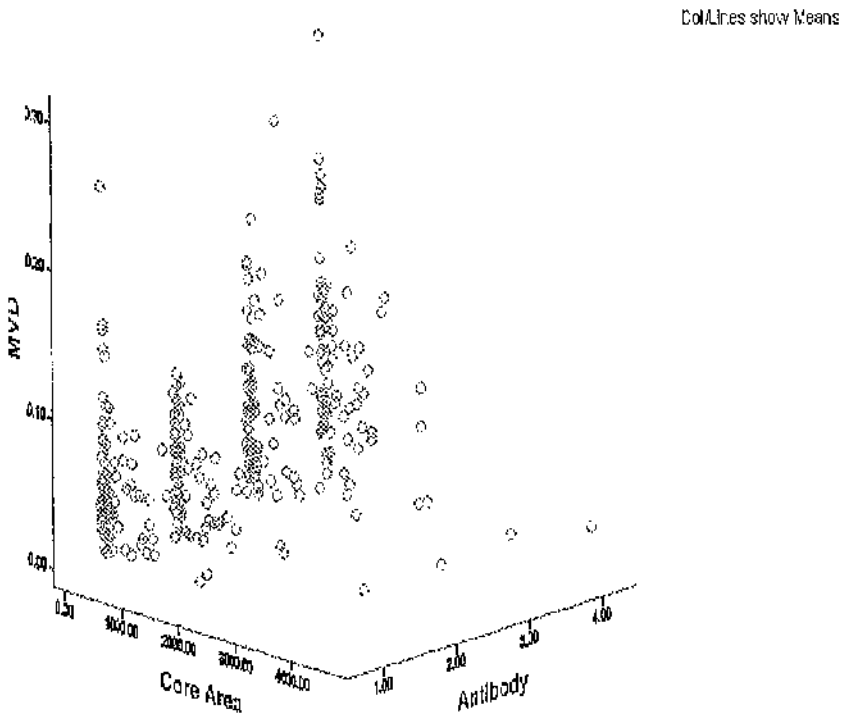
Relationship between MVD and core area

A significant negative correlation was noted between the core area of individual foci and MVD as determined with all four antibodies except when CD31 was used on cases of DCIS associated with invasion *(Figs. 9.7 & 9.8)*: as the area of the DCIS focus increased so the periductal MVD decreased.



Antibody	Correlation
vWF	p <0.001
CD31	p <0.001
CD141	p <0.001
CD34	p <0.001

Figure 9.7. Relationship between periductal MVD (vessels per 10^3 μm^2) and core area (10^3 μm^2) in* DCIS *without invasive carcinoma. (antibody 1=vWF, 2=CD31, 3=CD141, 4=CD34)



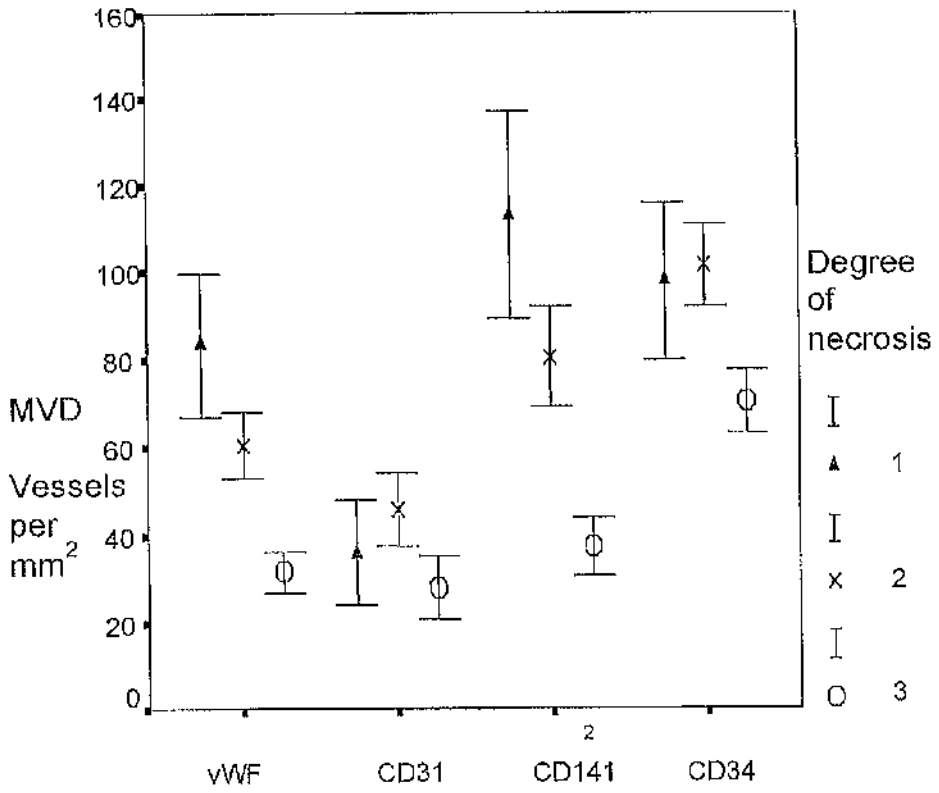
Antibody	Correlation
vWF	p <0.001
CD31	p =0.288
CD141	p <0.001
CD34	p <0.001

Figure 9.8. Relationship between MVD (vessels per 10^3 μm^2) and core area (10^3 μm^2) in **DCIS** *with invasive carcinoma. (antibody 1=vWF, 2=CD31, 3=CD141, 4=CD34)*

Relationship between MVD, core area and necrosis

As these analyses were restricted to high nuclear grade DCIS, cases with and without invasive carcinoma were pooled.

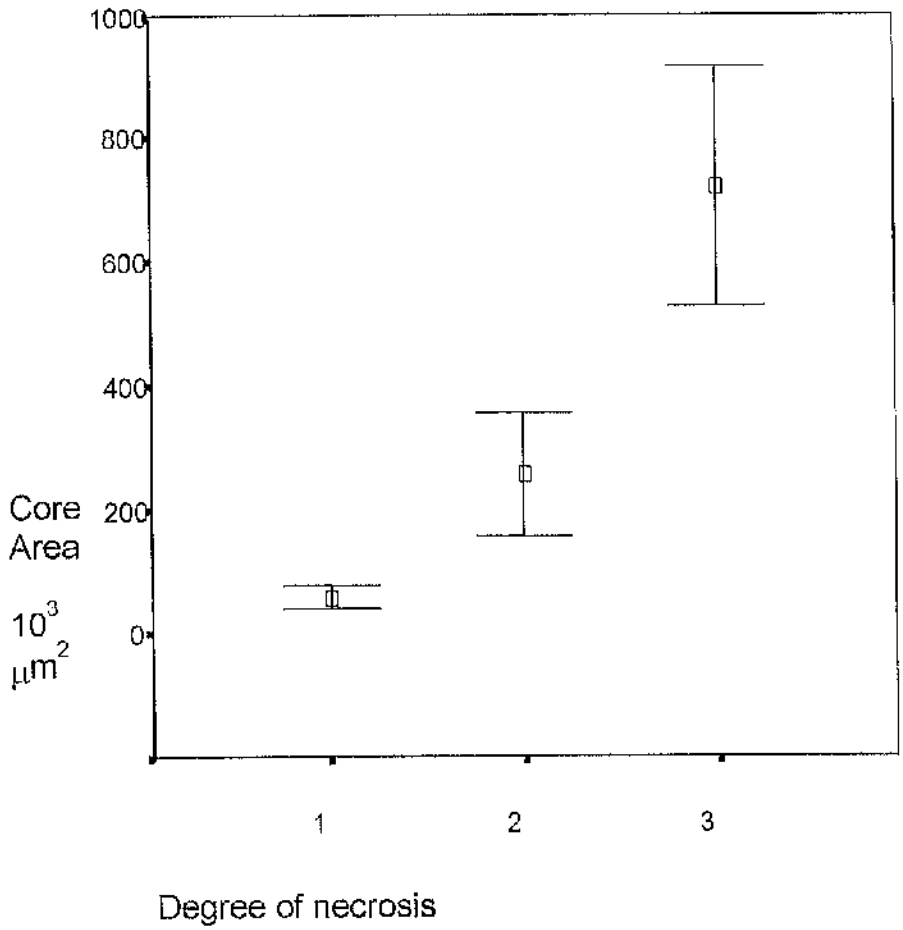
A significant negative correlation was observed between necrosis scores and MVD using all four antibodies: the more necrosis there was, the less vascularity there was. Using CD141 antibody, there was a significant difference in MVD from necrosis score 1 to 2 as well as between 2 and 3. Using anti-vWF and anti-CD34 antibodies, a significant difference was only seen between necrosis score of either 1 or 2 and 3 [*Fig. 9.9*].



vWF		CD31		CD141		CD34	
Comparison	p Value	Comparison	p Value	Comparison	p Value	Comparison	p Value
N 1 vs N 2	0.18	N 1 vs N 2	0.176	N 1 vs N 2	0.033	N 1 vs N 2	0.620
N 1 vs N 3	<0.001	N 1 vs N 3	0.057	N 1 vs N 3	<0.001	N 1 vs N 3	0.004
N 2 vs N 3	<0.001	N 2 vs N 3	<0.001	N 2 vs N 3	<0.001	N 2 vs N 3	<0.001

Figure 9.9. Relationship between periductal MVD and degree of necrosis in **DCIS** *with and without invasive carcinoma. (N in table represents the degree of necrosis)*

A significant positive correlation was observed between necrosis scoring and core area of the foci for both pure DCIS and DCIS associated with invasion: the larger the area of DCIS, the more necrosis was present [*Fig. 9.10*].



Comparison	p Value
Degree of necrosis 1 vs 2	<0.001
Degree of necrosis 1 vs 3	<0.001
Degree of necrosis 2 vs 3	<0.001

Figure 9.10. Relationship between the degree of necrosis and core area in* DCIS *with and without invasive carcinoma. (samples stained with CD34 were used for this assessment)

Relationship between MVD and ED

In this series, there was a significant and positive correlation between MVD and ED in both groups of patients (all p <0.001): as MVD increases, so does ED [*Fig.9.11a-d & 9.12a-d*].

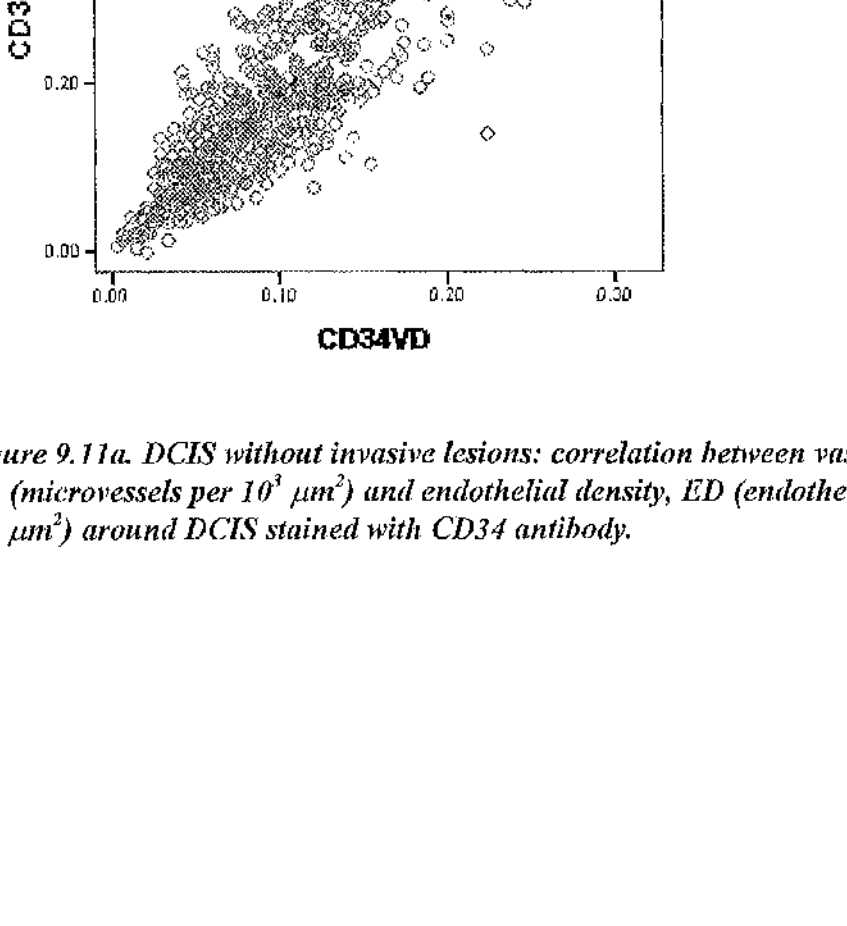


Figure 9.11a. DCIS without invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with CD34 antibody.

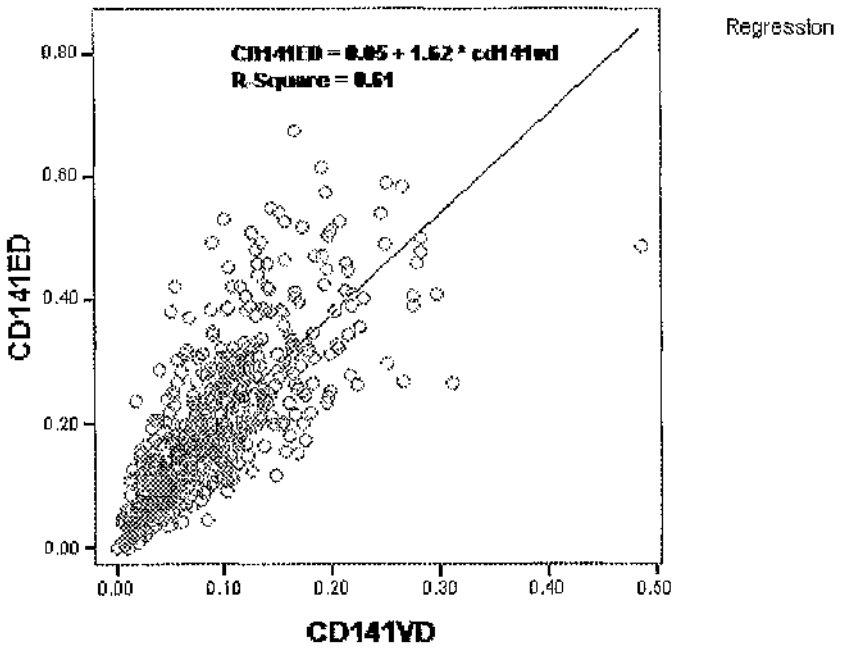


Figure 9.11b. DCIS without invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with CD141 antibody.

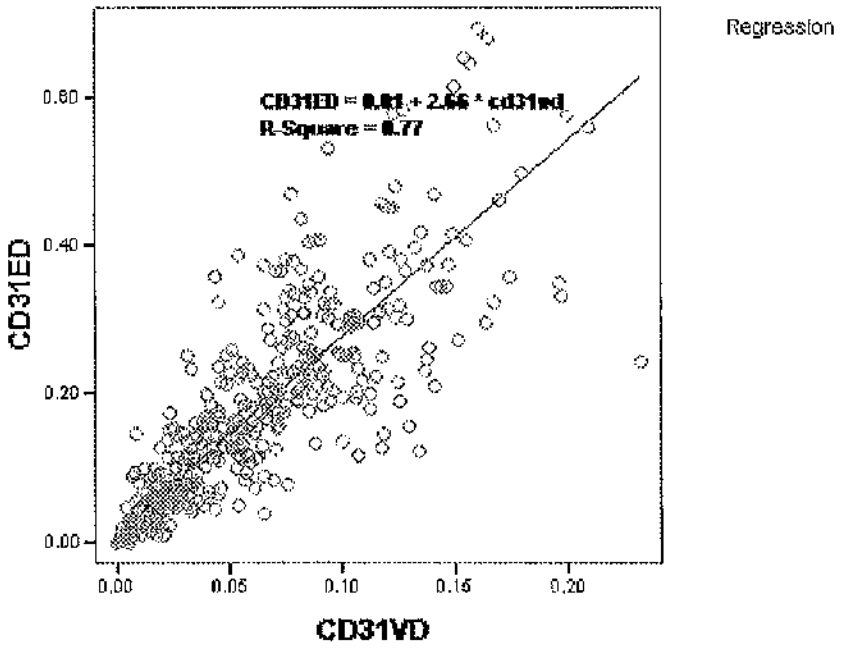


Figure 9.11c. DCIS without invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with CD31 antibody.

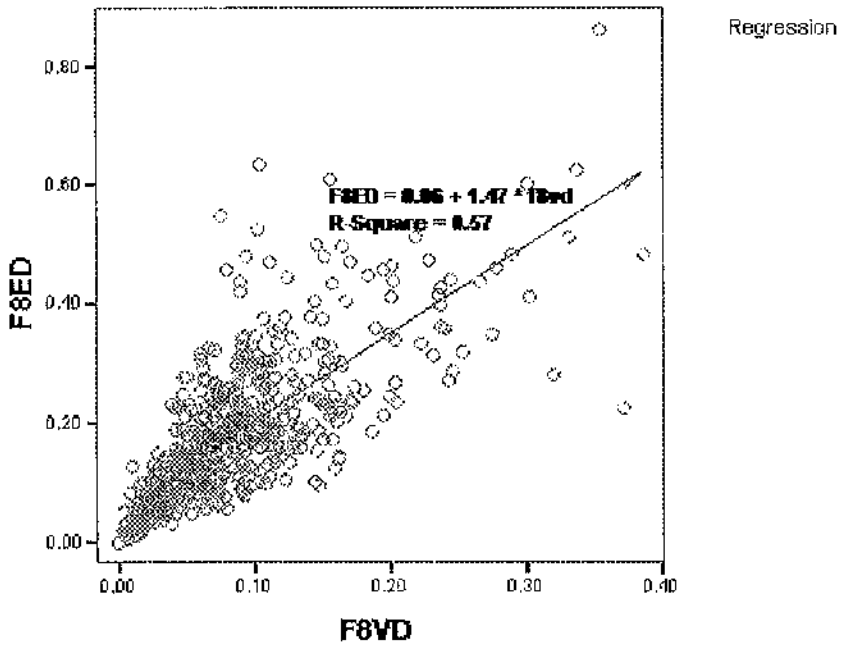


Figure 9.11d. DCIS without invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with vWF antibody.

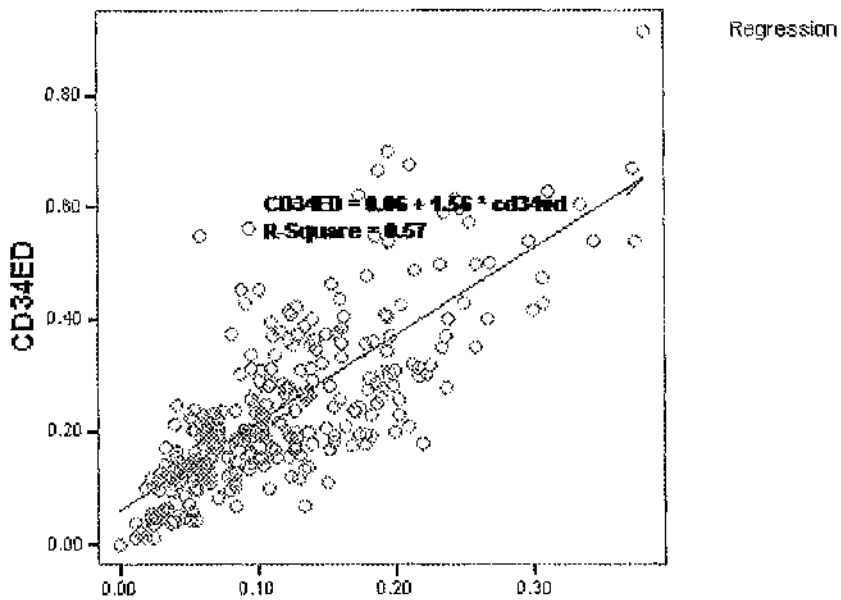


Figure 9.12a. DCIS with invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with CD34 antibody.

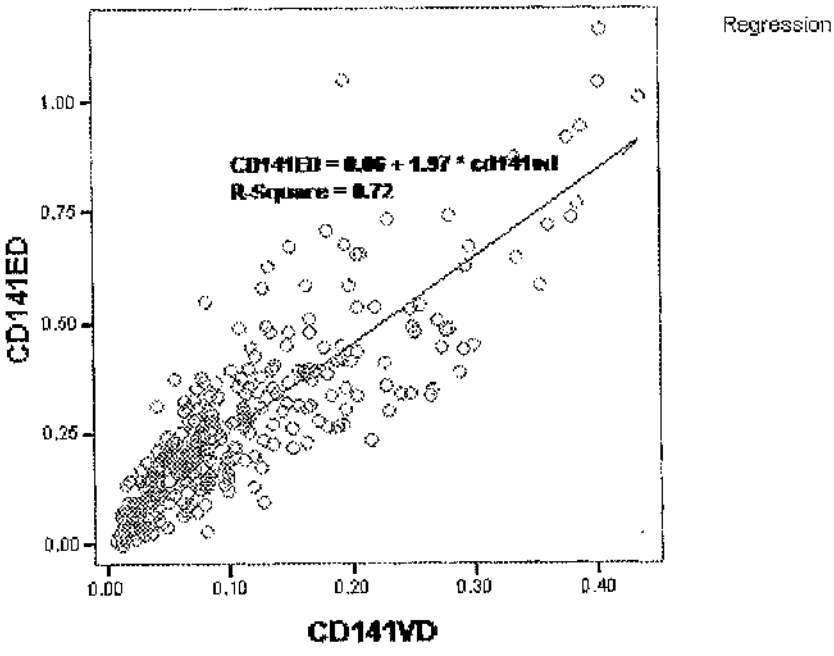


Figure 9.12b. DCIS with invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with CD141 antibody.

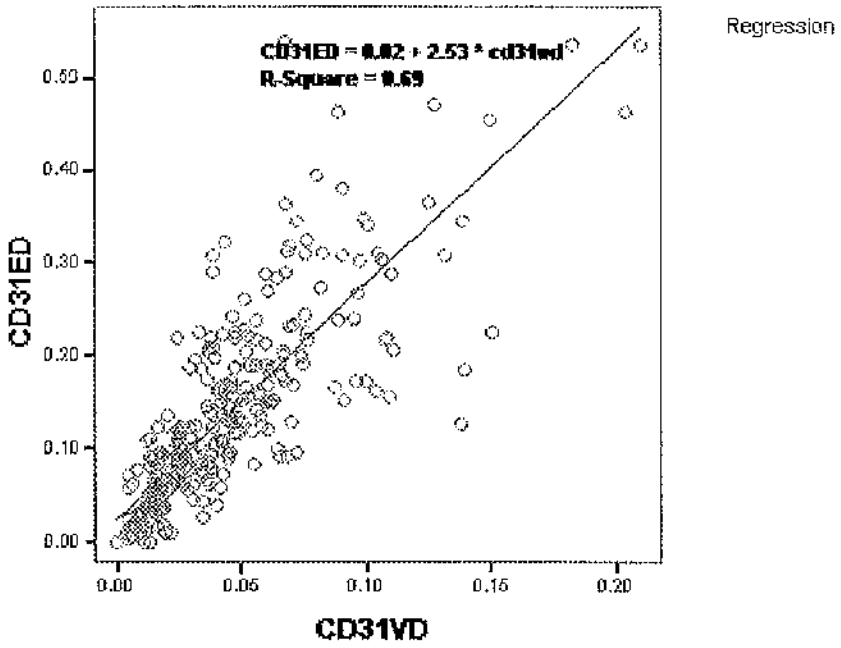


Figure 9.12c. DCIS with invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with CD31 antibody.

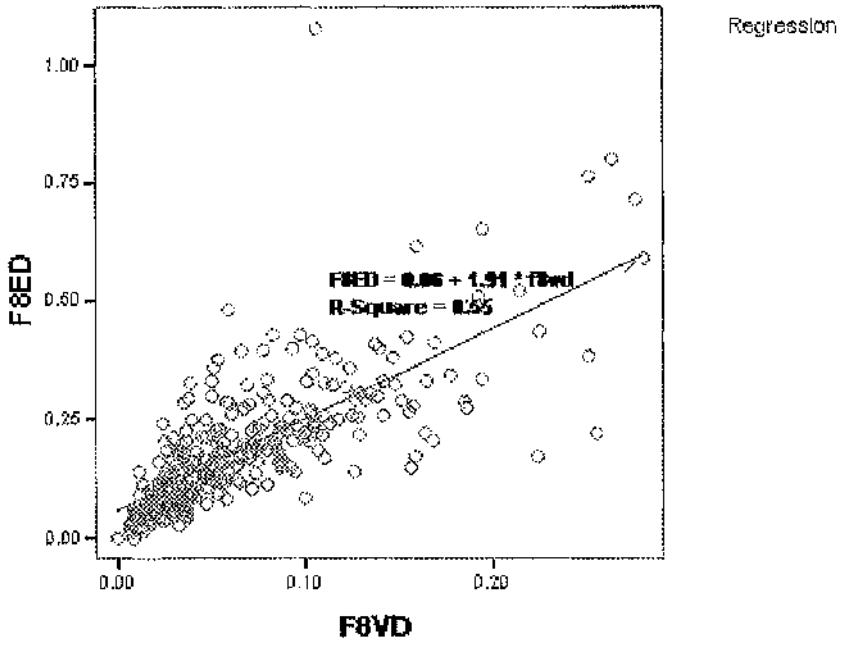


Figure 9.12d. DCIS with invasive lesions: correlation between vascular density, VD (microvessels per 10^3 μm^2) and endothelial density, ED (endothelial cells per 10^3 μm^2) around DCIS stained with vWF antibody.

Discussion

Several groups have previously investigated vascularisation of DCIS but none, to my knowledge, has compared vascular density around involved ducts and normal structures in cases with and without invasive carcinoma using morphometry and a panel of anti-endothelial antibodies. I am thus unaware of any previous reports of changes in MVD and vascular phenotype immediately surrounding DCIS and of their relationship with the presence of invasive carcinoma, the degree of necrosis and duct size.

For a tumour to grow beyond 1-2 mm3, it requires the formation of new blood vessels to supply oxygen and nutrients, and to excrete catabolites. There have been many studies of the process of angiogenesis in breast carcinoma but most have focused on invasive disease. Some have investigated DCIS and other precursor lesions [Lee AHS et al 1997&99, Guidi et al 1994&97, Engles et al 1997a&b, Sales et al 1999, Valtola et al 1999, Brown et al 1995] where 2 patterns of vascularity have been described: diffuse stromal vascularity and a vascular rim around the involved ducts. Several studies have shown that pre-malignant lesions of the breast can induce angiogenesis in animal experimental systems and in the human breast [Lichtenbeld et al 1998, Gimbrone et al 1976, Brem et al 1977, Maiorana et al 1978, Strum et al 1983, Heffelfinger et al 1996]. It thus seems possible that the pattern or extent of vascularisation around DCIS may be an important factor in determining the transformation from *in situ* to invasive carcinoma. It is likely that the periductal vessels are most important in this respect as incipient invasion is

most likely to be associated with changes in vessels in the immediate vicinity of the tumour cells.

The different antibodies gave different values for MVD both in normal lobules and DCIS indicating that they identified different sub-populations of small vessels. In pure DCIS, there was a significant increase above normal in CD34+ and CD31+ vessels but a reduction in those staining for vWF. These findings are in keeping with a change in phenotype as well as a change in vascular density although significant vascular proliferation must have taken place in order to maintain roughly normal density around greatly enlarged structures.

DCIS associated with invasive carcinoma showed a similar profile of vascular immunostaining but there were significantly greater numbers of CD34− and CD141+ vessels and fewer staining for vWF. It is not clear at present whether this higher periductal MVD reflects a greater predisposition to invade or whether the effect is due to the proximity of invasive carcinoma which induces angiogenesis. The latter possibility of the latter was minimised by evaluating DCIS at least 2mm from the nearest focus of invasive carcinoma but this would not exclude any effect of factors released by the tumour into the blood or lymphatic system. It is of interest in this context that the normal lobules from cases of DCIS with invasive carcinoma exhibited significantly lower numbers of vWF+ vessels than those from cases of pure DCIS. This finding is in apparent conflict with the work of Heffelfinger et al [1996] who found the vascularity of histopathologically normal epithelium to be greater in breast containing invasive carcinoma using an antibody to vWF.

The breasts without invasive disease in this study, however, included those with benign proliferative change as well as DCIS.

The different antibodies used gave different values for MVD in normal breast and showed dissimilar changes in DCIS. For patients with and without invasive disease, vWF+ vessels were at lower density in DCIS than in the normal breast. This is in keeping with observations on invasive tumours (colorectal carcinoma) where vWF immunostaining has been found to be absent from some of the capillaries in the tumour [Vermeulen et al 1995]. It has been shown that anti-vWF stains large vessels more strongly than small ones [Vermeulen et al 1996] and consequently our findings could reflect the immaturity of newly formed tumour-associated vessels. The density of CD34+/vWF- or CD141+/vWF- vessels could thus reflect the rate of tumour angiogenesis.

CD31 gave consistently lower values for MVD than the other antibodies. The molecule has a role in platelet adhesion in inflammation and wound healing [Parums et al 1990]and is expressed on large and small vessels in either normal or tumour tissue [Vermeulen et al 1996, Horak et al 1992, Toi et al 1993]. In contrast to vWF it appears not to be expressed on lymphatic vessels [Burgdorf et al 1981] and this could at least partially explain why determinations of MVD with the CD31 antibody were consistently lower in both normal lobules and DCIS. The possible contribution of lymphatics to neo-vascularisation in tumours has been largely ignored.

Values for MVD as determined using CD34 and 141 antibodies were roughly comparable both in normal lobules and DCIS. Both showed a significant increase in DCIS, with greater values in cases associated with invasive carcinoma.

CD34 is a cell surface adhesion glycoprotein expressed on human haemopoietic stem and progenitor cells and blood vessels. It has roles in leucocyte adhesion and endothelial cell migration [Fina et al 1990]. The antigen is a 105-120kDa single chain transmembrane protein which is expressed on most haematopoietic colony-forming cells from human bone marrow, including unipotent and multipotent progenitors [Civin et al 1984&89&90]. CD34 appears to be expressed at highest levels on the earliest progenitors, and to decrease progressively with maturation [Civin et al 1989]. Anti-CD34 antibodies are sensitive endothelial markers that stain small and large vessels in normal and tumour tissue equally [Vermeulen et al 1996]. Perivascular stromal cells and a proportion of lymphatic vessels are also stained [Vermeulen et al 1996]. My findings confirm those of Martin et al [1997], that CD34 antibodies give consistently higher vessel counts in breast carcinomas than antibodies against vWF or CD31.

CD141 antibodies bind thrombomodulin (TM), an endothelial cell-surface receptor that functions as a potent natural anticoagulant through its cofactor role in the thrombin-catalysed activation of human protein C [Bourin et al 1988, Preissner et al 1990]. It is widely distributed in normal human tissues, such as endothelial cells of blood vessels, lymph vessels and syncytiotrophoblast [Maruyama et al 1985]. It was recently reported by Calnek et al [1998] that the TM-dependent anticoagulant activity is regulated by VEGF,

an angiogenic factor. It has been reported that decreased TM expression could be associated with metastases of tumour cells [Tesuka et al 1995, Suehiro et al 1995, Collins et al 1992], something I was able neither to confirm nor refute in the present study.

I found an unexpected relationship between MVD and nuclear grade of DCIS. It might have been expected that MVD would simply increase with grade but in those with and without invasive disease, the MVD was significantly higher in intermediate than either low or high-grades. MVD was generally less in high than low grade DCIS but this often did not achieve statistical significance. There was also a significant negative correlation between vascular density and both the cross-sectional areas of the ducts involved and the extent of the necrosis of the tumour they contained. The last finding is in apparent conflict with the study of Guidi et al [1994]., who found that comedo-type lesions were more likely to be associated with higher microvessel density. In their study, however, vessels up to 500μm from the involved structures were included and only those from the 5 most vascular fields were recorded. Vessels forming cuffs around ducts were not studied quantitatively as they were found only in a minority of cases. These authors, however, identified vessels using an antibody to vWF only, which in my study stained significantly fewer vessels than the CD34 and CD141 antibodies. A likely explanation for my findings is that angiogenesis increases with growth rate but is unable to keep pace with the most rapidly growing lesions.

In this series, there was a significant positive correlation between MVD and ED in both groups of patients. It implies that it is sufficient to assess the MVD of the in-situ breast tumour as the results suggest that MVD represents the ED profile of the tumours examined. There were a few studies which examined the endothelial cell kinetics [Tannock et al 1970, Hirst et al 1982, Hobson et al 1984, Fox et al 1993, Vartanian et al 1994] in solid tumour angiogenesis. None had found any significant correlation between intratumoral endothelial cell proliferation with MVD [Vartanian et al 1994] even though it has become generally accepted that endothelial cell proliferate 30 - 40 fold faster in solid tumour blood vessels than in the vasculature of normal tissue [Fox et al 1993]. On the other hand, Simpson et al [1996] measured the total endothelial area (EA) of CD34 positive microvessels in invasive breast carcinoma using Samba 4000 image analyzer. Endothelial area was prognostically significant in univariate analysis of disease-free survival (DFS) and overall survival (OS). In multivariate analysis, EA remained as an independent predictor for OS in lymph node negative patients and for DFS in lymph node positive patients. Likewise, endothelial density would be a useful prognostic indicator if the study of this in-situ breast cancer is to be extended to a larger number of patients.

Chapter 10

Microvessel Density and Proliferating Activity in Ductal Carcinoma *in situ* of the Breast

Results

Relationship between either KD or MKD and KP

The mean value of Ki-67 density was 752 mm^{-2} . Ki-67 fraction ranged from 2 to 30.5% with a mean value of 12.5%. As part of the assessment of the validity of the second method of quantification of Ki-67 staining and whether this was representative of the proliferative activity of the tumour assessed, a significant positive correlation between KP and KD ($p<0.001$) [*Fig. 10.1*] as well as MKD ($p<0.001$) was demonstrated.

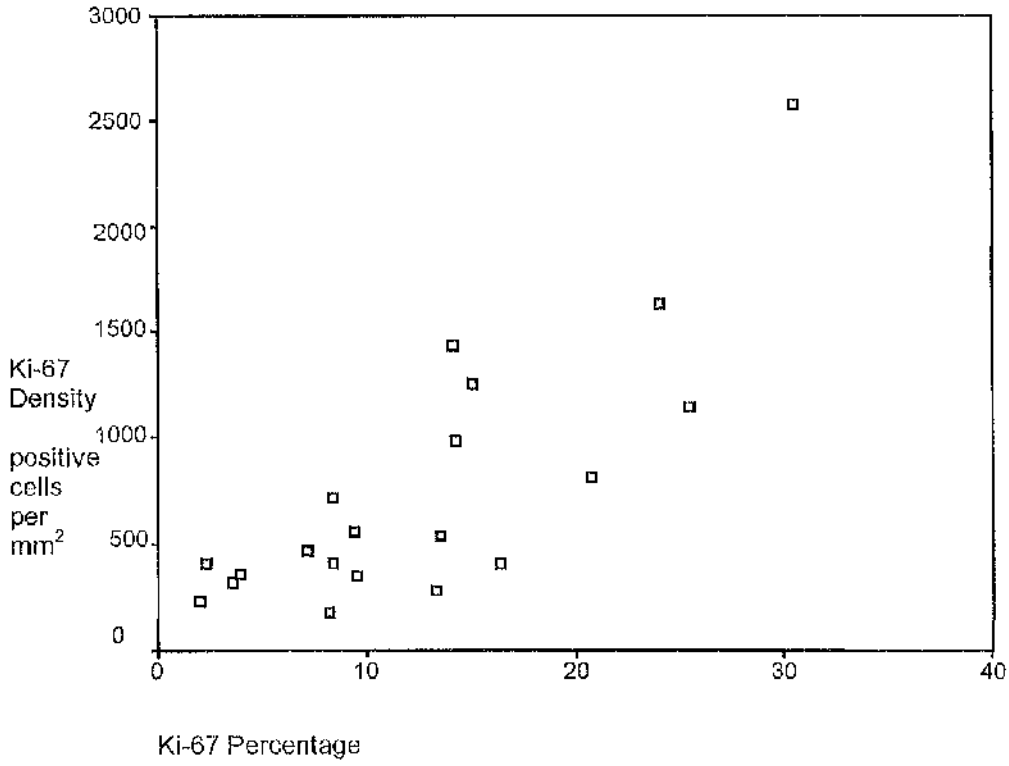


Figure 10.1. Correlation between Ki-67 percentage and Ki-67 density in DCIS.

Relationship between angiogenesis and proliferative activity

Mean microvessel density in each section of DCIS stained with a panel of endothelial markers (vWF, CD31, CD141 and CD34) from previous work were used for this assessment. There was no significant correlation between neither MKD nor KP and mean MVD using four endothelial markers.

Relationship between proliferative activity and histological features

Core area

The mean value core area of DICS was 171x $10^3 \mu m^2$. The individual core areas of DCIS were significantly and inversely correlated with the corresponding KD ($p<0.001$): as area of DCIS increased, the proliferation activity decreased.

Nuclear grade

There was a significant positive correlation between the nuclear grade and the corresponding KD ($p<0.001$). The proliferative activity increased from low to intermediate grade but decreased in high grade DCIS. There was a significant difference in KD between low grade and either intermediate ($p=0.003$) or high grade ($p<0.001$) lesions. However, there was no significant difference in KD between intermediate and high grade tumours [*Fig. 10.2*].

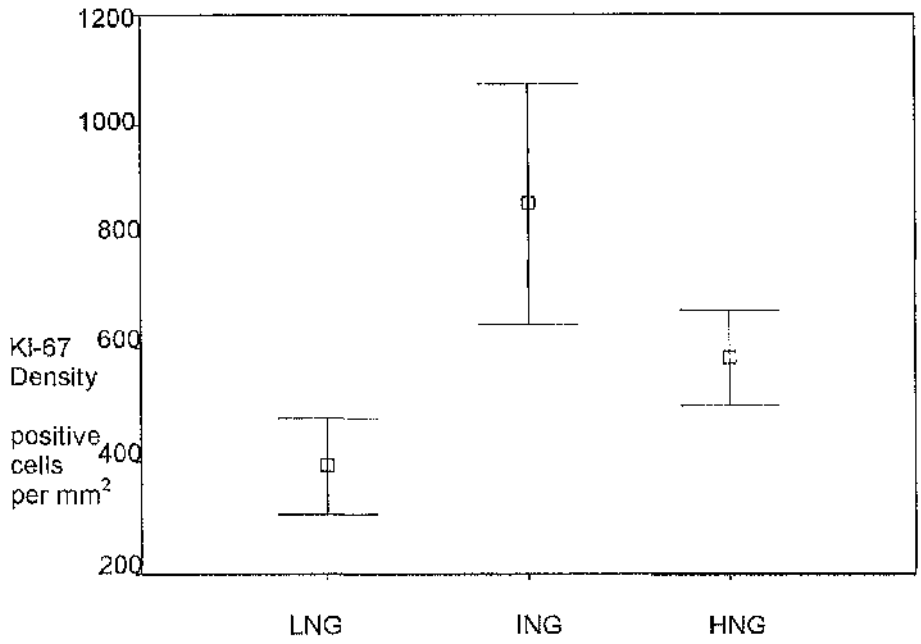


Figure 10.2. Relationship between nuclear grade and Ki-67 density in DCIS

Nevertheless, the nuclear grade was also significantly and positively correlated with KP ($p=0.016$). KP in high grade tumours was significantly higher than those in low grade lesions ($p=0.005$) whereas the difference in KP between intermediate grade and either low or high grade DCIS did not achieve statistical significance [*Fig. 10.3*].

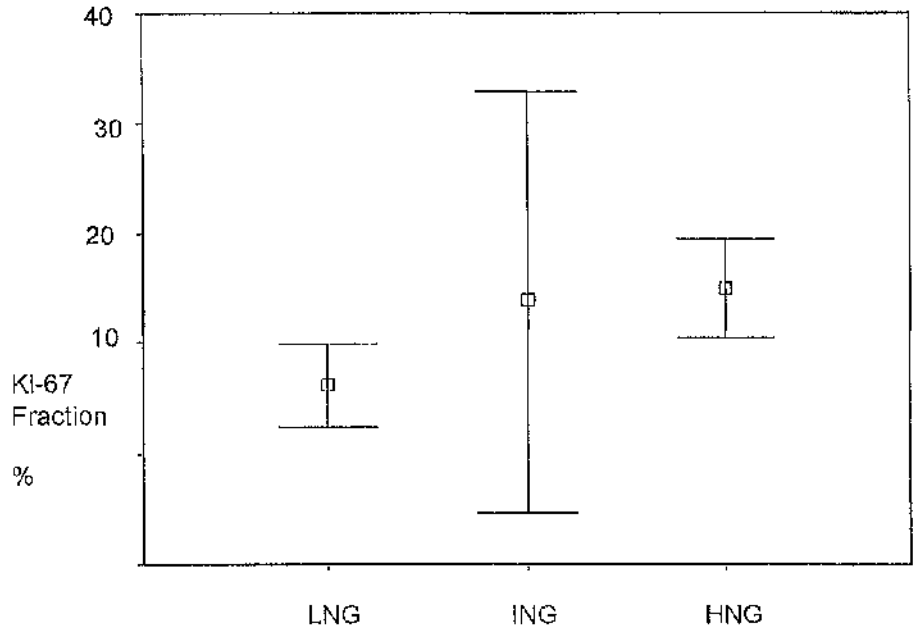


Figure 10.3. Relationship between nuclear grade and Ki67 percentage in DCIS.

Discussion

There have been some interests in the proliferation activity in DCIS as measured by anti-Ki67 antibodies [Zafrani et al 1994, Mack et al 1997, Gandhi et al 1998]. However, little is known of its significance in relation to angiogenesis in DCIS. Zolota et al [1999] investigated the relationship between periductal MVD (using anti-vWF antibodies)and proliferation activity (using anti-Ki-67 antibodies) semi-quantitatively. The rim of microvessels adjacent to the basement membrane was scored as an average of the percentage of the circumference occupied. Periductal MVD was scored as low when < 25% of the duct circumference was occupied by vessels, intermediate when it was 25% to 50% and high when it was > 50%. The percentage of neoplastic cells showing nuclear staining with Ki-67 was estimated with 10% taken as the cut-off level between low and high proliferating activity. An association was found between angiogenesis and over-expression of Ki-67.

In the previous chapter, I showed the microvessel density (MVD) in DCIS from patients with or without invasive carcinoma increased from low to intermediate grade but decreased in high grade DCIS. I also found a significant negative correlation between MVD and degree of necrosis as well as core areas in high grade DCIS. This suggests that the angiogenesis increases with the tumour growth rate but is unable to keep pace with the most rapidly growing lesions. To confirm this, I examined periductal vascularity in relation to proliferation activity using Ki-67 antibody in DCIS.

In this study, the proliferative activity was assessed quantitatively because I believed it represented a truer picture than semi-quantitative methods. As part of our quality assessment between two different methods of quantification of Ki-67 positive cells, I managed to show a positive and significant correlation between the two methods. This suggests KP does represent reasonable true picture of proliferation activity and its assessment is less time consuming compared with the one of KD.

KP ranged from 2% to 30.5% with a mean value of 12.5%. The mean percentage by other studies ranged from 7.2% to 22%. These differences can be partially secondary to different methods adopted. McGurrin et al [1987], who counted 1000 tumour cells in the section areas with the most dense labelling, found the highest mean percentage (22%). On the other hand, Wrba et al [1989] reported the lowest mean value of 7.2% by counting 1000 cells randomly. My results fell within the range from various groups and this confirmed the validity of my methodology.

I found a significant and positive correlation between nuclear grade and KD as well as KP. KP increased significantly and steadily from low to high grade DCIS. On the other hand, KD increased from low to intermediate grade but decreased in high grade DCIS at a level significantly higher than those in low grade lesions. I also showed that individual core areas of DCIS was significantly and inversely correlated with KD. These findings appeared to agree with my suggestion in previous chapter that angiogenesis around affected ducts in DCIS increases with the growth of the tumour but is unable to cope with most aggressive lesions, which contributes to the decreased proliferation activity in high grade lesions with

larger core areas. Previous studies showed the proliferation activity is related to tumour grade in breast cancer [Zafrani et al 1994, McGurrin et al 1987, Wrba et al 1989, Lell'e et al 1987, Barnard et al 1987, Walker et al 1988, Bouzubar et al 1989], so did our study.

Proliferation was also assessed within the microvessels around foci of DCIS as if there is any angiogenesis, this may be detected using the Ki-67 antibody. Also it has been shown that endothelial cells proliferate 30 - 40 fold faster in solid tumour blood vessels than in the vasculature of normal tissue [Fox et al 1993]. Thus if proliferation could be detected within these blood vessels, Ki-67 expression could possibly represent a quick method of assessing angiogenesis. Unfortunately, despite the reported increased proliferation rate within tumour associated blood vessels the rate remained too low to detect proliferating blood vessels in significant numbers.

Chapter 11

Vascular Density and Phenotype around Ductal carcinoma *in situ* of the Breast: relationship to Recurrences

Results

	DCIS with no recurrence	DCIS with *in situ* recurrence	DCIS with invasive recurrence
Number of cases	20	20	12
Age (years)			
Range	48-87	45-78	43-82
Mean	66.2	65.6	63.3
Follow up after treatment of DCIS (months)			
Range	29-138	9-98	7-52
Mean	94	43	48
Death	2 (both not related to breast cancer)	0	1 (died of breast cancer)
Operation			
Wide local excision	18	20	10
Mastectomy	2	0	2
Adjuvant Therapy			
None	9	9	5
Hormone	11	11	7
Radiotherapy	0	0	0
Others	0	0	0
Nuclear Grade			
Low	4	3	4
Intermediate	4	6	2
High	12	11	6
Van Nuys pathological grade			
1	2	3	3
2	6	6	3
3	12	11	6
Size (mm)			
Range	1-65	5-20	1-22
Mean	16.6	13.5	11.7
No of cases not assessable	0	9	3
Excision margin (mm)			
<1	7	4	3
1≤ to 10≥	4	4	0
>10	5	4	3
No of cases not assessable	4	8	6

Table 11.1. Clinical and pathological data from the three groups of DCIS patients.

Normal breast

The mean MVD around normal lobules was 79 vessels/mm^2 for CD 34 and 91 vessels/mm^2 for vWF. There was no significant difference in MVD for CD34 when normal lobules were compared in cases of DCIS with and without recurrence (Mann Whitney, P=0.5). In contrast, staining with vWF was more variable, the normal lobules of patients with DCIS that subsequently developed recurrent disease had a lower MVD than those that did not (Mann Whitney, P=0.024). In addition, MVD stained with anti-vWF antibody in cases with invasive recurrence was significantly lower than those that developed recurrence as *in situ* carcinoma (Mann Whitney, P<0.001).

DCIS

The MVD for the three groups of DCIS is summarised in *Table 11.2*.

	CD34 Mean MVD (vessels/mm^2)	vWF Mean MVD (vessels/mm^2)
Normal Lobules	79 (sd=60)	91 (sd=72)
DCIS (Non Recurrence)	95 (sd=49)	80 (sd=65)
DCIS (In-situ Recurrence)	119 (sd=66)	89 (sd=65)
DCIS (Invasive Recurrence)	126 (sd=59)	77 (sd=53)

Table 11.2. Microvessel density for CD34 and vWF for normal lobules and DCIS.

CD34

The highest MVD for CD34 was seen in cases of DCIS that subsequently developed an invasive recurrence and the lowest in cases of DCIS that were not known to have developed recurrent disease [*Fig. 11.1*]. The mean MVD was significantly higher around cases of DCIS that subsequently developed recurrent disease than those that did not (Mann Whitney $P<0.001$). The MVD was significantly higher in cases that developed an invasive recurrence than those that developed recurrent DCIS. However, the confidence intervals between these two groups largely overlaps. In addition, all three groups of DCIS had a higher MVD than that seen in normal breast (highest P = 0.001).

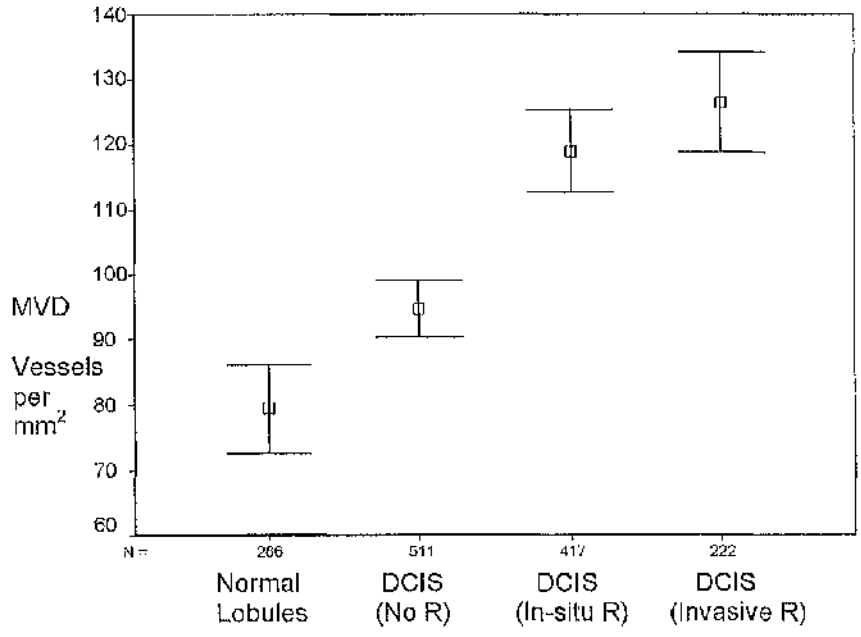


Figure 11.1. Comparison of CD34 microvessel density (MVD) among normal lobules and three groups of ductal carcinoma in situ (DCIS) [no recurrence (No R), recurrence with ductal carcinoma in situ (In situ R), recurrence with invasive carcinoma (Invasive R)]. The squares represent the mean values and the bars represent the 95% confidence intervals of the mean.

vWF

The data for vWF positive vessels was less clear cut. Normal lobules had the highest value of MVD, and DCIS with subsequent invasive recurrence the lowest [*Fig. 11.2*].

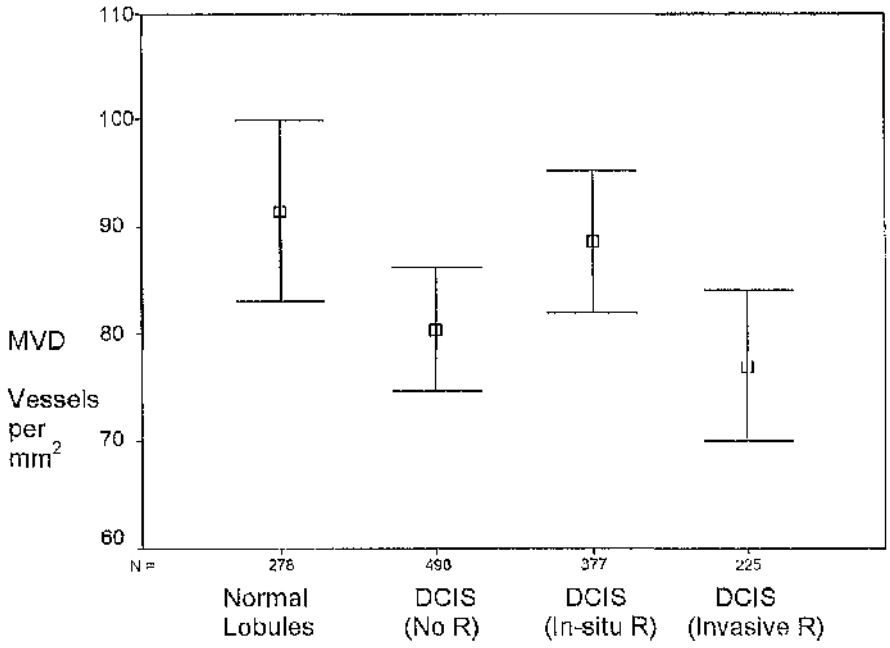


Figure 11.2. Comparison of vWF microvessel density (MVD) among normal lobules and three groups of ductal carcinoma in situ (DCIS) [no recurrence (No R), recurrence with ductal carcinoma in situ (In situ R), recurrence with invasive carcinoma (Invasive R)]. The squares represent the mean values and the bars represent the 95% confidence intervals of the mean.

Dual immunofluoresence for CD34 and vWF

On the 12 cases of DCIS on which dual immunofluoresence was performed three different immuophenotypes of blood vessels were identified around normal breast and DCIS: CD34+/F8-, CD34+/F8+ and CD34-/F8+.

Normal lobules

The mean MVD for CD34 positive vessels was 83 vessels/mm^2 , of these 45% coexpressed vWF. The mean MVD for vWF positive vessels was 56 vessels/mm^2 and of these 68% coexpressed CD34.

DCIS

The mean MVD for CD34 positive vessels around DCIS was 87 vessels/mm^2 , and of these 34% coexpressed F8. The mean MVD for vWF positive vessels around DCIS was 42 vessels/mm^2 and of these 72% coexpressed CD34. The MVD of CD34+/F8- vessels was significantly higher around DCIS than normal lobules (Mann Whitney $P<0.001$) [*Fig. 11.3*]. In contrast there was no difference between CD34+/F8+ or CD34-/F8+ vessels between normal breast and DCIS (Mann Whitney, lowest $P=0.4$).

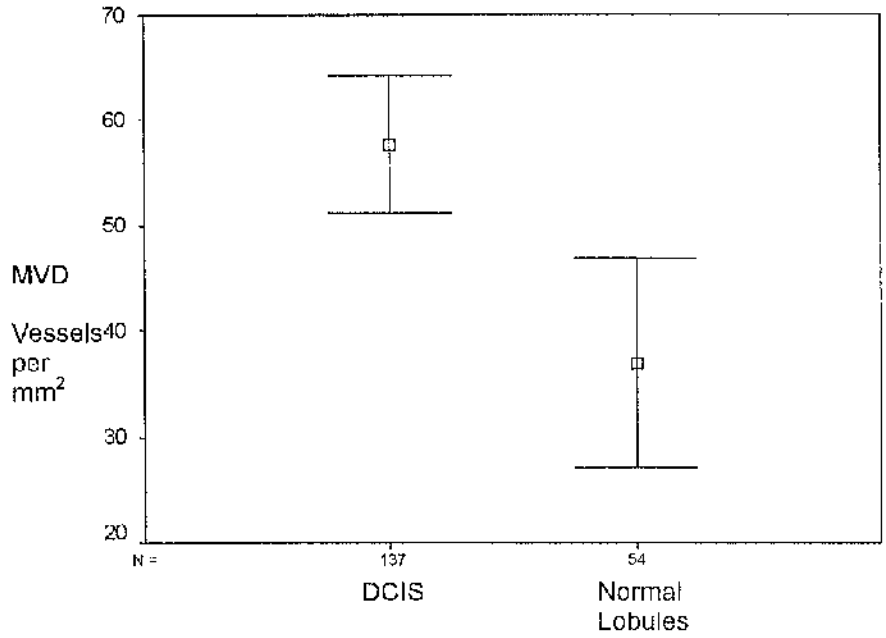


Figure 11.3. Comparison of microvessel density (MVD) for CD34+/vWF- vessels around ductal carcinoma in situ (DCIS) and normal lobules. The squares represent the mean values and the bars represent the 95% confidence intervals of the mean.

Discussion

In chapter 9, I have shown that there are differences in microvessel density between pure DCIS and DCIS associated with invasive cancer suggesting that increases in angiogenesis occur in cases that develop invasion. The aim of this study was to determine if the vascularity of DCIS at diagnosis was different in those who subsequently developed recurrent cancer compared to those who did not and thus whether angiogenesis could predict which women are likely to develop a recurrence. I have also shown in chapter 9 that in comparison to normal lobules, DCIS shows an increase in CD34 positive MVD and a decrease in vWF MVD. In this study, I carried out dual staining immunofluoresence to determine the phenotype of individual periductal blood vessel. This more precise method of determining the phenotype of vessels involved in angiogenesis may be able to give further insight into the role of angiogenesis in progression to malignancy.

This study demonstrates different populations of microvessels in DCIS and normal lobules using dual immunostaining. In DCIS, there was a significant increase above normal in CD34+ vessels, however for vWF the changes were less clear cut and overall were more in keeping with a drop in MVD rather than an increase. This agrees with my suggestion in previous chapter that there is a change in phenotype as well as vascular density from the transition from normal breast to DCIS, although significant vascular proliferation must have taken place in order to maintain roughly normal density around greatly enlarged structures.

The groups of DCIS that subsequently recurred had higher mean values for CD34+ MVD than those that did not. Increases in CD34 MVD thus not only correlated with the progression from normal to *in situ* neoplasia but also appeared to be able to detect cases that are likely to recur. Furthermore MVD was independent of the size, nuclear grade, and excision margin status as these were appropriately matched between the groups, however, the small numbers in each of these groups precludes further comment. In contrast the findings for vWF were more difficult to interpret. Differences were seen between normal lobules from the three different DCIS groups. It is not certain whether the changes in vWF MVD is due to the proximity of the DCIS, however, I attempted to minimise this by evaluating normal lobules at least 2mm from the nearest focus of DCIS. Furthermore, no such change was seen for CD34. Alternatively, staining with vWF may be more sensitive to the effects of tissue fixation and processing, the differences in staining reflecting these rather than definite changes in microvessel phenotype. DCIS also showed differences in MVD for vWF between the three groups. However, unlike that seen for CD34 a clear step-wise increase was not seen and instead there was a trend towards a decrease in MVD. When dual immunofluoresence was used the values for CD34 were comparable to those obtained by standard immunohistochemistry. However, far fewer vWF positive vessels were detected, again suggesting that this antibody may be affected by the methodology.

The dual immunofluoresence staining did, however, give further insight into the change in MVD. There was no difference between normal lobules and DCIS when vessels showing a CD34+/vWF+ or a CD34-/vWF+ phenotype were compared. In contrast, vessels with a CD34+/vWF- phenotype showed a significant increase between normal lobules and DCIS. This suggests that the increased vasculature around DCIS is primarily due to an increase in CD34+/vWF- vessels. This is in keeping with observations on colorectal carcinoma where vWF immunostaining has been found to be absent from some of the capillaries in the tumour [Vermeulen et al 1995]. It has been shown that anti-vWF stains large vessels more strongly than small ones [Vermeulen et al 1996]. In contrast, anti-CD34 antibodies are sensitive endothelial markers that stain small and large vessels in normal and tumour tissue equally [Vermeulen et al 1996]. Perivascular stromal cells and a proportion of lymphatic vessels are also stained [Vermeulen et al 1996], the former were excluded from my study by using a rigid definition for a vessel. The role of the latter in neoangiogenesis is unknown. Consequently my findings could reflect the immaturity of newly formed tumour-associated vessels. The density of CD34+/vWF- vessels could thus be related to the rate of tumour angiogenesis and the consequences it has for the biological behaviour of DCIS. It would therefore be interesting to know the phenotype of the blood vessels in the subsequent recurrences.

Solid tumours express genes which code for angiogenic stimulators [Klagsburn et al 1991, Leek et al 1994, Bicknell et al 1996, Bouck (b) et al 1996, Folkman (c) et al 1996], suggesting that the ability to form new vessels is part of the malignant phenotype. There is little doubt that an angiogenic phenotype develops during tumour progression, and that the development of a blood supply is vital in the escape from dormancy. Vessel density increases gradually with growth in some tumours, such as cervical squamous cell carcinoma [Smith-McClune et al 1994], whereas it may increase dramatically in others such as breast carcinoma [Stellmach et al 1996]. Oncogenes influence angiogenesis by coding for proteins and enzymes vital to the angiogenic process [Bouck (b) et al 1996, Bouck et al 1996, Volpert et al 1997]. Suppressor gene loss may, however, have a more profound effect [Stellmach et al 1996]. Increased production of proangiogenic factors appears to be necessary, but not sufficient, for the development of the angiogenic phenotype [Folkman (b) et al 1995], a decrease in inhibiting factors must also occur [Folkman (c) et al 1995, Dameron et al 1994]. Some neoplastic cells switch from an angiogenesis inhibiting to an angiogenesis stimulating phenotype upon transformation, as has been observed in cultured fibroblasts in patients with Li-Fraumeni syndrome [Dameron et al 1994]. Although the sequential events of vessel formation are similar, tumour vessels differ from those of normal tissue, in terms of permeability, cellular composition, stability and regulation of growth [Folkman et al 1976]. This suggests that antigenic differences may exist which may be exploited in specifically targeting tumour vasculature [Denckamp et al 1993, Fan et al 1995]. A number of antigens preferentially expressed in tumour vasculature have now been identified, includingEN 7/11 antigen, E9 antigen, TP-1, TP-3,

VEGF, Tie receptor kinase, Endosialin, BC-1 antigen, Integrins, and Endoglin. Gasparini and Chereah et al [2001] assessed the expression of intergin alphaVbeta3 in a cohort of primary breast cancers and in normal breast tissue as control. They found that LM 609 antibody preferentially immunostained proliferating vessels of small caliber within the tumour, whereas only weak expression of integrin alphaVbeta3 was observed on vessels of non-cancerous tissue.

A number of other studies have also looked at angiogenesis around pre-invasive disease. Ottinette et al [1988] examined vascular number and size within a 100 micrometer perimeter of proliferative breast disease and DCIS. They found an increased mean vascular size in proliferative breast disease and DCIS relative to normal epithelium but unlike the present study no increase in vessel numbers. Guidi et al [1994] used both a 1-3+ estimate of vascularity and quantitative counts of microvessels within 500 micrometer of DCIS. The vascularity was greater in comedo than non-comedo DCIS, was proportional to nuclear grade, and correlated with Her2/neu. Heffelfinger et al [1996] estimated angiogenic grade (0 to 4) according to the proportion of basement membrane of involved ducts touched by vessels and found that the vascularity of normal epithelium was higher in breasts containing invasive disease than in breasts lacking invasive disease; simple proliferative breast disease had a higher count than normal breast epithelium and, vascularity increased in proportion to epithelial lesion progression and relative risk of invasion. Interestingly, Guinebretiere et al [1994] showed that periductal vascularity in fibrocystic disease was a predictor of progression to invasive disease. In this study, I concentrated our investigations on periductal vascularity as I hypothesised that risk of

recurrence is likely to be associated with vascular changes in the immediate vicinity of the tumour foci.

In conclusion, this study shows that the blood vessels surrounding DCIS appear to have a different immunophenotype when compared with blood vessels surrounding normal breast lobules. Furthermore increases in vascular density, as detected with the CD34 antibody, correlates with recurrence and the development of invasive carcinoma.

Chapter 12

Stromal Vascularity and Thymidine Phosphorylase expression in Ductal Carcinoma in-situ of the Breast

Results

Comparison of stromal and periductal MVD

vWF

The mean stromal MVD and the mean periductal MVD when measured using anti-vWF antibody were both 160 vessels/mm^2 . A strong positive correlation was identified between stromal and periductal density regardless of how measured (eg. hotspot, mean of 3 highest stromal counts, mean of 5 highest stromal counts, highest periductal density, mean periductal density) [*Figs.12.1-12.6*]. The weakest correlation was between hotspot and mean periductal MVD (Pearson: lowest r=0.464, highest P<0.001).

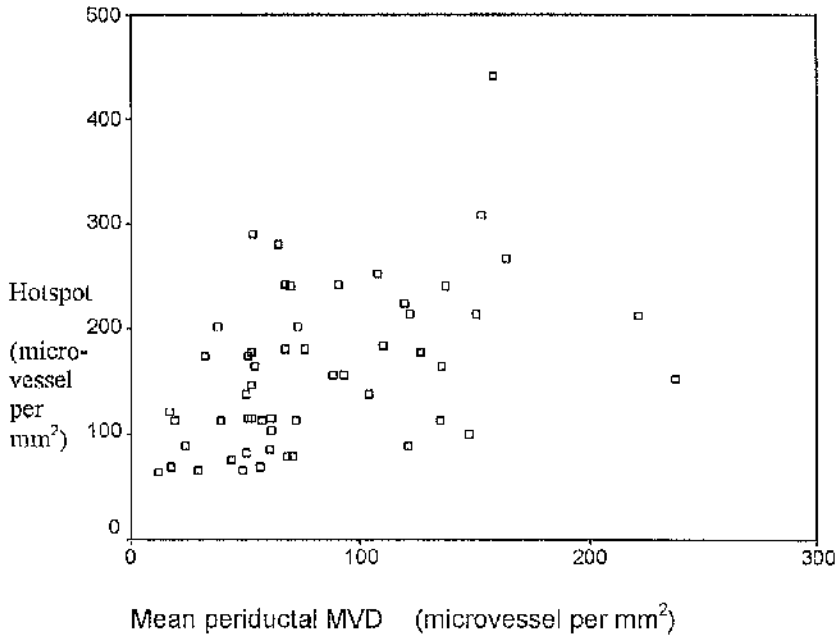


Figure 12.1. Relationship between hotspot and mean periductal MVD in all cases stained with vWF antibody

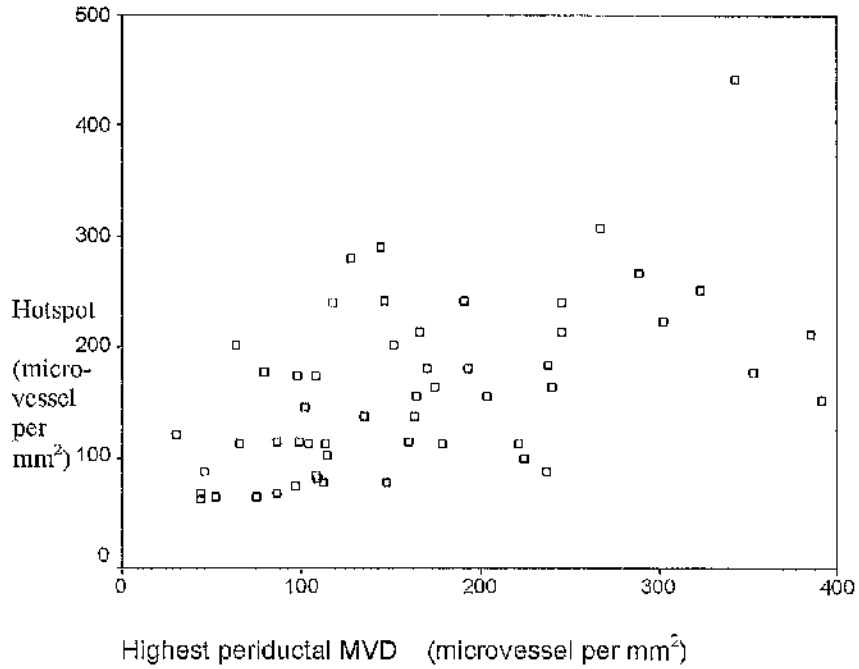


Figure 12.2. Relationship between hotspot and highest periductal MVD in all cases stained with vWF antibody

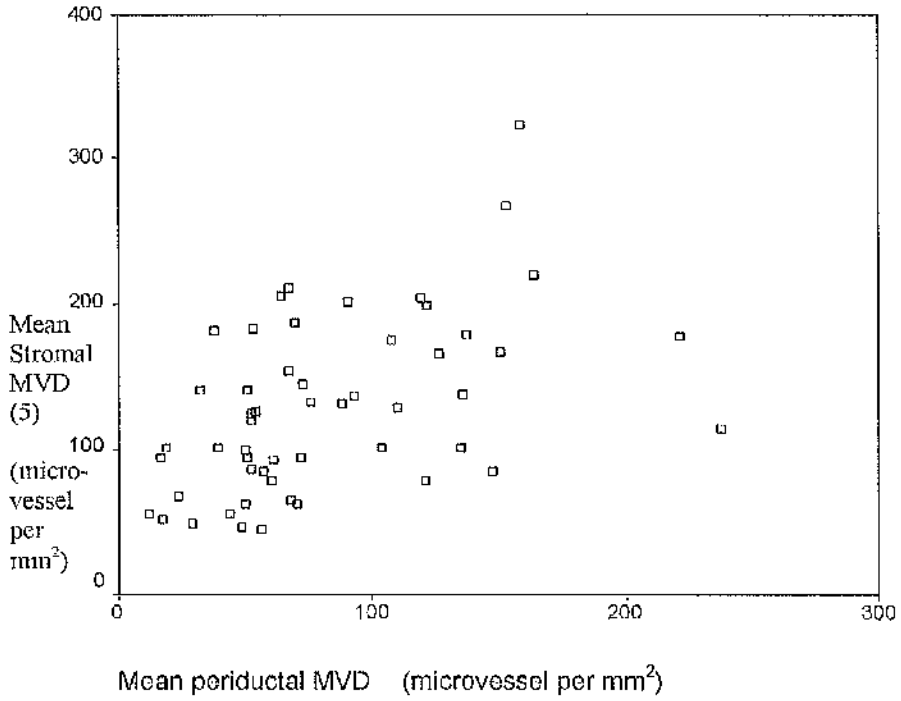


Figure 12.3. Relationship between mean stromal M(5)VD and mean periductal MVD in all cases stained with vWF antibody

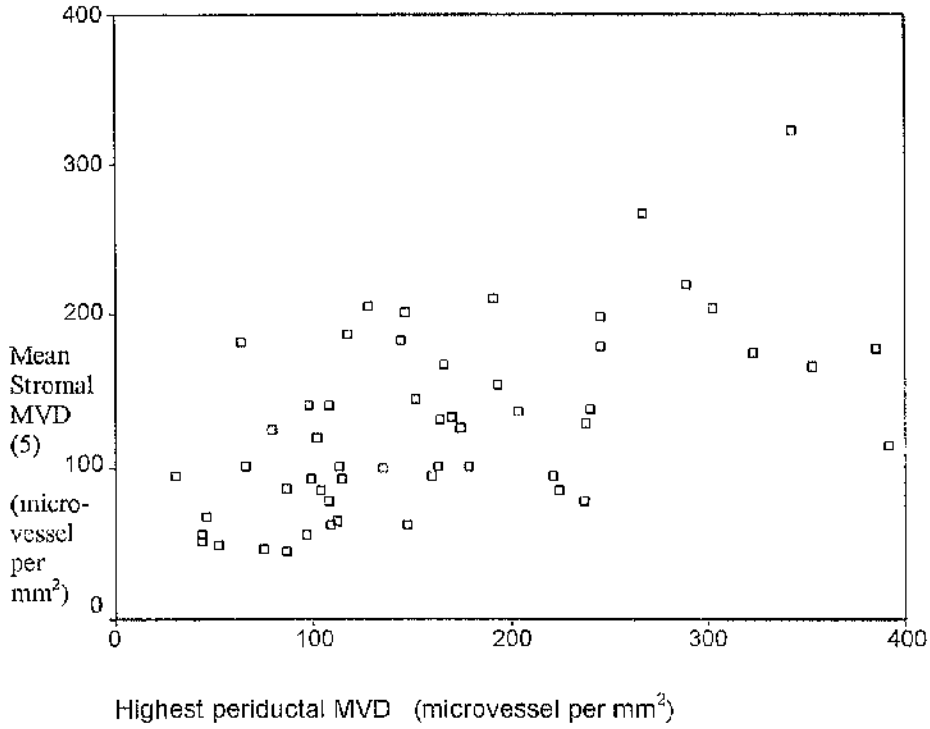


Figure 12.4. Relationship between mean stromal M(5)VD and highest periductal MVD in all cases stained with vWF antibody

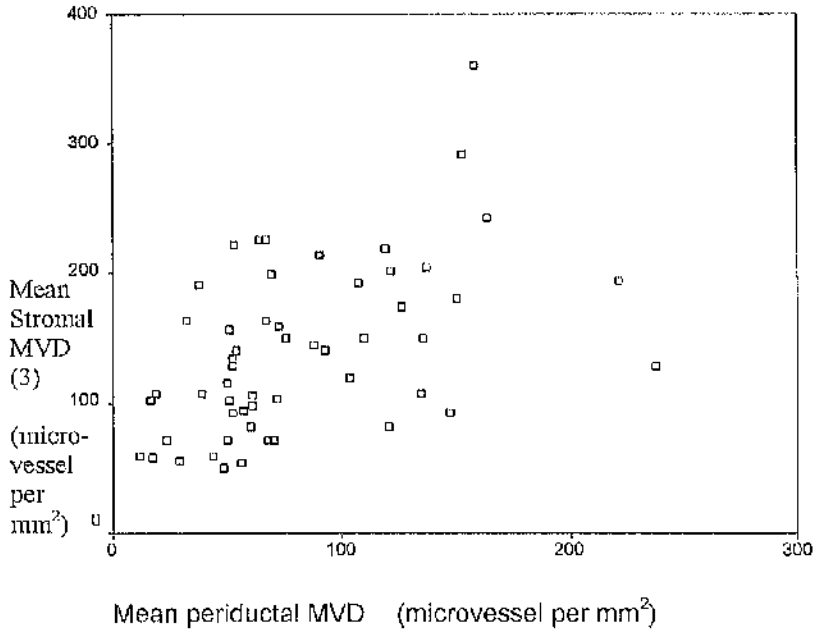


Figure 12.5. Relationship between mean stromal M(3)VD and mean periductal MVD in all cases stained with vWF antibody

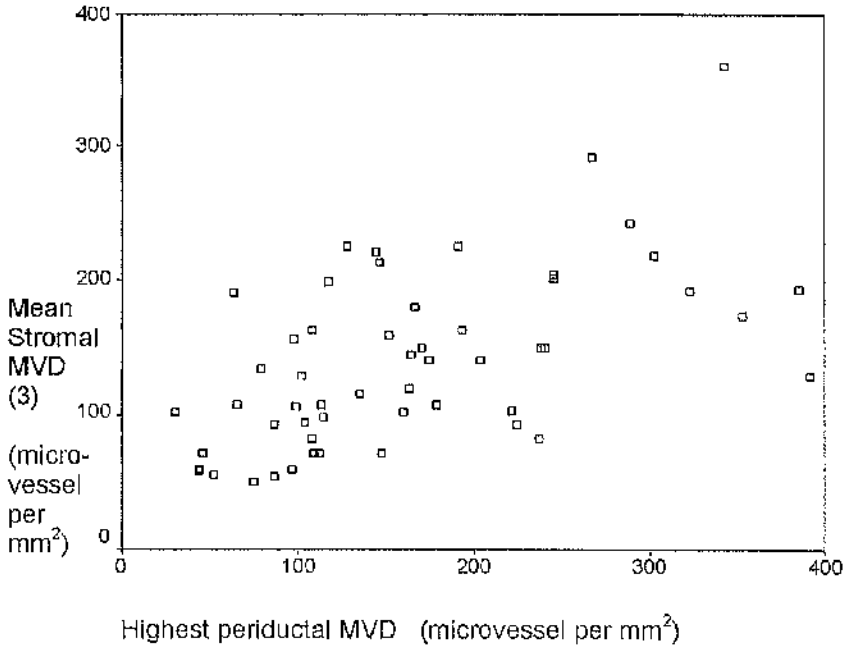


Figure 12.6. Relationship between mean stromal M(3)VD and highest periductal MVD in all cases stained with vWF antibody

There was no significant correlation between nuclear grade and stromal MVD in the entire group of DCIS (Spearman: lowest P=0.292). No significant difference was identified in stromal vascularity between DCIS cases which did and did not recur eventually (Mann-Whitney: lowest P=0.932).

CD34

The mean stromal MVD when measured using anti-CD34 antibody was 182 vessels/mm^2 and was higher than that seen with vWF (Mann-Whitney: P=0.037) [*Figs.12.7-12.9*]. The mean periductal density was 210 vessels/mm^2 and was again higher than that seen with vWF (Mann-Whitney: P<0.001) [*Figs.12.10-12.11*]. Furthermore, in contrast to that seen with vWF, the periductal MVD was higher than the stromal MVD regardless of how measured (Mann-Whitney: highest P=0.037). Unlike staining with vWF antibody, there was no significant correlation noted between stromal and periductal vascularity stained with CD34 antibody (Pearson: lowest P=0.101).

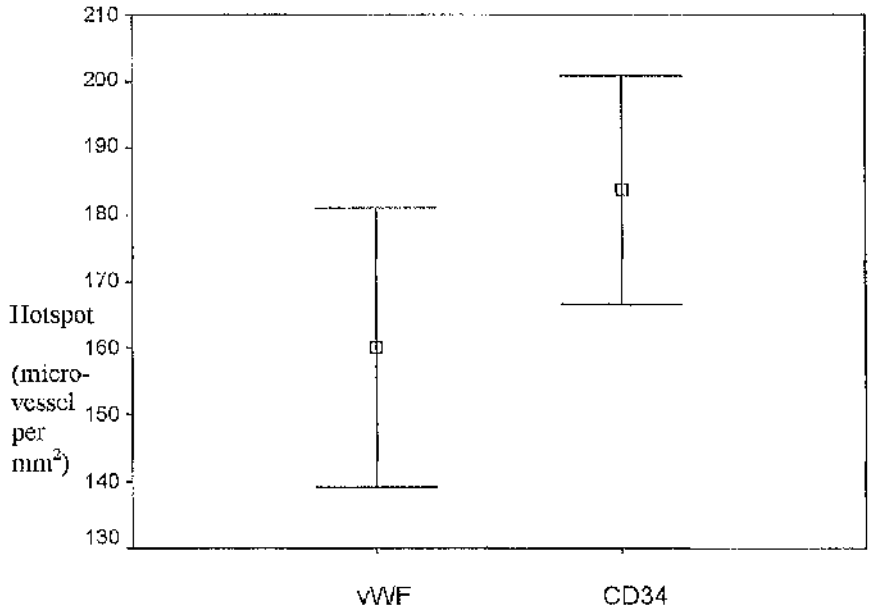


Figure 12.7. Hotspot in sections stained with vWF and CD34 antibodies (p=0.037)

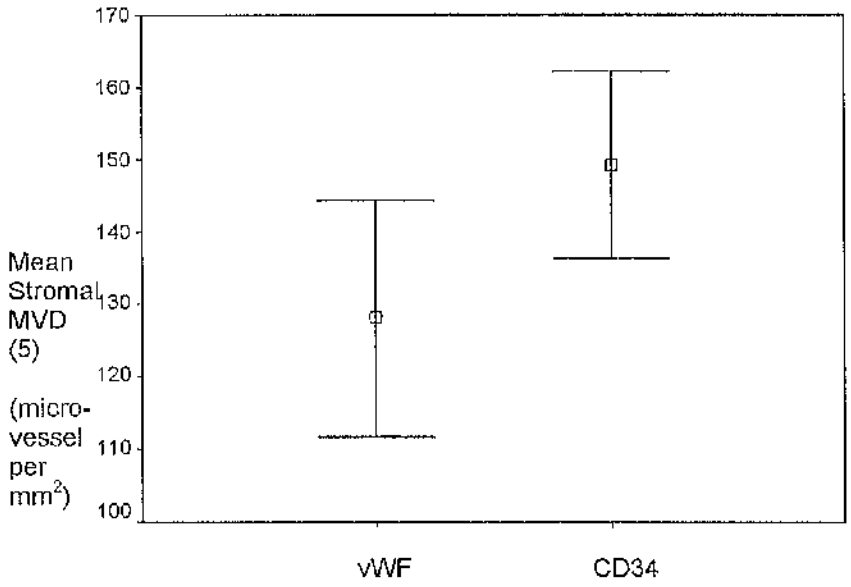


Figure 12.8. Mean stromal M(5)VD in sections stained with vWF and CD34 antibodies (p=0.019)

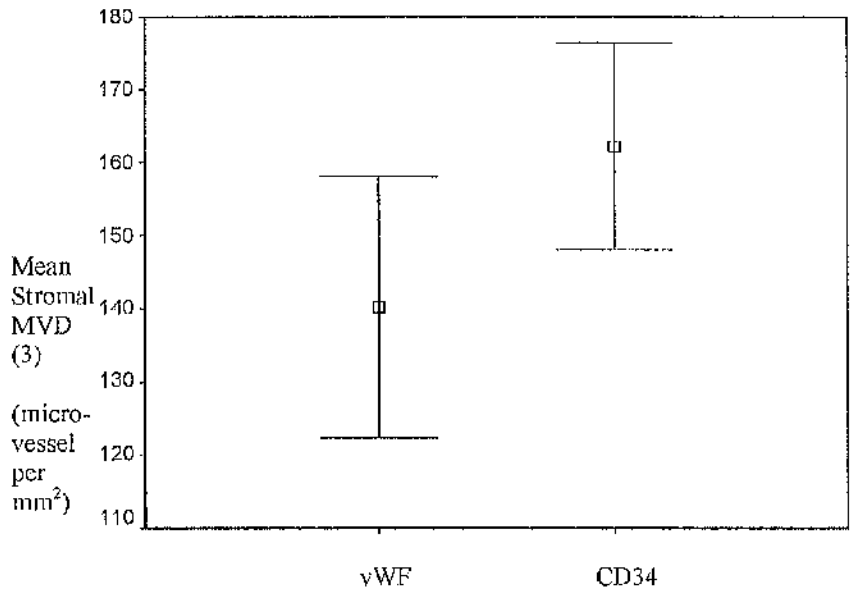


Figure 12.9. Mean stromal M(3)VD in sections stained with vWF and CD34 antibodies (p=0.025)

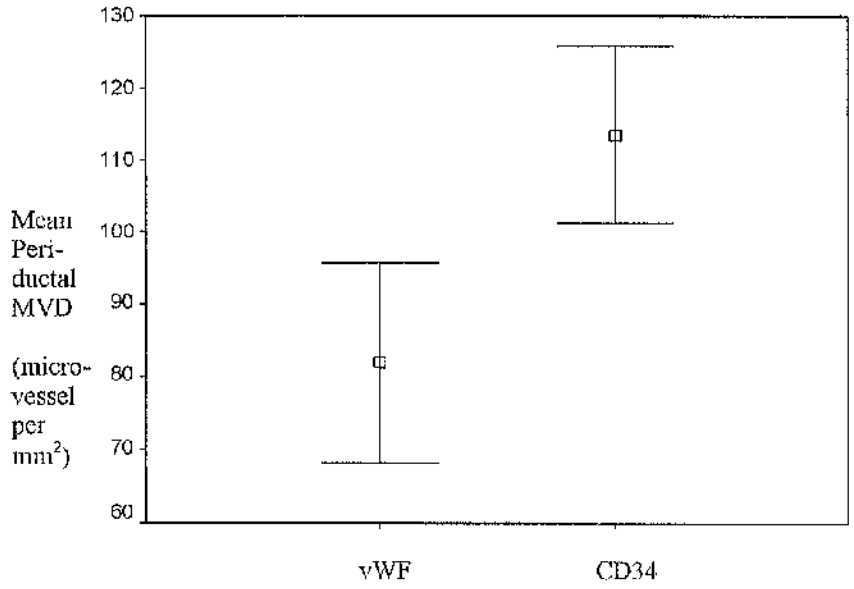


Figure 12.10. Mean periductal MVD in sections stained with vWF and CD34 antibodies (p<0.001)

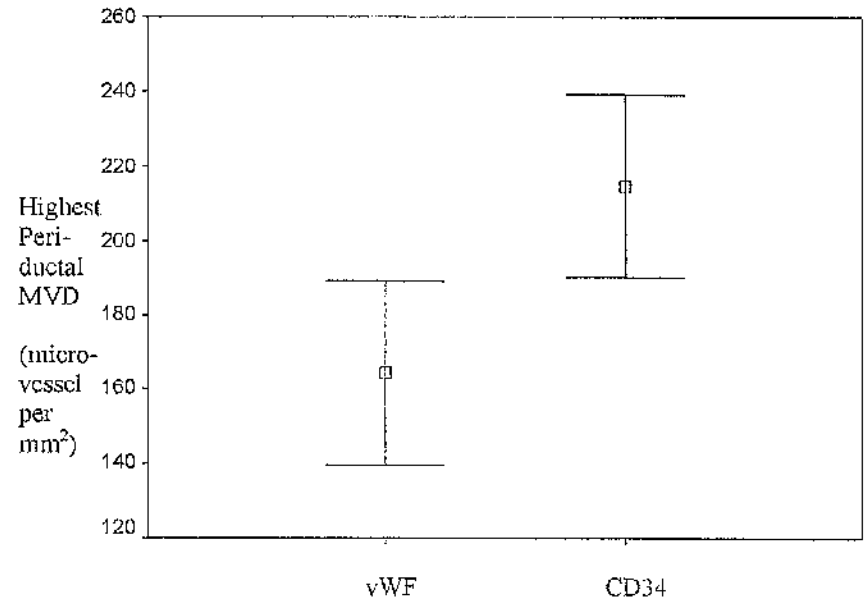


Figure 12.11. Highest periductal MVD in sections stained with vWF and CD34 antibodies (p=0.002)

Likewise as staining with vWF, no significant correlation was noted between nuclear grade and stromal vascularity (Spearman: lowest P=0.379). There was no significant difference in stromal vascularity between DCIS which did and did not recur after initial treatment (Mann-Whitney: lowest P=0.509).

Thymidine Phosphorylase staining in DCIS

No difference was identified when H-scores for TP staining were compared between cases of DCIS that subsequently recurred or did not recur (Mann-Whitney: P=0.181) [*Fig.12.12*]. A negative correlation was seen between the TP H-score and DCIS nuclear grade (Spearman: r=0.292, P=0.049). However, no significant difference in the TP H-score was identified between nuclear grade (Mann-Whitney: lowest P=0.061) [*Fig.12.13*]. The TP H-score also correlated with stromal (Pearson: highest P=0.035) but not periductal (Pearson: lowest P=0.091) vWF MVD. In contrast, for CD34, no significant correlation was identified between TP H-score and both types of vascularity (Pearson: lowest P=0.598).

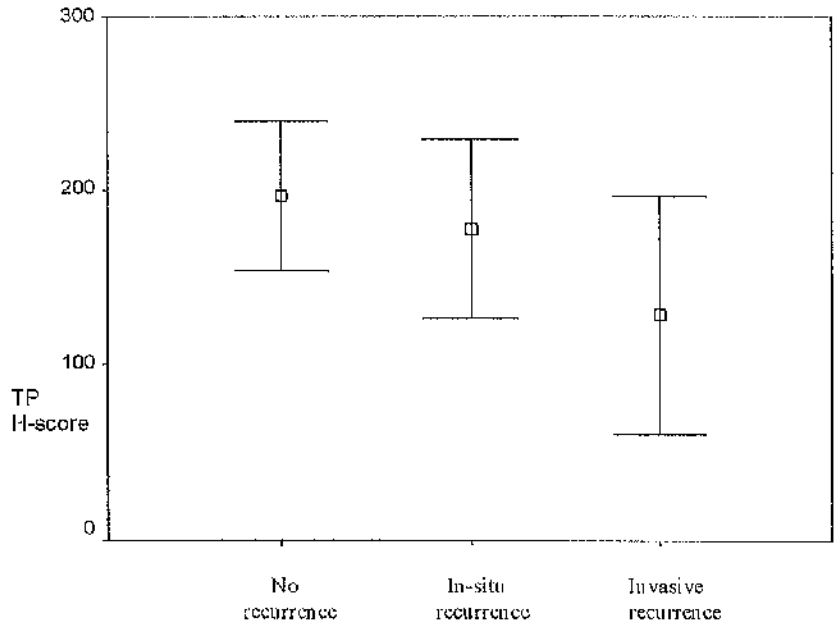


Figure 12.12. Relationship between TP expression and clinical outcome (TP=Thymidine Phosphorylase)

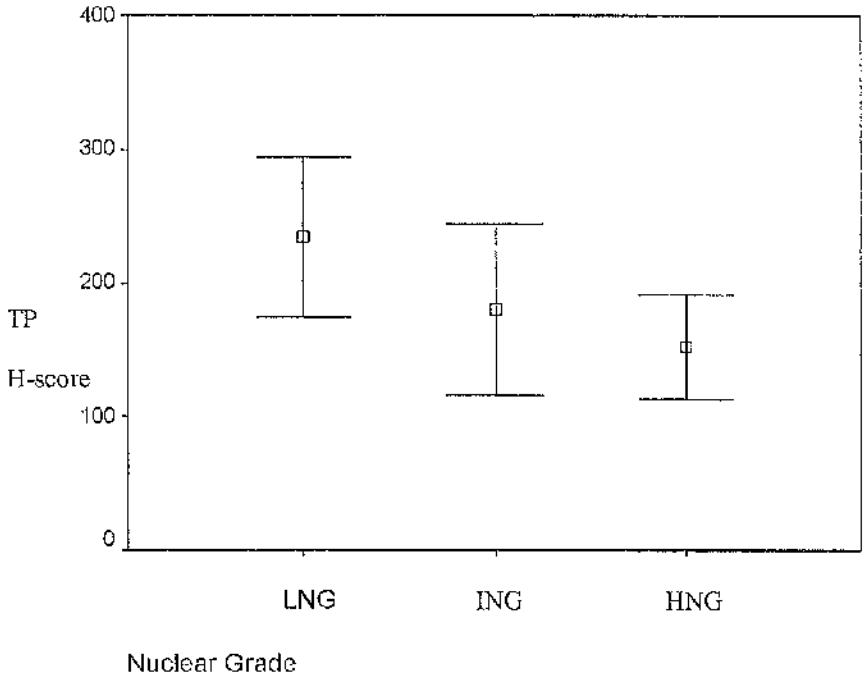


Figure 12.13. Relationship between TP expression and nuclear grade in all cases of DCIS (LNG=Lower nuclear grade, ING=intermediate nuclear grade, HNG=high nuclear grade; TP=Thymidine Phosphorylase)

Discussion

Angiogenesis is thought to play an important role in the progression of invasive cancer [Weidner et al 1991, Bosari et al 1992, Horak et al 1992, Weidner et al 1992, Karaiossifidi et al 1996, Heimann et al 1996]. It also appears to have a role in the development of hyperplastic and other precancerous lesions [Brem et al 1977, Gimbrone et al 1976, Guinebretiere et al 1994, Lichtenbeld et al 1998, Maiorana et al 1978, Strum et al 1983]. In DCIS two vascular patterns are described [Guidi et al 1994, Engels et al 1997a, Heffelfinger et al 1996], a diffuse stromal increase and an increase in periductal blood vessels (Pattern I and II). An increase in periductal MVD (Pattern II) is seen in 23-62% of DCIS cases [Guidi et al 1994, Engels et al 1997a, Heffelfinger et al 1996]. In my previous studies, I have shown that an increase in periductal MVD (Pattern II) around DCIS predicts for the development of a recurrence, particularly an invasive recurrence. An increase in vessels picked up by the antibody CD34 appear to be particularly important. It has also been proposed that the increased periductal MVD (Pattern II) may result from angiogenic factors secreted by tumour cells [Engels et al 1997a].

Although two patterns of vascularity (Pattern I and II) have been described in DCIS, the relationship between these two patterns is unclear. Guidi et al [1994] found increased stromal vascularity (Pattern I) in 25% of cases and the presence of a periductal vascular cuff (Pattern II) in 35%. However, they could not show an association between stromal (Pattern I) and perivascular (Pattern II) density. In contrast, I found with vWF a strong correlation between stromal (Pattern I) and periductal (Pattern II) vascularity with similar

values in each. CD34 on the other hand showed higher values than that seen for vWF . There was also a difference between stromal (Pattern I) and periductal (Pattern II) vascularity with the highest values being seen in the latter. The difference between the stromal (Pattern I) and periductal (Pattern II) counts is probably due to an increase in CD34/vWF – blood vessels as shown in our previous study. It thus appears that detection of periductal MVD (Pattern II) using the CD34 antibody is a sensitive predictive marker of DCIS recurrence and the development of invasion.

TP is thought to have a role in endothelial cell migration [Risau et al 1992] and differentiation [Klagsburn et al 1991] and has been shown to be associated with increased periductal MVD (Pattern II) in DCIS [Engels et al 1997b]. I therefore proposed that the increased periductal MVD (Pattern II) in those cases of DCIS that subsequently recurred was a result of TP expression. However, I found that TP expression in DCIS correlated with vWF stromal but not CD34 periductal MVD. It thus appears that TP may play a role in increased stromal vascularity (Pattern I) of vWF positive vessels, however, other factors may also be important and these may have a more important role. It would be interesting to see if other factors stimulated by TP, such as VEGF show a similar correlation.

TP in invasive breast cancer correlates with MVD and has prognostic value [Fajardo et al 1992, Folkman et al 1996,Fox et al 1996,Toi et al 1995]. It has also been shown to correlate with the relapse rate in DCIS although the same study did not show a significant correlation between relapse free survival and TP expression [Engels et al 1997b]. In the present study, when H-scores for TP expression were compared between patients that did

or did not subsequently develop recurrence, a difference was not seen. Although TP may have a role in vWF stromal MVD, its influence in the development of recurrence is probably less.

TP can also be induced by hypoxia and low pH [Shweiki et al 1992]. High grade DCIS with comedo necrosis may therefore be thought to be more likely to show TP expression. Surprisingly in our study we found that the TP H-score showed a negative correlation with grade even though most of our high grade DCIS cases showed at least focal comedo necrosis. Interestingly Lee et al [1999] also found no relationship between TP expression and recurrence. Possibly the hypoxia present in DCIS is not a strong enough stimulus to induce TP expression or the cells in high grade DCIS are so genetically deranged that they are unable to respond to the hypoxia by upregulation of TP.

In conclusion, stromal and periductal MVD measured by anti-vWF antibody correlates, but does not predict for recurrence. Stromal and periductal MVD measured by anti-CD34 antibody does not correlate, and the periductal MVD correlates with recurrence. This is important and supports my previous studies strongly: it is CD34+ periductal new microvessels that are important with regard to the risk of recurrence in DCIS after conservation treatment.

Chapter 13

Summary of results and Conclusions

Over 20% of screen-detected breast cancers are in-situ, the majority of those are treated by wide local excision, but as yet no molecular marker has been identified to predict for recurrences. I hypothesized that angiogenesis was important in the transformation of in-situ to invasive cancer and may predict an increased chance of recurrence.

The importance of angiogenesis has been demonstrated in that DCIS had a higher level of CD34 positive vessels compared to normal, interestingly not only on the number of vessels increased but also their phenotype. The vessels around DCIS tend to be positive for CD34 and negative for vWF, a finding not previously described.

The MVD continues to increase in DCIS associated with invasive cancer and once again these vessels are more likely to stain positively for CD34 and not vWF, suggesting that the vessels that are important in malignant transformation and growth are different from those found in normal breast lobules.

A correlation was noted between MVD (CD34) and nuclear grade with an increase in MVD in intermediate grade DCIS compared to low grade. It might have been expected that the MVD would continue to increase in high grade DCIS but this was found to be lower than intermediate grade DCIS. In an attempt to explain this, I measured MVD in relation to the area of the focus of DCIS and the degree of necrosis present. In both cases

MVD decreased. This provides a possible explanation for decreased MVD in high grade DCIS in that the vessel density increases with grade, but in high grade DCIS with high proliferation activity the new vessel growth fails to keep pace so the MVD decreases, and tumour necrosis occurs within the DCIS.

Increased CD34 positive vessel formation is important in malignant transformation as demonstrated above. It is not unreasonable to hypothesize that it may also act as a marker for recurrence in those who have had wide local excision. This proved to be the case with significantly increased periductal MVD in DCIS at diagnosis in those patients who subsequently developed a recurrence compared to those who did not.

Stromal (Pattern I) and periductal (Pattern II) vascularity had been described in DCIS and yet no one has identified the relationship between the two vascular patterns. I hypothesized that the relationship does exist and had also shown that both types of vascular network do correlate to one and another in sections stained with anti-vWF but not anti-CD34 antibody. The latter observation could be partly due to more immature new vessels (which stained better with anti-CD34 antibody) around DCIS compared to the distant stroma. Once again, these periductal vessels (which stained better with anti-CD34 antibody) had been shown to be reasonably related to the likeliness of recurrence as I could not show the relationship between the stromal vascularity and the risk of recurrence.

In conclusion, blood vessels surrounding DCIS appear to have a different immunophenotype when compared with blood vessels surrounding normal breast lobules. Increases in vascular density, as detected with the CD34 antibody, correlates with recurrence and the development of invasive carcinoma. Recurrent disease does not appear to be related to TP expression by DCIS. Periductal MVD appears to be more important than stromal MVD in predicting for recurrence in DCIS.

My work suggests that angiogenesis does play an important role in DCIS and periductal vascularity does have a potential value as a prognostic marker. The strength of my study is (1) the robustness of the methods, (2) the length of follow up of those patients who were treated for DCIS, and (3) usage of multiple endothelial markers to evaluate the microvessel density as well as vascular phenotype.

I was the only assessor of periductal and stromal vascularity after basic training with two consultant histopathologists in assessing microvessels for three months. I only started the project once our assessment of microvessels reached an agreement ratio of at least 70%. My study is transferable to another laboratory because:

1. The representative histological sections were selected by two consultant histopathologists,
2. The immunostaining was carried out with the standard method using the standard antibodies,
3. Adjacent non-cancerous lobules were used as internal controls,

4. During the assessment of microvessels, the histological sections were scanned in a systematic manner to avoid selection bias,
5. Strict criteria was adopted for assessment of microvessels,
6. The sole assessor was trained with two consultant histopathologists for three months, when the agreement ratio among three observers of microvessels reached at least 70%.
7. Overall, the methodology was robust

There are several weak points in my study. First of all, it is a retrospective study which involves a small number of DCIS cases with recurrence. Secondly, histological data for a few patients were not available during the study either due to inadequate histological assessment at diagnosis or poor record keeping. As for future work, a prospective study involving larger number of DCIS cases would be sensible. In addition, it would be interesting to compare the periductal vessel density between pure DCIS and DCIS with microinvasion. It would also be interesting to evaluate the phenotype of the blood vessels in the subsequent recurrences.

Chapters 14

Prognostic value of markers of angiogenesis

Before we look into the future, it is appropriate to review the values of current markers of angiogenesis as a prognotic indicator from recent literatures.

Breast cancer

Tumoural vascularisation

Up to mid 2000 [Gasparini et al 1998], studies had been published in peer-reviewed journal, on the association between tumoural vascularisation and clinical outcome (Table 14.1). All the studies were retrospective and used mainly the Weidner or Chalkley methods to determine and evaluate microvessel counts: 28 and 22 studies for relapse-free and overall survival, respectively, including multivariate analysis.

For relapse-free survival 31/41 studies and for overall survival 23/30 studies that evaluated the prognostic value of vascularity found a statistically significant association between microvessel counts and prognosis. In multivariate analysis, 23/29 and 20/23 showed that tumoral vascularistion is a significant an independent prognostic indicator relapse-free survival or overall survival, respectively. Among the studies with multivariate analysis that used antibodies to CD31 or CD34, most gave positive results on relapse-free survival (13/14) and all on overall survival (12/12) gave better results than with factor VIII-RA (relapse-free 8/13 and overall survival 7/10). Therefore, CD31 emerges as the panendothelial marker of choice for prognostic purposes (Table 14.1).

Tumour type	Number of studies	Number of patients	Stage	Median of follow-up (years)
Male urogenital tract				
Prostate	4	516	T1-2 Nx Mo (3), Gleason 6-7 (1)	3, 5-15
Bladder	3	314	Invasive stages	2.1-6.6
Renal	3	204	Invasive stages	1.9-3.0
Testicular germ-cell tumour	2	145	A-B	3.0-4.3
Female genital tract				
Cervix	9	684	FIGO IA-IVA	1.5-7.1
Endometrium	6	603	FIGO I-IV	2.6-11.5
Ovarian	7	323	FIGO I-IV (3), FIGO III-IV (4)	1.7-9.8
Gastrointestinal tract				
Oesophagus	5	393	Invasive stages	2.6-5.0
Gastric	5	723	Invasive stages	2.5-5.5
Colorectal	10	1247	Dukes A-C	1.3-6.3
Pancreas	1	40	Invasive	2.1
Other				
Head and neck	6	384	I-IV (5), I-II (1)	1.2-6.0
Lung	14	2450	I-IV (3), I-III (7), I-II (4), I (1)	2.0-8.5
Malignant melanoma	9	695	Invasive stages	3.1-10.0
Brain	4	238	Invasive stages	1.8-4.5
Breast	46	7345	I-II	2.1-16.0

Table 14.1. Studies on the prognostic value of intratumoral vascularisation.

The prognostic value of vascularity was first shown by two groups in 1992 [Horak et al 1992, Weidner et al 1992]. Others have since shown that the degree of vascularisation of the primary tumour correlates directly with presence of bone marrow micrometastases at diagnosis [Fox et al 1997], and that the presence of vascular 'hot spots' in axillary lymph nodes is associated with outcome [Guidi et al 2000]. Finally, the prognostic value of integrin alphaVbeta3 was investigated using the monoclonal antibody LM609 and immunohistochemisty [Gasparini et al 1998]. Determination of microvessel density by LM609 antibody was the single most significantly prognostic indicator for relapse-free survival in both node-negative and node-positive patients.

Angiogenic factors in breast tumours

Among proangiogenic factors, VEGF is the most extensively studied. 12 published retrospective studies, involving 3688 cases, have assessed the association between VEGF and clinical outcome in breast cancer. Among those using univariate analysis, 9/10 and 6/6 found a significant association between VEGF expression and relapse-free or overall survival. Among those with multivariate analysis, 7/9 and 6/6 found that VEGF is a significant and independent prognostic indicator for relapse-free or overall survival.

In a recent study by Linderholm et al [Linderholm et al 2000], of 363 consecutive node-positive patients, not only was the tumoral VEGF content of prognostic value, but also it predicted the anatomical site of first recurrence; the patients who developed brain, visceral, or soft-tissue metastases had a significantly higher VEGF content in the primary tumour than those with bone recurrences.

8 retrospective studies, of 1649 cases, evaluated the association between Thymidine Phosphorylase (TP) and prognosis; 4/7 studies and 4/5 found TP of significant prognostic value for relapse-free or overall survival, respectively, in univariate analysis [Toi et al 1999]. Most of the studies found that TP is a prognostic factor in the subgroups of patients treated with CMF adjuvant chemotherapy (cyclophosphamide, methotrexate, fluorouracil).

In the only published study on naturally occurring antiangiogenic factors [Gasparini et al 2000] there was no correlation of thrombospondins 1 and 2 with prognosis in 168 node-negative patients (Table 14.1).

Other tumours

Tumoral vascularisation

The prognostic value of vascularisation in non-small-cell lung cancer (NSCLC) has been evaluated in 2334 patients from 14 studies. In terms of overall survival, 10/14 studies were positive, including 7/8 with multivariate analysis (Table 14.1). Pastorino et al [Pastorino et al 1997] studied 515 cases of pathological stage 1 NSCLC, with median follow-up of 102 months. Several biological markers of angiogenesis were tested, including anti-CD31, and immunohistochemistry with Chalkley score. None of the biological markers emerged as independent prognostic factors, but in the subgroup of 137 patients with stage T1N0M0, both microvessel counts and EGFR expression were associated with relapse-free and overall survival in multivariate analysis. Fontanini et al [Fontanini et al 1997] examined

407 patients with stage S1-S3 NSCLC, with a median follow-up of 29 months. Tumour microvessel count stained with anti-CD34 antibody showed a significant and independent prognostic value for overall survival in multivariate analysis.

The largest study on melanoma [Massi et al 1999, Makitie et al 1999] showed that osteonectin, a glycoprotein involved in tissue mineralisation, regulation of endothelial cell shape, and proliferation during angiogenesis, was significantly correlated with clinical outcome in a series of 188 patients with thin cutaneous disease (<0.76mm).

In patients with choroidal and ciliary body melanoma, microvascular loops and altered networks were significantly and independent prognostic factors for overall survival. The majority of studies on cancers of the female genital tract revealed positive correlations between microvessel count and prognosis. However, owing to the heterogeneity of FIGO (Federation of International Gynaecological Oncology) stages included, the short follow-up time in some studies, and the scarcity of multivariate analysis, no definitive data can be obtained from these studies.

Most published studies on male urogenital-tract tumours reported positive correlations of microvessel count with prognosis in prostate cancer, with another six showing statistically significant correlations between highly vascularised cancers and the presence of metastases or extraprostatic spread at diagnosis. All three studies of the prognostic value of vascularisation in bladder invasive cancer reported positive associations with outcome, just as for renal cancer (Table 14.1).

Not all the studies showed that microvessel counts give independent prognostic information. Possible explanations for these discrepancies may be related to the small number of cases studied in certain tumour types and to potential methodological pitfalls, including criteria for selection of patients, heterogeneous therapy, inadequate follow-up, the choice of antibody used to stain endothelium, the vascular variable quantified and the area of tumour section assessed, the experience of the observer, and the appropriateness of statistical analysis. A consensus among experts has recently been proposed [Vermeulen (a) et al 1996].

Angiogenic factors

VEGF expression in oesophageal and gastric cancers has been shown to be an independent prognostic indicator in various multivariate analysis on overall survival (Table 14.2). A few studies with inconclusive results have been carried out on VEGF in other tumour types (Table 14.2).

2 studies examined TP expression in 330 cases of NSCLC both reported negative results on overall survival in multivariate analysis. Another 3 studies on patients with ovarian, head and neck, and endometrial cancers evaluated TP expression using multivariate analysis, and only those looking at head and neck cancer reported positive results (Table 14.2). Only 2 studies assessed the prognostic value of thrombospondin.

Tumour type	Number of studies	Number of patients	Stage	Median of follow-up (years)
VEGF				
Breast	12	3688	I-II	3.0-5.0
Bladder	2	116	Invasive	ND
Prostate	1	221	Invasive	3.5
Brain	1	27	Invasive	4.3
Head and neck	2	142	Invasive	6.1
Endometrium	2	107	FIGO I-IV	2.2-11.0
Ovary	2	126	FIGO I-II (1), I-II (1)	3.0-6.0
Melanoma	1	70	Invasive	3.3
Lung	4	493	I-IV	3.2-5.0
Colorectal	2	?	Dukes A-D (1), B (1)	3.0-4.4
Oesophagus	3	257	Invasive	2.6-5.0
Gastric	5	736	Invasive	3.0-5.5
Pancreas	1	40	Invasive	2.1
Testicular	1	80	I-II	4.3
TP				
Oesophagus	1	93	Invasive	3.0
Colorectal	3	156	A-C	1.6-4.5
Gastric	1	126	Invasive	3.0
Pancreas	1	40	Invasive	2.1
Breast	8	1649	I-II	3.0-9.8
Bladder	1	58	Invasive	2.0
Ovary	1	56	I-IV	3.0
Brain	1	27	Invasive	2.5
Head and neck	2	152	I-IV	3.0-4.0
Endometrium	1	109	I-IV	3
Cervix	2	146	Ib-II	3.6
FGFs				
Breast	4	658	I-II	3.0-3.9
Brain	1	26	Invasive	2.5
Head and neck	1	282	I-IV	2.3

Table 14.2. Studies on the prognostic value of angiogenic factors.

Chapter 15

The Future

To date, most studies examining therapeutic inhibition of angiogenesis have concentrated on advanced disease rather than prevention. Recently, there has been an increase in studies using genetically engineered mouse models, which themselves provide a general prototype of the pathways, parameters, and molecular mechanisms of multistage tumorigenesis. Such models provide opportunities for both the development of anti-cancer therapies as well as preventive measures that block the progression of pre-malignant lesions into tumours.

In general, antiangiogenic agents inhibit proliferation or invasion of blood vessels and may take days or weeks to produce an effect. Tumours may shrink proportionally to vessel regression, so there may be no reduction in vascular density and an increase in apoptosis [Bergers et al 1999]. Bergers et al [1999] studied the effects of four different angiogenesis inhibitors on RIP1-Tag2 mice at three distinct stages of pancreatic islet carcinogenesis. The experimental design included firstly, a prevention trial for the hyperplastic stage; secondly, an intervention trial for the early small asymptomatic cancers; and thirdly, a regression trial for advanced cancers. The four inhibitors examined were AGM-1470 (TNP470), BB-94 (batimastat), angiostatin, endostatin, and a combination of the latter two inhibitors. In the prevention trial, BB94, endostatin and the combination of endostatin/angiostatin reduced angiogenic switching by 50%, 61% and 63% respectively.

In the intervention trial, angiostatin, AGM-1470, BB-94 and endostatin reduced tumour burden by 60%, 82%, 83% and 88% respectively. Interestingly, histological analyses including vessel density didn't show a significant difference between the treatment and control groups.

On the other hand, drugs that affect the cytoskeleton of endothelial cells [Tozer et al 1999], or cause acute cell death, such as intra-arterial TNFa, produce effects within a few hours - rapid shutdown of blood flow and necrosis of large areas of tumour. Targeting coagulation factors to tumour vessels via upregulated specifically antigens, such as endoglin, also causes acute shutdown. Preclinical models indicate that antiangiogenic approaches work better on smaller tumours, as do synergies with conventional treatments [Teicher et al 1994]. The new agents are therefore likely to be used in combination therapy, which provides a rationale for early assessment of this approach.

The development of specific surrogate predictive marker for activity of inhibitors of angiogenesis is presently a major challenge of translational oncological research, taking into account that more than 40 antiangiogenic compounds are already under clinical evaluation [Gasparini et al 1997]. To rationalize the indications, monitoring of activity and to select the patients to be treated with inhibitors of angiogenesis, the availability of reliable, standardized and useful predictive markers of angiogenesis represents one of the major areas of research in the years to come.

Appendices

List of relevant publications

Teo N.B., Shoker B.S., Jarvis C., Holcombe C., Martin L., Sloane J.P.
Vascular Density and Phenotype around Ductal Carcinoma In Situ (DCIS) of the Breast: Relationship to Duct Size, Nuclear Grade, Comedo Necrosis and the Presence of Invasive Carcinoma
British Journal of Cancer 2002, 86: 905-911

Teo N.B., Shoker B.S., Martin L, Sloane J.P., Holcombe C.
Angiogenesis in Pre-invasive Cancers (Review Article)
Anticancer Research 2002, 22(4): 2061-2072

Teo N.B., Shoker B.S., Jarvis C., Martin L., Sloane J.P., Holcombe C.
Angiogenesis and invasive recurrence in ductal carcinoma in situ of the breast
European Journal of Cancer 2003 Jan; 39(1): 38-44

Teo N.B., Shoker B.S., Jarvis C., Sloane J.P. (late), Holcombe C
Thymidine Phosphorylase expression and Stromal Vascularity in Ductal Carcinoma In Situ of the breast
Journal of Clinical Pathology 2003 (accepted for publication)

List of relevant presentations

Angiogenesis in the Benign and Early Malignant Breast Cancer'
Oncology & Breast Cancer Research Meeting, Liverpool (2nd November 1998)

'Angiogenesis in DCIS of the Breast'
Oncology & Breast Cancer Research Meeting, Liverpool (4th October 1999)

'Merseyside DCIS of the Breast Audit'
Merseyside Breast Surgeons Meeting, Warrington (30th November 1999)

'Outcome of Treatment in 355 Cases of Pure DCIS of the Breast'
Oncology & Breast Cancer Research Meeting, Liverpool (7th February 2000)

'Outcome of Treatment of Pure DCIS of the Breast'
Association of Surgeons of Britain & Ireland and Surgical Research Society Conference, Cardiff (24th May 2000)

'Angiogenesis in Ductal Carcinoma in situ of the Breast is related to nuclear grade, comedo necrosis, the presence of invasive carcinoma and risk of recurrence'
British Breast Group Meeting, University of Oxford (7th July 2000)

'Treatment of DCIS of the Breast in Merseyside: A Review of 355 Cases'
Roger Croton Memorial Meeting, Warrington (10th July 2000)

'10 years of DCIS of the Breast diagnosed in Merseyside - Diagnosis, Outcome and Unit Comparisons'
Merseyside Breast Surgeons Meeting, Warrington (13th September 2000)

'355 cases of pure DCIS of the Breast'
2nd European Breast Cancer Conference, Brussels (27th September 2000)

'Angiogenesis in DCIS of the Breast'
2nd European Breast Cancer Conference, Brussels (27th September 2000)

'Angiogenesis in DCIS of the Breast and its relationship to the risk of recurrence'
British Association of Surgical Oncology Scientific Conference 2000, Royal College of Surgeons of England, London (27th November 2000)

'Changes in vascular phenotype with the development of Ductal Carcinoma in situ of the Breast'
British Association of Surgical Oncology Scientific Conference 2000, Royal College of Surgeons of England, London (27th November 2000)

'Angiogenesis and Ductal Carcinoma in-situ of the Breast'
23rd Annual San Antonio Breast Cancer Symposium, San Antonio, Texas, USA (9th December 2000)

'Angiogenesis in Ductal Carcinoma In Situ of the Breast is related to the presence of invasive carcinoma and risk of recurrence'
United States and Canadian Academy of Pathology Annual Meeting, Atlanta, GA, USA (6th March 2001)

'Angiogenesis in Ductal Carcinoma In Situ of the Breast is associated with the development of an invasive recurrence'
British Cancer Research Meeting, University of Leeds (2nd July 2001)

'Periductal and stromal angiogenesis in Ductal Carcinoma In Situ of the Breast (DCIS): its relationship to recurrence and thymidine phosphorylase expression'
British Cancer Research Meeting, University of Leeds (2nd July 2001)

'Angiogenesis in Ductal Carcinoma In Situ of the Breast (DCIS): its relationship to recurrence and thymidine phosphorylase expression'
Pathological Society of Great Britain and Ireland Meeting, Liverpool (4th July 2001)

'Treatment of DCIS of the breast in Merseyside: an audit of 355 cases'
7th Nottingham International Breast Cancer Conference, Nottingham (19th September, 2001)

'Angiogenesis in DCIS and the development of an invasive recurrence and Periductal and stromal angiogenesis in DCIS: its relationship to recurrence and Thymidine Phosphorylase expression'
7th Nottingham International Breast Cancer Conference, Nottingham (21st September, 2001)

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