Evaluation of laser Doppler flowmetry for the study of benign and malignant gastric blood flow in vivo

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Abstract

***Background*—Tumour vascularisation is a determinant of the development of metastases.**

***Aims*—To measure blood flow in normal stomach and gastric adenocarcinomas by laser Doppler flowmetry and correlate blood flow with vascularisation after immunohistochemical staining of resected specimens for CD31 and von Willebrand factor.**

***Patients*—Twenty two undergoing resection for gastric adenocarcinoma and 10 undergoing cholecystectomy.**

***Results*—Mean (SD) gastric blood flow was 208 (35) perfusion units (PU) in patients undergoing cholecystectomy and 190 (75) PU in the undiseased part of the stomach in patients with gastric adenocarcinoma. Gastric blood flow was higher in the border of gastric adenocarcinomas (322 (120) PU, p<0.01 *v* normal stomach) but lower in the centre (74 (27) PU, p<0.01 *v* normal stomach and tumour border). Blood flow was higher in tumours staged T⩾3 than in those staged T<3. Blood vessel density in normal stomach was 41 (8) stained cells/field viewed and was 1.9–3.4 times higher in gastric adenocarcinomas.**

***Conclusion*—Laser Doppler flowmetry is a valuable tool for studying the pathophysiological alterations of malignant blood flow in the human stomach in vivo.**

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Keywords: laser Doppler flowmetry; gastric adenocarcinoma; angiogenesis; CD31; von Willebrand factor

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Accelerated growth of malignant tumours is limited by an adequate vascular supply. Tumour progression is therefore inevitably accompanied by angiogenesis, a complex multistep process that involves extracellular matrix remodelling, endothelial cell proliferation, and migration, followed by capillary differentiation and anastomosis.[1–3] Circulating factors[4] as well as factors secreted by tumours in a paracrine fashion[5] have been identified in the propagation of angiogenesis. They include polypeptide growth factors,[6–9] lipids,[10] and smaller compounds like heparin and adenosine.[11 12] Not only has angiogenesis been associated with local tumour progression, it has been found to correlate with the metastasising potential of tumours in breast cancer, non-small cell lung cancer, and colorectal adenocarcinoma.[13–16] Also several clinical studies suggest that the activity of chemotherapeutic agents is more potent when their administration is adjusted to tumour blood flow.[17 18] Quantification of tumour blood flow therefore not only appears to be interesting from a pathophysiological standpoint but may have an impact on adjuvant treatment strategies. Clinical data on tumour blood flow, however, are lacking, mostly for methodological reasons. Methods of measuring local blood flow in vivo include microsphere and isotope washout studies and intravital video microscopy, but both of these methods are difficult to apply clinically. We here investigate the use of laser Doppler flowmetry for assessing blood flow in adenocarcinomas of the gastric corpus and antrum. Blood flow in gastric tumours correlated with the microvessel density and total vascular area in the resected specimens after immunohistochemical staining of a neoendothelial marker (CD31-PECAM1) and a conventional marker for endothelial cells (von Willebrand factor (vWF)).

Patients and methods

PATIENTS

After permission had been obtained from the local ethical committee, 22 consecutive patients (eight women, 14 men) were included in the study. Ten patients undergoing cholecystectomy without gastric pathology served as controls (eight women, two men; mean (SD) age 59.3 (20.3) years). The mean (SD) age of the 22 patients with gastric adenocarcinoma was 61.4 (14.4) years (range 26–92 years). Ten patients were classified as having a diffuse type adenocarcinoma according to Lauren, 10 patients had an intestinal type, and two had a mixed type adenocarcinoma. The median tumour stage was T2 (range T1–T4, mean 2.4).

All patients had general anaesthesia with sodium thiopentone, fentanyl, and pancuronium and maintenance with 0–70% N_2O in O_2 and isofluorane as well as repeated intermittent bolus doses of fentanyl and pancuronium. Standardised macrocirculatory and respiratory monitoring was performed, and blood pressure was maintained at a mean arterial pressure of 70–90 mm Hg during flow measurements by adjustment of anaesthetics or infusion of colloids and crystalloids. Arterial blood oxygen

Abbreviations used in this paper: PU, perfusion units; vWF, von Willebrand factor.

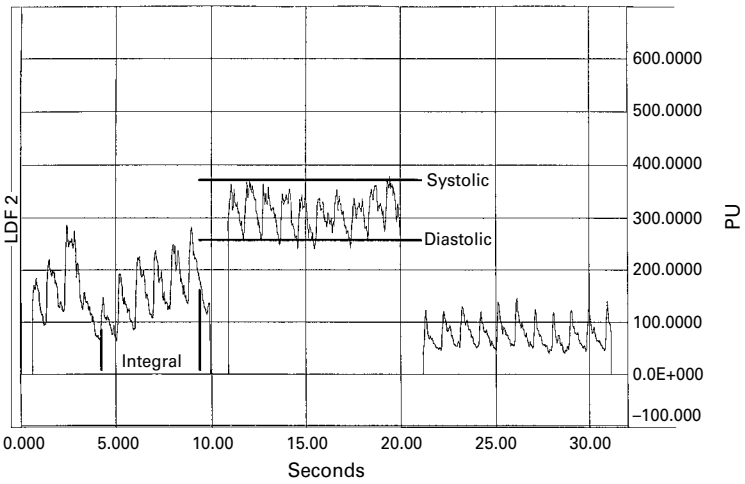

Figure 1 Laser Doppler blood flow reading of normal gastric blood flow and blood flow over the tumour border and the tumour centre (from left to right). Systolic, diastolic, and mean blood flow were calculated for each patient and measuring point over the entire sampling period using MP 100 WSW software. Furthermore pulse curves were analysed for the integral under the curve (over a 30 second period).

saturation was measured continuously by pulse oximetry and maintained above 95% during blood flow measurements.

Also, to avoid flow alterations as the result of mesenteric traction, blood flow was measured immediately after laparotomy, before further abdominal exploration.

BLOOD FLOW MEASUREMENT

In patients undergoing cholecystectomy, blood flow was measured in the gastric corpus (control). In patients with gastric cancer blood flow was measured in the undiseased stomach, over the centre of the tumour, and over the tumour border.

Flow was measured by laser Doppler flowmetry with a PF408 probe, and flow measurements were monitored on a Periflux 4001 Master (Perimed, Jarfälla, Sweden) as described previously.[19 20] In the gastrointestinal tract the penetration depth of the system used was shown to be 5–7 mm,[21] so that blood flow in the entire gastric wall was assessed.

Calibration was carried out as recommended by the manufacturer, by placing the probe in a standard latex solution. At a bandwidth of 12 kHz and a gain of 1 kHz, the flowmeter deflection was set to 25% of full scale. This level is defined as 250 perfusion units (PU). Analogue laser Doppler flow signals were digitalised with a UIM 100 (Biopac Inc, Goleta, California, USA) and processed on a personal computer with MP 100 WSW software (Biopac). Blood flow was recorded in the tumour as well as in normal tissue for at least 30 seconds, from the time at which a stable signal was obtained. Probe placement and analyser operation were performed by two different investigators (MKS, CR) to avoid observer bias and variability.[22] Data processing included calculation of systolic, diastolic, and mean blood flow as well as the area under the curve integrated over 30 seconds (fig 1).

After gastric resection, full thickness tissue samples from the area of previous blood flow measurements were collected and immediately placed in Bouin solution for further processing.

IMMUNOHISTOCHEMISTRY

Paraffin wax embedded tissue sections (2–4 μm thick) were immunostained for von Willebrand factor and CD31 receptor using the streptavidin-peroxidase/biotin technique (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA).[23 24] Tissue sections were submerged for 15 minutes in TBS buffer (10 mM Tris/HCl, 0.85% NaCl, pH 7.4) containing 0.1% (v/v) Triton X-100 and washed for five minutes in TBS buffer, as previously reported. Endogenous peroxidase activity was quenched by incubating the slides in methanol and in methanol/0.6% hydrogen peroxide, followed by washing in methanol and TBS/0.1% bovine serum albumin. After treatment with hyaluronidase (1 mg/ml in 100 mM sodium acetate, 0.85% NaCl), the sections were blocked for 30 minutes at 37°C with 10% normal goat serum before overnight incubation at 4°C with von Willebrand factor and CD31 receptor antibodies diluted in TBS containing 5% normal goat serum (von Willebrand factor) or a biotinylated goat anti-mouse IgG secondary antibody (CD31 receptor). Bound antibody was detected with a biotinylated goat anti-rabbit IgG secondary antibody and streptavidin-peroxidase complex (Kirkegaard & Perry Laboratories), followed by incubation with diaminobenzidine tetrahydrochloride (0.05%) as the substrate, and counterstained with Mayer's haematoxylin.

To ensure antibody specificity, control slides were either incubated in the absence of primary antibody or with an irrelevant IgG antibody. In both cases no immunostaining was detected.

BLOOD VESSEL QUANTIFICATION

After immunostaining of endothelial cells with a monoclonal antibody against CD31-PECAM-1 and a polyclonal antibody against von Willebrand factor, vascular density was quantified as described by Bosari and colleagues[14] and total vessel area was determined by computer assisted picture analysis. At a magnification of 40 ×, the three most vascularised areas were identified in each section. Microvessel density in these sections was then counted at a magnification of 200 × and is given as the number of positive stained vessels per field viewed (N_{CD31} or N_{vWF}). Stained single cells were considered to be sprouting endothelial cells and were also counted. Also microscopic images from tumours and normal tissues were transferred through a camera module connected to a computer screen by a Microsoft Windows Graphical User interface. Images were further processed using image-pro plus version 1.3 for Windows software. Vessels were marked at a magnification of 1:20 and the total vascular area per field (A_{CD31} or A_{vWF}) viewed was automatically calculated, and is given as pixels per field viewed. The software only recognised vessels with a diameter larger than 20 μm as luminar structures. At least ten fields per slide were viewed and calculated.

Table 1 Vessel density (per field, 1:200 magnification), total area (per field, 1:20 magnification) of blood vessels and blood flow in normal stomach and gastric adenocarcinoma

	Normal	*Centre*	*Periphery*
Vessel density			
vWF	41 (8)	78 (16)***	94 (31)*, ***
CD31	31 (8)	91 (28)***	104 (35)***
Vessel area			
vWF	17725 (2168)	20546 (3094)**	16456 (2327)
CD31	18499 (1461)	21546 (2673)***	24998 (1326)***
Blood flow			
Systolic	289 (108)	142 (52)**	461 (160)*, **
Mean	190 (75)	74 (27)**	322 (120)*, **
Diastolic	124 (58)	47 (20)**	226 (102)*, **
Area	861 (592)	418 (241)**	1085 (646)**

Results are expressed as mean (SD).
*p<0.05 *v* tumour centre, **p<0.01 *v* normal, ***p<0.001 *v* normal.
vWF, von Willebrand factor.

Table 2 Blood flow at the tumour border of gastric adenocarcinoma for different histological types and tumor stages

Tumour type/stage	*Mean blood flow (PU)*
Diffuse (n=10)	275 (78)*
Intestinal (n=10)	369 (122)
T1–2 (n=12)	212 (81)***
T3–4 (n=10)	398 (90)

Results are expressed as mean (SD).
*p=0.06 *v* intestinal type, ***p<0.001 *v* stage T3–4.
PU, perfusion units.

STATISTICAL ANALYSIS

Results are expressed as mean (SD) or as median and range. For statistical analysis Student's *t* test was used or analysis of variance with Bonferroni correction where applicable. Regression analysis was performed using a SigmaPlot and InStat software for Windows on a personal IBM compatible computer. Significance was defined as p<0.05.

Results

Mean gastric blood flow in patients without gastric pathology (patients undergoing cholecystectomy) was 208 (35) PU. Mean blood flow in the tumour-free gastric wall of patients with gastric cancer was 190 (75) PU. Blood flow was higher over the tumour border (322 (120) PU, p<0.01; analysis of variance) than in normal stomach, but significantly lower over the tumour centre (74 (27) PU, p<0.01, analysis of variance). Blood flow integrated over 30 seconds was 861 (592) PU in normal stomach, 1085 (646) PU over the tumour border, and 418 (241) PU over the tumour centre (table 1). Blood flow was higher in tumours staged T⩾3 (398 (90) PU) than in T<3 tumours (212 (81) PU, p<0.001) (table 2).

In undiseased stomach of gastric cancer patients, the N_{vWF} was 41 (8) and N_{CD31} was 31 (8) (fig 2). When compared with normal stomach, blood vessel density in the tumour centre was 1.9 (vWF) and 2.9 (CD 31) times higher and 2.3 (vWF) and 3.4 (CD 31) times higher in the border of gastric adenocarcinomas.

A_{vWF} in normal stomach was 17 725 (2168) pixels and A_{CD31} was 18 499 (1461) pixels and significantly higher in the centre of adenocarcinomas (table 1).

To study correlations between N and/or A with blood flow in the respective group, the variables were plotted in an x-y diagram and regression curves were calculated. When the manually counted N_{vWF} was plotted against N_{CD31}, a linear relation was found (r = 0.98, p<0.001). The negative y intercept (N_{CD31} = −25.5 + 1.4 N_{vWF}) indicates that, in gastric adenocarcinomas, relatively more CD31 posi-

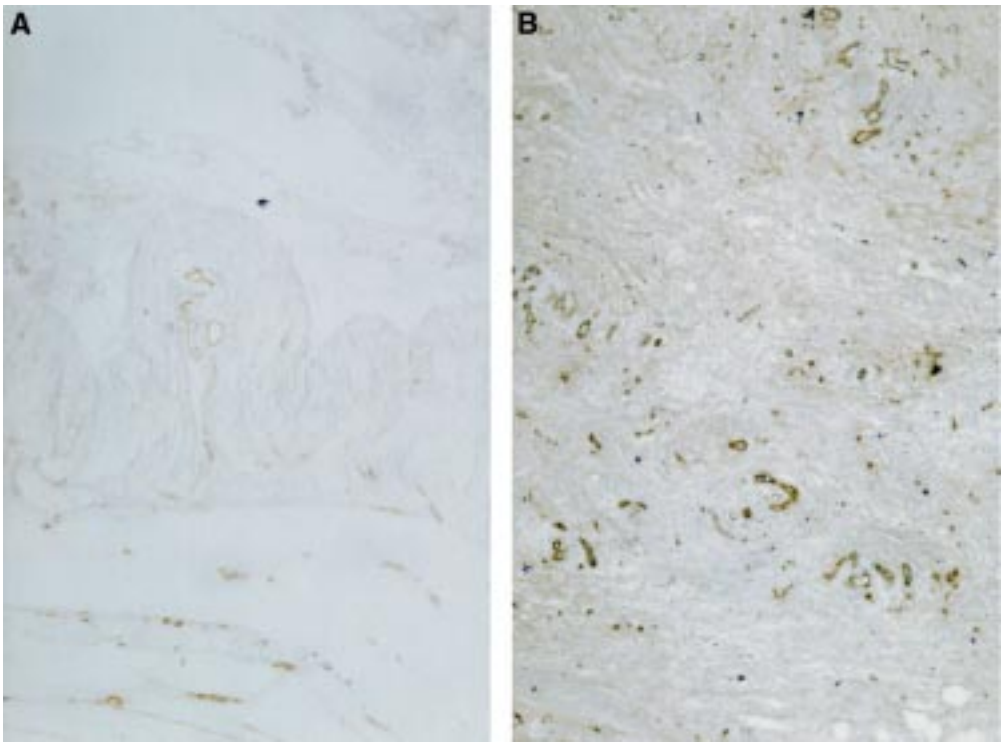

Figure 2 Immunohistochemical staining of CD31 in normal stomach (A) and gastric adenocarcinoma (B) (original magnification ×40). Note the high number of small positively stained vessels and cells in the submucous layer of the gastric cancer specimen.

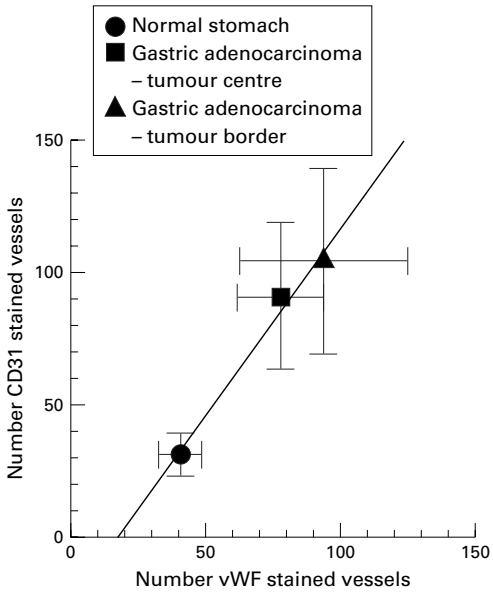

Figure 3 Linear correlation of the number of manually counted CD31 positive vessels with the number of vWF positive vessels ($N_{CD31} = -25.5 + 1.4\ N_{vWF}$).

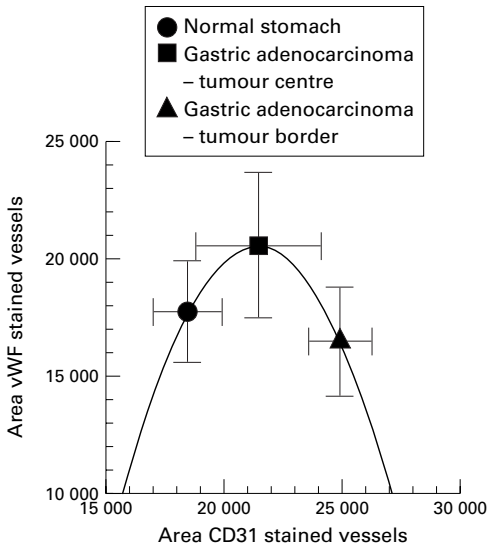

Figure 4 Area of CD31 positive vessels plotted against the area of vWF positive vessels.

tive vessels are found than in normal stomach (fig 3). Interestingly, when A_{vWF} was plotted against A_{CD31} (fig 4) no such linear relation was found, and a smaller A_{vWF} was found in the border of gastric adenocarcinomas than expected from A_{CD31}.

Also no linear relation was found when blood flow was plotted against $N_{vWF/\ CD31}$ (fig 5) or $A_{vWF/\ CD31}$ (data not shown), and blood flow in the centre of gastric adenocarcinomas was lower than expected from $N_{vWF/\ CD31}$ or $A_{vWF/\ CD31}$.

Discussion

Angiogenesis has been suspected to be an essential part of tumour growth since the beginning of this century.[25] The clinical significance of angiogenesis became apparent in colorectal,[15] breast,[14] and pulmonary[16] malignancies, when the degree of vascularisation

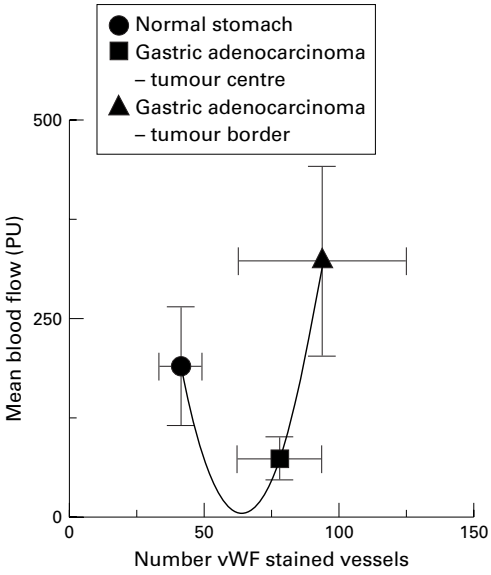

Figure 5 Mean blood flow plotted against the number of vWF positive vessels.

could be correlated with the development of metachronous metastases in patients with highly vascularised tumours.

Despite the importance of tumour vascularisation, there are no data on blood flow or blood flow regulation in human cancer in vivo. This may be attributed in part to the lack of clinically applicable methods of quantifying local perfusion without interfering with microcirculatory blood flow. In this study we quantified blood flow in gastric adenocarcinomas and the adjacent normal gastric wall by laser Doppler flowmetry, which has been used by us and others to measure gastrointestinal blood flow in a non-invasive clinically applicable manner. It correlates with other quantitative microcirculatory methods and has been shown to penetrate the whole gastrointestinal wall with an experimental set up comparable with that used in this study.[21] Blood flow in the gastric wall of control patients undergoing cholecystectomy was not different from blood flow in the undiseased part of the stomach of patients with gastric cancer and corresponded to normal gastric blood flow in previous studies.[19 20 26] In the centre of gastric adenocarcinomas, blood flow was lower than a linear relation between number of blood vessels and blood flow suggested and amounted to 40% of the blood flow in the normal gastric wall. In the tumour periphery, flow was 70% higher than in the normal stomach. It therefore appears likely that blood flow is redirected from the centre of gastric adenocarcinomas to the tumour periphery. Local blood flow is regulated by vasoactive substances secreted after various stimuli in a paracrine fashion. These substances, namely eicosanoids, nitric oxide, and adenosine, modulate the vasotonus by relaxing or constricting either the precapillary arteriolar or postcapillary venolar pericytes or smooth muscle cells. Neoangiogenetic tumour vessels are capillary in nature and lack pericytes or smooth muscle cells.[27] The findings of this study therefore suggest that gastric adenocarcinomas have

autogenous mechanisms for redistributing blood flow from the centre of the tumour to the infiltrating and expanding border probably by altering the vasotonus of postcapillary venules. It remains unclear which mediators are involved in this blood flow redistribution, but various experimental tumours have been found to contain large amounts of vasodilators like prostaglandin E_1,[28] adenosine, and adenosine diphosphate.[29] However, none of these factors has been studied in the regulation of blood flow in gastric adenocarcinomas.

Histopathological and methodological factors may account for the discrepancy between the linearity of the N_{CD31}/N_{vWF} correlation and the non-linearity of the A_{CD31}/A_{vWF} correlation in this study. Vascularisation in this study was determined manually after staining for von Willebrand factor and CD31 as suggested by Bosari *et al*,[14] and the total vascular area was calculated by computer assisted morphometry. The computer program only recognises vessels with a diameter larger than 20 μm as luminar structures, whereas the number count also included positively staining non-luminar cell aggregates and single cells as sprouting blood vessels. Identification of different vessel populations by von Willebrand factor or CD31 staining could be another reason for the discrepancy between vessel number and total vessel area. In macrovascular endothelia, high concentrations of von Willebrand factor are found in Weibel Palad bodies, but not in capillary endothelia.[30] Furthermore von Willebrand factor but not CD31 is expressed in lymphatic endothelia and in platelets. Horak and colleagues[30] therefore compared several antibodies directed to vascular endothelia with different specificities. CD31 (platelet endothelial cell adhesion molecule, PECAM-1) was found to be the most sensitive marker for neoangiogenetic endothelial cells, which stained more tumour vessels than von Willebrand factor or CD35 and CD34 antigen. It is only moderately expressed in normal endothelia but strongly expressed in proliferating endothelial cells.[31] Therefore the higher count of CD31 positive vessels in our study suggests that gastric adenocarcinomas contain mostly small newly formed blood vessels, especially in the periphery of tumours. These small vessels add relatively more to the total number of blood vessels than to the total vascular area.

In summary our data suggest that actual blood flow in gastric adenocarcinomas does not correlate with tumour vascularisation, and gastric adenocarcinomas appear to increase blood flow in the infiltrating periphery at the cost of the tumour centre. Finally laser Doppler flowmetry as applied here appears to be a valid method for studying and quantifying the effect of locally active or systemically applied vasodilators and their inhibitors on the microcirculation of gastric adenocarcinomas.

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