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Neovascularization in Human Cutaneous Melanoma: A Quantitative Morphological and Doppler Ultrasound Study

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Abstract—*A study of tumour blood flow in* 36 *patients with* 38 *malignant melanomas using Doppler Ultrasound flowmetry has shown that tumour blood flow can be detected in most melanomas more than* 0.9 mm *thick, and is absent in most melanomas less than this thickness. Histological quantitation of blood vessels using lectin staining to delineate vascular endothelium and automated image analysis has shown a high degree of correlation between vascularity at the tumour base and tumour thickness. Since it is likely that the development of a vascular plexus at the tumour base is a prerequisite for dissemination, the development of these blood vessels may prove to be an independent prognostic factor for thin melanomas. This work also provides a new model for dynamic,* in vivo *investigation of the vascularity of human tumours.*

INTRODUCTION

MALIGNANT melanoma of the skin has recently assumed great clinical and biological significance. Extensive clinical and experimental studies have shown that skin melanoma is one of the human cancers where all stages of neoplastic progression can be observed, diagnosed and treated in a highly predictable manner. At present its prognosis is best determined by histological tumour thickness, first described by Breslow [1] although the relationship of thickness with survival is not a linear one [2]. Experiments with melanoma cells and implants in animals have shown that the rapid growth of cells is dependent on the development of new blood vessels [3]. These are relatively large thin-walled tortuous tumour vessels which result in a high velocity turbulent blood flow. This flow pattern has recently been detected by Doppler ultrasound technique in a preliminary study of skin melanoma [4].

We now describe the results of Doppler flowmetry in 38 primary cutaneous melanomas and correlate the Doppler findings with the histological assessment of tumour vascularity by quantitative image analysis.

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PATIENTS AND METHODS

Thirty-six patients with 38 primary skin melanomas (stage I—34 patients, stage II—two patients) were examined by a continuous wave Doppler ultrasound flowmeter. There were 14 male and 22 female patients. The mean age was 56 yr with a range from 32 to 84 yr. The lesions were distributed in the following sites: head and neck—8; trunk—8; upper limb—5 and lower limb—17. The group comprised of 16 nodular melanomas, 15 superficial spreading melanomas, five melanoma-*in-situ* and two acral lentiginous melanomas. After wide excision without a preliminary biopsy the surgical specimens were fixed in formalin and H & E stained sections were used for routine histological assessment and tumour thickness measurement.

(a) Doppler flowmetry

A 10 MHz Doppler pencil probe was applied directly over the lesion. The frequency shift signals were displayed on the screen of a spectrum analyser (Angioscan, Unigon Inc., U.S.A.) and simultaneously recorded on an audio-cassette tape. The skin surrounding the lesion and at the mirror image site on the contralateral side of the body

was examined for comparison with the tumour signals.

(b) Vascular quantitation

The sections from the first 20 (10 Doppler flow positive tumours and 10 Doppler flow negative tumours) of the above 38 melanoma were chosen for the vascular quantitation. These sections were stained by Ulex europaeus agglutinin I (UEA–I) immunoperoxidase technique to delineate the vascular endothelium [5] and were examined with the IBAS –2 interactive image analysis system. Using × 16 or × 40 objective magnification at least four fields were analysed from each of the following sites: 1. within the melanoma itself (tumour); 2. at the junction of melanoma and underlying dermis (tumour base); 3. adjacent normal dermis (normal). The vessels were traced on the viewing screen and the following vascular parameters computed: 1. number of vessels per 100,000 sq microns of tissue cross-sectional area; 2. percentage of area occupied by vessels per field (PVA); 3. maximum vascular diameter (D– Max) in microns.

RESULTS

(a) Doppler flowmetry

Twenty-four primary lesions exhibited Doppler frequency shift signals. No flow signal could be detected in the other 14 cases. Maximum tumour thickness in the flow positive group was ≥ 0.9 mm in 23 lesions. The remaining lesion was 0.7 mm thick. Thirteen melanomas without flow signals were ≤ 0.75 mm in thickness. The remaining flow negative tumour was 2.00 mm thick. In 36 cases no flow signal was detected over the surrounding normal skin or on the contralateral side of the body. In two patients however—one with a lesion on the chest wall and the other with a subungual melanoma, Doppler signals were recorded from the mirror image sites of the body on the contralateral side. These signals were of lower peak systolic, mean systolic and minimum diastolic frequencies in comparison to the tumour signals.

(b) Vascular quantitation

Table 1 and Table 2 show the vascular counts in the tumour, tumour base and adjacent normal dermis. These vascular parameters are not normally distributed therefore non-parametric Mann–Whitney U-test and Wilcoxon paired sample test of statistical significance were applied. 1. Number of vessels—Doppler positive lesions contained a significantly greater number of vessels at the tumour base in comparison to the adjacent normal dermis (Wilcoxon $T- = 7$, $P < 0.05$). In the Doppler negative group however the number of vessels at the tumour base did not differ significantly from that of the normal dermis (Wilcoxon $T+ = 19.5$, $P > 0.2$). In both groups there were fewer vessels in the tumour itself than in the tumour base. 2. Per cent vessel area (PVA)—flow-positive lesions had a mean PVA of 9.04 ± 3.86% (mean ± 1 S.D.) at the tumour base and 1.22 ± 0.75% in the adjacent dermis (Wilcoxon $T-=0$, $P < 0.005$). The corresponding figures for the Doppler negative tumours were 3.30 ± 3.02% and 1.66 ± 0.88% (Wilcoxon $T- = 14$, $P = 0.2$). The Doppler positive lesions had a highly significant increase in vascularity at the tumour base over the Doppler negative cases (Mann–Whitney $U = 91$, $P < 0.002$). There was a good demarcation of the groups at a PVA level of 5.5% with a single exception in each group (Fig. 1). 3. Vascular diameter—The $D-$ Max at the tumour base did not differ in the Doppler flow-positive and Dopper flow-negative melanomas (Mann–Whitney $U = 70$, $P > 0.1$). However, the vessels at the tumour base had a significantly greater diameter in comparison to the vessels in the surrounding normal dermis in the same specimen in both the groups (Wilcoxon $T- = 3$, $P = 0.01$ for the Doppler-positive melanomas and $T- = 4.5$, $P < 0.02$ for the Doppler negative-tumours).

(c) Correlation of vascular parameters with tumour thickness and normal skin vascularity

1. Tumour thickness and vascularity—the maximum tumour thickness (mm) shows a positive correlation with the PVA at the tumour base.

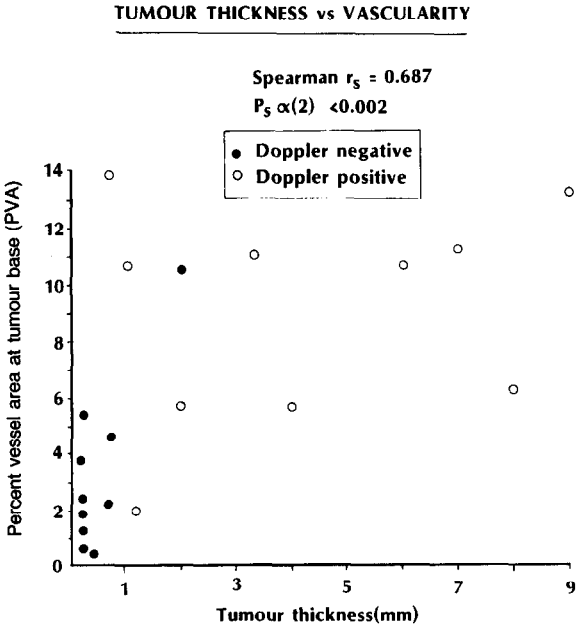

Fig. 1. Figure showing the relationship of tumour thickness and Doppler positivity to PVA at the tumour base.

Table 1. *Vascular quantitation in melanomas without Doppler signals*

S. No.	Tumour thickness (mm)	Number of vessels/100,000 μm²			Per cent vessel area			D–Max (μm)		
		Tumour	Tumour base	Normal dermis	Tumour	Tumour base	Normal dermis	Tumour	Tumour base	Normal dermis
1	2.0	2.6	12	8.6	1.6	10.5	1.2	20.2	32.2	22.2
2	0.2	3.1	10	5.8	1.4	1.8	1.5	57.6	64.6	53.4
3	0.19	1.3	5.6	8.4	0.2	3.8	2.8	14.3	61.2	52.4
4	0.2	2.1	9.7	12	0.2	2.4	3.4	18.5	34.2	31.3
5	0.2	4.2	8.1	9.7	0.9	5.4	1.2	26.7	45	26.3
6	0.7	1.9	3.9	2.3	0.6	2.2	2.2	41	41.6	35.6
7	0.2	5.8	9.7	9.7	0.8	1.3	0.8	24.7	16.3	19.2
8	0.2	5.8	5.1	7.1	0.9	0.6	1.5	14.2	17.5	17.8
9	0.4	0.6	2.3	3.6	0.1	0.4	0.6	29.8	22.3	20.3
10	0.75	3.8	7.9	9.8	1.5	4.6	1.4	40	46	18.8

Table 2. *Vascular quantitation in melanomas with Doppler signals*

S. No.	Tumour thickness (mm)	Number of vessels/100,000 μm²			Per cent vessel area			D–Max (μm)		
		Tumour	Tumour base	Normal dermis	Tumour	Tumour base	Normal dermis	Tumour	Tumour base	Normal dermis
1	7.0	5	6.3	10	2.6	11.2	2.3	30	58.4	25
2	1.02	10.5	16.3	6	1.9	10.7	0.7	30.8	68	17
3	3.3	6.3	12	5.9	3.2	11	0.7	37.5	46.4	28.7
4	0.7	3.1	10.5	1.6	3.3	13.9	0.3	21.9	61	13.9
5	4.0	3.2	9.1	3.5	1.5	5.7	0.8	45.3	56.1	30.4
6	9.0	1.5	4.9	6	0.5	13.2	2.6	39.9	46.4	28.6
7	6.0	0.8	6	4	0.9	10.7	1.4	71.6	40	24.3
8	1.2	9.1	10.6	4.7	1.4	2	0.5	24.8	18.5	20
9	8.0	1.5	2.5	2.3	2.9	6.3	1.2	68.8	37.6	38.2
10	2.0	2.3	4	2.7	0.7	5.7	1.6	19	48.5	30.4

Spearman rank correlation coefficient r being 0.687 at a $P < 0.002$ (Fig. 1.) 2. PVA at tumour base and in adjacent dermis—there is no correlation between PVA of adjacent normal dermis and that of tumour base (Spearman $r = -0.045$, $P > 0.5$). Thus tumour base vascularity shows a change independent of the vascularity of the surrounding skin.

DISCUSSION

It is now realised that research into tumour biology must go hand in hand with clinical diagnostic and therapeutic studies in cancer. Malignant melanoma is unique among human malignant tumours in its accessibility to detailed observation and study *in vivo*. Hence every piece of information which can be gleaned about this tumour *in vivo* is important in understanding human malignant disease in general.

Rapid cellular proliferation and increased metabolic demand by the cancer cells call for an increased supply of nutrients and oxygen. This increased demand is met with by simple diffusion of tissue fluid in the initial part of the tumour growth. However with continued growth this proves inadequate as the central part of the tumour becomes distant to the source of supply and the need for a well developed tumour vasculature of its own arises. This is initiated by the production of tumour angiogenesis factor(s) —TAF by the tumour cells [3, 6]. TAF appears to induce mitosis and migration of endothelial cells from the surrounding small venules. These vessels soon canalise and grow towards the tumour forming a complex network of tumour vasculature [7].

Vascularization of tumours in experimental animals is shown to be associated with an increase in growth rate and acquisition of metastatic potential [8]. Both melanoma cells and xenografts have been shown to produce angiogenesis factors and induce neovascularisation in the chorioallantoic membrance of the chick embryo, rabbit eye, hamster cheek pouch and athymic nude mice [8–12]. Rofstad [13] has grown human melanoma xenografts in nude mice and studied the relationship of tumour vascularity to the growth kinetics. He showed that the tumour volume doubling time and tumour cell loss factor decrease as the tumour vascularity increases. The fraction of cells in the *S*–phase and growth fraction were higher in melanomas having higher vascularity. Thus high vascularity is associated with rapid growth.

The demonstration of high velocity blood flow by the Doppler technique in melanoma of thickness greater than 0.9 mm, and the absence of detectable blood flow in lesions up to 0.75 mm in thickness correlates well with the animal experiments, which indicate the onset of angiogenesis at around 1 mm tumour size [3]. Detailed spectral analysis of the Doppler flow signals and Duplex scanning of the malignant masses suggest the presence of multiple blood vessels with different blood velocities flowing in different directions [14]. Moreover the centre of the tumour gives very feeble signals and dominant signals arise only from the periphery of the tumour mass (Woodcock, Srivastava and Hughes, unpublished data).

UEA–lectin staining and interactive image analysis give an objective measure of tumour vascularity. *Ulex europaeus* agglutinin is a sensitive and reliable stain for the vascular endothelial cells [15] and was used to delineate the vessels thus obviating the misinterpretation of tumour clefts and fixation artefacts as vascular spaces.

The presence of a significantly greater number of vessels with high PVA at the base of thick melanomas correlates very well with the Doppler detection of high velocity blood flow. Thus the significance of the Doppler signals in melanoma has been confirmed by correlation with objective demonstration of the increase in blood vessels in the tumour base. The study also confirms many of the findings regarding tumour angiogenesis which so far have been demonstrated only *in vitro* or animal experiments. The technique provides an unique model for the *in vivo* dynamic study of tumour vascularity in a clinical setting.

Of particular interest is the small number of tumours which do not conform to the common pattern. Three Doppler flow negative lesions had a marked increase in the PVA at the tumour base in comparison to the normal dermis. Perhaps the Doppler frequency shift becomes detectable only when flow velocity exceeds a critical value.

This may also explain the absence of signals in one thick melanoma. The strong correlation between tumour thickness and the percent vascularity ($P < 0.002$) together with Doppler results support the following hypothesis. Early radial growth phase melanoma is avascular. Its progression to vertical growth phase results in an increased vascularity which makes a tumour Doppler flow positive. It seems likely that the development of angiogenesis is critical to onset of a vertical growth phase with its requirement of profuse blood supply. Clark and co-workers [16] have shown that the radial growth phase melanoma is associated with a 5-yr disease-free survival of 100% whereas vertical growth phase is associated with metastasis and recurrence. The presence of thin walled blood vessels at the tumour base appears to be a prerequisite for the tumour dissemination, but not all melanomas with blood supply will metastasise. Presumably the blood vessels

provide a *modus operandi*—but the invasion of the vessel and establishment of metastasis would depend also on inherent properties of the cancer cells. If this hypothesis proves correct, the presence of blood supply in thin melanoma may mean that that particular tumour is capable of metastasis.

At present our number of cases is too small and follow-up too short for us to confirm the above hypothesis. But there is an urgent need to determine adverse prognostic factors for melanoma of intermediate thickness.

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