ULTRASOUND BACKSCATTER MICROSCOPE ANALYSIS OF MOUSE MELANOMA PROGRESSION

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Abstract—The incidence and mortality rate of cutaneous melanoma continue to increase throughout the world, making the study of melanoma biology an important area of current research. While recent breakthroughs in transgenic mouse technology have led to promising mouse skin models of melanoma, there is presently no technique available for quantitatively studying subsurface melanoma progression, in vivo. We demonstrate the first application of an imaging method called ultrasound backscatter microscopy (UBM) for imaging early murine melanomas with spatial resolution of 30 μm axial and 60 μm lateral. Murine B16 F10 melanomas have been imaged from their earliest detection, over several days, until they are 2 to 5 mm in diameter. Melanoma dimensions measured by UBM were found to be in excellent agreement with those determined histopathologically on the excised tumours. The relative rms errors in UBM-determined melanoma height and width were found to be 8.7% and 4.2%, respectively. The mean rate of increase in tumour height of early murine melanoma was found to be 0.37±0.06 mm/day. Computer-generated volumetric renderings of melanomas have been produced from three-dimensional image data, allowing quantitative comparisons of tumour volumes to be made. Using a priori assumptions of ellipsoid tumour shape, the relative error in UBM-determined volume was shown to be less than 17%. These results should be of considerable interest to investigators studying melanoma biology using mouse skin models, and have implications in the use of high frequency ultrasound imaging for the clinical assessment of cutaneous melanoma.

Key Words: Ultrasound backscatter microscope, High frequency ultrasound imaging, B16 F10 melanoma, C57BL/6 mice.

INTRODUCTION

Cutaneous melanoma is now a common form of cancer, the incidence and mortality rate of which continue to grow throughout the world (Koh 1991). The underlying biology of melanoma has been the subject of much recent research, which may ultimately result in more effective approaches to the treatment and control of this disease. In this endeavor, the use of animal models for cutaneous melanoma is essential for studying the effects of growth factors and therapeutic agents.

An easily accessible, in vivo imaging technique for assessing the growth characteristics of cutaneous melanoma in these animal models could be particularly useful in quantitative studies of the effects of various agents and for elucidating the dynamics of melanoma progression under different conditions. Ultrasound backscatter microscopy (UBM) is one approach that has the potential to address this problem.

UBM is a method for subsurface imaging of biologic tissues with high resolution. UBM employs the principles of pulse-echo ultrasound imaging at much higher frequencies (40 to 100 MHz) than most clinical ultrasound (3 to 10 MHz) (Foster et al. 1993). A UBM system developed for imaging skin has been described previously (Turnbull et al. 1995). This scanner uses mechanically scanned, focused transducers operating be-
between 40 and 100 MHz. Two-dimensional (2D) images can be produced at close to real-time frame rates (five images per second), and three-dimensional (3D) image data can be acquired as a series of 2D images in approximately 1 min. At 50 MHz, the UBM system has a measured spatial resolution close to 50 µm and a penetration depth of 3 to 4 mm in skin.

In this article, we describe the use of the skin UBM system for imaging murine melanoma and its progression, in vivo. The growth of a number of tumours was readily visualized over several days, and quantitative data on melanoma growth was obtained from the images. Tumour dimensions measured by UBM were compared to histopathologic measurements, and were found to be in excellent agreement. Finally, several 3D image data sets have been analyzed to show that quantitative measurements of tumour volume are also possible with UBM. Taken together, these results show great potential for the use of UBM in studying the dynamic progression of melanoma in a mouse skin model.

**MATERIALS AND METHODS**

*Ultrasound backscatter microscope*

Full details of the UBM system used in this article have been given previously (Turnbull et al. 1995) and so will only be described briefly. The scanner produces 2D images which are 512 × 512, 8-bit pixels covering an area of 8 mm × 8 mm. Volumetric (3D) image data were collected as a series of adjacent 2D images. Superficial tumours were imaged using a highly focused, 62-MHz transducer with a measured axial (depth) resolution of 20 µm and lateral (horizontal) resolution of 35 µm. To obtain adequate penetration (3 to 4 mm) to image deeper tumours, a 50-MHz transducer was used, with measured resolution of 30 µm in the axial direction and 60 µm in the lateral direction.

Dimensions of the tumours and skin were measured with the electronic calipers on the UBM scanner or offline (next section, below), assuming an ultrasound velocity of 1515 m/s. This velocity represents an intermediate value between the dermal (1580 to 1595 m/s) and subcutaneous fat (1440 m/s) ultrasound velocities which have been measured in human skin (Moran et al. 1995; Serup 1992). While this assumed ultrasound velocity is probably not correct for any of the individual tissues (epidermis, dermis, fat, muscle) or the tumour, the figure is an average, or effective value for the skin as a whole. Based on measurements made in human skin, the speed of sound is unlikely to differ from our assumed value by more than ±5%. Our assumed sound speed is also close to previous measurements made at room temperature in excised mouse skin samples: 1512 m/s in 2-month-old mice and 1540 m/s in 27-month-old mice (Bhagat et al. 1980).

*Animal procedures*

Animals used in this study were maintained according to protocols approved by the Institutional Animal Care and Use Committee at the Sunnybrook Health Science Centre. Eight-week-old female C57BL/6 mice were purchased from Charles River (Kingston, NY, USA). One group of nine mice were injected with B16 F10 murine melanoma cells at the age of 9 weeks, while a second group of 16 mice were injected at the age of 24 weeks. In each mouse, 1 × 10⁵ melanoma cells were injected as superficially as possible after anesthetizing the mouse using 10% (vol/vol) sodium pentobarbital in phosphate buffered saline (PBS) administered at a dose of 10 µL/g of body weight. The injections were intended to be intradermal, but subsequent histologic analysis indicated that most of the injections were most likely subcutaneous (see Results). Previous investigations have shown that mouse melanomas produced by this method can usually be detected within 2 weeks after the injection, and that the vascularized tumours grow rapidly, doubling in weight every 1 to 2 days (McKenzie et al. 1994). A patch of skin (2 to 3 cm in diameter) was wet shaved on the side of each mouse and the B16 cells were injected in the center of the shaved region, which was kept clear of hair during the course of the experiment.

UBM was used to image the injection sites on day 0, and every 2 to 5 days subsequently. The mice were also visually inspected two or three times per week for signs of tumour development. UBM images were obtained by anesthetizing the mice and fitting a small water bath to the skin, over the injection site, to provide a fluid coupling medium between the transducer and the skin. The water bath was produced by making a well with thick ultrasound gel, approximately 20 mm in diameter and 10 mm in height, and filling this well with distilled water. In this way, the water bath was made without applying pressure to the skin, thus avoiding skin distortion during ultrasound imaging. Along with the B16-cell-injected mice, a group of four mice in each of the two age groups were maintained as controls. These animals were given a sham injection of PBS rather than the B16 cells, but were otherwise treated the same as the experimental animals, being anesthetized, shaved and imaged with UBM as part of the protocol. After detection of a tumour by UBM or visually, the tumour was imaged daily with UBM to analyze the growth characteristics. At a maximum tumour diameter of 2 to 5 mm, the anesthetized animal was killed by cervical dislocation and the tumour excised for subsequent histologic examination. The tumours were harvested to produce a range of tumour heights between 1 and 4 mm, allowing a reasonable com-
comparison between the UBM and histologically determined tumour dimensions over this range.

For each tumour, the UBM image plane was oriented visually perpendicular to the dorsal-ventral (D-V) axis of the mouse, close to the center of the tumour, until a maximum tumour height was measured with the electronic calipers of the scanner. A separate motorized motion stage was then used to move the real-time UBM scan plane up and down the D-V axis to locate the lateral edges of the tumour. An automatic 3D image acquisition protocol on the UBM scanner, which synchronously steps the scan plane and captures a 2D image repeatedly, was used to collect a complete volumetric data set as a series of adjacent 2D images from the dorsal edge to the ventral edge of the tumour. Tumour dimensions were then measured off-line on a Macintosh computer, searching through the 2D image data for the maximum tumour height, using NIH Image 1.55 (public domain software, National Institutes of Health, Bethesda, MD, USA) to make the measurements.

Histology

Excised skin samples were immediately fixed in 10% buffered formalin. The fixed skin samples were embedded in paraffin within 1 to 4 weeks after UBM imaging, and sectioned to a thickness of 5 to 10 μm. Histologic sections were taken from the center of the tumour, generally the location where tumour width and height were maximal. The histologic sections were stained with hematoxylin and eosin (H&E) and examined by an experienced dermatopathologist. Comparisons of tumour dimensions were made between the histologic sections from the center of the tumour, and the UBM images also made at the center of the tumour. At the start of the experiment the only parameter being compared was maximum tumour height, with no attempt being made to obtain exact correspondence between the UBM and histologic sections. Later, six samples were pinned flat and the D-V direction marked to allow the histologic sections to be made at the same location and orientation as the UBM images. These samples were used to compare UBM and histopathologic tumour width as well as height.

Three-dimensional (3D) image analysis

Three-dimensional UBM data were collected as described above, by mechanically translating the 2D image plane across the tumour volume and collecting serial scans. The spacing between image planes in the 3D data sets was 100 μm. The number of images from one side of the tumour to the other ranged from 10 to 50, corresponding to tumour widths between 1 and 5 mm. Volumetric renderings of several murine melanomas were produced on a 3D image workstation (Alle-
ellipsoid shape, symmetric in the lateral (width) directions, a simple formula for tumour volume can be written (Beyer 1976):

\[ V = \frac{4}{3} \pi \left( \frac{H \cdot W^2}{2} \right) = \frac{1}{6} \pi HW^2 \]  

(2)

Using this formula for tumour volume, it is straightforward to show that the relative error in the UBM-derived volume is bounded by the sum of the relative errors in the axial and lateral directions (Dahlquist and Bjorck 1974):

\[ \left| \frac{V_e - V_h}{V_h} \right| \leq E_H + 2E_W \]  

(3)

where \( V_e \) and \( V_h \) are the UBM and histology-determined tumour volumes [eqn (2)], respectively, and the rms errors in UBM-determined tumour height, \( E_H \), and width, \( E_W \), are given by eqns (1a) and (1b).

**RESULTS**

Histologic examination of the tumour samples showed significant dermal involvement in 6 of the resulting 16 tumours, indicating that the B16 cell injections were intradermal. In the other 10 mouse melanomas, histologic examination revealed deeper tumours, indicating that the injections were most likely subcutaneous, into the layer of fat below the dermis. All tumours were used in the subsequent analysis, regardless of whether they had a significant dermal component. The main difference between the two age groups was the time taken before the tumours were first detected. The mice injected at 9 weeks of age developed tumours with a mean time to first detection of 8.4 days (range: 6 to 15 days), while the 24-week-old mice developed tumours considerably slower, with a mean time to first detection of 16.2 days (range: 11 to 24 days). The efficiency of tumour induction by the injection method also appeared to be reduced in the older mice, where 7 of 16 mice failed to develop a tumour over the course of the experiment (6 weeks after injection), whereas only 1 of the 9 younger mice failed to develop a tumour. One mouse died during the course of experiment and the tumour could not be used for histologic analysis. At the end of the experiment, we had UBM and histopathologic data from 16 melanomas. Six melanomas were detected when the tumour height was less than 1 mm, either visually or from UBM images of the injection site. The remaining 10 tumours were detected with a height > 1 mm, and were either harvested immediately after imaging or allowed to grow for 1 day after initial detection. The growth rate after detection was similar in both age groups, with a mean rate of increase in tumour height of 0.37 mm/day (see below). The murine melanoma tumours were quite regular in shape and well defined in UBM images.

One interesting unexpected finding was the induction of pigmentation and thickening of the skin in the shaved region. Figure 1 shows a UBM image of the skin of one mouse where there was a marked boundary between pigmented and unpigmented regions in the shaved patch. The thickness of the pigmented skin is clearly greater than that of the adjacent unpigmented skin, the difference being measured easily with UBM. We observed that the average skin thickness in the mid-side region of our injection sites increased from approximately 300 \( \mu m \) to 600 \( \mu m \) in these pigmented regions of skin. Slominski et al. (1991) provide an excellent discussion of this phenomenon of melanogenesis and skin thickening induced by hair removal and the subsequent synchronous anagen (hair growth) phase in the C57BL/6 mouse. The control mice in our study (see Materials and Methods) also developed thickened, pigmented regions of skin, but no tumours.

In order to validate the tumour dimensions measured in vivo by UBM, the dimensions of each excised tumour were also measured histopathologically. For each tumour sample, the following measurements were obtained from central sections where tumour height was maximized: distance from the skin surface to the tumour; the height of the tumour; and the width of the tumour. Figure 2 shows an example of a mouse melanoma imaged on
day 19 (after injection) with UBM (Fig. 2A) and the corresponding matched histologic section (Fig. 2B). The UBM appearance of the murine melanomas was similar in all cases, with the tumour tissue at a lower average gray level than the surrounding dermal and subcutaneous tissues. The uniform appearance on UBM seemed to reflect the histopathologic properties of the tumours, which consisted of a uniform density of B16 melanoma cells with well-defined margins. Tumour dimensions were measured both during the UBM scanning procedure and on 3D image data, off-line (see Materials and Methods). All UBM measurements were made by one person, experienced in using the system. Several preliminary experiments were performed, comparing UBM and histologic images side by side, to gain confidence in the interpretation of the UBM images. In tests using the off-line measurement system, the dimensions measured in the same tumour data sets were repeatable to within 100 μm. Interobserver variations in tumour measurements were not assessed in this study. All histopathologic tumour dimensions were determined, independently of UBM measurements, by one experienced dermatopathologist. No attempt was made to quantify inter- or intraobserver repeatability in the histologic measurements.

The height of each of the 16 tumours determined by UBM and histopathology is shown in Fig. 3A. Good agreement was found between the tumour height measured using both methods (linear regression analysis, \( r = 0.98 \)). One tumour in which the UBM determined height was 25% greater than the height determined by histology had a large hemorrhage in the center, which underwent a reduction in size after dissection (linear regression analysis of UBM versus histology tumour heights excluding this case, \( r = 0.99 \)). The surface-to-tumour depths measured by UBM and histology agreed to better than 100 μm in all cases (Fig. 3B) (linear regression analysis, \( r = 0.93 \)). In some cases where this dimension becomes vanishingly small, the surface-to-tumour depth measurement borders on the resolution limits of the UBM system. In six of the tumours, the excised specimens were marked to enable the histologic sections to be made in the same orientation as the UBM images, as described in the Materials and Methods section. For these tumours, we also found excellent agreement between the maximum tumour width determined by UBM and that determined histopathologically (Fig. 3C) (linear regression analysis, \( r = 0.99 \)).

Unlike histologic examination, which gives very high resolution data of the dimensions and cellular composition of a tumour at a single fixed time, UBM is an in vivo imaging method which can provide dynamic data on tumour growth in a single melanoma. This is demonstrated in Fig. 4 where tumour height is shown over 2 to 4 days of growth for the six melanomas that were first detected with tumour heights less than 1 mm. The growth rate for each tumour appeared to be similar, with a mean rate of increase in tumour height ± standard deviation of 0.37 ± 0.06 mm/day. It is not possible to obtain dynamic information of this type using histology. Interesting structural changes in single tumours can also be observed with UBM, such as the growing together of two branched regions of a single melanoma (Fig. 5).

The ability to acquire 3D image data with UBM
UBM data. The tumour is shown embedded in the mouse skin and subcutaneous tissues, with the skin overlying the tumour rendered semitransparent to allow the tumour to be visualized. Quantitative data on tumour volume change as a function of time can be obtained using computer-generated volumetric renderings. The volume of the melanoma shown in Fig. 6 changed from 1.4 mm³ to 6.3 mm³ on the third day of imaging, when the animal was killed and the tumour excised for histologic examination. Complete verification of the UBM-derived tumour volume would require that the volumetric UBM data be quantitatively compared to similar volumetric renderings made from histologic images. As described in the Materials and Methods section, we have derived a simple estimate for the relative error in UBM-determined tumour volume based on measured rms errors in the UBM-determined tumour heights and widths. Using the UBM and histology-determined heights from the 16 tumour samples, the relative rms error was estimated from eqn (1a) to be 0.087. The relative rms error for UBM-determined tumour width was estimated to be 0.042 using eqn (1b) for the six tumour samples in which UBM and histologic orientation was matched. These error estimates were then used in eqn (3) to determine that the relative error in UBM tumour volume was less than 0.171 (17%). Therefore, an upper bound on the error in the UBM-determined tumour volume for the melanoma shown in Fig. 6 varies from 0.24 mm³ (at 1.4

![Fig. 3. Quantitative comparisons of tumour dimensions determined from UBM and histology. (A) B16 melanoma height measurements for 16 tumours. The measurements for one tumour with a large central hemorrhage (△) showed a significant difference between UBM and histology. (B) Skin surface to tumour (S-T) depth measurements for the 16 melanoma samples. (C) B16 melanoma width measurements for six tumours in which the orientation of UBM and histology were matched. In each of (A), (B) and (C), the line shown is that of identity (i.e., UBM parameter = histology parameter).](image)

makes it possible to quantify volumetric changes in a growing tumour. Figure 6 shows the volumetric rendering of a murine B16 melanoma, produced from 3D

![Fig. 4. Dynamic tumour growth data measured by UBM. Melanoma height measurements, made from UBM images, are shown for six tumours over 2 to 4 days of tumour progression. Day 0 is the first day of tumour detection.](image)
mm$^3$ volume) to 1.1 mm$^3$ (at 6.3 mm$^3$ volume). Using the histologic measurements of height (1.8 mm) and width (2.7 mm) of the excised tumour in eqn (2), the ellipsoid model predicts a volume of 6.9 mm$^3$, which differs from the in vivo UBM measurement (6.3 mm$^3$) by 8.7%.

**DISCUSSION AND CONCLUSIONS**

Cutaneous melanoma is a disease which has been increasing in incidence and mortality over the past several decades. Realistic animal models of human melanoma have long been sought in order to gain more insight into this serious disease. Recently, promising mouse models exhibiting many of the characteristics of human melanoma have been produced using transgenic approaches (Mintz and Silvers 1993). The characteristics of these transgenic mouse models include distinct radial and vertical growth phases, the hallmark of superficially spreading melanoma which is the most common form of human melanoma. In this article, a simpler mouse melanoma model has been used to demonstrate the feasibility of using UBM to obtain quantitative information on tumour growth, in vivo. This model, produced by injecting B16 F10 murine melanoma cells into the skin of C57BL/6 mice, has been used recently to study the effect of various growth factors on melanoma progression (McKenzie et al. 1994). The UBM system should be particularly useful in studying early melanoma progression when tumours are less than several millimeters in height. New insights on early human melanoma progression may develop from the use of UBM to image transgenic mouse melanomas, in vivo, from the earliest formation of a skin tumour.

Clinically, the assessment and management of cutaneous melanoma is often difficult. The most prognostic feature of melanoma has been found histopathologically to be tumour thickness or height (Breslow 1970). In this investigation, excellent correlation has been demonstrated between the maximum height of the mouse melanomas determined by UBM and histology. The relative rms error in UBM-determined tumour height was determined to be 8.7%. Preliminary clinical studies have shown that human melanoma height can also be determined in vivo using UBM, although the thickness is sometimes overestimated when inflammatory cells exist at the base of the melanoma (Semple et al. 1995). Complications due to inflammation were not present in the mouse melanomas described in this study, probably because the tumours were only allowed to progress to a height less than 4 mm and a lateral size of 2 to 5 mm; i.e., before the point when inflammation and regression might be expected. In general, the UBM appearance of the B16 murine melanomas is simple compared to images of human melanomas, with more regular shape and more easily identified margins, reflecting to some extent the need for more realistic animal models of the human disease (Mintz and Silvers 1993).

Volumetric renderings are tedious to produce at this time since the tumour boundaries have to be manually outlined in a series of 2D images. Efficient and robust algorithms for segmenting the tumours from surrounding tissues need to be developed before the full potential of 3D UBM will be realized. In particular, better speckle reducing filters must be found to avoid computer-derived contours from bleeding into adjacent regions of the image through speckle elements at the periphery of the tumour region being segmented. Motion artifacts, due to respiratory motion of the anesthetized mouse during the data acquisition are also evident in some of the volumetric renderings. These could probably be eliminated, or at least reduced, by synchronizing the UBM data acquisition to the mouse's respiratory cycle. Problems notwithstanding, the 3D image data provides an in vivo measure of tumour volume.
and may yield new parameters related to tumour progression which could be useful for experimental animal models of melanoma growth. Additional verification of the accuracy of the UBM-derived tumour volume measurement is required before this parameter can be used in experimental systems or clinical studies. Such verification will require validation of the UBM acquisition and computer reconstruction algorithms, probably making use of tissue-mimicking phantoms. In addition, validation of the UBM volume measurements will require comparison with 3D histologic measurements, taking into account the distortions involved in fixing, cutting and digitizing a series of histologic sections. None of these tests have been performed at this time. In this study, we have shown that in the case of an ellipsoidally shaped tumour with major and minor axis dimensions determined from histopathologic sections, the relative error in UBM-determined tumour volume is at most 17%. Partial validation of this estimate was given in a comparison of the volume derived from the ellipsoid model using histologically determined dimensions (6.9 mm$^3$) with the in vivo UBM measurement of volume in the same tumour (6.3 mm$^3$).

Boundary definition of melanoma using UBM is clearly feasible, given the excellent correlation between histology and UBM tumour heights (relative rms error = 8.7%) and also tumour widths (relative rms error = 4.2%) when the orientations were matched. In vivo parameters of tumour growth can be measured from UBM images. The mean rate of increase in B16 melanoma height ± standard deviation was determined to be 0.37 ± 0.06 mm/day. The use of 3D UBM imaging has been described, which can be used to determine quantitative, volumetric parameters of melanoma progression. It may be that tumour volume or 3D shape factors, which can be derived from UBM images, will provide new clinical prognostic features in addition to the well-established feature of melanoma height. The ability to quantify tumour volume should be of great interest to investigators studying the effects of various agents on melanoma progression or regression. These results indicate great potential for UBM to be used to aid in the study of melanoma biology, as well as the possible application of UBM in the clinical management of cutaneous melanoma.

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