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THE IMPORTANCE OF DETERMINING NECROTIC FRACTION WHEN STUDYING THE EFFECT OF TUMOUR VOLUME ON TISSUE OXYGENATION

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The relationship between tumour tissue oxygenation and necrosis at different tumour sizes was investigated in a C3H mammary carcinoma implanted in the feet of female CDF1 mice. Experiments were performed using tumours that ranged in size from 80 to 800 mm^3. Necrosis was estimated histologically. Tumour tissue oxygenation (pO_2) was estimated with an Eppendorf electrode. Our results showed that as tumour volume increased there was a corresponding increase in necrotic fraction ranging from 1% in small tumours up to 51% in large tumours. The percentage of pO_2 values ≤ 5 mmHg increased from 2% up to 79%, in small and large tumours respectively. After correcting for necrosis, the apparent, significant increase in the % of pO_2 values ≤ 5 mmHg was lost. We conclude that correcting for necrotic fraction in this tumour model is necessary when attempting to measure tumour oxygenation using electrodes.

Solid tumours are known to contain hypoxic cells that compromise the success of radiotherapy (1). Identifying and quantifying tumour hypoxia has thus been an important task in the last 3 decades (2). One of the most direct methods to achieve this is to determine the oxygen partial pressure (pO_2) distribution in tumours using polarographic-needle electrodes (3). Clinical studies using this procedure have shown that the least responsive tumours were those with the lowest pO_2 values (4–7). Experimental studies trying to correlate the pO_2 measurements with the actual percentage of clonogenic hypoxic cells showed that the correlation existed only when measurements were performed in one tumour model in which the hypoxic fraction was modified by different methods (8). No correlation was seen when hypoxic fraction and pO_2 were measured under normal conditions in a range of different tumour models grown in various sites at different sizes (9, 10). This lack of a correlation could be the result of variability in tumour necrosis across the different tumour models.

Tumour necrosis is known to change as a function of tumour volume (3, 11). In the current study, we have therefore investigated the importance of necrotic fraction determination on tissue oxygenation in relation to changes in tumour volume.

Material and Methods

Mice and tumours. C3H mouse mammary carcinomas were implanted on the feet of 10–14-week-old female CDF_1/Bom mice. The derivation and maintenance of the tumour system has been previously described (12). Tumour size was determined by the formula $\pi/6 \times D_1 \times D_2 \times D_3$ where Ds are the three orthogonal diameters, ranged from 80 to 800 mm^3. All experiments were performed on restrained non-anaesthetized animals. Different groups of mice were used for the different endpoints.

Necrotic fraction estimation. Isotropic uniform random sections (13) were obtained from formalin-fixed paraffin-embedded tumours. Four to six equi-distant sections, 3μ thick, were stained using haematoxylin and eosin. Sections were examined using a projecting light microscope. Each section was projected on a grid with 105 points and all

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fields were systematically scanned. Each field was assessed separately for the total number of points hitting the field (nT), the number of points hitting viable tissue (nV) and the number of points hitting necrosis (nN). Necrosis was defined as areas which stained red with the eosin stain and lacked any blue haematoxylin-staining material. The necrotic fraction for each slide was determined by $\Sigma(nN)/\Sigma(nT)$, and the necrotic fraction for the whole tumour represented the average necrotic fraction of all the slides.

Tumour oxygenation measurements. Measurement of tumour oxygenation were performed using a polarographic needle electrode (Eppendorf, Hamburg, Germany). The mouse was restrained with the tumour bearing foot exposed and loosely taped to the jig, such that the normal blood supply to the foot was not interrupted. The needle electrode was inserted up to 1 mm depth in the tumour and then moved automatically in forward steps of 1 mm followed by a 0.3 mm backward step prior to the measurement. The length of the track was determined for each tumour according to its diameter. Three to seven random parallel electrode tracks were performed in each tumour, yielding a total of 50–100 measurements per tumour. The percentage of values $\leqslant 5$ mmHg was selected and plotted against tumour volume.

Statistical methods. Data were calculated as means and standard error (SE). The statistical program (SPSS/PC + V4.01) was used to calculate the weighted least square regression line with the slope and intercept values for the estimated necrosis data as well as the t-statistic and the corresponding significance level whenever stated in the paper.

Results

Necrotic fraction estimation. Fig. 1 shows representative examples of necrosis in C3H mammary carcinoma of different size. In small tumours necrosis develops as small patches which increase in size as the tumour volume increases. The relationship between the logarithm of tumour volume and necrotic fraction is shown in Fig. 2. Necrotic fraction increased from 1% in the smallest tu-

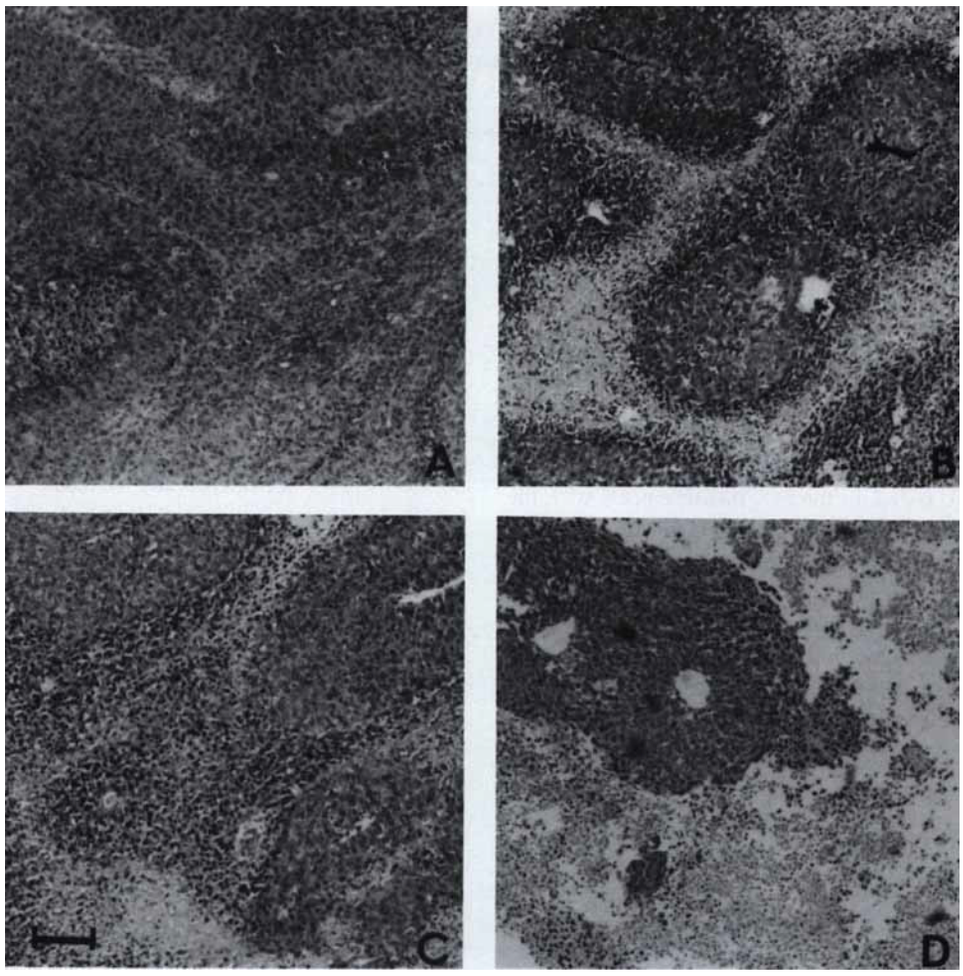

Fig. 1. Microscopic pictures of different sized tumours (A) 105 mm^3, (B) 323 mm^3, (C) 551 mm^3 and (D) 726 mm^3. Bar $\approx$ 100 μ.

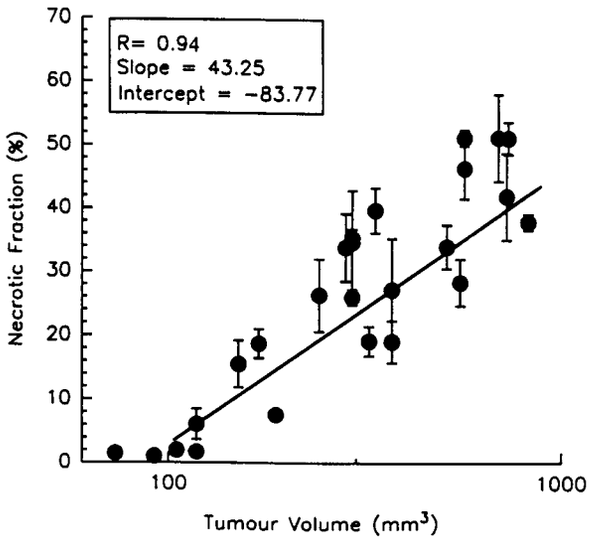

Fig. 2. The relationship between the tumour volume and the necrotic fraction. Each point represents the average value from different sections of one tumour and the error bars are ±1 SE.

mours up to 51% in the largest. The line fitted to this data shows an excellent correlation (R = 0.94). Thus, a straight line equation can be used to summarize this relationship. The slope and intercept obtained from this line were used to predict the necrotic fraction in the other tumours using the formula;

$$\text{predicted necrotic fraction} = -83.77 + 43.25 \times \log \text{Vol.}$$

Tumour tissue oxygenation. As tumour size increased the pO_2 distribution shifted to lower values and became less variable (data not shown). The percentage of pO_2 measurements with values ≤5 mmHg are summarized in Fig. 3a. Clearly, as tumour volume increased, the percentage of values ≤5 mmHg also increased from 2% to 79%; the slope of the fitted regression being significantly different from zero ($p = 0.001$). Since necrotic tissue is likely to contribute to the low pO_2 values, the formula described previously was used to estimate the necrotic fraction in each tumour. In each tumour a number of pO_2 readings corresponding to the predicted necrotic fraction were then subtracted from the readings ≤5 mmHg as well as from the total number of measurements and a new percentage of values ≤5 mmHg was calculated. When the pO_2 values are corrected for necrosis the apparent significant change in percentage of values ≤5 mmHg was lost, such that the slope of the fitted regression line was not different from zero $p = 0.4$ (Fig. 3b).

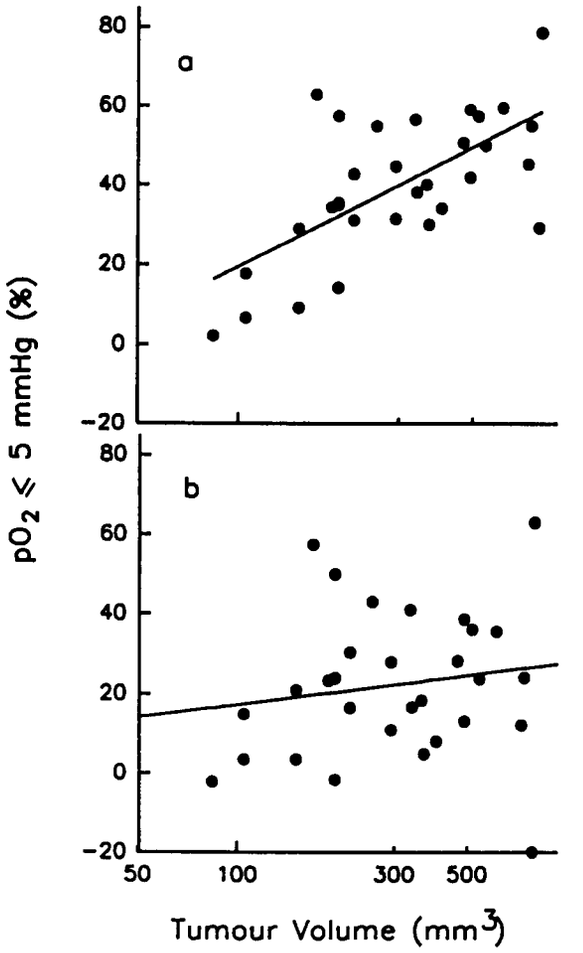

Fig. 3. The relationship between the percentage of pO_2 values ≤5 mmHg and tumour volume either before (a) or after (b) correcting for necrosis.

Discussion

Previous histological studies using a similar tumour model had shown tumour tissue cords with a single capillary in the centre and necrosis around the outside (14). In our study it was found that the necrotic fraction of this tumour model increased as a function of tumour volume, which is in agreement with other reports using other tumour systems (15–17). Necrotic areas were not always located in the centre of the tumour, but were randomly distributed along the histological section (data not shown). Hilmas & Gillette (15) also showed that tumour necrosis was not always located in the centre of the tumour. However, they mentioned a non-random distribution of necrotic areas, which could be the result of their definition of necrotic tissue as 'areas which contained no colloidal carbon filled vessels'. In our study, we counted all evident necrotic areas whether they contained vessels or not.

The reduction in tumour tissue oxygenation with increasing tumour volume, demonstrated in this study, has been reported previously with other tumours (11). We selected the percentage of values ≤5 mmHg as the endpoint since our preliminary studies showed the strongest correlation with tumour hypoxia (18). Effective measurements of hypoxia should include tumour cells that are viable and capable of recurring the tumour after radiation. The pO_2 measured by the polarographic needle electrode

gives the average partial pressure of oxygen in the tumour tissue, irrespective of whether it is in a viable or non-viable tumour, and this could limit the use of this method as a predictive assay for tumour radio-responsiveness. In spite of the fact that necrosis is expected to contribute to the low values of the pO_2, no attempts were previously made to correct for its effect. When we take necrosis into account, the apparent effect of tumour volume on the oxygenation status of this tumour model was lost. Studies with the pO_2 electrode in the same tumour model in our laboratory revealed a larger proportion of hypoxic cells, as indicated by pO_2 values ≤ 5 mmHg, than was estimated from a clonogenic hypoxic assay (19, 20). Based on our current results, this difference can be seen as over estimation of the clonogenic hypoxic cells by the pO_2 electrode. We are currently estimating the radiobiological hypoxic fraction in the same tumour model at different volumes. The preliminary results suggested a stable hypoxic fraction in the range of tumour volumes studied.

In conclusion, pO_2 measurements could overestimate tumour hypoxia if the tumour contains necrosis. The suggested correction for the necrotic fraction will yield a more realistic value for tumour hypoxia. We are aware of the difference between the experimental and clinical situations yet, we believe that correction for necrotic fraction could up-grade our knowledge of tumour hypoxia and its real influence on radiation response.

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