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Is the grading of breast carcinomas affected by a delay in fixation?*

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Summary. The effect of delay in fixation on the modified Bloom and Richardson grade of eight breast carcinomas was investigated. Topologically shuffled samples of each tumour were immersed in fixative at times of 0.5, 2, 4, 6, 18 and 24 h after surgical removal. The grade of each tumour was assessed at delays of 0.5 and 6 h. The tubule formation and nuclear pleomorphism components of the grade showed no change with a delay in fixation of 6 h. The number of mitotic figures declined by a mean of 53% over the same period and this resulted in a decrease in the histological grade of one of the tumours. The implications of these findings for the handling of breast specimens in a diagnostic histopathological laboratory are discussed.

Key words: Breast carcinoma – Grading – Fixation delay

Introduction

The grading of breast carcinomas using the method of Bloom and Richardson (1957) or the "Nottingham" modification of this (Elson 1987) has been shown to be a useful prognostic predictor which may be used when considering different therapeutic options (Anderson et al. 1981; Haybittle et al. 1982; Elston et al. 1982; Parl and Dupont 1982; Elston 1984) and has been combined with tumour size and lymph node staging to produce an overall prognostic index (Todd et al. 1987). This grading method assesses tubule formation, nuclear pleomorphism and mitotic rate; the mitotic rate has been shown to be the most important component and may represent a better prognostic indicator on its own than as part of a combined histological grade (Mann et al. 1985).

Animal studies have shown a sharp decline in the number of observable mitotic figures if fixation of tissues is delayed (Bullough 1950; Edwards and Donalson 1964; Graem and Helweg-Larsen 1979). A similar decline has been reported in normal human colonic mucosa (Cross et al. 1990), but there are no studies known to us which demonstrate this phenomenon in human breast carcinomas. This study investigates the effect of delay in fixation on the grading of breast carcinomas with emphasis on the mitotic component of this grade.

Materials and methods

Eight mastectomy specimens containing breast carcinomas were received in the operating theatre directly after removal from patients. The time of removal of each specimen was noted and this was designated as time zero. The tumour was identified and 21 cubes of tissue each measuring approximately $5 \times 5 \times 5$ mm were obtained. These cubes were then shuffled to prevent topological sampling bias, divided into groups of 3 and left at a constant temperature of 20° C, which approximates to the average working temperature in our laboratory. At 0.5, 1, 2, 4, 6, 18 and 24 h after removal at surgery, a group of 3 cubes were immersed in 4% formaldehyde solution. After a further period of 24 h each cube was bisected and routinely processed into paraffin wax. Sections 6 μm thick were cut from the exposed face of the block and stained with haematoxylin and eosin.

The total number of mitotic figures in 60 high power fields was counted independently on each section by two observers (RDS and MSF) using a Leitz SM Lux microscope with a high power field ($\times 40$) area of 0.196 mm^2. Figures in all phases of mitoses were counted and care was taken to distinguish these from pyknotic nuclei and lymphocytes. The samples were counted in random temporal sequence and all the windows on the counter were occluded except for that displaying the number of fields counted. The number of mitotic figures per 60 high power fields at each time interval was recorded and from this the mean of the two observations was calculated. The distribution of the results was approximately Gaussian and the paired Students' *t*-test was used to assess statistical significance of the results. Observer variation was measured by randomisation tests using a two-way analysis of variation on the differences in observations between time intervals and also between specimens.

The tumours were graded using the modified Bloom and Richardson method (Elston 1987). Tubule formation and nuclear

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pleomorphism was assessed by two observers (RDS and JHFS) and were combined with mean number of mitoses per 10 high power fields to obtain the grade. Grading assessment was performed on each tumour after fixation delays of 0.5 and 6 h.

Results

The results are summarized in Fig. 1 and Table 1.

The only factor used in the determination of tumour histological grade which varied with delayed fixation was the number of observable mitoses (Table 1); nuclear pleomorphism and tubule formation were unaffected.

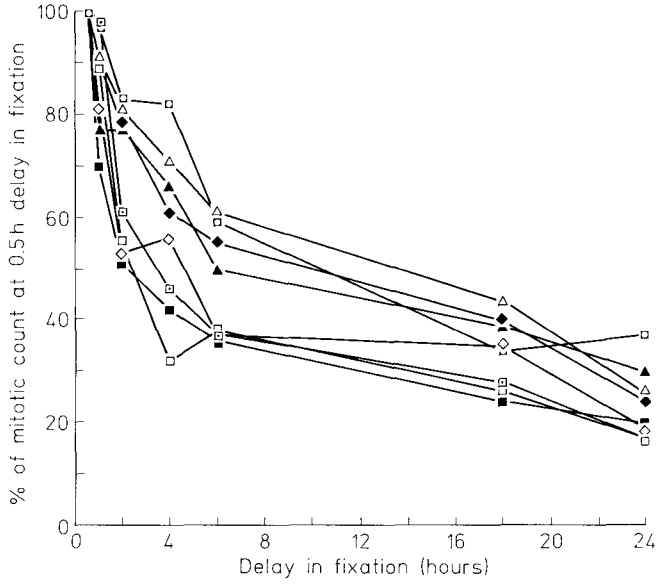

Fig. 1. Graph showing the percentage decline in the number of mitotic figures for each specimen with the varying delays in fixation. —⊡— specimen A; —◆— specimen B; —◘— specimen C; —◇— specimen D; —■— specimen E; —□— specimen F; —▲— specimen G; —△— specimen H

Although the numbers of mitotic figures in the 0.5 h specimens showed considerable variation (range 167–706) the numbers declined in all eight specimens. The samples in which there had been a fixation delay of 6 h showed a variable decline in the number of mitotic figures (mean 53%, range 39–64%) and the difference in numbers at 0.5 and 6 h was statistically significant ($0.001 < P < 0.01$). The decrease in mitotic figures was not proportional to the original level of mitotic activity in the tumours. There were no significant differences between the two observers ($0.001 < P < 0.01$).

At a fixation delay of 0.5 h, seven of the eight carcinomas had grades of 3. One of the grade 3 tumours (specimen E) was subsequently assessed as a grade 2 tumour at a fixation delay of 6 h and the mitotic score component of the grade of another tumour (specimen F) was reduced with no alteration of tumour grade. The remainder of the specimens showed no change in score or grade.

Discussion

There are several potential sources of error when counting mitotic figures (Kempson 1976; Norris 1976; Scully 1976; Silverberg 1976; Ellis and Whitehead 1981; Donjuijsen 1986). Mitotic counts must be performed over defined areas in a prescribed pattern using good quality sections if an acceptable degree of consistency is to be achieved (Baak 1990; Quinn and Wright 1990). The effect of delayed fixation on mitotic counts in a human tumour has not been reported.

This study shows a decline in the number of mitotic figures in human breast carcinoma with delayed fixation with a subsequent change in the histological grade of one specimen. The decline in observable mitotic figures between 0.5 and 6 h delay in fixation (mean 53%) is similar to that observed in normal human colonic mucosa (mean 49%) by Cross et al. (1990). The reasons for this decline in observable mitotic figures have been dis-

Table 1. The effects of delayed fixation of 0.5 and 6 h on the histological grade of breast tumours

Specimen	Fixation delay (h)	Tubule formation	Nuclear pleomorphism	Mitoses per 10HPF	Total score	Tumour grade
A	0.5	3	3	3	9	3
	6	3	3	3	9	3
B	0.5	3	3	3	9	3
	6	3	3	3	9	3
C	0.5	3	3	3	9	3
	6	3	3	3	9	3
D	0.5	2	3	3	8	3
	6	2	3	3	8	3
E	0.5	2	3	3	8	3
	6	2	3	2	7	2
F	0.5	2	2	3	7	2
	6	2	2	2	6	2
G	0.5	3	3	3	9	3
	6	3	3	3	9	3
H	0.5	3	2	3	8	3
	6	3	2	3	8	3

cussed previously (Cross et al. 1990), but the recent study by Donhuijsen et al. (1990) suggests that delays in fixation do not produce significant changes in the proliferative fractions of the cell cycle and that the decrease is secondary to poor identification of mitotic figures in a background of autolytic tissue.

Seven of the eight carcinomas were assessed as grade 3 tumours at a fixation delay of 0.5 h; this is probably an over-representation of poorly differentiated carcinomas but the tumours had to be large enough to obtain the 21 cubes and leave material for diagnosis. All the carcinomas had mitotic counts (mean 69 mitotic figures per 10 high power fields, range 27–117) at 0.5 h which were well above the 20 per 10 high power fields that is the lower limit of a mitotic score of 3 in the modified Bloom and Richardson system. These high initial mitotic counts mean that the effect on the overall grade is less marked than might be expected from the demonstrated decline in observable mitotic figures. If the same rate of decline occurred in tumours with a mitotic score of 2 (10–19 mitoses per 10 high power fields) then a delay in fixation of 18 h (with reduction in mitotic figures by a mean 66%) would lower the mitotic score to 1 in virtually all these tumours. The methodology used in this study did not allow us to examine smaller, and possibly better-differentiated tumours, because the sampling required might have prejudiced the diagnostic assessment of such tumours. Even with the preponderance of grade 3 tumours with high mitotic activity there was still a drop in grade from 3 to 2 with a delay in fixation of 6 h in one of the tumours.

Formaldehyde (4%) penetrates tissue by 3.8 mm in 24 h (Medawar 1942; Baker 1960) so most unsliced specimens of breast carcinoma will not have been fixed within that period since most lesions will be more than 3.8 mm from the nearest resection margin. Our results show that the number of observable mitotic figures is likely to have declined by 76% in 24 h and this could affect the histological grade. This finding has important implications for the accuracy and validity of such grading systems given the inevitable variation in fixation delays of surgical specimens during routine procedures. Tissue fixation procedures in laboratories must be standardised if a histological grading system is to achieve consistency. The validity of any multi-centre study involving the comparison of tumour pathology may be seriously compromised if there is failure to observe a defined system of tissue fixation. This concept receives brief and imprecise attention in the recent Breast Cancer Screening Draft Guidance (1990) and may undermine any proposed pathological audit within the current National Breast Cancer Screening Programme.

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