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Quantitative Cytochemistry of Glucose-6-phosphate Dehydrogenase in Benign and Malignant Breast Tumours

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Glucose-6-phosphate dehydrogenase (G6PD) activity was quantified cytochemically in mammary epithelial cells within frozen tissue sections from 38 patients with breast cancer and 44 with benign breast disease. G6PD activities were measured under atmospheres of both N_2 and O_2. The mean (S.E.) G6PD value 2.5 (0.23) IE U/min measured in N_2 in mammary epithelial cells from the group of malignancies was significantly greater than that of 1.6 (0.37) IE U/min in the benign group ($P < 0.001$), but there was considerable overlap between individual values. G6PD measured in O_2 was detectable in 84% of malignancies compared to only 14% of benign biopsies and the group mean of 1.3 (0.18) IE U/min in the former was significantly greater than that of 0.35 (0.20) IE U/min in the latter ($P < 0.001$). Significant correlations between G6PD activities measured in N_2 and O_2 were observed in both groups. The techniques present a sensitive method of identifying increases in G6PD activity in mammary epithelial cells and provide an assay that in a majority of cases permits the separation of malignant from benign tissues.
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INTRODUCTION

QUANTITATIVE CYTOCHEMISTRY, by relating biochemical activity to detailed histology, allows the study of metabolic changes within selected cells of a target tissue. These techniques have been successfully applied to the study of a number of malignancies [1]. The enzyme glucose-6-phosphate dehydrogenase (G6PD) has frequently been selected for study because of its involvement in the production of NADPH required in many growth processes [2–4]. G6PD activity has tended to be greater in mammary epithelial cells from malignant biopsies than in those from benign tissues [5, 6], although wide variations between tumours and often considerable overlap between benign and malignant tissues have been reported.

Despite the heterogeneity of these findings, measurement of G6PD has been used to differentiate between benign and malignant tissues in a variety of malignancies by measuring activity under an atmosphere of oxygen and studying the oxygen-sensitivity of the hydrogen transfer mechanisms [6–10]. Differences in oxygen-sensitivity and reaction rates of G6PD activity between cells from benign and malignant rat mammary tissue have also been demonstrated in our laboratory [11].

The objectives of the present investigation were to apply techniques developed in the rat model to the study of G6PD activity in human breast, to compare activities between benign tissues and malignant neoplasms and to investigate the oxygen-sensitivity phenomenon.

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MATERIALS AND METHODS

Tissue preparation

38 specimens of malignant biopsy material and 44 of non-malignant were investigated. Of the malignant tissues 71% were histologically identified as invasive with the remaining 29% comprising a histologically heterogenous group. All such specimens were obtained at mastectomy or following excision biopsy. The benign breast specimens consisted of 63% with fibrocystic disease, 20% fibroadenomata and 13% histologically normal. The remaining 4% were miscellaneous benign tumours. Within the group of fibrocystic disease tissues, 27% contained elements of hyperplasia of which five were identified as atypical, including two specimens with focal epitheliosis and apocrine cyst formation. These specimens were obtained following excision biopsies. Normal tissue was obtained from the area surrounding fibroadenomas. Samples were obtained from consecutive biopsies peformed at the Pathology Department, St Vincent's Hospital, Dublin, Ireland. Histological diagnosis was made from parallel sections by pathologists at the same department.

Specimens were dissected into blocks measuring approximately 5 mm^3. These blocks were supercooled by "snap-freezing" to −70°C in n-hexane (BDH) using a dry carbon dioxide/methylated spirits ice-bath. Once chilled, tissue was stored (for a maximum of two days) at −70°C until further analysis.

Enzyme reaction

Quantitative cytochemical measurement of G6PD activity was performed using previously described methods [12, 13]. Briefly, tissue blocks were mounted in ice and sections cut at a set thickness (~10 μm) in a Slee TE cryostat (Slee Medical Equipment, London) equipped with an automatic motorised cutting drive to minimise section variation. The cryostat temperature was maintained at −30°C throughout with the microtome knife maintained at −70°C. Initial sections from each block were stained with methylene blue to establish the presence of epithelial

cells prior to further analysis. Duplicate slides were analysed per experimental point, each slide containing three sections. Parallel sections were cut and stained with haemotoxylin and eosin (H+E). These sections were used to identify histologically the different cell populations and also for histopathological examination.

Slides were removed from the cryostat and allowed to reach room temperature. The "open ring" technique of incubation was used. A perspex ring wide enough to encircle the sections was fixed to the slide by a thin film of vaseline and the slides then placed in the reaction chamber and allowed to equilibrate to 37°C. The reaction medium was poured into the area enclosed by the ring and thus into contact with the sections. The incubation medium consisted of 50 mmol/l glycylglycine buffer (Sigma) containing 30% w/v polyvinyl alcohol (Grade G04/140), 5 mmol/l neotetrazolium chloride (Serva, Heidelberg), 0.65 mmol/l phenazine methasulphate (Sigma), 5 mmol/l glucose-6-phosphate (Sigma), 3.2 mmol/l NADP (Sigma) and 1% calcium chloride (Analar).

The final pH of the medium was 8.0. The reaction medium was divided into two lots and bubbled with oxygen-free nitrogen and 95% oxygen/5% carbon dioxide for 3 minutes, respectively. After the appropriate reaction interval the rings were removed and the slides soaked for 5 minutes in warm distilled water to wash off any remaining medium. Sections were air-dried and mounted in Farrants medium pH 6.5 (Raymond Lamb, London) and allowed to stand overnight.

Quantitation

The reaction product was quantified using a Vickers M85 scanning and integrating microdensitometer. Measurements were made at 585 nm; the isobestic point of the neotetrazolium formazan. The mask size employed depended on the size of the cells within the individual section. A 100 × objective and a flying spot of 0.22 μm were used. Between 25 and 50 cells were scanned per experimental point. To eliminate operate bias, cells occurring in a randomised grid pattern were measured. The machine units were transformed into absolute integrated extinction units (IE ×100) and finally expressed as IE ×100/minute (i.e. the amount of formazan deposited per unit time). A detection limit was established (0.2 IE ×100) below which accurate identification and quantification of cells was not possible due to the very low levels of formazan deposited. All values falling below this limit were designated zero.

Statistical methods

The Wilcoxon matched-pairs signed-ranks test was used for comparisons between matched data, e.g. enzyme activity in nitrogen and activity in oxygen. The Mann–Whitney *U* test was used to compare values for G6PD activity in mammary epithelial cells from malignant biopsies and that in benign biopsies. Correlation was estimated between activity in nitrogen and activity in oxygen using the Pearson correlation coefficient, *r*.

RESULTS

Reaction time courses

The effects of increasing incubation time on the G6PD activity in mammary epithelial cells measured under atmospheres of both N_2 and O_2 in a benign and a malignant breast biopsy specimen are shown in Fig. 1. Formazan deposition was shown to be linear in all cases. A lag period of 17 minutes occurred before formazan deposition in oxygen in the benign specimen, while no such lag period occurred in the malignant specimen.

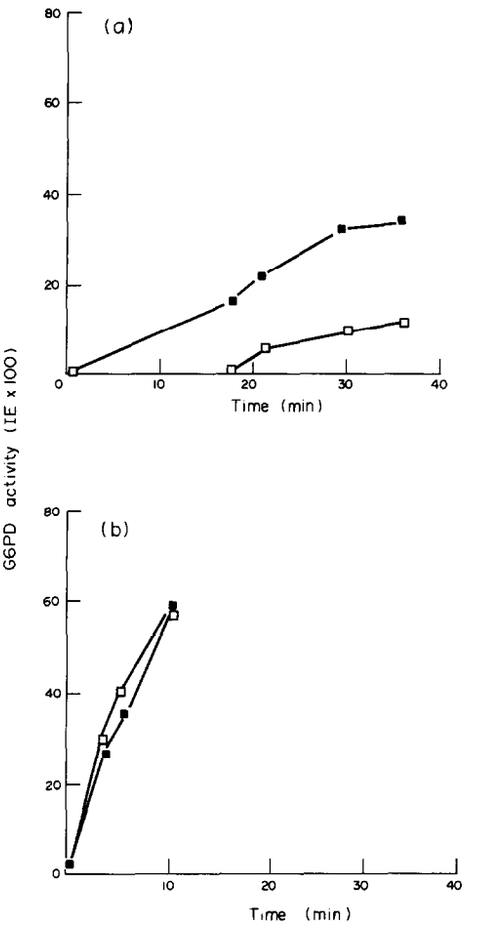

Fig. 1. The effect of increasing reaction time on G6PD activity in (a) a benign breast biopsy specimen and (b) a malignant breast specimen. The reaction was carried out in an atmosphere of nitrogen (■) and oxygen (□).

Activity was detected in all malignant specimens within 5 minutes of incubation in oxygen. Formazan deposition in the malignant specimen was rapid, reaching significant levels by 10 minutes after which time quantitation of further increases in chromophore deposition was not possible. In contrast, the reaction was considerably less in the benign specimen and this difference in activity was consistent in all specimens studied. Such time courses were used to establish appropriate single incubation times for the analysis of mammary epithelial cells from both benign and malignant specimens.

G6PD activity in breast tissue

Figure 2 compares the G6PD activity measured in atmospheres of both nitrogen and oxygen in 38 malignant specimens to that measured in 44 benign specimens. It can be seen that individual G6PD values measured in nitrogen showed considerable overlap between mammary epithelial cells from malignant and benign tissues. However, the mean (S.E) activity of 2.5 (0.23) IE ×100/minute obtained in the group of malignancies was significantly greater than that of 1.6 (0.37) IE × 100/minute in the benign biopsy group ($P < 0.0001$). G6PD activity measured in oxygen was detectable in mammary epithelial cells from 32/38 (84%) of malignant tissues but was undetectable in 38/44 (86%) of those from benign tissues. The mean value of 1.3 (0.18) IE × 100/minute in malignant tissues

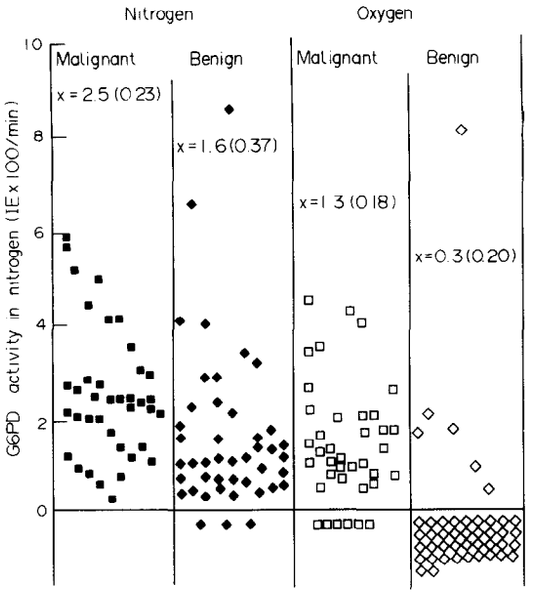

Fig. 2. G6PD activity in 38 malignant biopsy specimens and 44 benign breast biopsies measured under atmospheres of nitrogen and oxygen. All points below the horizontal line were defined as not detectable.

was significantly greater than that of 0.35 (0.20) IE × 100/minute in benign ($P < 0.001$).

The relationship between G6PD activity in nitrogen and that measured in oxygen in the individual biopsy specimens was investigated by regression analysis. Figure 3 shows the correlation between these two values for each biopsy in both groups, benign and malignant. A strong positive correlation was found in both groups: $r = 0.80$ and $r = 0.70$, respectively ($P < 0.001$). The comparison between activity of G6PD in nitrogen and oxygen in histologically different types of breast tissue is further illustrated in bar chart form in Fig. 4. Activity in nitrogen in the malignant group ($n = 38$) was significantly greater than in fibrocystic disease ($n = 27$), fibroadenoma ($n = 10$) or normal tissue ($n = 7$); $P < 0.001$ in each case. There was no significant difference between activity measured in fibrocystic and fibroadenoma specimens, while mean G6PD activities in both groups were significantly greater than that in histologically normal tissue. As shown in Fig. 4, 52% of G6PD activity was retained in the malignant epithelial cells in oxygen compared with 22% in fibrocystic tissues, while no activity was detected in either fibroadenoma or histologically normal tissue.

DISCUSSION

This study demonstrates that techniques of quantitative cytochemistry can be used to identify quantitative differences in G6PD activity between types of mammary tissues. It was clearly demonstrated that in mammary epithelial cells from malignant tumours the mean activity of this enzyme is significantly elevated over that observed in those from the histologically normal and benign breast biopsy specimens. Secondly, the phenomenon of oxygen-sensitivity as discussed by Altman *et al.* [13] was investigated in these tissues. It has been shown that in mammary epithelial cells from normal tissue formazan production is greatly decreased in the presence of oxygen, while in malignant tissues, inhibition is significantly less [6, 8, 10, 14–16]. It has been suggested that this phenomenon may have some significance in the investigation of G6PD activity in malignancy [13].

Previous investigations into G6PD activity indicated that relative or absolute amounts of G6PD, comparable to or greater than that found in normal tissue, would appear to be characteristic of most animal and human neoplasia [2, 4]. Subsequent histochemical and cytochemical techniques allowed a more precise study of enzymes in neoplastic transformation. Bannasch [17] showed that in the progression from normal to malignant hepatocytes there was a series of morphological and biochemical changes, including increased G6PD activity, indicating early activation of the pentose phosphate pathway and glycolysis.

It has been demonstrated [15, 17, 20] that aberrant G6PD activity may appear at very early stages of dysplasia, which would suggest that irreversible malignant changes have occurred, presumably at the level of altered gene expression, even before the stage of cell proliferation. Several studies have shown that glycolytic activity is related to mitotic activity. A positive correlation between G6PD activity and synthesis of intermediary metabolites in DNA production [21–23] showed that a marked increase in G6PD activity preceded DNA syn-

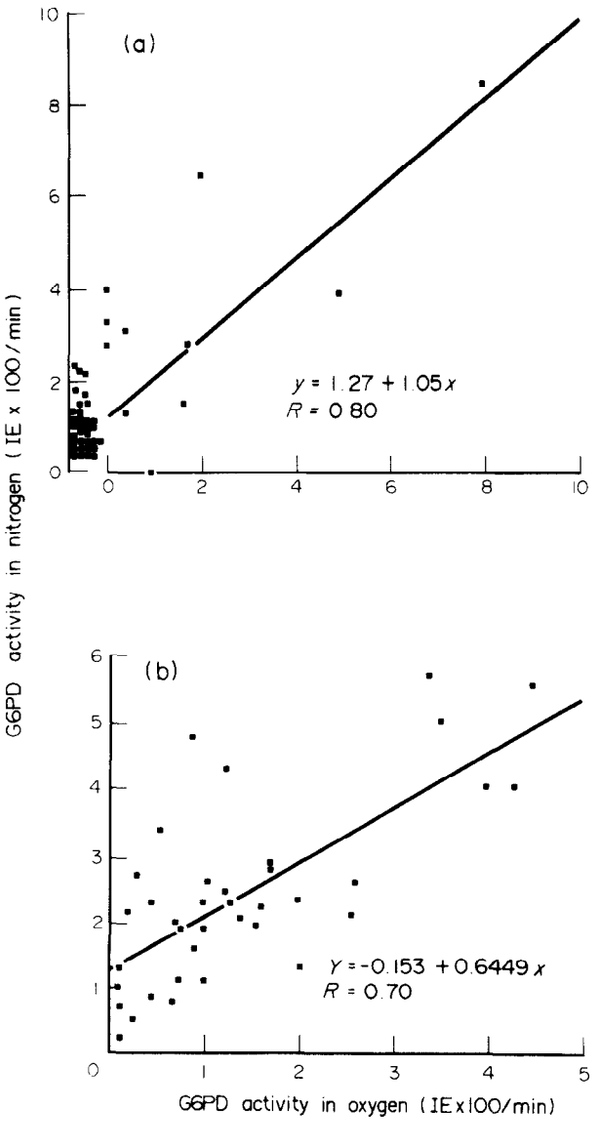

Fig. 3. Correlation between G6PD activity measured in nitrogen and that measured in oxygen in (a) 44 specimens of benign breast disease and (b) measured in nitrogen and that measured in oxygen, in 38 specimens of malignant breast disease.

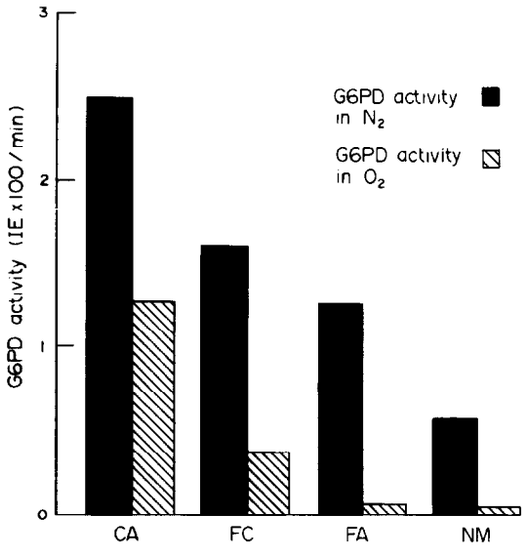

Fig. 4. A comparison between mean G6PD activities in nitrogen and oxygen in histologically different types of breast tissue. CA = malignant biopsies, n = 38; FC = fibrocystic disease, n = 27; FA = fibroadenoma specimens, n = 10; NM = normal breast, n = 7.

thesis in wound healing. The direct association between enzyme activity and proliferation is not always obvious. In the present study, in a number of instances, foci of intraductal proliferation exhibited greater activity than adjacent foci of infiltrating cells. In a number of biopsies of fibrocystic disease, a striking increase in G6PD activity was observed and it was demonstrated that this increase was associated with hyperplastic epithelial proliferation. Foci of apocrine hyperplasia were shown to have greatly elevated G6PD activity consistent with a strong correlation between proliferation and G6PD activity which is in agreement with observations previously made in epitheliosis and apocrine metaplasia [5, 6, 20, 25].

An interesting corollary of this evidence for elevated G6PD activity in malignant cells is the possible protective effect against cancer of G6PD deficiency, a hereditary condition transmitted by a sex-linked gene which causes interference with normal cell function and replication [26].

A number of authors have suggested that the retention of G6PD activity in an atmosphere of oxygen is, to a certain degree, characteristic of malignancy. Oxygen was found to exert a greater inhibitory effect on formazan production in mammary epithelial cells from normal compared to malignant tissue in the liver and in squamous carcinoma of the bronchus [7, 8]. The possibility of functional difference between epithelial cells from malignant and benign tissues is further suggested by the work of Ibrahim *et al.* [10], in which it was demonstrated that normal and non-malignant colonic and gastric specimens were clearly different from malignant specimens with respect to retention of activity in an atmosphere of oxygen. The results of the present study show that in agreement with the above, significantly greater activity was retained in mammary epithelial cells from the malignant biopsy group. However, analysis of the data indicates that a strong correlation exists between the level of activity measured in nitrogen and that obtained in oxygen in both groups studied, suggesting that activity in oxygen or degree of inhibition by oxygen is strongly correlated to the initial G6PD activity in nitrogen.

It was previously demonstrated in this laboratory that in the rat mammary gland, greater activity was measured in both nitrogen and oxygen in the lactating gland than in either DMBA-induced mammary tumour or virgin mammary gland [11]. These results are consistent with the greatly elevated biosynthetic activity of lactating tissue and the possibility of a simple quantitative relationship between activity in nitrogen and activity in oxygen. A similar system using oxygen-saturated reaction medium has been used to identify malignant and premalignant squamous cells of the skin [15]. Positive reactions were detected with very early premalignant changes and increased in intensity with the appearance of more advanced changes. The findings in the present study both of elevated G6PD activity in malignant cells of neoplastic tissues and a significantly greater measure of enzyme activity in these cells under an atmosphere of oxygen are in agreement with published observations in similar studies. The interpretation of these results is not fully resolved as to whether this represents a qualitative or quantitative difference between malignant and benign cells of mammary tissues. The recent work of Best *et al.* [27] in epithelial cells from colonic tissues demonstrates comparable results with respect to elevated G6PD activity in malignancy but considers a decrease in activity of the enzyme superoxide dismutase to be responsible for the increased measure of enzyme activity in an atmosphere of oxygen when compared to non-malignant tissues.

It would appear that the cytochemical techniques using incubation in an atmosphere of oxygen present a more sensitive method of identifying unusually elevated G6PD activity which is not affected by false results and in many cases permits the separation of neoplastic tumours from benign tissues.

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The Role of Nucleolar Organiser Regions as Prognostic Factors in Breast Cancer

Matti J. Eskelinen, Pertti K. Lipponen, Yrjö Collan and Kari J. Syrjänen

Nucleolar organiser regions (NORs) were stained in paraffin-embedded biopsy specimens of 80 female breast carcinomas by the silver (Ag) technique. The patients were prospectively followed up for a mean of 12.4 years (range 11.5–13.3). The number of different types of Ag-NORs was correlated with the histological grade, clinical stage, DNA ploidy, S-phase fraction (SPF) and clinical outcome. Grade III tumours showed higher Ag-NOR counts than low grade tumours. The total number of Ag-NORs ($P = 0.0059$) and the number of dispersed Ag-NOR ($P = 0.0199$) were significantly related to DNA ploidy aneuploid tumours showing higher Ag-NOR counts. The number of aggregated Ag-NORs was predictive ($P = 0.0413$) for the development of metastatic disease during follow-up. On the other hand, crude, cancer-related or recurrence-free survival could not be predicted significantly by the Ag-NORs. The results suggest that the number of Ag-NORs is clearly related to the proliferative activity in breast cancer, but the prognostic value of Ag-NOR counting is inferior to the previously recognised prognostic factors.
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INTRODUCTION

A NUMBER OF well-established prognostic factors have been elucidated by now to predict the clinical course of breast cancer. Such factors include the clinical stage [1–6], histological type [4], histological grade [7–9], hormone receptors [10], parameters measured by quantitative histology [9, 11], mitotic index [9], DNA aneuploidy [12–16] and S-phase fraction (SPF) [14, 16]. Tumour-associated antigens seem to be of some assistance in evaluating the malignancy of breast cancer as well [17–19].

Nucleolar organiser regions (Ag-NORs) represent the loops of DNA actively transcribing to ribosomal RNA (rRNA) [20]. The technique to demonstrate NORs by a simple silver (Ag) staining was described 15 years ago by Goodpasture *et al.* [21]. In 1986, Ploton *et al.* introduced a modification, which made method applicable for formalin-fixed, paraffin-embedded tissue sections [22]. This has prompted a large number of studies on Ag-NORs as possible prognostic factors in a variety of malignant

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