ELSEVIER

European Journal of Cancer 40 (2004) 2269–2273

European Journal of Cancer

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Protein kinase C alpha expression in normal breast, ductal carcinoma *in situ* and invasive ductal carcinoma☆

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Received 20 April 2004; received in revised form 27 May 2004; accepted 24 June 2004
Available online 26 August 2004

Abstract

The purpose of this study was to determine if Protein Kinase C alpha (PKC alpha) is altered in expression or localisation in normal breast, ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC). We obtained 14 mixed cases of invasive ductal carcinoma (IDC) and DCIS, 36 pure DCIS cases and 25 cases of normal breast. The sections were stained immunohistochemically for PKC alpha expression. Staining was cytoplasmic. The results showed a progressive reduction in staining intensity from normal breast to invasive ductal carcinoma. The staining pattern was heterogeneous in the cytoplasm of DCIS and IDC, but homogeneous in the cytoplasm of normal breast ductal epithelium. Interestingly, mitotic cells and cells with aberrant nuclear morphology showed increased cytoplasmic staining in DCIS and IDC. PKC alpha activity is altered in dividing or abnormal cells, but overall expression is reduced in IDC. This raises the possibility of an alteration in the subcellular localisation of PKC alpha which may relate to changes in desmosomal adhesive state.

Keywords: PKC alpha; DCIS; Invasive ductal carcinoma

1. Introduction

The aim of this study was to assess the immunohistochemical expression of the serine/threonine kinase Protein Kinase C alpha (PKC alpha) in normal breast, ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC).

PKC is a multifunctional serine and threonine kinase that phosphorylates a wide variety of proteins. PKC has a number of important roles in cellular growth and differentiation, cellular metabolism and transcriptional activation of normal and transformed breast epithelial cells. Total PKC activity is seen to be increased in breast cancer [1]. Little is known about the biological activity of PKC alpha in DCIS.

The apparent importance of PKC in growth control is seen in studies with phorbol esters that resemble diacylglycerol – a lipid molecule that remains in the plasma membrane following its formation by inositide -specific phospholipase C (PI-PLC) beta. There it recruits PKC that phosphorylates serine and threonine residues on target proteins. Phorbol esters activate PKC in a variety of cultured cells, causing them to lose growth control and behave temporarily as malignant cells. When the phorbol ester is removed from the medium, the cells recover their normal growth properties. In contrast, cells that have been genetically engineered to constitutively express PKC exhibit a permanent malignant phenotype

☆ This work was supported financially by Bolton Hospitals NHS Trust.
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doi:10.1016/j.ejca.2004.06.027

in cell culture and cause tumours in susceptible mice. Phorbol esters activate several PKC isoforms and stimulate growth inhibition and differentiation of some mammary epithelial cell lines [2]. PKC alpha is sensitive to both phorbol esters and calcium.

Wallis and colleagues examined PKC alpha activity in desmosomal adhesion in MDCK cells. They found that upon monolayer wounding, PKC alpha translocates rapidly to the cell periphery and becomes concentrated in lamellipodia. This is succeeded by a total increase in PKC activity of the wounded fraction and associated with desmosomes reverting to calcium dependence [3].

PKC alpha has been implicated in other pathways potentially involved in malignant transformation, such as the raf/ERKl/2 pathway. Endothelial cell cyclic strain causes PKC alpha to translocate from the cytoplasm to the membrane. Subsequent activation with PKC epsilon causes raf/ERKl/2 activation [4] – a pathway involved in tumorigenicity.

MCF-7 breast cancer cells stably transfected with PKC alpha show a more aggressive phenotype and increased tumorigenicity in nude mice [5]. These cell lines also show reduced expression of Wnt 1 and adhesion molecules, E-cadherin and beta-catenin.

The apparent importance of transcriptional events associated with stably transfected MCF-7 cells with PKC alpha is highlighted in Carey's paper [6] cDNA's were isolated from mRNA differential display from the parent MCF-7 cell line to the MCF-7-PKC alpha cells. One cDNA was found to be upregulated, GBDR1, and four cDNA's were downregulated. They were histone 3.B, integrins alpha3 and alpha6 and G2Q. G2Q is found in normal breast and downregulated in breast cancer. Antisense oligonucleotides to G2Q in MCF-7 cells increased their invasive capacity.

O'Brian and colleagues [7] demonstrated elevated PKC activity in eight out of nine spontaneous human breast tumours compared with normal breast tissue. They indicate PKC activity may be a potential marker for malignant disease. PKC activity may also be altered in DCIS as these cells have potential for malignant transformation.

Hence, we hypothesised that examining the immunohistochemical expression of PKC alpha in IDC, DCIS and normal breast will show relative differences in their levels of expression. No studies have previously examined PKC alpha expression in DCIS.

2. Patients and methods

2.1. Formalin-fixed paraffin embedded sections

We identified 50 patients from the Royal Bolton Hospital BASO database who had undergone surgery for primary breast cancer; either mastectomy or wide local excision. Archival tissue was obtained for these patients. Full ethical committee approval and informed consent for the use of patient tissue was obtained prior to the study. Wide local excision specimens were processed by margin inking and parallel serial slicing into 3–4 mm blocks. The number of blocks produced depended on the size of specimen resected. Mastectomy specimens were processed similarly. However, the mastectomy specimen was first radiographed to identify the main lesion required for block processing. Reassessment of Haematoxylin and Eosin staining of the sections from the paraffin blocks showed that 14 of the 50 cases had associated IDC, 25 of the 50 cases had adjacent normal ductal epithelium, 36 out of the 50 cases had pure DCIS and 5 of the cases had areas of epithelial hyperplasia of usual type. Each section was formalin-fixed for 24 h and paraffin-embedded for archival storage. No block older than 5 years was used for tissue retrieval.

2.2. Immunohistochemistry

Five micron sections were microtomed from each paraffin block and placed immediately onto glass slides. Staining took place within one week of cutting tissue from the block in order to minimise loss of antigenicity. Slides were stored at 4 °C. Immunohistochemical staining was carried out using the indirect immunoperoxidase method with the primary monoclonal antibody PKC alpha developed in rabbits (P4334 from SIGMA laboratories). The sections were immersed in xylene for 7 min to dissolve the paraffin and rehydrated to water via 99% and 95% isopropyl alcohols. Endogenous peroxidase activity was inhibited by immersing in 3% hydrogen peroxide for 5 min. Adequate antigen retrieval was achieved by microwaving at 900 W for 20 min and allowing a further 30 min cooling in 10 mM citrate buffer pH 6.0. Non-specific epitope binding sites were blocked with 20% swine serum (X0901 from DAKO) in tris-buffered saline (TBS) for 5 min. The primary PKC alpha antibody was applied at a 1/1000 concentrations diluted in antibody diluent (S2022 from DAKO) for 1 h at room temperature. The slides were washed with TBS solution pH7.4 for 5 min. For negative controls, the primary antibody was omitted. The blocking peptide (P4584 from SIGMA) was combined with PKC alpha in selected cases to demonstrate specificity of PKC staining. The universal secondary antibody developed in the horse made up in 5% swine serum in antibody diluent was applied for 30 min. Vectastain ABC kit (P6200) was used as the tertiary agent. Binding of the primary antibody was visualised using the standard avidin–biotin complex method with diaminobenzidine as the chromogen. The staining was intensified by immersing in 0.5% copper sulphate solution for 5 min. The sections were then briefly counter-

stained with Harris' haematoxylin, dehydrated in a graded alcohol series, fixed in xylene and mounted.

2.3. Evaluation of staining

Evaluation of the intensity of staining for the sections was carried out independently by two different observers. Where there was a major discrepancy for the staining score for an individual case, the result was not used for analysis. However, there was only one case where this occurred. Where there was a minor difference between the staining scores for a case, the average score was taken. The results were classified semi-quantitatively into five categories:- level 0 absent, level 1 weak, level 2 mild, level 3 moderate and level 4 strong. The cytoplasmic staining in normal breast ductal epithelium was used as the positive control for level 4 strong staining. Negative control staining was used as the reference for level 0 staining. A staining score and description was given for all the pathologies in each case. One slide per patient was evaluated and given a score for its staining in normal breast, DCIS and IDC. Negative controls with primary antibody omitted and a section of normal breast for positive controls were run with each set of slides. The specificity for staining of PKC alpha was demonstrated by using the PKC alpha blocking peptide with each set of slides.

3. Results

The results are summarised in Table 1 to show a comparison of average staining intensity, median staining value and inter-quartile ranges for each component of the DCIS slides. This data are also shown in Fig. 1 as Box and Whisker plots. It can be seen that there is strong overall staining for normal breast and epithelial hyperplasia of the usual type (Figs. 2(a) and (d)) and a progressive reduction in intensity of staining for DCIS (Fig. 2(c)) and then for IDC (Fig. 2(b)). DCIS associated with IDC tended to have a lower staining intensity compared with DCIS alone. This data was analysed using the non-parametric Kruskall-Wallis test. A comparison of the staining differences between the following groups

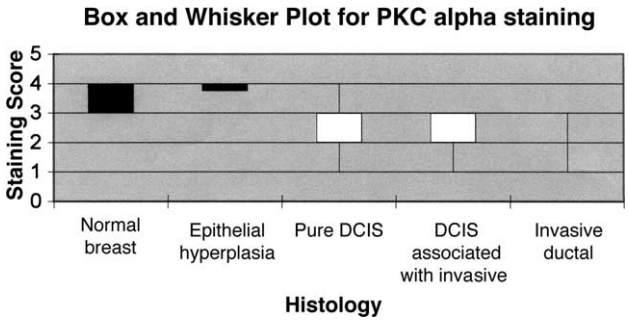

Fig. 1. Box and Whisker plot for PKC alpha staining.

was found to be statistically significant: normal breast and pure DCIS ($P < 0.0001$), epithelial hyperplasia and pure DCIS ($P = 0.008$), normal breast and IDC ($P < 0.0001$), pure DCIS and IDC ($P = 0.001$), DCIS associated with IDC and pure DCIS, ($P = 0.009$). There was no statistical difference in staining between normal breast and epithelial hyperplasia.

Immunohistochemistry is ideal for demonstrating staining patterns within cells. Staining was exclusively cytoplasmic in all of the sections. Staining in normal breast tended to be located peripherally (Fig. 2(a)). Staining was heterogeneous in the cytoplasm of DCIS (Fig. 2(c)) and invasive ductal carcinoma (Fig. 2(b)) compared with a more homogeneous appearance in normal breast (Fig. 2(a)) and epithelial hyperplasia of usual type (Fig. 2(d)). There were focal cytoplasmic increases in mitotic cells in DCIS and IDC (Fig. 2(c)).

4. Discussion

Loss of cell–cell adhesion is the initial crucial step in tumour motility leading eventually to metastasis [8]. The understanding of and ability to identify factors involved is potentially important both for scientific understanding, but also in mapping the progress from normal to pre-malignant and malignant lesions. Such knowledge could be helpful in the separation of entities such as a typical ductal hyperplasia (ADH) and DCIS. PKC alpha expression does appear to vary between normal breast, DCIS and IDC. The changes appear

Table 1
Data showing the intensity of PKC alpha staining

Description	Number of cases	Average staining score Maximum = 4 Minimum = 0	Median staining score	Inter-quartile range of staining score
Normal breast	25	3.75	4	3 to 4
Epithelial hyperplasia of usual type	5	3.8	4	3.75 to 4
Pure DCIS cases	36	2.96	3	2 to 3
DCIS associated with IDC	14	2.32	2	2 to 3
IDC	14	2.27	2	2

DCIS, ductal carcinoma *in situ*; PKC alpha, Protein kinase C alpha; IDC, invasive ductal carcinoma.

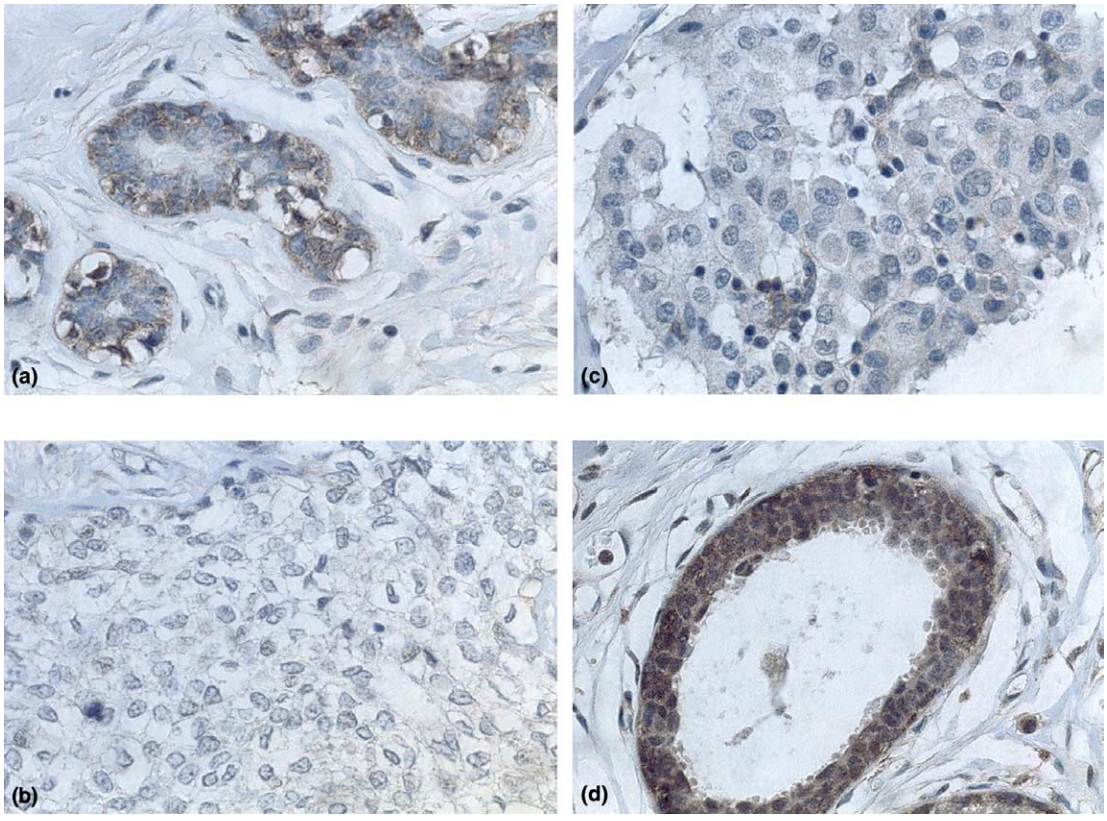

Fig. 2. (a) Normal breast ducts from a reduction mammoplasty showing strong level 4 increased peripheral cytoplasmic staining. (b) Grade III Invasive ductal carcinoma showing mild level 2 granular heterogeneous cytoplasmic staining. (c) Intermediate grade cribriform DCIS showing moderate level 3 granular staining increased focally in the cytoplasm of mitotic cells. (d) Epithelial hyperplasia of usual type showing strong level 4 heterogeneous cytoplasmic staining.

to occur at a stage between epithelial hyperplasia of the usual type and DCIS, as shown by the significant difference in staining between epithelial hyperplasia and DCIS, $P = 0.008$. Its association with desmosomal function suggests a potentially important role in the transition from benign hyperplasia to atypia and frank carcinoma.

The use of specific PKC alpha inhibitors or antisense oligonucleotides such as Affinitak may have a role in combination with traditional chemotherapy agents in the treatment of breast cancer; but so far no role in isolation has been found [9,10].

The present study suggests a trend that would fit with perceived clinical behaviour. The limitation in this study is that immunohistochemistry is not particularly useful for accurate quantitation or subcellular localisation. Hence, staining may not reflect true expression. Further work using techniques such as real-time polymerase chain reaction (PCR) to better define its level of expression and immunofluorescence or immunogold labelling to identify sub-cellular localisations, is required in these various pathological stages. However, the focal increases of PKC alpha seen in mitotic nuclei of cancer cells may indicate an upregulation of PKC alpha DNA.

Ethics

This work fully complies with its ethical approval. Details from the Royal Boltan Hospital Ethical Committee Approval can be obtained by contacting Mr. John Winstanley, Consultant Surgeon, Royal Bolton Hospital, Bolton, BL4 OJR.

Conflict of interest

There were no financial or personal relationships with other people that could inappropriately bias the work of this paper.

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