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Types of Cyclic AMP Binding Proteins in Human Breast Cancers

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Total level and type of cyclic AMP binding proteins have been measured in 117 breast cancers. Six major molecular species of binding proteins were detected. The pattern and relative proportion of binding proteins varied between individual tumours. However, there were highly significant correlations between the expression of different binding proteins, including positive relationships between 52 and 67 kD proteins, 43 kD and both 39 and 37 kD proteins and inverse correlations between 48 and 52 kD, 37 and 67 kD proteins. The expression of three binding proteins (48, 43 and 39 kD) was also positively related to total binding whereas that of the remaining three bindings proteins (67, 52 and 37 kD) was negatively correlated with total levels. It may be that differential expression of certain types of binding protein are the underlying rationale for our previously published finding that tumours with high levels of high binding protein are associated with poor prognosis.
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INTRODUCTION

TUMOUR LEVELS of cyclic AMP binding proteins have been reported to relate to prognosis in patients with breast cancer [1] and, in association with oestrogen receptors, to predict for hormone sensitivity [2, 3]. However, amongst binding proteins which are the regulatory subunits for cyclic AMP-dependent protein kinases, there are major subtypes RI and RII [4]. These may differentially affect cellular proliferation and differentiation [5]. It, therefore, was of interest to determine the major types of binding proteins within breast cancer cytosols and investigate potential relationships with each other and total binding activity.

MATERIALS AND METHODS

Tissue

This study was carried out on 117 primary breast tumour biopsies obtained from patients presenting without evidence of distant metastatic disease to the Breast Clinic in Edinburgh. Tumour was obtained at mastectomy or by biopsy and was immediately transported on ice to the laboratory. Following removal of tissue for histopathological diagnosis and oestrogen receptor analysis, material was stored in liquid nitrogen until assayed.

Measurement of cAMP binding proteins

The method for quantitation of total cAMP binding proteins was as described previously [6]. In brief, tumour cytosols were prepared by homogenisation at 0°C in a 20 mmol/l Tris buffer (w/v 1:10), followed by ultra centrifugation at 105 000 *g* for 1 h. Duplicate aliquots (50 μl) of the resulting supernatant were used as cytosols and were incubated with 5′8′-[^{3}H] cyclic AMP (100 μl, 25 nmol/l) in the absence and presence of varying amounts of radioinert cyclic AMP (100 μl, 10, 20, 40, 80 and 1000 nmol/l) for 3 h at room temperature. Bound cyclic AMP was separated from free nucleotide by filtration through Millipore filters (HAWP 0.45 μmol/l) under negative pressure. The filters absorbing protein-bound cyclic AMP were dried in scintillation vials. Micellar fluor NE260 (Nuclear Enterprises) was added to each and radioactivity measured on a Tricarb liquid scintillation counter (Packard). The number of binding sites and dissociation constants of binding were determined by Scatchard analysis [7]. Results were expressed as fmol binding proteins/mg of cytosol protein, protein content being assessed according to the method of Bradford [8].

Photoaffinity labelling

Photoaffinity labelling of individual binding proteins was achieved by using an adaptation of the method of Pomerantz *et al.* [9]. Cytosol samples (50 μl) prepared as above were incubated with 8-azido ^{32}PcAMP (0.4 μmol/l sp. act. 2.07–2.29 TBq/mmol, ICN Radiochemicals) diluted in buffer containing 0.27 mol/l morpholino ethane sulphonic acid (Sigma) and 53 mmol/l magnesium chloride in 96-well microtitre plates at room temperature for 1 h in the dark. The reaction mixtures were then irradiated for 30 s at 254 nmol/l by placing a Mineralight UVS-11 hand lamp directly over the plate. The reactions were stopped by the addition of SDS–PAGE electrophoresis buffer (3% SDS, 15% 2-mercaptoethanol, 30 mmol/l Tris, 30% glycerol, 1% bromophenol blue). The samples were heated to 90°C for 3 min, and the proteins resolved electrophoretically on 12% SDS–PAGE according to the method of Laemmli [10] for 3.5 h at 35 mA per gel, with one track per gel assigned to ^{14}C-labelled molecular weight reference markers. The gels were fixed overnight in 40% methanol, 10% acetic acid, 10% glycerol, dried in a gel drier (Model 583- Bio-rad) under vacuum, and exposed to preflashed X-ray film (Kodak X-omat AR, or Fuji) for 5–15 h at −80°C in autoradiography cassettes fitted with intensifier screens (Hi-speed X-Genetic Research International). The autoradiograms were processed in Kodak X-ray developer and fixer.

Quantitation of cAMP binding protein types

Autoradiograms were scanned by densitometry. Peak areas of the resolved bands were assessed by computer analysis following subjective assignation of their positions on the scan. The results were then expressed as a percentage of the total scan.

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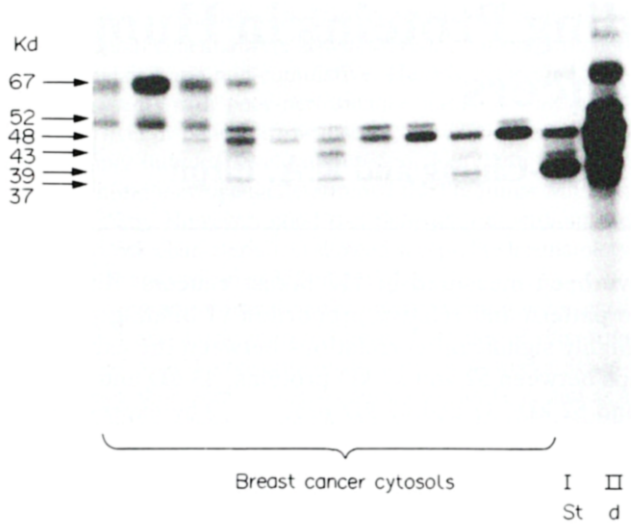

Fig. 1. Autoradiogram of 12 different breast cancer cytosols photoaffinity labelled with $8N_3[^{32}P]$ cyclic AMP. Std I and Std II are cyclic AMP dependent protein kinase standard as supplied by Sigma (codes P4890 and P3891, respectively). Methods as described in the text.

Statistical methods

Correlations between total cAMP binding and individual binding proteins were analysed by the Spearman correlation coefficient test.

RESULTS

Total binding

Cyclic AMP binding was detected in all tumours cytosols, levels varying between 759 and 12 467 (median 4729) fmol/mg cytosol protein. The dissociation constants ranged between 0.97 and 4.38 $\times$ 10^{-8} mol/l.

Typing by photoaffinity labelling

Following photoaffinity labelling with [^{32}P]8-azido cAMP and separation by polyacrylamide gel electrophoresis, binding proteins with molecular weights of 67, 52 (this band was sometimes diffuse such that the extremes of molecular weight could vary between 50 and 56 kD), 48, 43, 39 and 37 kD were identified (Fig. 1). The 52 and 48 kD proteins were present in all cytosols whereas the other bands appeared in most, but not all, tumours (Table 1). The pattern and relative proportion of binding proteins differed markedly between individual tumour cytosols (Table 1). It was, therefore, of interest to correlate the presence and proportion of individual bands with each other and also the level of total binding of cAMP.

Table 2. Correlation coefficients and P values for relationships between % expression of different binding proteins and total cAMP binding

	Correlation coefficients						
	67 kD	52 kD	48 kD	43 kD	39 kD	37 kD	Total
67 kD	—	0.243	−0.532	−0.159	−0.415	−0.027	−0.510
52 kD	0.009	—	−0.340	−0.377	−0.448	0.107	−0.327
48 kD	<0.001	<0.001	—	−0.149	−0.973	−0.313	0.661
43 kD	0.088	<0.001	0.110	—	0.371	0.314	0.004
39 kD	<0.001	<0.001	0.299	<0.001	—	0.055	0.157
37 kD	0.774	0.252	0.001	0.001	0.556	—	−0.237
Total	<0.001	<0.001	<0.001	0.967	0.093	0.011	—
	P values						

Correlation between individual binding proteins and total binding

The correlation coefficients for comparisons between individual binding proteins and total binding are shown in Table 2. Significant positive correlations were observed between 52 and 67 kD proteins, 43 kD and both 39 and 37 kD proteins. Inverse correlations were observed between (i) 48 and 52 kD proteins and (ii) 37 and 67 kD proteins. With regard to total binding, significant positive correlations were observed with the 48 kD species (Fig. 2) and negative correlations with the 67, 52 (Fig. 3) and 37 kD proteins.

DISCUSSION

The present study represents the first in which types of cyclic AMP binding proteins have been characterised and related to levels of total binding in an extended series of human breast

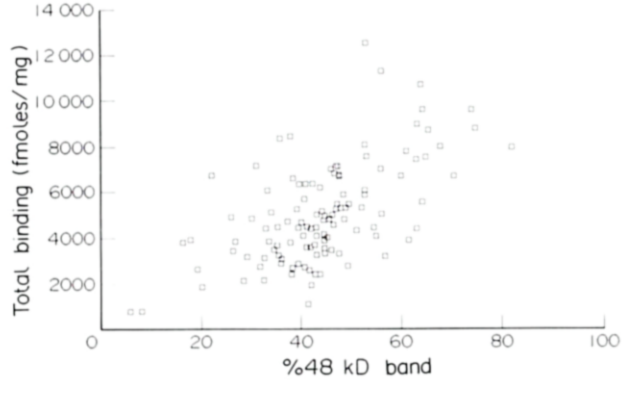

Fig. 2. The relationship in breast cancer cytosols between total cyclic AMP binding and the % of binding detected as a 48 kD band following photoaffinity labelling.

Table 1. The incidence and differential expression of individual cAMP binding protein in cytosols of 117 breast cancers

Molecular weight	67 kD	52 kD	48 kD	43 kD	39 kD	37 kD
Number in which identified	79	117	117	102	113	67
% of total	68	100	100	87	97	57
% of total scan						
Median	5.2	18.3	43.3	13.1	12.0	6.2
Range	0.5–49.8	2.9–42.1	8.1–81.8	3.9–26.6	2.1–44.7	1.2–30.5

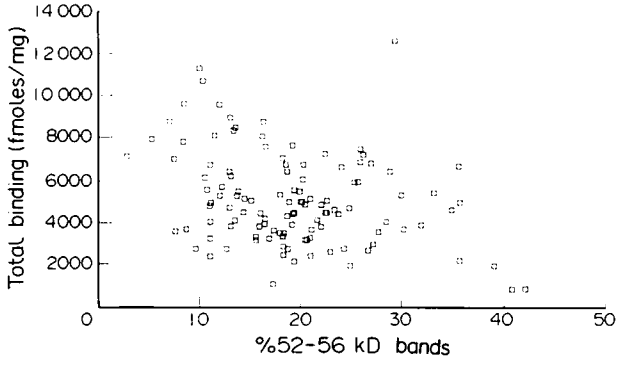

Fig. 3. The relationship in breast cancer cytosols between total cyclic AMP binding and the % of binding detected as a 52–56 kD band following photoaffinity labelling.

cancers. The results confirm our previous finding that cyclic AMP binding protein levels vary considerably between individual tumours [6] and indicate that, following photoaffinity labelling with 8 azido cyclic AMP and polyacrylamide gel electrophoresis, distinct binding proteins with differing molecular weights can be detected. Binding proteins with molecular weights of 48 kD and 52–56 kD were detected in all tumour cytosols and species with molecular weights of 39 and 43 kD were present in 97 and 87% of the tumours, respectively; a cyclic AMP binding protein with an apparent molecular weight of 37 kD was found in just over one half of tumour cytosols. The majority of tumours also possess a binding protein with a migration corresponding to a molecular weight of 67 kD but, unlike the other proteins, binding for 8-azido [^{3}P] cyclic AMP could only be partially blocked with excess radioinert cyclic AMP. Similar types of cAMP binding proteins have been reported in a smaller number of breast cancer cytosols [11]. These authors further suggested that the 48 kD protein represented the RI regulatory subunit of protein kinase A, the 52 kD protein was the RII regulatory subunit and the 39 and 37 kD proteins were proteolytic cleavage products. The identification of the 43 kD protein remains unresolved. The 67 kD protein is also incompletely characterised but it would seen, at least in part, to comprise of albumin, 8-azido cyclic AMP binding being incompletely blocked by radioinert cyclic AMP and displaying a distinct mobility change when electrophoresis was performed under non-reducing conditions (both characteristics of albumin); tumour cytosols possessing the 67 kD species were also found to contain albumin as determined by rocket immunoelectrophoresis.

The other major finding in the present study was the observation that individual breast cancers display distinctive patterns and proportions of cAMP binding proteins. Because of the relatively large number of tumours investigated it was possible to look for associations between the various species of binding proteins. As a result several highly statistically significant relationships were detected. Firstly, there was a positive relationship between 52 and 67 kD proteins. Assuming these are largely comprised of type II regulatory subunit and albumin, repectively, there is no apparent reason for such an association. Secondly, there were positive relationships between 43, 39 and 37 kD proteins which might be explained if all are proteolytic products of native binding protein [11]. Lastly, there was an inverse relationship between 52 and 48 kD proteins. Since in the majority of tumours these were the most abundant binding proteins, this observation could simply reflect the mathematics of proportionality but the association with total binding protein level suggests that this is not the case. Thus it was shown that there was a strong positive correlation between total binding and the proportion of 48 kD protein and an equally strong inverse correlation between total binding and the 52 kD band. This suggests that there is a tendency for tumours to express either 48 or 52 kD binding proteins rather than co-express both and that in general tumours with low binding tend to express the 52 kD protein whereas tumours with high levels of high binding protein, are more likely to predominately display a 48 kD phenotype.

If the 52–56 kD proteins reflect type II regulatory subunits of protein kinase A and the 48 kD proteins type I regulatory subunit, this might have important biological implications. It has been suggested that type I binding proteins programme for proliferation and type II are concerned with differentiation [5, 12]. We have already presented evidence that tumours with high cyclic AMP binding are associated with a more aggressive natural history than those with lower levels of binding protein [1]. The underlying reason for this may be that, as is shown in the present study, tumours with high binding proteins have a relative overexpression of type I to type II regulatory subunits. It is, therefore, of immediate clinical importance to determine whether patterns of cAMP binding proteins have a particular prognostic significance and add to the discriminatory powers of total binding sites in predicting the outcome of patients with breast cancer.

1. Miller WR, Elton RA, Dixon JM, Chetty U, Watson DMA. Cyclic AMP binding proteins and prognosis in breast cancer. *Br J Cancer* 1990, **61**, 263–266.
2. Kvinnsland S, Ekanger R, Doskeland SO, Thorsen T. Relationship of cyclic AMP binding capacity and oestrogen receptor to hormone sensitivity in human breast cancer. *Breast Cancer Res Treat* 1983, **3**, 67–76.
3. Watson DMA, Hawkins RA, Bundred NJ, Stewart HJ, Miller WR. Tumour cyclic AMP binding proteins and endocrine responsiveness in patients with inoperable breast cancer. *Br J Cancer* 1987, **56**, 141–142.
4. Doskeland SO, Ogreid D. Binding proteins for cyclic AMP in mammalian tissues. *Int J Cancer* 1981, **13**, 1–19.
5. Cho-Chung YS. Cyclic AMP and its receptor protein in tumour growth regulation *in vivo*. *J Cyclic Nucleotide Res* 1980, **6**, 163–177.
6. Miller WR, Senbanjo RO, Telford J, Watson DMA. Cyclic AMP binding proteins in human breast cancer. *Br J Cancer* 1985, **52**, 531–535.
7. Scatchard F. The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 1949, **51**, 660–672.
8. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dyne binding. *Analyt Biochem* 1976, **72**, 248–254.
9. Pommerantz AH, Rudolph SA, Haley BE, Greengard P. Photoaffinity labelling of a protein kinase from bovine brain with 8-azidoadenosine 3',5' monophosphate. *Biochemistry* 1975, **14**, 3858–3862.
10. Laemmli UK. Cleavage of structural protein during the assembling of the head of bacteriophage T4. *Nature* 1970, **227**, 680–685.
11. Handschin JC, Handloser K, Takahashi A, Eppenherger U. Cyclic adenosine 3':5', monoposphate receptor proteins in dysplastic and neoplastic human breast tissue cytosol and their inverse relationship with oestrogen receptor. *Cancer Res* 1983, **43**, 2945–2954.
12. Cho-Chung YS. Role of cyclic AMP receptor proteins in growth, differentiation and suppression of malignancy: new approaches to therapy. *Cancer Res* 1990, **50**, 7093–7100.

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