ADENOSINE 3′, 5′-CYCLIC-MONOPHOSPHATE DEPENDENT PROTEIN KINASE AND CYCLIC-AMP-BINDING IN HUMAN MAMMARY TUMORS*

U. EPPENBERGER, K. TALMADGE, W. KÜNG, E. BECHTEL, J. PREISZ, P. HUBER, R. A. JUNGMANN+ and A. SALOKANGAS

Hormone Laboratory and Experimental Endocrinology, Dept. Gynecology, University Clinic Medical School, 4004 Basel, Switzerland and *+Dept. of Biochemistry, Northwestern University Medical School, Chicago, Illinois 60611, USA*

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1. Introduction

The presence of cell-specific steroid hormone receptors in certain human mammary carcinomas has been related to the regulation of neoplastic cell proliferation. The absence or presence of such steroid hormone receptors has been used as a diagnostic tool for the identification of hormone-dependent mammary carcinomas, and in successful remission by endocrine therapy of breast cancer in women [1]. Few data, however, are available concerning the potential effects on neoplastic cell growth of several non-steroid hormones such as catecholamines, polypeptide and glycoprotein hormones which also assume important regulatory functions in growth regulation of human mammary epithelial cells.

In contrast to steroid hormones, the molecular mechanism of a great number of non-steroid hormones involves the direct participation of adenosine 3′, 5′-monophosphate (cyclic AMP) and cyclic AMP-regulated protein kinases [2–4]. Through the action of cAMP-dependent protein kinases** non-steroid hormones, via cyclic AMP, achieve a phosphorylative and possibly functional modification of specific cellular proteins [3,4]. The potential significance of a cyclic AMP-mediated nuclear protein phosphorylation in the regulation of RNA synthesis in rat mammary gland has become evident through studies by Turkington and his co-workers [5–7]. Additionally, Maller and Krebs [8] have recently established an important functional correlation between activation of cyclic AMP-dependent protein kinase and the regulation of cell replication.

In view of the experimental evidence implicating cyclic AMP and cyclic AMP-dependent protein kinase in the regulation of gene transcription and of cellular proliferation, we have assessed cyclic AMP-dependent and -independent protein kinase activities and cyclic AMP-binding activity in normal, dysplastic and neoplastic human breast tissue. We have determined that progressive neoplastic dedifferentiation can be correlated with increases of the specific activities of cyclic AMP-dependent protein kinase and cyclic AMP-binding, as well as increases of cyclic AMP-independent protein kinase specific activity.

2. Materials and methods

All biochemical reagents were obtained from Sigma Chemical Company. Protamine sulfate was from Merck. [γ-^{32}P]ATP (4–10 Ci/mmol) and cyclic [^{3}H]AMP (37 Ci/mmol) were purchased from New England Nuclear.

Breast tissue specimens were obtained from patients during mastectomy, from biopsies (2–6 g

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** Enzyme: cAMP-dependent protein kinase or ATP: protein phosphotransferase (EC 2.7.1.37).

Address correspondence to: U. Eppenberger, Hormone Laboratory and Experimental Endocrinology, Dept. of Gynecology, University Clinic Medical School, 4004 Basel, Switzerland

tissue) and from patients undergoing plastic surgery. Specimens were frozen immediately after excision and stored at −70°C. For the various biochemical assays the breast tissue was pulverized in liquid nitrogen. The powdered tissue was homogenized in 5 parts (w/v) of ice-cold 10 mM Tris/HCl buffer, pH 7.4 – 1.5 mM EDTA – 5 mM theophylline with a Polytron PT-20 (Brinkmann Instruments) at low speed (setting 4). The homogenate was subsequently filtered through cheesecloth and centrifuged for 90 min at 105 000 × g. The supernatant fraction was recovered and used for protein kinase and cAMP-binding assays.

Protein kinase was assayed by measuring the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into protamine (9). Binding of cyclic [^{3}H]AMP to protein was determined by the membrane filter assay as described previously [10]. The heat-stable protein kinase inhibitor was isolated from rabbit skeletal muscle by the method of Walsh et al. [11]. Protein was determined by the method of Lowry et al. [12].

Polyacrylamide gel electrophoresis was performed in a multiphase buffer system B [13] containing 0.2% Triton X-100. Polymerization of the polyacrylamide gels and electrophoresis were carried out according to Chrambach et al. [14].

Morphological characterization of the tissues was carried out by macroscopic examination at the time of surgery and microscopic examination by a pathologist. The tissues were classified according to the criteria given by the WHO (1968): Simple dysplasia with extensive fibrosis = MI-F; simple dysplasia = MI; proliferative dysplasia = MII; fibroadenomas (FA) and primary carcinomas (CA) [15]. Simple dysplasia with extensive fibrosis was regarded as normal tissue. The cellular density was evaluated by counting the number of nuclei per 1000 μm^2 tissue in 3 to 4 different tissue areas per biopsy. No distinction was made between epithelial and stroma nuclei. The number for each biopsy was 4–6 × 10^3 nuclei.

3. Results

3.1. *Protein kinase activity*

Cytosol cyclic AMP-dependent and -independent protein kinase activities were examined in 283 samples of normal, dysplastic and neoplastic human breast tissues. The specific enzyme activities (SA) of the various morphological groups are given in table 1. Cyclic AMP-dependent protein kinase activity of primary carcinomas exhibited a 2.5-fold higher SA

Table 1
Cyclic AMP-dependent and independent protein kinase activity in human breast tissue

Tissue	Number of patients	Protein kinase activity			p value
		Skeletal muscle inhibitor			
		None		200 μg	
		− Cyclic AMP	+ Cyclic AMP	+ Cyclic AMP	
		pmol phosphate incorp./min/mg protein ± SD			
Simple dysplasia with extensive fibrosis (M I-F)	60	34 ± 15	91 ± 42	24 ± 11*	< 0.001
Simple dysplasia (M I)	78	46 ± 25	121 ± 62	30 ± 13*	< 0.001
Proliferative dysplasia (M II)	60	54 ± 26	143 ± 60	69 ± 19	< 0.001
Carcinoma (CA)	69	86 ± 40	227 ± 89	75 ± 35*	–
Fibroadenoma (FA)	16	84 ± 32	200 ± 94	104 ± 52	not signific.

The protein kinase assay was carried out with 50–100 μg cytosol protein. Each mean value of the inhibitor study represents either 4 or 8 experiments. The significance was calculated by comparison with the CA-group.

(227 ± 89) than normal human breast tissue exhibiting a SA of 91 ± 42. Interestingly, no significant difference of SA was identified between fibroadenoma and carcinoma tissues.

An important correlation can be established when the protein kinase specific activities (table 1) are expressed as kinase activity per cell. Such correlation (using the cellular densities listed in table 3) reveals significantly lower (2 to 3-fold lower) cellular cyclic AMP-dependent protein kinase levels in primary carcinoma tissue than in breast tissues with simple or proliferative dysplasia.

Cyclic AMP-independent protein kinase activity can be identified in the presence of saturating concentrations of heat-stable protein kinase inhibitor from rabbit muscle. The inhibitor combines specifically with the catalytic subunit of cyclic AMP-dependent protein kinase, forming inactive catalytic subunit–inhibitor complexes [16], and does not affect cyclic AMP-independent kinase activity. Interaction of saturating amounts of the inhibitor with the cytosol fractions of the different histopathological groups resulted in varying degrees of protein kinase inhibition demonstrating the presence of varying amounts of cyclic AMP-independent protein kinase (table 1). Similar to the cyclic AMP-dependent activities, cyclic AMP-independent protein kinase levels were also significantly increased in carcinoma and fibroadenoma tissues as compared to breast tissue displaying simple dysplasia.

3.2. *Substrate specifity*

The ability of cytosol protein kinase activity to phosphorylate different protein substrates was examined by testing several substrates present in the assays in equal concentrations by weight. No difference in substrate preference was detected in the fifteen tissue samples selected (3 of each histopathological group). In all cases, protamine was found to be the best substrate followed by total histone and histone F1. Casein was a relatively poor substrate and bovine serum albumin was not phosphorylated at all.

3.3. *Cyclic AMP-binding activity*

For binding studies, cytosol fractions of the various tissues were tested as described by Walton and Garren [8]. The specific cAMP-binding activity (SB) increased gradually from normal (MI-F) to neoplastic tissue (CA) with intermediate cAMP-binding values in the dysplastic groups (Table 2). The 49 primary tumors (CA) exhibited a 2 to 3-fold higher SB as compared to the 45 cytosol fractions of MI-F ($p < 0.01$). Small differences in cAMP-binding were observed between MI and MI-F, whereas MII and the fibroadenomas revealed significantly higher binding values at the $p < 0.002$ and $p < 0.01$ level respectively, when compared to MI-F.

3.4. *Polyacrylamide gel electrophoresis*

Representative cytosol fractions of MI-F, MI, MII

Table 2
Levels of cyclic AMP-binding in human breast tissue

Tissue	Number of patients	Mean ± S.D. (pmole cAMP bound/mg protein)	p value
Simple dysplasia with extensive fibrosis (M I-F)	45	1.08 ± 0.40	–
Simple dysplasia (M I)	37	1.25 ± 0.86	not signific.
Proliferative dysplasia (MII)	49	1.54 ± 0.62	< 0.02
Carcinoma (CA)	49	2.33 ± 1.23	< 0.001
Fibroadenoma (FA)	10	3.33 ± 1.89	< 0.001

The cAMP-binding was assayed with 100–200 μg cytosol protein. The significance was calculated by comparison to normal tissue (MI-F).

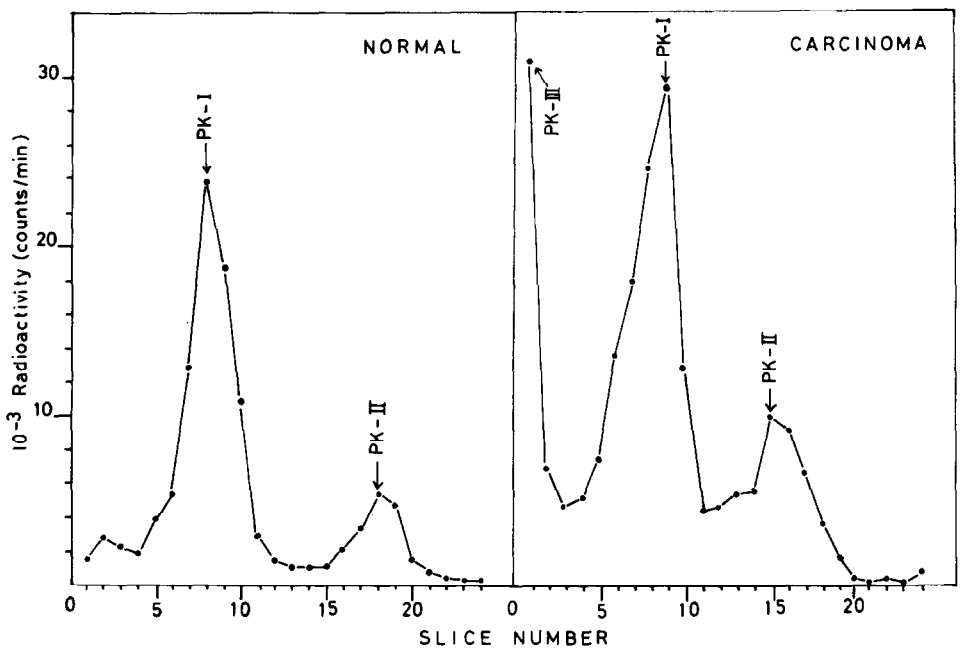

Fig.1. Electrophoretic separation of different protein kinases of normal (MI-F) and neoplastic (CA) tissues: Electrophoresis was carried out at pH 10.2, 0°C, with 2% bisacrylamide, 8% acrylamide, 0.2% Triton X-100. After electrophoresis the gels were sliced into 1 mm sections, extracted overnight and aliquots were analyzed for enzyme activity, R_F-values of PK-I and PK-II ranged from 0.18–0.20 and 0.35–0.40, respectively. Recovery of protein kinase activity was 60% to 70% of the applied total activity.

and CA were analyzed for different protein kinase species by polyacrylamide gel electrophoresis at pH 10.2 according to Salokangas et al. [13]. All groups exhibited 2 enzymatically active species which were designated PK-I and PK-II (fig.1). In addition to PK-I and PK-II a slowly migrating enzyme form (PK-III) was detected in most CA samples. All protein kinases were cAMP-dependent with PK-I being the major peak. Electrophoretic analysis at various gel concentrations revealed approximate molecular weights of 213 000 for PK-I and 80 000 for PK-II.

3.5. *Histometry*

Cell density measurements were carried out on samples in each of the various tissue groups. The data are shown in table 3. The results point out marked variations in cellularity of the various tissues. The cell density in the carcinomas was 5–6 times higher than in normal tissue (simple dysplasia with extensive fibrosis). The proliferative dysplasia exhibited intermediate levels of cellularity.

4. Discussion

Our results indicate that cyclic AMP-dependent and -independent protein kinase activity as well as cyclic AMP-binding activity are significantly higher in human breast cancer tissue than in normal mammary tissue provided the protein kinase and cyclic AMP-binding activities are compared per unit weight of cellular protein. These values do not take into account the relative cellular densities of the different tissues (see table 3). A more representative picture of the changes of cellular protein kinase and cyclic AMP-binding activities has been obtained by correlating the activities with the cellular densities. When this correlation is considered, the relative cellular levels of protein kinase and cyclic AMP-binding activity are markedly lower in the neoplastic than in the dysplastic and normal tissues. This is in agreement with the recent report by Majumder [17] who determined the protein kinase activities per unit weight DNA and found considerably lower levels of cyclic AMP-dependent

Table 3
Cellular density of normal and neoplastic human breast tissue

Tissue	Number of patients	Mean ± S.D. μm^2 (Number of cells/1000 μm^2)
Simple dysplasia with extensive fibrosis (M I-F)	9	0.75 ± 0.49
Simple dysplasia (M I)	10	1.81 ± 0.64
Proliferative dysplasia (M II)	11	3.89 ± 1.43
Carcinoma (CA)	18	5.60 ± 2.29
Fibroadenoma (FA)	7	4.59 ± 1.16

No separation of epithelial and stroma nuclei was carried out. Sample size per biopsy: $4-6 \times 10^3$ nuclei. Each value is the mean ± S.D., an average of 6 histometry measurements.

protein kinase in mouse mammary carcinoma tissue than in normal mouse mammary gland.

Our data, considered in context with previous findings implicating cyclic AMP-dependent protein kinase in the regulation of cell proliferation [8], may imply major alterations of the mechanism controlling cell growth in neoplastic tissues. Recent experiments demonstrating an inverse relationship between the cellular concentrations of estrogen receptors and cyclic AMP-binding activity [18] also suggest the involvement of the cyclic AMP - protein kinase system in the growth control of hormone-dependent mammary tumors in the rat.

Thus, the cyclic AMP-protein kinase system in addition to the effect exerted by steroid hormones need to be considered in the regulation of normal and neoplastic growth.

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