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No tumor-specific expression levels of protein kinase C isoenzymes and of c-fos in human breast cancer cell cultures

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Epithelial cells derived from 46 human breast tissue samples of patients suffering from breast cancer have been cultivated. Twenty-five of these cell cultures stemmed from normal and 21 from tumor tissues. Moderate to large variations of protein levels of three protein kinase C (PKC) isoenzymes (α, δ and ϵ) were found among the various cell cultures. The cell cultures also exhibited very heterogeneous basal as well as inducible levels of c-fos mRNA. However, none of these variations could be correlated with the character of the original tissue nor with any clinical parameter of the respective patient. Our results suggest that altered levels of PKC isoenzymes or of the protooncogene c-fos *per se* cannot serve as an indication for a transformed behavior of the epithelial cell fraction of human breast tissue.

Introduction

Breast carcinoma is the commonest type of cancer occurring in women and is the main cause of death from cancer in Europe. The relationship between growth of breast cancer and endocrine regulatory mechanisms governed by steroids is well established (1). Steroid hormones play an important role in growth regulation of both normal human breast tissue and breast tumors (2). Estrogen is known to induce mRNA expression and secretion of polypeptide growth factors like TGFα, PDGF, and IGF-II (3). Since many cells also possess receptors for GFs an autocrine pathway is indicated.

Stimulation of growth factor receptors by growth factors activates protein kinase C (PKC*) via production of the second messengers Ca^{++} and diacylglycerol (4). Members of the PKC family are recognized as key enzymes in signal transduction, cell differentiation and tumor promotion (5). PKC modulates the synthesis of the epithelial growth factor receptor (EGF-R) as well as its binding to EGF or TGFα (6). Therefore it has been suggested that high levels of EGF-Rs displayed by the late stage of breast cancer may be caused by the high levels of PKC activities.

Altered levels of PKC activities or protein levels have been reported also for other types of cancer. Decreased levels of PKC activities have been found for human colon tumors compared to normal mucosa (7). Elevated PKC activities have been described in human gastric cancer (8), lung cancer cells (9) and in neoplastic squamous epithelia cancer from the upper aero digestive tract (10). The expression of PKCα is increased in glioblastoma cells compared to the low levels of the isoenzyme in glia cells of normal tissue (11). In contrast, PKCα as well as nPKCϵ are downregulated in neuroblastoma which in turn showed increased expression of cPKCβ (12). Distinct PKC isoforms have therefore been suggested to be involved in the tumorigenesis of different brain tumors (13).

The expression of the protooncogene c-fos has been implicated in the control of both cell proliferation and differentiation (14,15). Agents which stimulate cell cycle transitions and cell proliferation such as peptide growth factors, phorbol esters, cyclic nucleotides and Ca ionophores all can activate expression of c-fos (16,17). c-Fos binds together with c-Jun protein as a heterodimer to AP-1 recognition sites mediating induction of the controlled genes (18). AP-1 activity is positively modulated by phorbol esters, cytokines and growth factors that activate protein kinase C by a variety of mechanisms inducing transcriptional activation of the c-jun and c-fos genes and posttranscriptional modifications of the protein products (19). We studied protein levels of six PKC isoforms (α, β, γ, δ, ϵ and ζ) using cultures of epithelial cell fractions from individual breast carcinomas or from normal breast tissue. Also basal c-fos mRNA levels as well as inducibility of this message by the phorbol ester TPA in these cultures were measured with respect to a possible correlation to the character of the original tissue or to clinical parameters.

Materials and methods

Preparation and culturing of epithelial cells from human breast tissue

Preparation and culturing of human epithelial breast tissue was basically performed according to Stampfer *et al.* (20–23). Tissues from human primary breast tumors and from normal human breast tissues derived from patients suffering from breast cancer were provided by the Institute of Pathology (J.Torhorst) and by the Department of Gynecology and Obstetrics of the University Hospital of Basel. Data about the patients and the respective tumor tissues are listed in Table I. The tissue samples (1 g) were cut, digested at 37°C for 12–15 h with collagenase CLS III (10 000 U/ml, Flow Laboratories) and hyaluronidase Type III (10 000 U/ml, Sigma, St Louis) and finally seeded for culturing into medium containing Dulbecco's modified Eagle medium (DMEM) and Ham's F12 (1:1). The medium was supplemented with 5% horse serum and various additions as described (23). Enrichment of the cultures in epithelial cells was achieved by repeated trypsinizations during which fibroblasts come off the plastic faster than epithelial cells. After 2–3 passages only the epithelial cell fractions remained and could be expanded to the cell volumes needed for the preparation of mRNA and protein fractions.

Immunofluorescence staining

Cells were grown on glass slides using the medium described (23). The slides were washed with PBS, fixed with 3.7% of formaldehyde in PBS and stained with the anti-cytokeratin antibody (clones AE1/AE3, Boehringer, Mannheim) for 45 min. After three washes with PBS an FITC conjugated rabbit anti mouse antibody was added and incubated for another 45 min. The slides were then washed with water, dried and embedded.

Immunoblotting

Cytosol and membrane protein fractions were prepared, separated by PAGE, transferred to nitrocellulose and exposed to antibodies using the Western technique as described (24). The antibodies used were as follows: anticytokeratin antibody (AE1/AE3, Boehringer, Mannheim); monoclonal antibodies specific for α- (MC5, Amersham), β- and γ-PKC (Seikagaku Kogyo, Tokyo); polyclonal antibodies specific for δ-, ϵ- and ζ-PKC (25).

Northern analysis

Total mRNA was prepared following the procedure described by Chomczynski and Sacchi (26). Formaldehyde agarose gels, blotting of RNA to Zeta Probe (Biorad) and hybridisations were as described (27). The 1.3 kb *EcoRI/HindIII*

*Abbreviations: PKC, protein kinase C; EGF-R, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle medium.

Table I. Clinical data of patients and the respective tumors

	Age	TNM	Estrogen receptor (ER)	Progesterone receptor (PR)
NO-1-17044	46	pT_1 pN_1		
NO-1-17967				
CA-1-CG039				
CA-1-21101	74	pT_2 pN_1 M_0	279 p	22 p
NO-1-21277	75	pT_2 pN_0 M_0		
CA-1-21277				
NO-1-21329	35	pT_3 pN_0 M_0	5 n	5 n
CA-1-22256	53	pT_3 pN_1 M_0	431 p	52 p
NO-2-06494	52	pT_1 pN_0 M_0	276 p	800 p
CA-2-06494				
NO-2-07965	58	pT_2 pN_1		
CA-2-07965				
NO-2-08576	51	pT_3 pN_1 M_0	29 p	38 p
NO-2-09360	40	mastopathy		
NO-2-09818	39	pT_2 pN_0 M_0	30 p	63 p
CA-2-10259	77	pT_2 N_x	1 n	1 n
NO-2-13864	55	pT_2 pN_1 M_0	123 p	96 p
NO-2-14055	54	pT_2 pN_1 M_0	368 p	90 p
CA-2-14055				
NO-2-14686	48	pT_2 pN_2 M_1	105 p	159 p
CA-2-14686				
CA-2-14811	52	T_2	27 p	6 n
CA-2-15056	80	pT_2 pN_2 M_x	595 p	491 p
CA-2-15068	57	pT_2 pN_0 M_0	42 p	36 p
NO-2-15070	74	pT_2 pN_0 M_0	19 p	10 p
NO-2-15456				
NO-2-16047	37	pT_3 pN_0 M_0	7 n	5 n
NO-2-16419	82	pT_3 N_x M_x	15 p	10 p
CA-2-16438	66	pT_2 pN_1		
NO-2-18807	63		112 p	72 p
CA-2-20538	79	pT_2		
NO-2-21441	65	pT_4		
CA-2-21441				
NO-2-21988	72	pT_2 pN_1 M_0	50 p	6 n
CA-2-21988				
NO-2-23215	60	pT_4 pN_1 M_1	456 p	9 p
CA-2-23215				
NO-2-23722	35	pT_2 pN_1 M_0	99 p	63 p
CA-2-23722				
NO-3-01177	73	pT_1 pN_1 M_0	210 p	420 p
CA-3-01177				
CA-3-01204	68	pT_4 pN_0	11 p	20 p
NO-3-01354	84	pT_2 pN_1		
CA-3-01354				
NO-3-01372	51	pT_2 pN_1 M_0	23 p	29 p
NO-3-01764	45	pT_2 pN_1 M_0	320 p	4 n

Normal (NO) and tumor (CA) tissue was taken from patients suffering from breast cancer (except 2-09360).
T, tumor size; N, nodal status; M_0, no metastases; M_1, metastases diagnosed. Index x, not examined. p, diagnosed by histological methods. ER and PR (fmol/mg protein). Tumor tissues exhibiting ER or PR values higher than 10 fmol/mg are considered to be positive (p), those with values below negative (n).

DNA fragment of the plasmid pRNfos (27) was used as a probe. The intensities of the 18/28 S rRNA (stained by ethidium bromide) bands were used for standardisations.

Results and discussion

Cultivation of epithelial cell fractions of normal human breast tissue and of primary breast tumors

The cultivation of epithelial breast tissue cells was basically performed as described (20–33) but some important modifications had to be introduced in order to obtain large and homogeneous cultures of the epithelial cell fraction from the tissues. Cell cultures that initially consisted of a mixture of fibroblasts and of epithelial cells (Figure 1) were stepwise enriched in epithelial cells by fractionated trypsinization as well as by the use of a relatively high Ca^{++} concentration (0.3 mM). Homogeneous epithelial cell cultures were obtained after 2–4 passages (Figure 1b). To verify the epithelial character of these cell cultures immunostaining was performed using anticytokeratin antibodies (see Materials and methods). The cells that were assumed to be epithelial became stained by this antibody, whereas fibroblasts obtained from these tissues did hardly bind the antibody (Figure 2). Using the same antibody in a Western blot analysis revealed in addition that mainly the membrane fractions of the epithelial cells exhibited several proteins in the range between 30 and 50 kDa that are recognized by the anticytokeratin antibody whereas fibroblasts exhibited only very weak and unspecific hybridization, mainly to proteins of the cytosolic protein fraction (Figure 3). These results demonstrate that the distinction between fibroblasts and epithelial cells judged by morphological criteria is reliable enough.

Forty-six epithelial cell cultures were grown that stemmed from breast tissues of patients suffering from breast cancer. Twenty-five of them were derived from normal breast tissue and 21 from breast tumors of these patients. Twenty-two of them represented pairs of carcinoma and of normal tissue respectively, of eleven patients. Within 6–9 weeks (5–7 passages) the cells were grown to high cell volumes (5×10^8 cells). When using complete growth medium (supplemented with horse serum, hydrocortisone, insulin and EGF) all cell cultures exhibited very similar growth rates independent of the nature of the original tissue (normal or tumor). Ten per cent of the cultures exhibited no drastic but still distinct specific differences in their cellular morphologies but these differences could not be connected with features of the respective tissue or patient. Some typical morphologies of the 46 cultures are shown in Figure 1b.

Besides these 46 cultures there were 25 cultures whose cells ceased growing after few passages. Ten of them were derived from normal and 15 from tumor tissues. We conclude that epithelial cell cultures exhibited about the same probability to grow to higher passage numbers independent of the character of the original tissue.

Taken together, our observations suggest that cell morphologies and growth properties of epithelial short-term cell cultures derived from breast tissue exhibited some individual but no tumor-linked variations.

Protein levels of PKC isoenzymes

Using the primary cell cultures described in the previous section the content of various PKC isoenzymes was determined. The goal was to analyze possible differences in the expression levels of various PKC isoenzymes comparing normal and tumor tissues. Using monoclonal or polyclonal antibodies protein levels of the PKC isoforms α, β, γ, δ, ϵ and ζ respectively, were determined by Western blot analysis. Protein fractions (cytosol and membrane) of each individual culture were prepared before and after the addition of the phorbol ester and PKC activator TPA. Figure 4 shows the typical pattern of the down-regulation of the various PKC isoenzymes upon treatment with TPA. αPKC is very efficiently translocated to the membrane and subsequently down-regulated upon treatment of the cells by the phorbol ester. ϵ-PKC is only partially translocated to the membrane and is very slowly down-regulated. The efficiency of the translocation as well

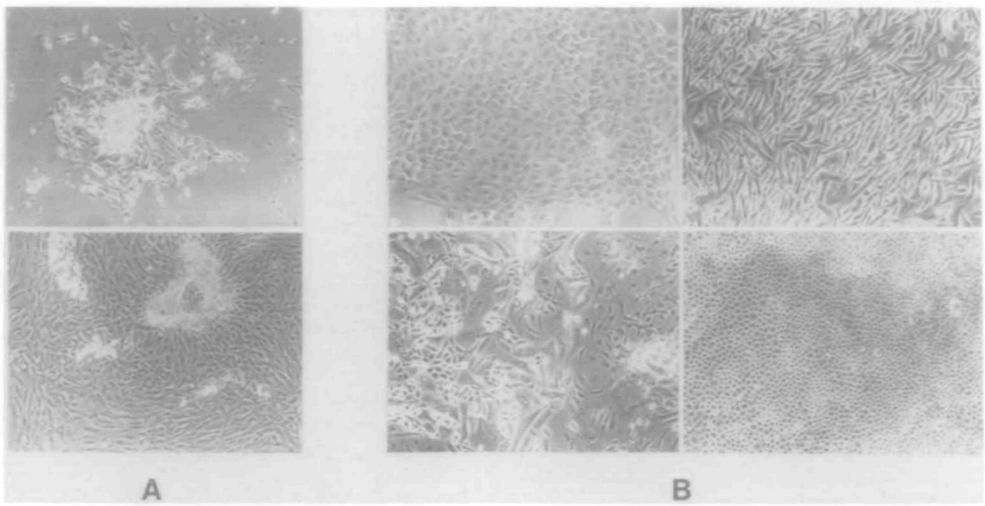

Fig. 1. Cell cultures from various tissues and culturing times. (**A**) Upper: cell culture NO-2-15056 10 days after initial seeding of the normal breast tissue. Epithelial cells in the middle, fibroblasts on the top right. Lower: the same culture 10 days later. (**B**) Cell cultures CA-2-21441 (top left), NO-2-18807 (top right), NO-1-15644 (bottom left) and CA-2-20538 (bottom right) after 4 weeks exhibiting different morphologies. The cells were grown as described in Materials and methods.

as the subcellular distribution of this subspecies varied from culture to culture. δ- and ζ-isoenzymes did not at all translocate or down-regulate upon the addition of TPA in any of the cultures.

Basal PKC protein levels exhibited by the individual cell cultures are shown in Table I and discussed in the following. No β- or γ-PKC could be found at detectable levels in any of the cultures. The levels of ζ-PKC were found to be very similar in all of the cultures. Comparing the cell cultures differences of the total protein levels (membrane plus cytosol) of up to a factor of five were found for each of the three isoenzymes, α-, δ- and ϵ-PKC. No positive or negative correlation between expression levels of the three subspecies in the individual cultures could be detected suggesting that there is no mutual dependence of expression levels of PKC isoenzymes. Cell cultures exhibiting high levels (relative values of 3−5 in Table II) of any of these three PKC isoenzymes occurred as frequently with cultures originating from tumors as with those from normal tissues: high α levels with 4/25 of the NOs versus 3/21 of CAs, high δ levels with 7/25 of the NOs versus 4/21 with CAs and high ϵ levels with 7/25 NOs versus 7/21 CAs. This indicates that high levels of specific PKC isoenzymes did not correlate with tumor tissues.

Finally we asked the question whether there was a connection of high PKC protein levels and clinical parameters like age, tumor size, nodal or estrogen or progesterone receptor status. Groups of patients (like 9 out of 32 patients for $T \geq 3$, 13 patients for $N \geq 1$, 11 patients with high ER or 10 patients with high PR) were compared with strong PKC expressors but not even one significant correlation could be found. Our results suggest that PKC protein levels feature naturally random variations and that epithelial cell fractions of carcinomas and of normal breast tissues can therefore not be simply distinguished by such protein levels. Our finding is opposing reports that describe altered PKC levels or enzyme activities in various types of tumors (7−13,28) but the finding is in accordance with the report by Regenass *et al.* (23) where no difference in PKC enzyme activities could be found when primary cell cultures derived from normal human breast tissue had been compared to those derived from breast carcinomas. However, specific isoforms of this kinase might still be involved in the development of breast cancer but rather on the level of their enzymatic activation along the signal transduction pathways to which they belong.

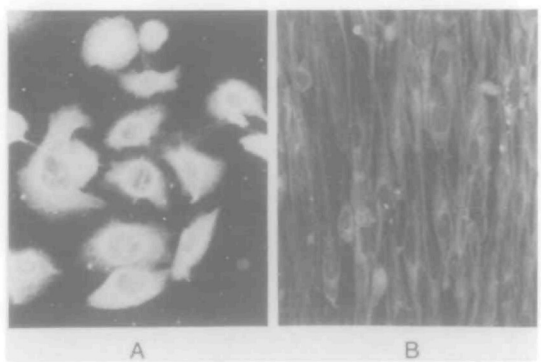

Fig. 2. Immunostaining of epithelial cells and of fibroblasts by anticytokeratine antibodies. The epithelial (**A**) and the fibroblast (**B**) cell fractions of the cell culture of CA-2-21441 were stained with the anticytokeratine antibody (AE1/AE3) as described in Materials and methods.

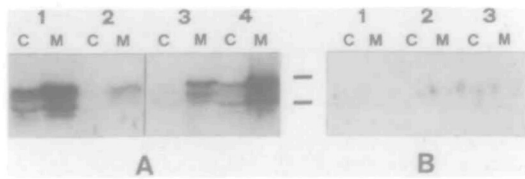

Fig. 3. Western blot analysis. 70 μg of fractionated proteins (C = cytosol, M = membrane) of the various cell cultures were separated on a 12% SDS−PAGE, blotted to nitrocellulose and hybridized to the anticytokeratine antibody (AE1/AE3, Boehringer, Mannheim). (**A**) Epithelial cell fraction of NO-2-14686 (1), CA-2-14686 (2), NO-2-14055 (3) and CA-2-14055 (4). (**B**) Fibroblast cell fraction of the same cultures as under (A). No fibroblast fraction could be obtained from culture CA-2-14055. The bars represent marks for proteins with mol. wts of 35 kDa (lower) and 45 kDa (upper).

Basal and inducible levels of c-fos mRNA

It is known that activation of PKC leads to the induction of nuclear proteins like c-FOS (19). The induction of FOS as a component of the transcription factor AP-1 is an essential event for cell cycle progression and mitosis (16,17,29). Up- and down-regulation of c-fos mRNA upon TPA induction has been analyzed using 41 of the 46 cell cultures derived from 24 normal and from 17 carcinoma tissues (Figure 5a). The very heterogeneous indu-

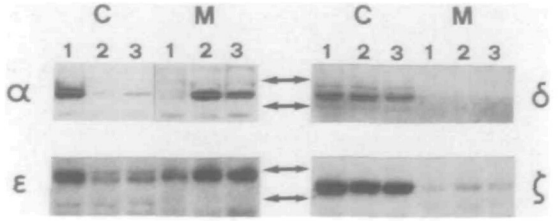

Fig. 4. Western blot analysis for PKC isoenzymes. Using the cell culture NO-1-21329 a time course experiment was performed to follow the various PKC isoenzymes upon the addition of the phorbol ester TPA to the cells. 70 μg of fractionated proteins (C = cytosol, M = membrane) were separated by SDS–PAGE (8%), blotted to nitrocellulose and hybridized to the PKC isoenzymes-specific antibodies against α-, δ-, ε- and ζ-PKC (see Materials and methods). Without phorbol ester TPA (1), 30 min (2) and 4 h (3) after the addition of TPA. Arrows represent mol. wt markers: 93 kDa (upper) and 68 kDa (lower).

Table II. PKC isoenzymes and c-fos

	PKC				c-fos mRNA		
	α	δ	ε	ζ	0′	45′	4 h
NO-1-17044	2	3	1	1	0	2	0
NO-1-17967	3	2	3	1	0	1	0
CA-1-CG039	5	1	2	1	0	0	0
CA-1-21101	1	5	1	1	n.d.	n.d.	n.d.
NO-1-21277	2	3	1	1	0	1	1
CA-1-21277	1	2	5	1	0	0	0
NO-1-21329	5	2	5	1	0	3	0
CA-1-22256	2	4	3	1	0	1	0
NO-2-06494	1	3	2	1	0	2	1
CA-2-06494	1	5	2	1	0	3	0
NO-2-07965	1	3	2	1	1	4	1
CA-2-07965	2	5	3	1	n.d.	n.d.	n.d.
NO-2-08576	2	2	2	1	0	0	0
NO-2-09360	1	1	2	1	0	3	4
NO-2-09818	1	2	1	1	0	2	1
CA-2-10259	1	5	5	1	n.d.	n.d.	n.d.
NO-2-13864	2	1	1	1	0	2	1
NO-2-14055	2	3	3	1	0	3	0
CA-2-14055	3	2	2	1	0	1	0
NO-2-14686	1	1	3	1	1	1	1
CA-2-14686	1	1	3	1	2	2	3
CA-2-14811	2	2	2	1	0	1	0
CA-2-15056	2	1	2	1	0	2	0
CA-2-15068	2	1	2	1	1	1	1
NO-2-15070	2	1	1	1	0	1	0
NO-2-15456	3	2	1	1	3	4	3
NO-2-16047	1	1	1	1	0	1	2
NO-2-16419	1	2	5	1	1	2	1
CA-2-16438	1	2	2	1	0	1	1
NO-2-18807	2	4	2	1	0	3	0
CA-2-20538	2	1	2	1	0	3	0
NO-2-21441	1	1	2	1	0	1	0
CA-2-21441	2	2	2	1	3	4	3
NO-2-21988	1	1	3	1	0	3	1
CA-2-21988	1	2	2	1	0	2	0
NO-2-23215	2	2	3	1	0	1	0
CA-2-23215	1	2	1	1	n.d.	n.d.	n.d.
NO-2-23722	1	1	2	1	1	1	3
CA-2-23722	3	2	3	1	1	1	3
NO-3-01177	2	4	2	1	0	1	1
CA-3-01177	1	2	3	1	0	2	1
CA-3-01204	2	2	1	1	0	1	1
NO-3-01354	3	2	1	1	n.d.	n.d.	n.d.
CA-3-01354	1	2	2	1	1	1	1
NO-3-01372	1	2	1	1	1	1	0
NO-3-01764	1	1	1	1	1	1	2

Relative protein levels of four isoenzymes of PKC and relative levels of c-fos mRNA (before and after induction by the phorbol ester TPA were determined in the various epithelial cell cultures stemming from normal human breast tissue (NO) or from breast carcinoma tissue (CA). The figures represent relative levels determined by densitometric measurements of protein or mRNA bands obtained after Western analysis for the PKC isoenzymes (see also Figure 4) and after Northern analysis for c-fos mRNA (0′ = without TPA, 45′ and 4 h after the addition of 100 nM TPA). The figures for the PKC isoenzymes are understood as relative levels (densitometric determination) of each isoenzyme among the various cell cultures. This means that only the figures in each column can be compared with each other but not the figures within lines. All figures can be compared with each other for the relative levels of c-fos mRNA. n.d., not determined.

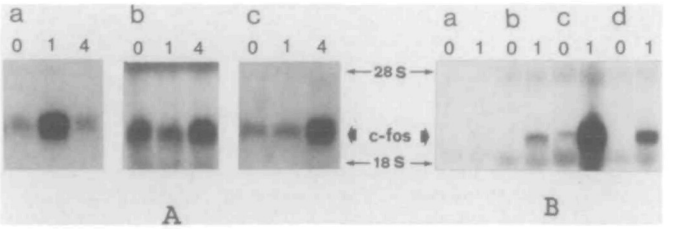

Fig. 5. Northern analysis after induction of c-fos mRNA by phorbol ester TPA. Cell cultures were analysed for their basal levels of c-fos mRNA (lanes 0) and for the respective c-fos mRNA levels 1 and 4 h after the addition of the phorbol ester TPA (100 nM). Total mRNA was prepared and 10 μg were separated on an agarose gel (see Materials and methods). The gel was transferred to Zeta-Probe (Biorad) and hybridized against the c-fos probe as described in Materials and methods. (**A**) represents the cell cultures NO-2-14055 (a), CA-2-14686 (b) and NO-2-09360 (c). (**B**) represents the established breast carcinoma cell line MDA-MB-134 (a), the breast carcinoma cell line ZR-75-1 (b), the breast carcinoma breast cell line MCF-7 (c) and the normal breast cell line HBL-100 (d).

cibilities of c-fos mRNA by TPA are shown in Table I and are summarized in the following. (i) In three cases (two tumors and one normal tissue) c-fos mRNA could not be induced at all by the phorbol ester. (ii) A 'normal' time course of c-fos mRNA induction—that is a very pronounced peaking at around 45 min followed by a rapid down-regulation—was observed in 17 cases (8 normal tissues, 7 tumors). (iii) In 11 cases (8 normal tissues, 3 tumors) c-fos mRNA was induced but was not rapidly down-regulated. (iv) The remaining 12 cultures (7 normal tissues, 5 tumors) were identical to (iii) but exhibited, in addition, relatively high basal c-fos mRNA levels. We can therefore state that basal c-fos mRNA levels as well as the inducibility of this message by the phorbol ester TPA are very heterogeneous among the cell cultures. The ratios NOs versus CAs in these groups demonstrate that there are no significant correlations between any of these groups and the tumor or the normal tissues. The four groups have also been compared to tumor and patients' data (Table I) but basically no interconnections could be detected. The most promising—although weak—correlation was that 6 out of a total of 12 cultures exhibiting high basal c-fos levels (group iv) belonged to the group of 15 patients with $N \geq 1$.

In this context it is worthwhile to mention that the established cell line HBL-100 (normal breast tissue) as well as the breast carcinoma cell line ZR-75-1 exhibit an induction pattern of the c-fos gene like group ii (Figure 5b). In contrast, c-fos can hardly be induced in MDA-MB-134 (breast carcinoma cell line) by TPA whereas MCF-7 (breast carcinoma cell line) already exhibits high basal c-fos mRNA levels and very high levels of this message are reached upon the addition of TPA. It can therefore be stated that the very heterogeneous levels of c-fos mRNA and its inducibility by TPA as well as the independence of this pattern

from the character of the original breast tissue are not only a feature of the 41 primary cell cultures but also of established human breast cell lines.

Our results suggest that altered cellular growth regulation in human breast tumors is not connected with altered expression levels of specific isoenzymes of PKC nor with an altered PKC-driven expression of c-fos.

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