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Tumorigenicity-associated expression of protein kinase C isoforms in rhabdomyosarcoma-derived cells

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Cell sublines were derived from Ni-induced rat rhabdomyosarcoma which differed in their degree of tumorigenicity. We compared the expression of protein kinase C isoforms α, β, γ and ε in three sublines developing tumors in syngeneic rats (Sy^+) and in two sublines devoid of tumorigenicity in these animals (Sy^-), with that of normal skeletal muscle. Northern and Western blotting experiments showed that PKC α was dramatically overexpressed in Sy^- cells whereas it was underexpressed in Sy^+ cells. Southern blot analysis provided evidence for a 3-fold increase of PKC α-related DNA fragments in Sy^- cells. Steady-state levels of the PKC ε-related transcript were also markedly decreased in Sy^+ cells. However, the expression of PKC β-related RNA was increased in these cells. Our data support the concept that the differential expression of the PKC isoforms may play a critical role in determining the neoplastic phenotype.

Protein kinase C isoform; Gene expression; Muscle; Rat rhabdomyosarcoma

1. INTRODUCTION

Protein kinase C (PKC) plays a key role in signal transduction. Extracellular ligand binding to specific surface receptors results in phospholipid breakdown which yields diacylglycerol, the physiological activator of PKC. Enzyme activation proceeds through close interactions with membranes, presumably at the sites of DG formation. Membrane-bound PKC becomes a substrate for calpain-related proteases and is rapidly down-regulated [1–3]. Protein kinase C consists of a family of closely related proteins expressed in a tissue-specific manner. These isoforms exhibit differences in enzymatic characteristics (e.g. substrate specificity, susceptibility to down-regulation, dependence on Ca^{2+}) which reflect their functional heterogeneity [4–7]. As a rule, several isoforms are expressed in a given cell type which may relate to distinct signalling pathways.

A number of tumor promoters, including phorbol esters, activate PKC through an interaction with the DG-binding site or unknown sites, and mimick the action of the physiological effector (as reviewed in refs. [8] and [9]). Phenotypic changes associated with induction of differentiation have been related to alterations in the expression of specific PKC isoforms [10–12]. In tumors, activities are generally found to be impaired; they are either reduced or increased when compared to parent normal tissues [13–16]. In addition, cDNA transfection and stable expression of an individual PKC isoform may affect growth and tumorigenicity in cell lines [17–19]. The above data suggest that the levels of expression of individual PKC isoforms may contribute to specify the cell phenotype.

In the present work, we examined the expression of PKC α, β, γ and ε isoforms in skeletal muscle and rhabdomyosarcoma-derived sublines exhibiting various degrees of tumorigenicity. We report severe alterations in the expression of these isoforms which were associated with cancer cell phenotype.

Abbreviations: PKC, protein kinase C; GAPDH, gluceraldehyde-3-phosphate dehydrogenase; EGTA, ethyleneglycol-bis (b-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride

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2. MATERIALS AND METHODS

2.1. *Cell lines and tumors*

The parental RMS 9-4/0 cell line was established in culture from a Ni-induced rat rhabdomyosarcoma. Cell sublines with differing tumorigenicity were selected, as described in refs. [20] and [21]. RMS 9-4/0, S4 and F21 cell lines were tumorigenic in syngeneic rats where R9 and C8 cell lines were not tumorigenic but formed tumors in nude mice. The present study was performed on solid tumors obtained after injection of these sublines. Herein, we refer to the first three as Sy^+ and the last two as Sy^-.

2.2. *RNA isolation and Northern blot analysis*

Total RNA was isolated from tumors and rat quadriceps muscle by the guanidinium–cesium chloride method and poly(A^+)RNA was selected by retention on an oligo(dT) cellulose column. Poly(A^+)RNA was analyzed by electrophoresis and Northern blot hybridization ac-

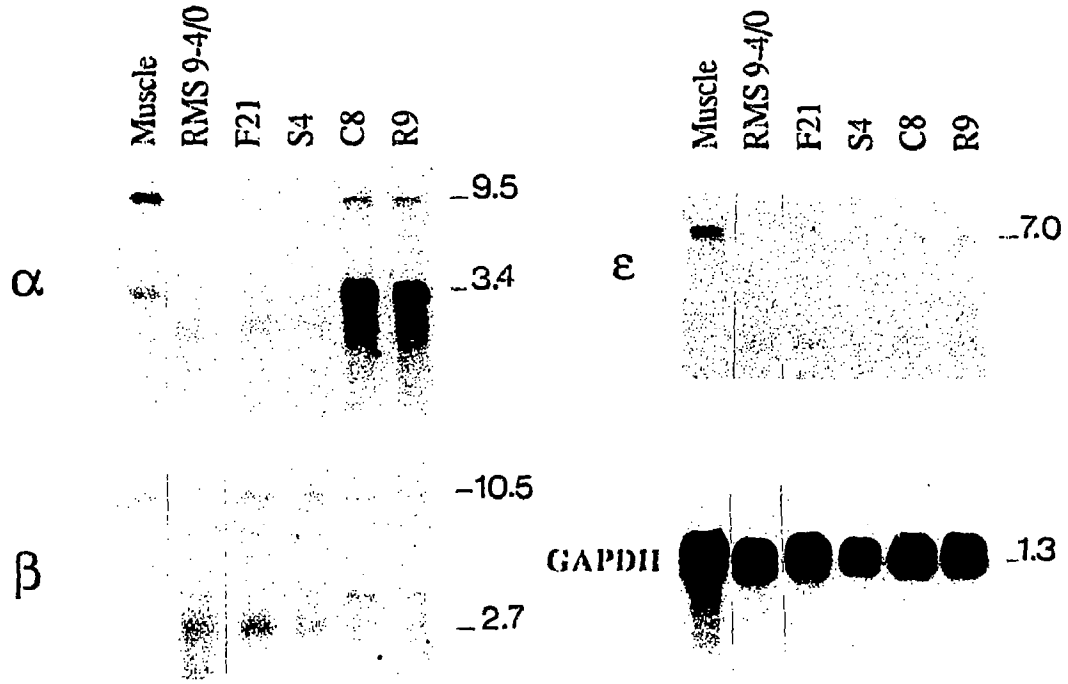

Fig. 1. Expression of PKC α, β, ε-related transcripts in muscle, in three sublines which are tumorigenic in syngeneic rats (Sy⁺), RMS 9-4/0, F21, S4 and in two sublines (C8, R9) which are non-tumorigenic in syngeneic rats (Sy⁻). Poly(A⁺) RNA were analyzed by agarose gel electrophoresis and by successive hybridizations to randomized ^{32}P-labeled PKC α, β, ε cDNA probes (0.5–1 × 10⁹ cpm/μg DNA). Exposure time of autoradiograms was, respectively, 15, 60 and 40 h for PKCα, β and ε probes. RNA blots were hybridized to a GAPDH cDNA probe to assess the amounts of RNA present in each well. Ribosomal RNA 18S and 28S were used as size markers.

cording to a standard procedure [22] with the appropriate probes. The PKC α probe was a 1.77 kb *Bam*HI fragment; the PKC β_1, β_2 probe was a 1.22 kb *Pst*I fragment; the PKC γ probe was a 2.1 kb *Bam*HI–*Xba*I fragment, the PKC ε probe was a 2 kb *Eco*RI fragment; the GAPDH probe was a 1.3 kb *Pst*I fragment.

2.3. *DNA isolation and Southern blot analysis*

DNA extracted from tumors and quadriceps muscle were digested with 2–3 U/μg DNA of *Eco*RI. DNA fragments were fractionated by electrophoresis on 0.7% agarose gels according to a standard procedure [25]. λ DNA-*Hin*dIII digest was used as size marker. DNA fragments were transferred overnight by blotting to nitrocellulose membrane. Hybridization was carried out overnight with DNA probes for PKC α and PKC β in the conditions used for Northern blot analysis. Autoradiograms were quantitated by scanning and integration using Joyce and Loebl densitometer, model Chromoscan 3.

2.4. *Immunoblot analysis of PKC α and PKC β isoforms*

Deep frozen tumors and muscle were homogenized in 20 mM Tris buffer (pH 7.4) containing 10 mM EGTA, 2 mM EDTA, 0.5% Nonidet P-40, 2 mM PMSF, and 10 mg/ml leupeptin. Detergent-soluble protein extracts were fractionated by SDS-PAGE and transferred to nitrocellulose membrane overnight by electrophoretic transfer. Nonspecific binding was blocked by incubating the blots with 10% horse serum containing 5% dried milk for 1 h and soaked twice for 15 min in 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 0.05% (v/v) Tween 20. Membranes were incubated for 2 h with 1 mg/ml PKC α-specific monoclonal antibody MC-3a [24] or polyclonal anti PKC β-specific antipeptide diluted 1:100 [10]. Washed blots were then incubated for 1 h with horseradish peroxidase conjugated sheep anti-mouse IgG (PKC α) or goat anti-rabbit IgG (PKC β) diluted 1:2000. The blots were developed using ECL Western blotting detection (Amersham). Rat brain PKC, purified as previously described [25] migrated in parallel.

3. RESULTS

Northern hybridization analysis of poly(A⁺) RNA showed striking changes in the expression of PKC α-related transcripts in rhabdomyosarcoma-derived cells when compared to skeletal muscle where two transcripts of 9.5 and 3.4 kb were present (Fig. 1). In Sy⁻ cells, the smaller transcript was overexpressed whereas both transcripts were barely detectable, even after a 2-day exposure in Sy⁺ cells. PKC β was poorly expressed in muscle and tumors. However, the expression of the two transcripts (10.5 and 2.7 kb) was enhanced in Sy⁺ cells when compared to that in skeletal muscle (Fig. 1). The weak 3.4 kb band detected by the PKC β probe only in Sy⁻ cells was likely due to a non-specific hybridization of this probe to the PKC α 3.4 kb transcript overexpressed in these cells. The PKC ε-related transcript (7.0 kb) was detected in skeletal muscle. This transcript was less strongly expressed in rhabdomyosarcoma-derived cells than in muscle, and was virtually undetectable in Sy⁺ cells. PKC γ was not expressed in any sublines. No transcript could be observed even after 5-day exposure.

Southern blot analysis of *Eco*RI-digested DNA from these materials showed an increased hybridization in Sy⁻ cells with PKC α probe whereas PKC β probe hybridization was similar in all cells. Fig. 2A and B display the autoradiograms of major DNA fragments of 13.8 kb and 8.5 kb detected with PKC α and PKC β probes,

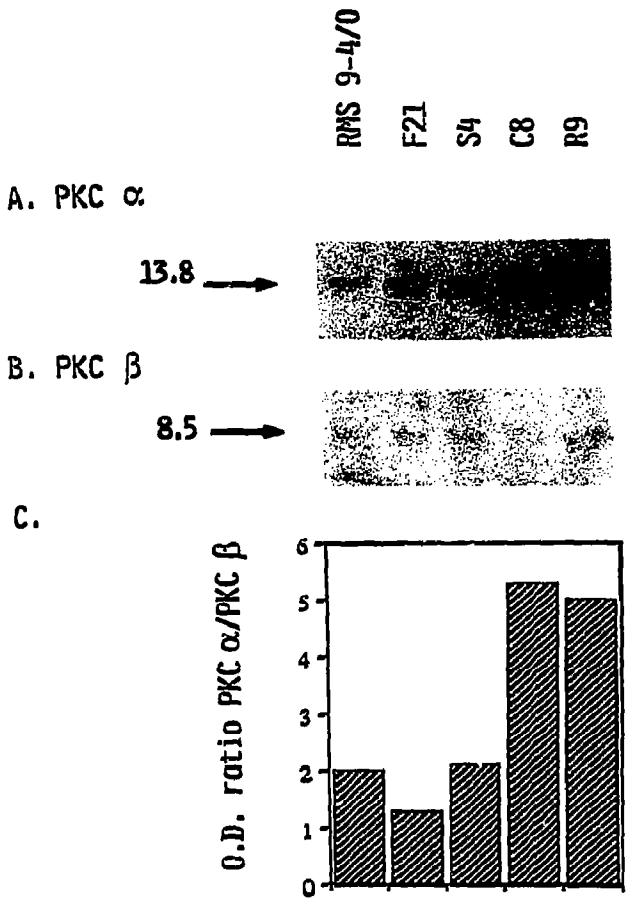

Fig. 2. PKC α and PKC β cDNA hybridization to *Eco*RI restricted DNA from muscle, Sy^+ (RMS 9-4/0, F21, S4) and Sy^- cells (C8, R9). *Eco*RI-restricted DNA fragments were resolved by agarose gel electrophoresis and hybridized to randomized ^{32}P-labeled PKC α and PKC β cDNA probes as described in the legend to Fig. 1. The two probes were hybridized to the same blots which were exposed for 24 (PKC α) and 48 h (PKC β). A and B: major DNA fragments related to PKC α and PKC β of 13.8 and 8.5 kb, respectively. Autoradiograms were scanned by densitometry and the ratio PKC α/PKC β probe hybridization is displayed in C.

respectively. Hybridization was quantitated by densitometric scanning and the ratio PKC α/PKC β suggested that the PKC α gene is 3-fold amplified in Sy^- cells, unless chromosomal abnormalitites are present (Fig. 2C).

Immunoblotting experiments were performed in muscle, F21 and R9 cells, using antibodies raised against isoforms α and β. As a control, we used PKC purified from rat brain which expressed all isoforms. As shown in Fig. 3, Sy^- cells R9 strongly expressed PKC α while we failed to detect the enzyme in muscle and Sy^+ cells F21. In contrast PKC β was expressed similarly in F21 and R9 cells, while this isoform was not detectable in muscle. However, several muscle proteins ($>10^5$ kDa) were detected with antibody raised against PKC β.

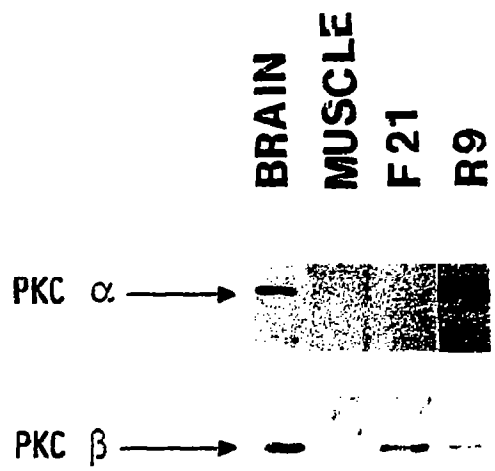

Fig. 3. Immunoblot analysis of detergent-soluble protein extracts (approximately 100 μg protein) from muscle, Sy^+ F21 cells, Sy^- R9 cells and 20 ng of purified rat brain PKC with specific antibodies raised against PKC α (A) or PKC β (B). The arrow indicates the major reactive species the size of which is 80 kDa.

4. DISCUSSION

This study provides the first demonstration of a correlation between the expression of some PKC isoforms in a series of rat rhabdomyosarcoma-derived sublines and tumor cell growth in syngeneic animals. Northern blot analysis has shown that Sy^- cells which do not grow in syngeneic animals overexpressed PKC α whereas the expression of this isoform was decreased in Sy^+ cells. Interestingly, one of us (N.H.) recently reported that Sy^- but not Sy^+ cells, strongly expressed *c-sis* related transcripts which encode for the B-chain of PDGF [20]. The observed changes in the expression of PKC α and PKC β are consistent with the characteristics of fibroblast R6 cells overproducing PKC β [17] or PKC α [18]. Indeed, R6 cell derivatives carrying an integrated copy of PKC β_1 cDNA insert, became partially or completely transformed upon overproduction of PKC β_1, while cell derivatives carrying the PKC α cDNA insert failed to grow in soft agar, though these cells strongly expressed PKC α. However, it should be stressed that the induction of individual PKC isoform-specific phenotypic changes appears to be cell line- and/or tissue-specific, since overexpression of PKC β_1 in the colon cancer cells HT29 suppressed the neoplastic phenotype [19].

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REFERENCES

[1] Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. (1982) J. Biol. Chem. 257, 13193–13196.
[2] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[3] Fournier, A. and Murray, A.W. (1987) Nature 330, 767–769.
[4] Yoshida, Y., Huang, F.L., Nakabayashi, H. and Huang, K.P. (1988) J. Biol. Chem. 263, 9868–9873.
[5] Cooper, D.R., Watson, J.E., Avecedo-Duncan, M., Pollet, R.J., Standaert, M.L. and Farese, R.V. (1989) Biochem. Biophys. Res. Commun. 161, 327–334.
[6] Fournier, A., Hardy, S.J., Clark, K.J. and Murray, A.W. (1989) Biochem. Biophys. Res. Commun. 161, 556–561.
[7] Ken-Ichi, K., Yasuhiro, K., Hisashi, F., Masatoshi, H., Hiroyoshi, H., Yasuo, F. and Yoshimi, T. (1989) Biochem. Biophys. Res. Commun. 161, 1020–1027.
[8] Castagna, M. (1987) Biol. Cell 59, 3–14.
[9] Castagna, M. and Martelly, I. (1990) Bull. Cancer 77, 489–494.
[10] Makowske, M., Ballester, R., Cayre, Y. and Rosen, O.M. (1988) J. Biol. Chem. 263, 3402–3410.
[11] Obeid, L.M., Okazaki, T., Karolak, L.A. and Hannun, Y.A. (1990) J. Biol. Chem. 265, 2370–2374.
[12] McSwine-Kennick, R.L., McKeegan, E.M., Johnson, M.D. and Morin, M.J. (1991) J. Biol. Chem. 266, 15135–15143.
[13] Guillem, J.G., O'Brian, C.A. Fitzer, C.J., Forde, K.A., Logerfo, P., Treat, M. and Weinstein, I.B. (1987) Cancer Res. 47, 2036–2039.
[14] Baum, C.L., Wali, R.K., Sitrin, M.D., Bolt, M.J.G. and Brasitus, T.A. (1990) Cancer Res. 50, 3915–3920.
[15] O'Brian, C.A., Vogel, V.G., Singletary, S.E. and Ward, N.E. (1989) Cancer Res. 49, 3215–3217.
[16] Hagiwara, M., Hachiya, T., Watanabe, M., Usuda, N., Iida, F., Tamai, K. and Hidaka, H. (1990) Cancer Res. 50, 5515–5519.
[17] Housey, G.M., Johnson, M.D., Hsiao, W.-L.W., O'Brian, C.A., Murphy, J.P., Kirschmeier, P. and Weinstein, I.B. (1988) Cell 52, 343–354.
[18] Borner, C., Filipuzzi, I., Weinstein, I.B. and Imber, R. (1991) Nature 353, 78–80.
[19] Choi, P.M., Tchou-Wong, K.-M. and Weinstein, I.B. (1990) Mol. Cell Biol. 1, 4650–4657.
[20] Sweeney, F.L., Pot-Deprun, J., Poupon, M.F. and Chouroulinkov, I. (1982) Cancer Res. 42, 3776–3782.
[21] Hanania, N., Boyano, M.D., Mangin, C., Poupon, M.F. (1991) Anticancer Res. 11, 473–480.
[22] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, p. 202, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
[23] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[24] Onoda, K., Hagiwara, M., Hachiya, T., Usuda, N., Nagata, T. and Hidaka, H. (1990) Endocrinology 126, 1235–1240.
[25] Da Silva, C., Fan, X.T., Martelly, I. and Castagna, M. (1990) Cancer Res. 50, 2081–2087.