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Expression of protein kinase C-alpha (PKC-α) and MYCN mRNAs in human neuroblastoma cells and modulation during morphological differentiation induced by retinoic acid

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It is known that PKC is differently expressed in brain and the peripheral nervous system and is involved in cellular differentiation. We have analyzed 9 human neuronal-derived crest-cell lines for PKC-α mRNA. Seven out of nine expressed 9.0 kb and 4.0 kb PKC-α mRNAs, but three had high level of 9.0 kb transcription. The different expression of the two messenger RNAs may result from alternative splicing and a different degree of cell maturation. The same cell lines were studied for MYCN gene expression. A possible relation between the two genes is discussed. One cell line expressing high levels of both PKC-α mRNA was treated with 10^{-6} M retinoic acid (RA). The expression of both messenger RNAs was suppressed when the cells achieved a morphological differentiation and showed neurite-like processes. A decrease of PKC-α gene expression was associated to down regulation of MYCN mRNA. These preliminary results suggest that PKC suppression of PKC-α mRNA is associated with reversion of the malignant phenotype.

Neuronal differentiation; Human neuroblastoma; PKC-α; Gene expression; Retinoic acid

1. INTRODUCTION

Protein kinase C (PKC), a phospholipid- and calcium-dependent kinase, is the natural cellular receptor for the phorbol ester tumor promoter (TPA). PKC is involved in many functions including control of cell proliferation [1]. There is also evidence that PKC is involved in transformation caused by oncogenes in vitro [3–5] and persistent activation of PKC has been associated with a transformed phenotype [6]. The PKC-α gene is coded by multifamily genes since at least 3 different genes were isolated from bovine, rat, and human cDNA libraries [7–9]. Different PKC-α mRNAs have been demonstrated in rat and in human cells and probably the related protein have different functions [7,10,11]. In the rat at least two different mRNAs of 9.0 and 4.0 kb have been identified and their different expression in various anatomical regions suggest distinct functions [10]. Northern blot analysis shows PKC-α mRNA in the brain and in the spinal cord of the rat and in the brain but not in the spinal cord of the monkey (reviewed in [1]).

We have investigated the PKC-α gene expression in 9 human neuroblastoma cell lines; a neuroblastoma being a tumor derived from the neuroectodermal tissue which very often expresses MYCN oncogene. Two PKC-α mRNAs, respectively of 9.0 and 4.0 kb were differently expressed in these tumor cells. PKC-α expression was found in 7 out of 9 cell lines. To investigate if the PKC-α expression is maintained during cell differentiation we have also studied the expression of PKC-α in SK-N-BE(2)C cells treated with retinoic acid (RA). In fact, it is known that neuroblastoma cells can be induced to

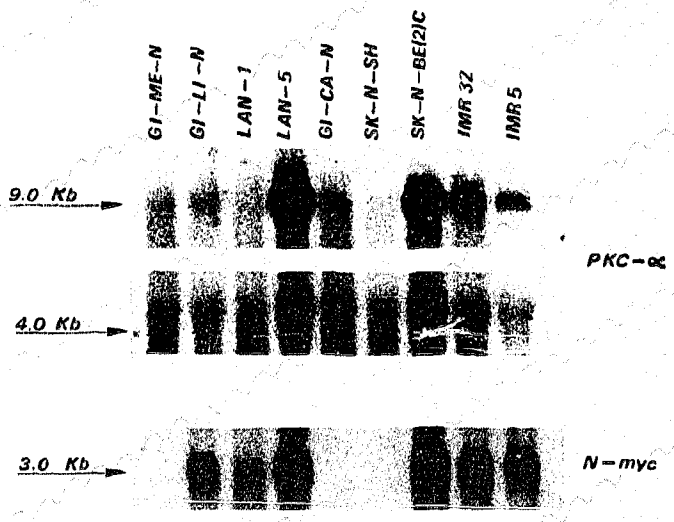

Fig. 1. Expression of PKC-α and MYCN genes in human neuroblastoma cell lines.

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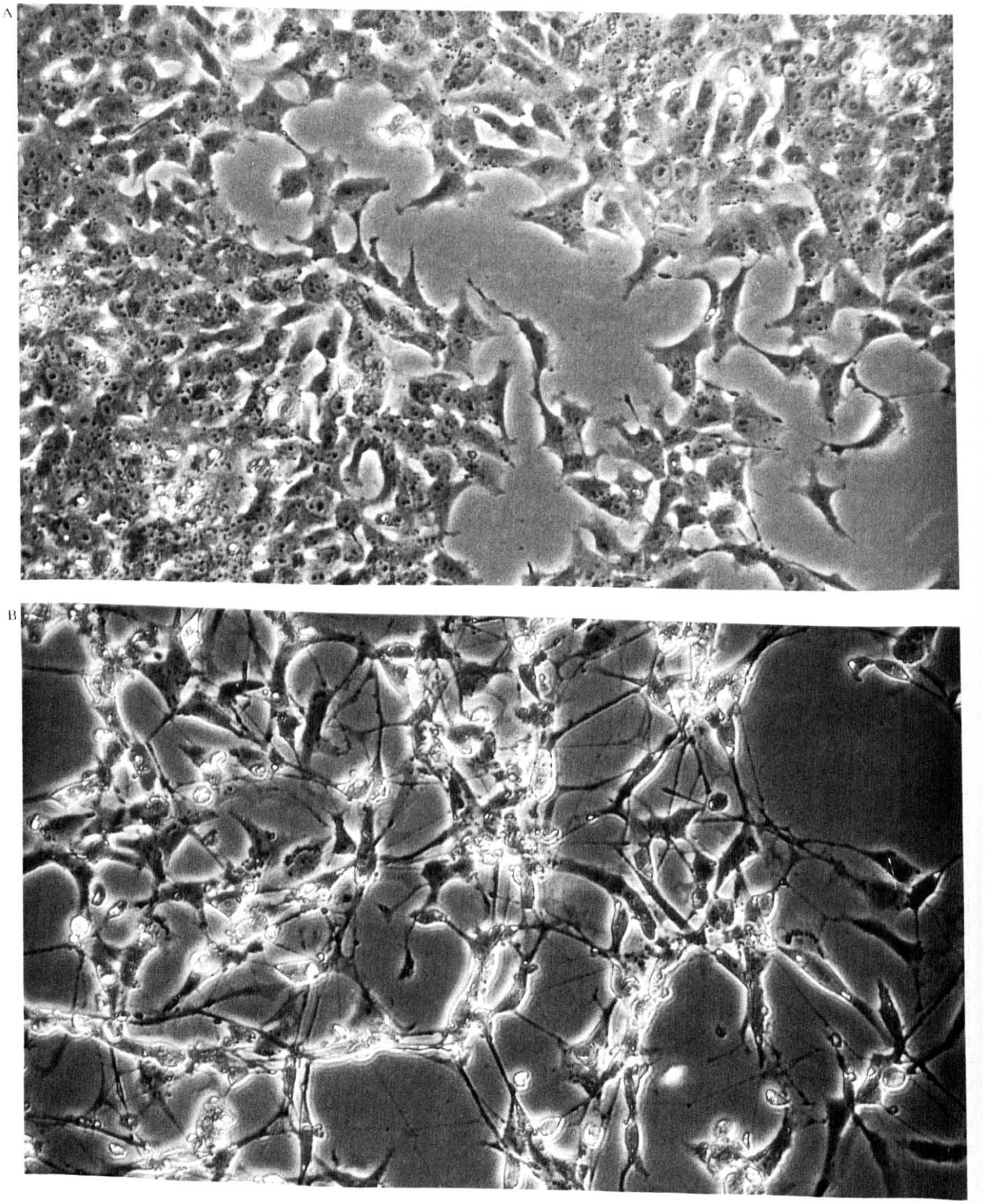

Fig. 2. Morphological change and neuritis outgrowth in SK-N-BE(2)C cells treated with 10^{-5} M RA. A. Control; B. RA-treated. Magnifications 40×.

differentiate in vitro employing several chemical or natural agents [12-14]. Our results show that PKC-α is not expressed in all neuroblastoma cells suggesting a different molecular pattern of PKC-α transcription in this tumor. Moreover, PKC-α mRNA is inhibited during neuroblastoma cell differentiation and it appears that PKC-α is involved in neural cell differentiation.

2. MATERIALS AND METHODS

2.1. *Cell lines and cell cultures*

LAN-1, LAN-5, (provided by Dr R. Seeger, CA), IMR-5, IMR-32, SK-N-SH, SK-N-BE(2)C (provided by Dr V. Ciccarone and J. Biedler, NY), GI-ME-N, GI-CA-N, and GI-LI-N [15-17] cell lines were cultured in RPMI 1640 medium (Gibco, Scotland, UK) supplemented with 15% fetal calf serum (FCS) (Gibco, Scotland, UK), 2% penicillin-streptomycin (Flow Laboratories, Milano, Italy), 1% glutamine (Gibco). SK-N-BE(2)C (3×10^6 cells/flask 170 cm^2, Becton-Dickinson, NY) were treated twice with 10^{-7} M RA (Sigma, St. Louis, MO) and observed through a contrast-phase microscope until the morphological differentiation was achieved. The cells were mycoplasma-free (Mycoplasma test kit Dapi, Boehringer-Mannheim, West Germany).

2.2. *RNA Northern blot analysis*

RNAs were extracted from cell cultures as previously described [18]. Briefly, 20 mg of total RNA was run in a 1.2% formaldehyde agarose gel electrophoresis and transferred onto nitrocellulose filters (BRL, Bethesda, USA). Filters were hybridized with [α-^{32}P]dCTP (3000 mCi/mM, Amersham, UK) *Eco* RI fragment labeled human PKC-α (provided by Dr J. Knopf, Genetics Inst.) at 42°C with 50% formamide, washed and exposed to Kodak S-Omat film. The filters were subsequently boiled and hybridized again with [α-^{32}P]dCTP MYCN 2.0 *Eco* RI fragment from plasmid pUC9-N-myc (NB-19-21) provided by Dr F. Alt (Columbia University, NY) and again with [α-^{32}P]dCTP 28S ribosomal RNA (rRNA) probe and finally exposed at the same conditions described above. All probes were labeled with [α-^{32}P]dCTP using the random priming technique [19] employing a Multiprime DNA labeling system (Amersham, UK).

3. RESULTS

Expression of PKC-α mRNA in human neuroblastoma cell lines

Different neuroblastoma cell lines were tested for expression of PKC-α mRNA. The results of a representative experiment are shown in Fig. 1. Every cell line tested expressed a 9 kb and 4 kb PKC-α transcript to a variable degree. LAN-5 and SK-N-BE(2)C demonstrated the highest level of expression, in two cell lines (LAN-1 and SK-N-SH) the 9 kb band was either negative or at the limit of detection depending on the experiment. Interestingly, the cell lines could be divided into two categories on the bases of the relative expression of the two mRNA species. In 4 of them (GI-ME-N, LAN-1, GI-CA-N, SK-N-SH) the 4 kb band was more intense than the 9 kb band; in the remaining 6 the 9 kb band was similar (GI-LI-N) or predominant. These results demonstrated that neuroblastoma cell lines express PKC-α mRNA and differ in the relative expression of the two main transcripts.

The same cell lines were tested for the expression of MYCN. The results shown in Fig. 1 indicate that MYCN was expressed in but 6 of the 9 cell lines tested. Among the MYCN-positive cells, LAN1 consistently expressed MYCN mRNA 10-50 times less than the other cell lines. It is interesting that a strong expression of MYCN was associated with the predominant expression of the 9 kb PKC-α mRNA while poor or no expression of MYCN was found in the cell lines expressing predominantly the 4 kb PKC-α mRNA. The same blot hybridized to a 28S rRNA probe confirmed that equal amounts of total RNA were blotted. These results suggest a correlation between 9 kb PKC-α mRNA and MYCN expression.

The SK-N-BE(2)C cell line was selected for further studies on the expression of PKC-α mRNA during the neuronal differentiation induced by RA. RA induces differentiation of SK-N-BE(2)C cells as shown by the induction of neurite formation (Fig. 2). A differentiated phenotype became evident after 2 days of exposure to RA and reached a maximum after 5 to 7 days depending on the experiment. The differentiation process was associated with a concomitant decrease in the expression of PKC-α and MYCN mRNA (Fig. 3). The

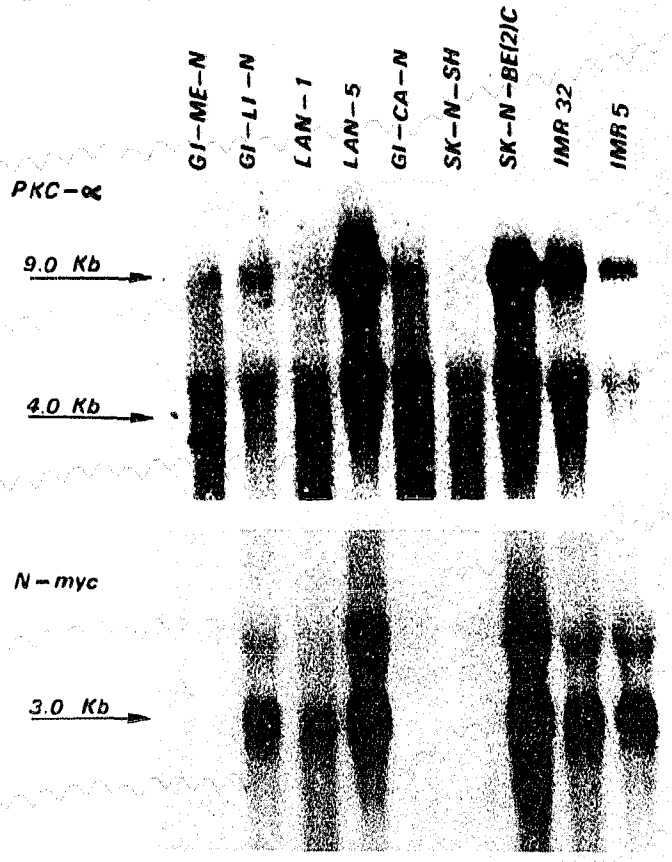

Fig. 3. Changes at 24, 48 and 72 h (from right to left) of the expression of PKC-α and MYCN in SK-N-BE(2)C treated with RA. The 28S rRNA expression was used to check the amount of mRNAs loaded on the gel.

9 and 4 kb PKC-α mRNAs were undetectable after 2 days of exposure of the cells to RA. A decrease in MYCN, but not in PKC-α mRNA expression was also observed in control cultures when they reached confluence after about 3 days of culture. However, after two days of culture a clear decrease of MYCN expression in RA-treated cells relative to controls was evident.

4. DISCUSSION

Our studies on the expression of PKC-α mRNA demonstrated a variable degree of suppression of this gene among various neuroblastoma cell lines, perhaps reflecting the heterogeneity of these cells. The expression of MYCN was also variable with 5 lines expressing high levels, one low level and 3 undetectable levels of MYCN mRNA. However, a comparison between MYCN and PKC-α mRNA levels in the different cell lines indicated the interesting association between high expression of MYCN mRNA and predominant expression of the 9 kb, relative to the 4 kb band of PKC-α mRNA. There are indications that the two species of PKC-α mRNA may derive from alternative splicing and/or processing of PKC-α mRNA precursors and that both species code for a 76 kDa polypeptide recognized by anti-PKC antibodies. Our results may indicate that different pathways of PKC-α mRNA are predominantly utilized by individual neuroblastoma lines. The fact that the pathway of maturation of PKC-α mRNA to the 4 kb species may also inhibit MYCN expression is an interesting possibility that is presently under investigation. However, a more intensive analysis of other neuroblastoma cell lines is necessary to support the hypothesis of a casual relationship between the two phenomena.

A recent report indicates that PKC plays a key role in the control of neural cell differentiation. Inhibition of PKC by 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H7) is associated with neurite outgrowth in murine neuro-2a cells and in human neuroblastoma cells [20,21]. Previous reports indicate that PKC is inhibited by treatment with RA in transformed rat cell lines [22] and in mouse neuroblastoma cell lines [23]. We employed RA treatment to induce neural differentiation of the SK-N-BE(2)C.72 h after continuous RA treatment the transcription of both PKC-α mRNAs were completely inhibited and MYCN mRNA was detectable at low levels. SK-N-BE(2)C cells changed dramatically their morphology, and neurite structures became evident like in the mature neuron with several interconnections. Thiele et al. [24] and our previous observation [25] indicated that the decrease of MYCN gene expression was not related to RA-induced growth arrest. Data reported here confirm that the down-regulation of MYCN expression was associated with the neurite formation and neural differentiation. Moreover, PKC-α gene expression is inhibited during neuronal differentiation. PKC-α mRNA decreases to undetectable levels during the RA-induced differentiation of NB cells. We suggest that down-modulation of PKC-α gene expression is associated with the reversion of malignant neuroblast to a more mature neuronal-like phenotype.

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REFERENCES

[1] Nishizuka, Y. (1988) Nature 334, 661-665.
[2] Weinstein, B. (1987) Cell. Biochem. 33, 213-214.
[3] Nishizuka, Y. (1984) Nature 308, 693-698.
[4] Ashendel, C.L. (1985) Biochim. Biophys. Acta 822, 219-242.
[5] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
[6] Fleishman, L.F., Chahawala, S.B. and Cantley, L. (1986) Science 231, 407-410.
[7] Parker, P.J., Coulens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233, 853-859.
[8] Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U., Ullrich, A. (1986) Science 233, 859-866.
[9] Knopf, J.L., Lee, M.H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Hewick, R.M. and Bell, R.M. (1986) Cell 46, 491-502.
[10] Brandt, S.J., Niedel, J.E., Bell, R.M. and Young III, W.S. (1987) Cell 49, 57-63.
[11] 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.
[12] Sidell, N., Altman, A., Haussler, M.A. and Seeger, R.C. (1983) Exp. Cell. Res. 148, 21-30.
[13] Parodi, M.T., Varesio, L. and Tonini, G.P. (1989) Cancer Chemother. Pharmacol. 25, 114-116.
[14] Di Martino, D., Avignolo, C., Marsano, B., Di Vinci, A., Cara, A., Giaretti, W. and Tonini, G.P. (1990) Cancer Lett. 52, 101-106.
[15] Donti, E., Longo, L., Tonini, G.P., Verdona, G., Melodia, A., Lanino, E. and Cornaglia-Ferraris, P. (1988) Cancer Genet. Cytogenet. 30, 225-231.
[16] Cornaglia-Ferraris, P., Ponzoni, M., Montaldo, P., Mariottini, G.L., Donti, E., Di Martino, D. and Tonini, G.P. (1990) Pediatr. Res. 27, 1-6.
[17] Cornaglia-Ferraris, P., Sansone, R., Mariottini, G.L., Longo, L. and Tonini, G.P. (1990) Int. J. Cancer 45, 578-579.
[18] Tonini, G.P., Radzioch, D., Gronberg, A., Clayton, M., Blasi, E. and Varesio, L. (1987) Cancer Res. 47, 4544-4547.
[19] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13.
[20] Minana, M.D., Felipo, V. and Grisolia, S. (1989) FEBS Lett. 255, 184-186.
[21] Parodi, M.T., Varesio, L. and Tonini, G.P. (1990) FEBS Lett. 269, 4-6.
[22] Vanier, M.C., Banerijee, D. and Mukherjee, B.B. (1988) FEBS Lett. 241, 154-158.
[23] Vada, H., Ohono, S., Kubo, K., Taya, C., Tsuji, S., Yonehara, S. and Suzuki, K. (1989) Biochem. Biophys. Res. Commun. 165, 533-538.
[24] Thiele, C.J., Reynolds, C.P. and Israel, M.A. (1985) Nature 313, 404-406.
[25] Di Martino, D., Ponzoni, M., Cornaglia-Ferraris, P. and Tonini, G.P. (1990) Cell. Mol. Neurobiol. 10, 459-470.