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The differential expression of protein kinase C genes in normal human neonatal melanocytes and metastatic melanomas

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Expression of protein kinase C (PKC) genes (α , β , γ and ϵ) was measured in cultured normal human neonatal melanocytes and metastatic melanoma cell strains. Three of the PKC isotypes (α , β and ϵ) were constitutively expressed in neonatal melanocytes. Protein kinase C β RNA transcripts were induced in neonatal melanocytes cultivated in medium with serum and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In contrast, PKC α and ϵ RNA transcripts were detected in melanocytes cultivated in medium without serum and TPA, but were repressed in melanocytes cultivated in medium with serum and TPA. Only PKC α and ϵ RNA transcripts were detected in the melanoma cell strains and the PKC RNA transcript expression levels varied among the five metastatic melanomas. In four metastatic melanoma cell strains, PKC α and ϵ RNA transcript expression levels were repressed by serum, but in one melanoma cell strain, PKC α and ϵ RNA transcript expression levels were induced by serum. Protein kinase C γ RNA transcripts were not detected in either the melanocytes or melanoma cell strains. These data suggest an alteration of PKC isotype gene expression in the progression of primary melanocytes to metastatic melanoma. The absence of the PKC β RNA transcripts and altered expression of PKC α and ϵ isotypes in particular may be a feature in the transformation of human primary melanocytes.

Introduction

Within the past decade, the cultivation of human primary melanocytes *in vitro* has become possible. The requirements for growth were phorbol esters (e.g. 12-*O*-tetradecanoylphorbol-13-acetate, TPA*), fetal calf serum, calcium ions, agents that elevated cAMP levels, insulin, epidermal growth factor and basic fibroblast growth factor (1,2).

The intracellular receptor of phorbol esters is protein kinase C (PKC). This enzyme is a serine/threonine protein kinase which interacts with calcium ions, phospholipids and diglycerides to form a complex associated with a cellular membrane structure (3). PKC represents a multigene family and seven different cDNA clones have been isolated to date (4). Northern blot analysis has suggested tissue-specific expression of the PKC subspecies (5–7).

PKC has been implicated in the regulation of many cellular processes including growth, differentiation, neuronal function and gene expression (4,8,9). Although the role of each PKC isotype in cellular processes is unknown, investigations of the over-

expression of a PKC gene (10,11) or expression of a mutated PKC gene in mouse fibroblasts (12) have demonstrated either altered growth regulation or complete transformation of the transfected cells. Protein kinase C has also been implicated in the *in vitro* transformation processes induced by the oncogenes *ras*, *sis*, *fms*, *src*, *fps* and *fes* (13–15). Researchers have observed either elevated levels of *sn*-1,2-diacylglycerol or phosphorylation of a transformation-related protein and a PKC substrate in cells transformed by these oncogenes.

To investigate the role of the PKC isotypes in the transformation of human primary neonatal melanocytes, Northern blot analysis was carried out on total RNA isolated from human primary neonatal melanocytes and metastatic melanoma cell strains. We demonstrate the expression of PKC α , β and ϵ RNA transcripts in proliferating melanocytes. In contrast, PKC β RNA transcripts were undetectable in human metastatic melanomas, and were not inducible by serum.

Materials and methods

Culture of melanocytes

The method used is a combination of the procedures developed by Eisinger and Marko (16) and Halaban and Alfano (17). Foreskin samples were collected from newborn infants, melanocytes isolated, and transferred to a T-75 flask. Primary newborn melanocytes were cultivated in MCDB 153 medium (Irvine Sci., Irvine, CA) as described by Halaban *et al.* (1) and modified by Kath *et al.* (18) and designated melanocyte complete medium. Fibroblast contamination was suppressed by adding geneticin (250 μ g/ml) to the growing medium for 2 days. Melanoma cell strains (c81-46a, c81-46c, c81-61, c81-61x and c83-2cy) were cultured in F-10 with 5% fetal calf serum, 5% calf serum, 1% glutamine, penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (19) and designated as melanoma complete medium. The passage number for the various cell strains used in these experiments was <8. Cell strains c81-46a, c81-46c, c81-61 and c82-2cy have been previously described (19–21). Cell strain c81-61x was isolated from a nude mouse following injection of the parental metastatic melanoma c81-61. Melanoma cell strains were initially cloned through soft agar and will form tumors in nude mice. Viable cell counts were determined by trypan blue exclusion.

Experimental conditions

Primary melanocytes and melanoma cells were cultivated in melanocyte complete medium or melanoma medium respectively, until 70–80% confluent. Melanocytes were washed and incubated with prewarmed medium with or without serum and TPA. Melanoma cells were incubated with prewarmed medium with or without serum. Incubation of melanocytes in medium without serum and TPA and melanomas in medium without serum induces the cells to proliferate at a very low rate. There was no change in cell morphology when the human melanocytes and melanomas were cultivated in the medium without either serum and TPA or serum for 24 h. Melanocytes are 80% viable when cultivated in medium without serum and TPA for 1–3 days. Melanomas are 100% viable if kept in medium without serum for 24 h. Following incubation for 24 h, the cells were isolated and total RNA was recovered.

Isolation of RNA and Northern blot analysis

The procedure was a modification of that described by Chirgwin *et al.* (22). Two to six T-75 flasks were used per growth condition. The cells were pelleted and lysed using a 4 M guanidine isothiocyanate/1% Sarkosyl solution. The sheared homogenate was layered on top of a 5.7 M CsCl cushion and centrifuged at 55 000 r.p.m. for 3 h. The RNA pellet was resuspended in TE with 0.1% SDS, extracted, ethanol precipitated and resuspended in sterile water. The Northern blot was prepared as described by Fourney *et al.* (23). Total RNA (10 μ g/sample) was electrophoresed on a denaturing formaldehyde agarose gel, transferred by capillary action overnight to a nylon filter (Nytran, Schleicher and Schuell, Keene, NH), and prehybridized for 2–4 h at 42°C. The probe was labeled by method of Feinsberg and Vogelstein (24) using a random priming kit (Promega, Madison,

*Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MELC, murine erythroleukemia cells; HMBA, hexamethylene bisacetamide.

WT). Fresh hybridization solution containing 1×10^6 c.p.m./ml of radiolabeled probe was added to the filter as described by manufacturer's procedures. Hybridization was done overnight followed by two or three stringent washouts (50–60°C with 0.1% SDS and $0.1 \times$ SSC). The filter was exposed to Kodak X-OMAT film from 2 to 20 h at –80°C. The filters were stripped of probe by using two washes of 0.1% SDS, $0.1 \times$ SSC at 80°C. For quantitation of individual RNA transcripts, films were exposed for time periods which produced band intensity that was linear with respect to time. Films were then scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA) and RNA transcript abundance was determined from the area of the peak corresponding to each RNA transcript. Values for PKC gene expression were corrected for RNA loading using an 18S rRNA probe. RNA standards were used to determine RNA sizes (0.24–9.5 kb RNA ladder, BRL, Gaithersburg, MD).

Probes

The PKC α , β and γ probes have been described by Coussens *et al.* (25). The PKC α data were obtained with a 1.3 kb *EcoRI* restriction fragment from the plasmid, phPKC- α 7. An 0.9 kb *EcoRI* restriction fragment from the plasmid, phPKC- β 1-15-*EcoRI*2, was used to probe for PKC β expression. The PKC γ data was obtained with a 1.0 kb *BamHI* restriction fragment from the plasmid, phPKC- γ 6. The PKC ϵ data was obtained by isolating a 1.5 kb *PstI/EcoRI* restriction fragment from the plasmid, pmt-PKC- ϵ (26,27). The 18S probe was isolated by an *EcoRI* restriction digest and isolation of the 5.6 kb insert from the plasmid, pB (28).

Results

PKC α RNA transcript expression

The expression of the PKC α , β , γ and ϵ genes in human primary neonatal melanocytes and metastatic melanoma cell strains was measured. The densitometric scans of the Northern blots of the PKC data (Figures 1–4) are summarized in Table I.

Northern blot analysis indicated that the PKC α gene was expressed as two major RNA transcripts of 9.5 and 4.0 kb with the 9.5 kb RNA transcript more abundant. Primary melanocytes expressed both PKC α RNA transcripts in medium without serum and TPA (Figure 1). A decrease in the expression of the 9.5 kb RNA transcript (by 30%) and in the 4.3 kb RNA transcript (by 20%) was observed when the melanocytes were cultivated in medium with serum and TPA compared to medium without serum and TPA.

Expression of both PKC α RNA transcripts was different in the metastatic melanomas cultivated in medium with serum. Expression of PKC α 9.5 kb RNA transcript was repressed (45, 76 and 80% respectively) in melanoma strains (c81-46a, c81-46c and c81-61x) when the cells were cultivated in medium with serum compared to medium without serum. Expression of the PKC α 4.3 kb RNA transcript was also repressed in three melanoma strains [c81-46a (59%), c81-46c (72%) and c81-61x (49%)] when the cells were cultivated in medium with serum compared to medium without serum. In contrast, c83-2cy cells expressed both PKC α RNA transcripts at a similarly low level when the cells were cultivated in medium with or without serum. Interestingly, in c81-61 cells the expression of the PKC α RNA transcripts was induced [9.5 kb RNA transcript (83%), 4.3 kb RNA transcript (37%)] when the cells were cultivated in medium with serum compared to medium without serum.

Expression of both PKC α RNA transcripts was different in the melanomas compared to melanocytes. High basal expression levels of PKC α RNA transcripts were detected in cell strains c81-46a and c81-61x compared to primary melanocytes. An increase in the PKC α 9.5 kb RNA transcript in c81-46a cells (7.6-fold) and in c81-61x cells (5-fold) was observed in the melanomas cultivated in medium without serum compared to primary melanocytes cultivated in medium without serum and TPA. Also the PKC α 4.3 kb RNA transcript was increased in the melanomas [c81-46a (9.9-fold) and c81-61x (7.4-fold)] compared to primary melanocytes cultivated in medium without

Table I. Quantification of PKC RNA transcripts in primary melanocytes and metastatic melanomas

	PKC α		PKC β		PKC γ	PKC ϵ
	9.5 kb	4.3 kb	10.0 kb	4.0 kb		
Melanocytes						
–	1.0	1.0	1.0	1.0	ND	1.0
+	0.7	0.8	1.7	1.7	ND	0.3
Melanomas						
c81-46a –	7.6	9.9	ND	ND	ND	1.6
+	4.2	4.1	ND	ND	ND	0.8
c81-46c –	4.2	3.5	ND	ND	ND	0.7
+	1.0	1.0	ND	ND	ND	0.2
c81-61 –	0.6	2.7	ND	ND	ND	1.1
+	1.1	3.7	ND	ND	ND	1.5
c81-61x –	5.0	7.4	ND	ND	ND	2.9
+	1.0	3.8	ND	ND	ND	0.9
c83-2cy –	0.2	0.5	ND	ND	ND	1.0
+	0.2	0.6	ND	ND	ND	0.8

Primary melanocytes were cultivated in medium without serum and TPA (–) or with serum and TPA (+). Metastatic melanoma cell strains were cultivated in medium without serum (–) or with serum (+). Values are standardized to PKC RNA transcript expression levels in primary melanocytes cultivated in medium without serum and TPA. ND, not detected.

Fig. 1. Expression of PKC α RNA transcripts in various growth medium for primary melanocytes and metastatic melanomas. Cells were cultivated and treated for 24 h in various conditions described in Materials and methods. Melanocytes were cultivated in complete medium with (+) or without serum and TPA (–). Melanomas were cultivated in medium with (+) or without serum (–). Total RNA (10 μ g) was hybridized with the 32 P-labeled restriction of the PKC α cDNA insert. Numbers on the left show the positions of migration by RNA standards with indicated kilobase length.

serum and TPA. In melanoma c83-2cy, expression of the PKC α RNA transcripts was reduced. Reduction in the expression of the PKC α RNA transcripts [9.5 kb (80%), 4.3 kb (at least 30%)] were detected in cell strain c83-2cy cultivated in medium with or without serum compared to melanocytes cultivated in medium without serum and TPA.

Passage of cultured melanoma cells (c81-61 cell strain) through a nude mouse changed the expression level of PKC α RNA transcripts. A similar level of both PKC α RNA transcripts was detected in c81-61x cells compared to c81-61 cells cultivated in

Fig. 2. Expression of PKC β RNA transcripts in various growth medium from primary melanocytes and metastatic melanomas. Experimental details are similar to those given in the legend to Figure 1, except that the RNA samples were probed with a PKC β cDNA insert.

Fig. 3. Expression of PKC ϵ RNA transcripts in various growth medium from primary melanocytes and metastatic melanomas. Experimental details are similar to those given in the legend to Figure 1, except that the RNA samples were probed with a PKC ϵ cDNA insert.

medium with serum. However, an increase in the PKC α RNA transcripts [9.5 kb RNA transcript (8.3-fold), 4.3 kb RNA transcript (2.7-fold)] was detected in c81-61x cells compared to c81-61 cells when both melanoma cell strains were cultivated in medium without serum.

PKC β RNA transcript expression

Expression of PKC β RNA transcripts were detected only in primary melanocytes. The PKC β 4.2 and 10 kb RNA transcripts were observed with similar expression levels for both transcripts. An increase (70%) in the expression of the PKC β 4.2 and 10 kb RNA transcripts was detected in melanocytes cultivated in medium with serum and TPA compared to medium without serum and TPA. No PKC β RNA transcripts were detected in any of the melanomas cultivated in medium with or without serum.

PKC γ RNA transcript expression

The expression of a 3 kb PKC γ RNA transcript was examined in melanocytes and melanomas. A 3 kb PKC γ RNA transcript was detected in rat brain RNA, which served as a positive control (data not shown). No 3 kb PKC γ RNA transcripts were detected in melanocytes cultivated in medium with or without serum and TPA nor in any of the metastatic melanomas cultivated in medium with or without serum (data not shown).

PKC ϵ RNA transcript expression

The 7.1 and 9.3 kb PKC ϵ RNA transcripts were observed in the adult rat brain and glioblastoma U138. Expression of PKC ϵ RNA transcripts was detected in primary melanocytes and metastatic melanoma cell strains. Only the 7.1 kb RNA transcript was detected in primary melanocytes and metastatic melanoma cells. Protein kinase C ϵ RNA transcripts were observed in melanocytes cultivated in medium without serum and TPA, but were repressed when the melanocytes were cultivated in medium with serum and TPA and were not detectable.

Expression of PKC ϵ RNA transcripts was also detected in melanomas. Melanoma c81-61 expressed higher levels of PKC ϵ RNA transcripts (by 36%) when cultivated in medium with serum compared to medium without serum. Repression of PKC ϵ RNA transcript expression levels was observed in the other four melanomas [c81-46a (50%), c81-46c (72%), c81-61x (69%) and c83-2cy (20%)] cultivated in medium with serum compared to medium without serum. Protein kinase C ϵ RNA transcripts were faintly detected in c81-46c cells when cultivated in medium with serum.

Discussion

We have investigated the expression of PKC (α , β , γ and ϵ) RNA transcripts in human primary neonatal melanocytes and metastatic melanoma cell strains. The constitutive expression of three PKC isotypes (α , β and ϵ) was measured in normal human melanocytes. PKC α and β RNA transcripts were detected in melanocytes cultivated in medium with or without serum and TPA. PKC γ RNA transcripts were not observed in primary melanocytes cultivated in medium with or without serum and TPA. PKC ϵ RNA transcripts were detected in melanocytes cultivated in medium without serum and TPA, but were undetectable when the cells were cultivated in medium with serum and TPA.

The expression of PKC isotypes in metastatic melanoma cell strains was different from normal human melanocytes. PKC β and γ RNA transcripts were undetectable using Northern blot analysis in any of the melanomas cultivated in medium with or without serum. Weinstein has shown that overexpression of the PKC β gene in murine fibroblast cell lines induced altered growth control (10). However, using HT29 colon cancer cells, overexpression of the PKC β gene was found to act as a tumor suppressor gene (29).

Studies on PKC genes have also found that the level of PKC β protein increases upon cell differentiation. An increase in PKC β protein was observed in HL-60 cells following differentiation induced by TPA, dimethyl sulfoxide and retinoic acid (30,31). Decreased expression of PKC β protein was observed in a HL-60 variant cell line resistant to TPA-induced differentiation (32). In murine erythroleukemia cells (MELC), hexamethylene bisacetamide (HMB) induced differentiation has been found to result in an increase in PKC β RNA transcript expression levels (33). Introduction of purified PKC β protein, but not purified PKC α protein into permeabilized MELC accelerates HMB

induced differentiation (34). Thus expression of the PKC genes could vary in their transforming or tumor suppressor/differentiation activity depending on the target cell. Potentially, the PKC β gene may act as a tumor suppressor gene in human melanomas. Further studies using transfection of the PKC β gene into human metastatic melanoma cells may elucidate the functional role of this gene.

Expression of the PKC α and ϵ isotypes were of three distinct patterns. In the first case (c81-46a, c81-46c and c81-61x), PKC α and ϵ RNA transcript expression levels were repressed when the melanomas were cultivated in medium with serum compared to medium without serum. In the second case (c83-2cy), PKC α and ϵ RNA transcript expression levels were expressed at a low level and may reflect another type of metastatic melanoma. This melanoma strain (c83-2cy) may utilize another signal pathway, since the expression of all four PKC isotypes were either undetectable (PKC β and γ) or expressed at a low level (PKC α and ϵ) relative to normal melanocytes. In the third case (c81-61), PKC α and ϵ RNA transcript levels were induced when the melanoma cell strain was cultivated in medium with serum. Melanoma cell strain c81-61 was initially isolated from a pregnant woman and may have been influenced by exogenous growth factors. We have also observed the induced expression of *c-fos* and *c-jun* oncogenes in this melanoma (D.T.Yamanishi *et al.*, in preparation), which suggests that genes can be induced via exogenous growth factors.

Interestingly, the expression of PKC isotype RNA transcripts was different in the cell strain c81-61 (isolated from soft agar) compared to cell strain c81-61x (isolated from a nude mouse). Although there was little difference in PKC gene expression for both melanomas cultivated in medium with serum, there were distinct changes in PKC gene expression when the melanomas were cultivated in medium without serum. In the low proliferation state (medium without serum), higher levels of PKC α and ϵ RNA transcripts were detected in c81-61x compared to c81-61. This change in PKC gene expression levels may allow a tumor to proliferate in low nutrient conditions; changes which may be required for growth in a nude mouse.

Similarities and differences were also detected in PKC gene expression in the melanoma cell strains isolated from independent sites within the same patient (c81-46a and c81-46c). Both tumor cell strains displayed a similar repression of PKC α and ϵ RNA transcripts when the cell strains were cultivated in medium without serum compared to medium with serum. However, cell strain c81-46a had higher levels (at least 100%) of PKC α and ϵ RNA transcripts compared to cell strain c81-46c. These data would suggest that due to tumor heterogeneity, different metastases display similarities in general aspects of gene expression, but each metastasis is distinct with respect to specific aspects of gene expression.

Investigation of PKC isotype expression in other cell types has shown different patterns (5–7,29). PKC enzyme activity was observed to be, in general, higher in normal or non-transformed cells compared to their malignant or transformed cell counterpart (35). Altered growth control has been observed in cells transfected with either a mutated PKC α gene or an overexpressed PKC α or γ gene (10–12,36). We speculate that the overexpression of the PKC α and ϵ genes, and the repression in the expression of the PKC β gene may be one step in the transformation of human melanocytes. Further investigation into the protein phosphorylation targets and the regulatory mechanisms of the PKC isotypes is necessary to elucidate the functional role of the PKC genes in the transformation of human melanocytes and their potential use as a target for clinical treatment.

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