Immunocytochemical detection of p21ras, Raf-1, ERK1/MAP kinase and PKC isoforms in a 20-methylcholanthrene-induced transformed murine embryonal fibroblast cells in culture

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An immunocytochemical study using antibodies against p21ras, Raf-1, MAP kinase/ERK1 and PKCα, β, γ, δ, ε, ζ, isoforms were performed on a 20-methylcholanthrene-induced transformed murine embryonal fibroblast cells in both *in vitro* and *in vivo* growth conditions. Altered expression of p21ras, Raf-1, MAP kinase in this particular cell line strongly supported the previous findings of the activation of one component of signal transduction under the influence of the other in the MAP kinase cascade of signal transduction during neoplastic transformation and which also seemed to be involved in CNCI-PM-20 cell line. The altered expression of PKCα, β, and δ was thought to be an epigenetic event occurring under the indirect influence of other changes in these cells. Host physiology and metabolism did not have much impact on the expression of these gene products after biological incubation of these cells in syngenic host.

Key words: Chemical carcinogenesis, components of MAP kinase cascade, PKC isoforms.

Overexpression of several genes implicated in the mitogen-activated protein kinase signaling cascade (mitogen-activated protein kinase, MEK-MAP kinase, Raf-1, Ras) seemed to be most likely responsible for initiated cells acquiring a proliferating phenotype, which facilitated the accumulation of structural changes in additional genes resulting in the generation of autonomously growing preneoplastic and neoplastic lesions [12]. The highly conserved family of ras genes in the MAP kinase cascade had been detected as transforming genes in a wide variety of naturally occurring tumors [16] and in *in vivo* and *in vitro* experimental models after carcinogenic insult [4, 32]. Although multiple ras effector pathways had been identified, the Raf protein kinases which lied downstream of ras were believed to be the primary mitogenic effectors [23] and also the raf protein were upregulators of the mitogen activated protein kinases (MAPK/Erk) [8, 11]. The constitutive upregulation of this pathway by oncogenic ras was thought to promote cellular transformation [4].

Protein kinase C, a ubiquitous family of eleven related isoforms were another group of signal transducing molecules deeply implicated in carcinogenesis [3]. Protein kinase C isoforms were usually divided into three main classes; classical PKC (α, β, γ), new PKC (δ, ε, η, θ) and novel PKC (ζ, λ/t). The level of total PKC and phosphorylated PKC got altered in several types of malignancies [3] and one important concomitant of PKC activation was the intracellular redistribution, overexpression and downregulation of different PKC isoforms in different compartment of the transformed cells in comparison to non transformed cells [21].

The present study was undertaken to identify the status of the various components of MAP kinase cascade (MAP kinase, Raf-1, Ras) as well as non phophorylated forms of different protein kinase C isoforms α, β, γ, δ, ε, ζ to determine how and at what extent the expression of these components behaved during the stepwise development of a 20-

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methylcholanthrene-induced transformed fibroblast cell line CNCI-PM-20 developed from 20 days old Swiss mouse embryo in culture. Only those *in vitro* passages (P20, P27, P32, P36, P42) of this cell line which showed significant variations in expression of their other previously studied characters were chosen for the present investigation [27]. The impact of host physiology and metabolism was also studied by establishing the primary cultures of the tumors formed after the biological incubation of these cells in syngenic host.

Material and methods

Chemicals. Mouse monoclonal antibody against ras; goat polyclonal antibodies against Raf-1 & MAP kinase/ERK1 were purchased from Santa Cruz Biotech,Inc, USA. Anti mouse IgG and anti goat IgG were purchased from Sigma, USA. Anti-Protein Kinase C Isozyme sampler set, Alkaline phosphatase conjugated goat anti rabbit IgG antibody, NBT-BCIP mix substrate were purchased from Gibco, BRL, USA). All other chemicals used in this study were of reagent grade.

Cell and culture procedure. CNCI-PM-20 cell line was routinely maintained in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum (FBS) at 37 ˚C in a humidified atmosphere of 5% CO_2 and 95% air. Refeeding with fresh growth medium was done once a week [27].

During the course of this study cells from different passages at exponential growth phase were cryopreserved in liquid nitrogen by following standard technique as described by PAUL [31]. The freezing mixture comprised of growth medium containing 10% DMSO and 10% FBS. Each 2 ml cryovial contained 5×10^6–1×10^7 cells/ml.

Immunocytochemical detection.

a) P21ras, Raf-1 kinase & MAPKinase/Erk1. The cells of different *in vitro* passages grown on glass coverslips to 70% confluency, were serum starved for 2 hours and then fixed in cold methanol at –20 ˚C for 10 minutes, incubated at 37 ˚C with 3% BSA to block non specific binding sites, followed by incubation with mouse monoclonal and goat polyclonal primary antibodies (1 μg/ml) overnight at 4 ˚C. The coverslips were then treated with FITC conjugated antimouse and antigoat IgG antibodies (1:100 and 1:160, respectively) for 1 hour at 37 ˚C. Each incubation step was followed by several washing with PBS. The mounted coverslips were observed under microscope equipped with epifluorescence.

b) PKC isoforms α, β, γ, δ, ε, ζ. In this experiment similar procedures as described above were followed for growing, fixing, blocking of the cells against non specific binding and treating the cells with primary antibodies (1 μg/ml) in Tris buffer (50mM Tris pH 7.5, 0.15 mM NaCl, 0.05% Tween 20). The cells were treated with alkaline phosphatase conjugated goat anti rabbit IgG antibody (1:200) at 37 ˚C for one hour and colorimetric development was achieved with NBT-BCIP mix substrate solution for 30 minutes in dark. The reaction was stopped by washing the cells with water. Mounting was done in DPX after dehydrating through graded alcohol (30%–100%) and xylene.

In all cases semiquantitation of positivity were done by counting at least 500 cells selected randomly from different fields under microscope and the results were expressed in percentage. The corresponding mean values of the results between the primary cultures were compared with the results of the studied passages by t-statistics.

Results

ras expression. The cells of different *in vitro* passages of CNCI-PM-20 cell line were found to be positive for ras expression as the immunofluorescence signals were predominantly cytoplasmic, whereas the primary culture of normal fibroblast cells did not show the presence of any such ras expressing cells (Fig. 1A). The percentage of total ras positive cells in different *in vitro* passages was found to be almost same (97% to 99.4%) (Tab. 1, Fig. 1B). It was interesting to note that in all the passages a certain population of positive cells showed a higher intensity of immunoreactive signals in comparison to the rest of the population (P20, 15.7%; P27, 18.6%; P32, 19.5%; P36, 26.3%; P42, 34% and P42 (*in vivo*), 44.7% (Tab. 1).

Raf-1 expression. Qualitatively the cells of different *in vitro* passages were positive in their Raf-1 expression as the immunoreactive signals were confined to the membrane and cytoplasmic compartment of the cells. Absence of Raf-1 expression in primary culture of normal fibroblasts cells was observed (Fig. 1C) whereas 98% or more cells of different *in vitro* passages of CNCI-PM-20 cell line expressed Raf-1 (Tab. 1). Among the total positive cells, from all the passages, a varying amount of population of cells exhibited intense fluorescein immunostaining than that of the surrounding cells (Fig. 1D, Tab. 1). This population of highly fluorescing cells grew in size with advancement of passages (P20, 16%, P27, 17%, P32, 19%, P36, 25.5%, P42, 29.4% and P42 (*in vivo*), 45%) (Tab. 1).

MAP kinase/ERK1 expression. Cells of CNCI-PM-20 cell line were said to be expressing MAP kinase /ERK 1 since some population of cells exhibited cytoplasmic as well as nuclear localization and other showed either nuclear or cytoplasmic immunostaining (Fig. 1F). The cells of normal embryonal fibroblast did not show any MAP kinase/ERK 1 expression (Fig. 1E). When immunofluorescein staining of CNCI-PM-20 cells of different passages were observed it was noted that in all cases the percentage of total cells expressing MAP kinase/ERK1 was above 95% (Tab. 1). The

Table 1. Analysis of p21 *ras*, Raf1, MAP kinase/ERK 1, PKCα, PKCβ & PKCδ expressing cells in different *in vitro* passages of CNCI-PM-20 cell line using immunocytochemical methods of detection

Gene product	Percentage of immunopositive cells (results are mean $\pm$ SD)						
	P0	P20	P27	P32	P36	P42	P42 (*in vivo*)
p21 RAS	0	97.0 ± 1.2 $(15.7 \pm 1.8)^{*}$	97.6 ± 1.6 $(18.6 \pm 2.6)^{*}$	98.0 ± 2.1 $(19.5 \pm 5.9)^{*}$	97.1 ± 1.3 $(26.3 \pm 1.1)^{*}$	99.4 ± 1.4 $(34.1 \pm 1.8)^{*}$	98.5 ± 2.8 $(44.7 \pm 3.7)^{*}$
RAF-1	0	98.5 ± 2.8 $(16.0 \pm 1.2)^{*}$	97.1 ± 3.1 $(17.0 \pm 1.5)^{*}$	99.0 ± 1.2 $(19.3 \pm 1.3)^{*}$	98.7 ± 2.3 $(25.5 \pm 4.8)^{*}$	97.7 ± 1.5 $(29.4 \pm 0.6)^{*}$	98.3 ± 3.1 $(45.0 \pm 2.9)^{*}$
MAP KINASE	0	97.4 ± 2.7 $(17.6 \pm 1.5)^{*}$	98.9 ± 1.2 $(17.0 \pm 2.6)^{*}$	96.0 ± 1.8 $(19.5 \pm 3.3)^{*}$	98.0 ± 2.1 $(25.3 \pm 5.0)^{*}$	97.0 ± 1.1 $(28.2 \pm 2.6)^{*}$	97.8 ± 3.1 $(42.6 \pm 2.7)^{*}$
PKCα	99.0 ± 1.9 (4.0 ± 1.1)	$99. \pm 1.8$ $(35.5 \pm 1.6)^{*}$	98.7 ± 1.8 $(38.2 \pm 1.7)^{*}$	97.6 ± 2.1 $(50.6 \pm 1.9)^{*}$	98.5 ± 2.2 $(61.4 \pm 1.8)^{*}$	98.7 ± 2.1 $(63.4 \pm 1.6)^{*}$	99.0 ± 2.3 $(66.0 \pm 2.2)^{*}$
PKCβ	99.0 ± 1.1 $[0]$	98.0 ± 1.0 $(28.2 \pm 1.4)^{*}$	99.0 ± 1.2 $(30.1 \pm 1.2)^{*}$	99.1 ± 1.5 $(35.0 \pm 1.5)^{*}$	99.0 ± 1.7 $(40.0 \pm 1.7)^{*}$	99.3 ± 1.3 $(50.0 \pm 1.2)^{*}$	99.0 ± 1.9 $(58.0 \pm 1.5)^{*}$
PKCδ	99.0 ± 1.1 $[0]$	99.2 ± 1.8 $[25.2 \pm 1.3]^{*}$	98.9 ± 1.2 $[31.1 \pm 1.5]^{*}$	98.5 ± 2.8 $[32.0 \pm 1.2]^{*}$	99.0 ± 1.9 $[47.0 \pm 2.0]^{*}$	98.0 ± 1.0 $[48.0.0]$	97.4 ± 2.7 $[58.0 \pm 1.5]$

Figures in the parenthesis indicate the percentage of cells showing higher immunopositivity in comparison to the rest of the positive cells. Figures in square brackets indicate percentage of cells with lower immunopositivity in comparison to the rest of the cells. P – denotes passage number, * – indicates p<.01.

percentage of highly fluorescing cells were found to be 17.0% in P20 and 27, 19.5% in P32, 25.3% in P36, 28.2% in P42, 42.6% in P42(*in vivo*) (Tab. 1).

PKCα expression. PKCα expression of normal as well as chemically transformed cells (CNCI-PM-20) are shown in Figure 2A and 2B and Table 1. The results demonstrated that almost all fibroblast cells of normal primary culture showed staining of PKCα though the staining intensity of these cells seemed to be lower than that of the cells of CNCI-PM-20 (Fig. 2A). PKCα was mainly localized in the cytoplasm, in areas of cell to cell contact and in the perinuclear region in the different *in vitro* passages (i.e. P20, P27, P32, P36, P42, P42(*in vivo*) (Fig. 2B). In all the passages including primary cultures of normal fibroblast cells, more than 98% of the cells were positive but variations in terms of percentage of highly positive cells did persist among the cells of different *in vitro* passages (Tab. 1, Fig. 2B). Only about 4% of normal embryonal fibroblast showed high expression of PKCα (Fig. 2A).

PKCβ expression. The result showed that PKCβ was predominantly localized in the cytoplasm of the normal embryonal fibroblast cells (Tab. 1, Fig. 2C). But overexpression of PKCβ with higher immunoreactive staining was observed in both cytoplasm and nucleus of the cells of different *in vitro* passages of CNCI-PM-20 cell line. Though more than 98% (Tab. 1, Fig. 2D) cells in all the observed passages of CNCI-PM-20 cell line were found to be showing cytoplasmic and

nuclear staining, a gradual increase in the highly stained cells for PKCβ was observed with advancement of passage (Tab. 1).

PKC γ, δ, ε, ζ expression. PKC γ, ε, ζ expressions were found to remain unaltered in the cells of different *in vitro* passages of CNCI-PM-20 cell line when compared to control cultures (Fig. not shown). PKC δ expression was found to be down regulated in the cells of this cell line in comparison to the normal embryonal fibroblast cells since lower level of immunoreactive signals were evident in the cells of CNCI-PM-20 cell line (Fig. 2E and 2F, Tab. 1).

Discussion

High content of immunocytochemically defined p21ras protein and its downstream components of signal transduction pathway (i.e. c-Raf-1 and mitogen-activated protein kinase/ERK 1) was present in the cells of different *in vitro* passages (P20 to P42) and after *in vivo* growth of P42 cells of CNCI-PM-20 cell line (Fig. 1B, 1D and 1F, Tab. 1).

Unlike other proto-oncogenes normal p21 ras protein was consistently expressed throughout the development of mouse embryo [6]. Though the antibody to p21 ras used in the present study did not differentiate between Ha-ras, Ki-ras, N-ras or wild type from mutated form, the absence of detectable reactivity of this protein in non-transformed primary cultures of Swiss mouse embryo (Fig. 1A) might be
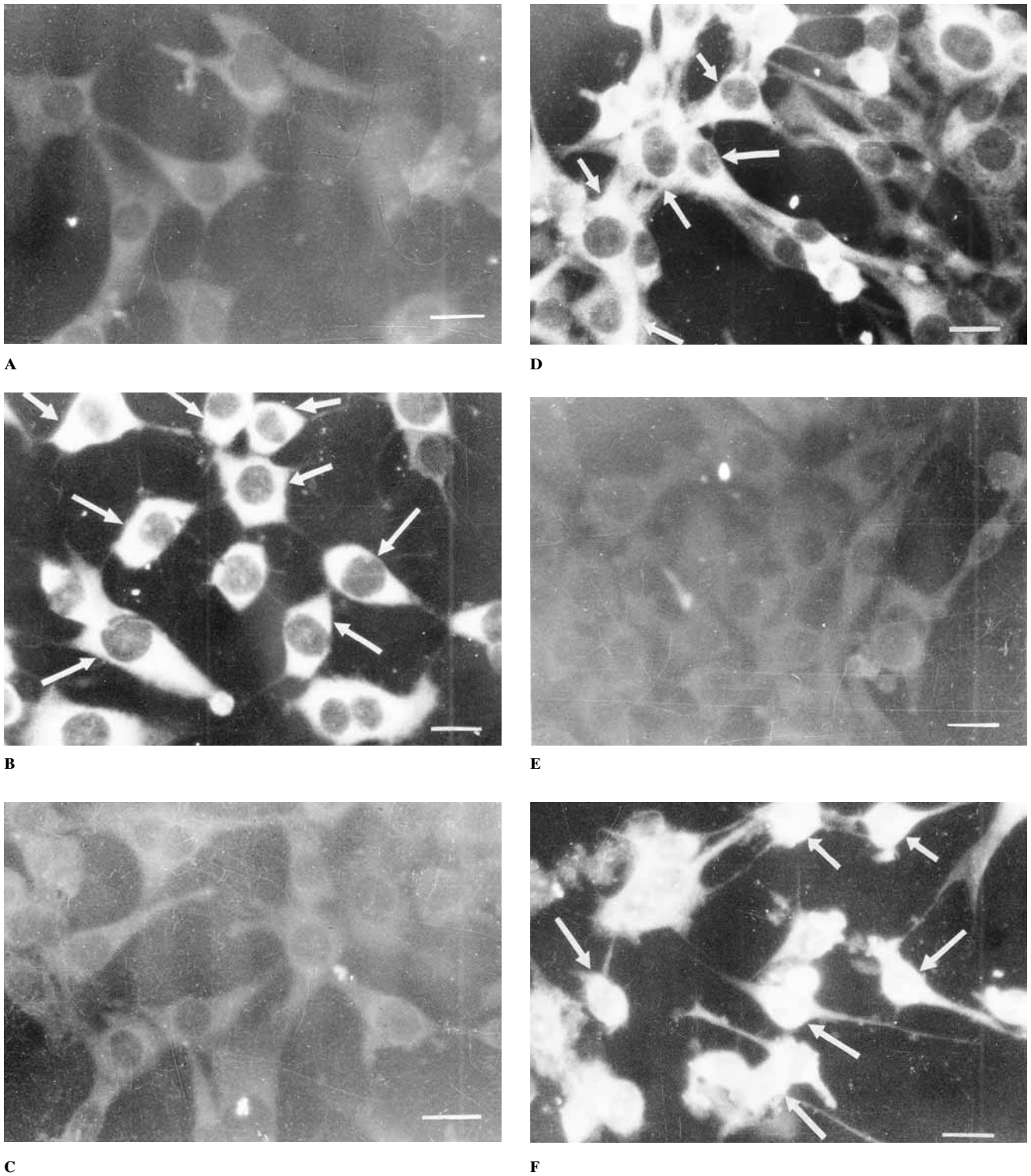
Figure 1. Immunocytochemical detection of p21 ras protein, Raf-1 and MAP kinase protein, respectively by indirect immunofluorescence staining. (A) normal diploid fibroblast cells with no immunostaining for p21ras; (B) cells of passage 42 showing altered expression of p21 ras; (C) normal diploid fibroblast cells with no immunostaining for Raf-1; (D) cells of passage 42 showing altered expression Raf-1; (E) normal diploid fibroblast cells with no immunostaining for MAP Kinase/ERK1; (F) cells of passage 42 showing altered expression for MAP kinase/ERK1. Arrows indicate highly immunostained cells. Bar = 3 μm.
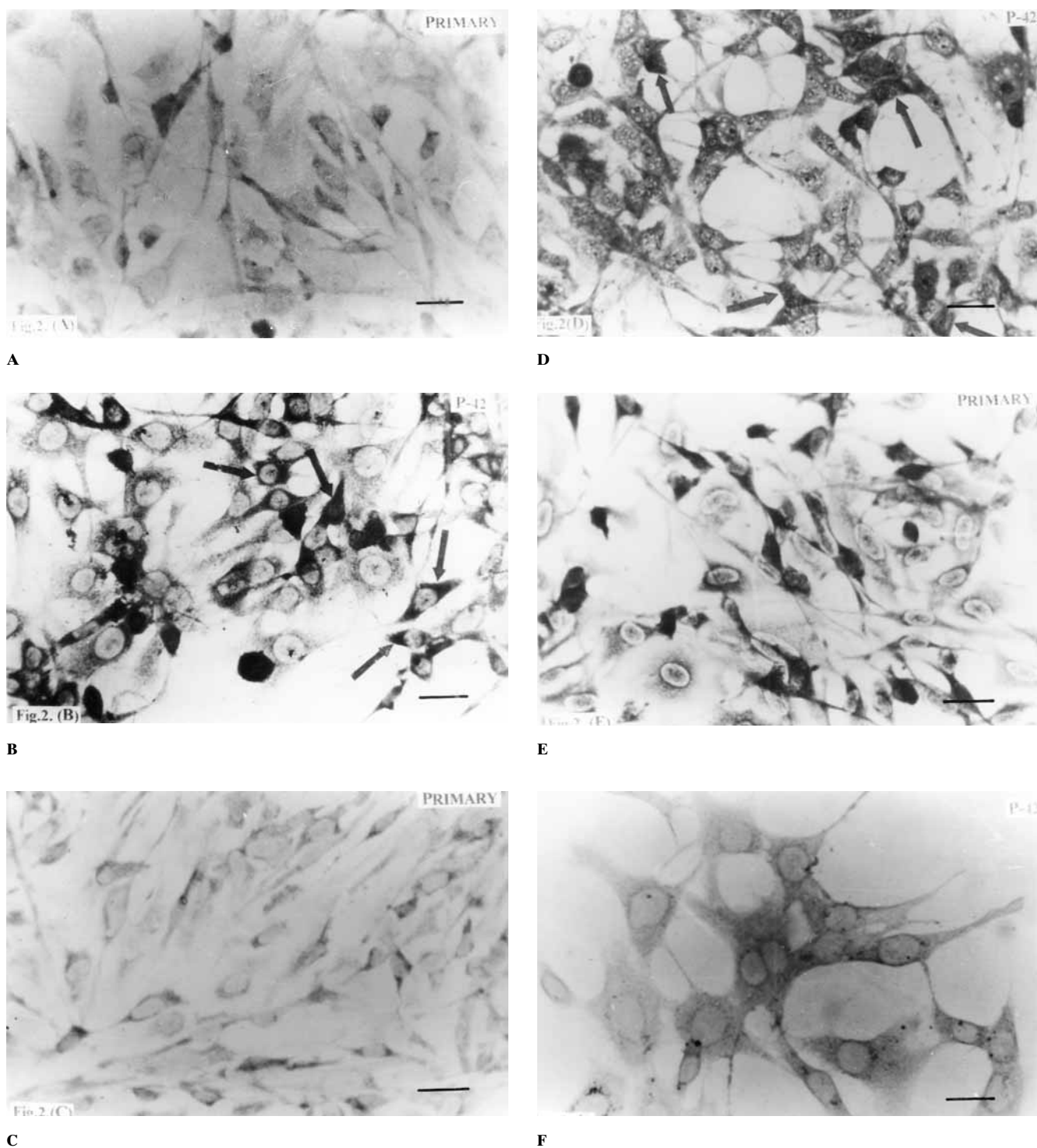
Figure 2. Immunocytochemical analysis PKCα, β and δ isoforms by indirect immunostaining. (A) normal diploid fibroblast cells with immunostaining for PKCα; (B) cells of passage 42 showing higher immunostaining for PKCα; (C) normal diploid fibroblast cells with only cytoplasmic immunostaining for PKCβ; (D) cells of passage 42 with cytoplasmic and nuclear immunostaining for PKCβ; (E) normal diploid fibroblast cells with immunostaining PKCδ; (F) cells of passage 42 with relatively lower immunostaining PKCδ. Arrows indicate highly immunostained cells. Bar = 3 μm.

beyond the sensitivity limits of our immunocytochemical assay. Equal or rather excess immunofluorescein staining intensity for p21 ras in the cells of different passages of this cell line to that of the positive control (i.e T-24 cell line, data not shown) undoubtedly suggested that *in vitro* transformation of the present cell system was accompanied by the overexpression of p21 ras protein (Fig. 1B), along with gradual increase in the percentage of strongly positive cells with advancement of passages. Since the possibility of the role of p21 ras in regulating proliferation was evidenced in a study where Ki-ras regulated proliferation at the G0/G1 transition in kidney cells [13] and also a high Ha-ras p21 content was related to the so-called dedifferentiation associated with developmental, regenerative and carcinogenic processes in liver [14], it could be suggested that overexpression of p21 ras protein of CNCI-PM-20 cells was possibly correlated with high proliferative activity or high growth rate of these cells [27]. The elevation in the level of expression of p21 ras was noted from passage 20 in this particular cell line. The ability of the cells of this cell line to survive without senescence for several passages before reaching passage 20 revealed that immortalization of cells had occurred prior to reaching passage 20. Though the control cultures of normal fibroblasts and MCA treated cells of this cell line were maintained under identical growth condition, the normal cultures did not survive after fourth passage, while just after eight days of MCA treatment primary cultures showed several morphological changes along with the formation of small foci which grew into colonies and when passaged, they formed monolayers. Thus the present data ruled out the involvement of any background factors for the observed changes in the treated cells [27]. Therefore it could be assumed that chemical carcinogen MCA worked as initiator of carcinogenesis in normal fibroblast cells giving rise to immortality and the ras gene possibly played a role to increase the malignant phenotype. Since p21 overexpression was observed from passage 20, its phenotypic consequences of activation might substantiate the speculation that ras activation was probably not a very late event in progressive neoplasia of this cell line.

Though the present study was confined only to c-Raf-1 and MAP kinase expression, yet involvement of other signal transduction pathway needs further investigation. The positive signals of the Raf-1 kinase in the cells of different *in vitro* passages of CNCI-PM-20 (Fig. 1D) pointed towards the overexpression of the active kinase form of the Raf-1, since the antibody used here was directed against the active form of this protein. The similarity in the trend of Raf-1 and p21 ras expression also indicated the possibility of the activation of cellular Raf-1 as a consequence of p21ras protein expression in this cell line. Experiments involving expression of a dominant inhibitory mutant of c-Raf-1 and of c-Raf antisense RNA in p21 ras transformed cells demonstrated that Raf-1 was required for ras-induced transformation [19].

It had been reported that mutant p21 ras expression was sufficient to activate Raf-1 in some cell line [26], whereas p21v-ras expression was not sufficient in others [35]. Also the pathways for transformation and transcriptional activation by increased level of normal ras and raf might be important in tumors that showed overexpression but lacked mutationally activated form of these two protooncogenes [10].

MAP kinase was considered to play a central role in diverse cellular events including carcinogenesis and tumor progression. Indeed expression of tyrosine phophorylated MAP kinase was altered in different types of carcinogen-induced transformation, as it was found in case of CNCI-PM-20 cell line (Fig. 1F). The general tendency of having greater number of highly positive cells in successive passage was in compliance with previous study with ras and raf-1 expression. An increase of MAP kinase was reported in adenocarcinoma after MNNG administration [5], in mammary tumors initiated by v-Ha-ras [2], in the adenoma to carcinoma sequence in human colorectal tumorigenesis [30], in breast cancer [22] while MAP kinase signaling was not upregulated in pancreatic cancer [36].

In situ analysis of classical PKCβ isoforms revealed its high level of expression in CNCI-PM-20 cell line (Fig. 2E). Increased expression of PKCβ isoform in thyroid tumors [15] and in colon carcinogenesis were reported [28]. Fibroblasts of rat displayed several disorders in growth control due to overexpression of PKCβ [17], whereas in HT29 colon cancer and in erythroleukemia this overexpression inhibited growth and accelerated differentiation, respectively [9, 24]. PKCβ was thus found to have dual functions in regulation of growth and differentiation. The probability of direct or indirect functional involvement of PKCβ in the progressive neoplasia of CNCI-PM-20 cell line was revealed by the occurrence of over expression of this isoform in this cell system (Fig. 2E). The overexpression of PKCβ might be a consequence of the production of diacylglycerol which is possibly generated from some unknown growth factor expression or might be an overflow effect of p21 ras expression observed in this study, or overexpression of PKCβ made this cell system susceptible to ras-induced transformation [17, 18, 20, 29]. Further investigations are required in this direction.

Relatively higher expression of quiescent cytosolic PKCα than control culture was observed in the cells of CNCI-PM-20 cell line (Fig. 2A, 2B and Tab. 1). Similar observation with respect to the overexpressing inactive state of cytosolic PKCα in the glioma and glioblastoma cell lines was reported [37]. From the finding that c-Ha-ras oncogene transformed rat fibroblast cell line which overexpressed PKCβ and displayed several fold increase in the expression of the endogeneous PKCα [34], it may be assumed that enhanced expression of PKCα in CNCI-PM-20 cell line might have resulted due to the overexpression of PKCβ in these cells.

PKCδ expression showed a lower intensity in transformed cells (Fig. 2H) than its normal counterpart (Fig. 2G). Overexpression of PKCδ resulted in cell division arrest in CHO cells [33] and in TPA induced NIH3T3 cells showed decreased growth rate [25], whereas under similar condition it was involved in differentiation of mouse myeloid progenitor cell line [25]. Therefore the downregulation of PKCδ in this cell system may be influencing the process of de-differentiation and probably its altered expression was due to some epigenetic changes.

Therefore it could be summarized that the MAP kinase cascade was playing a pivotal role in the progression of the cells in this model system. From this study it appears that all the above studied gene product expressions were occurring simultaneously from passage 20 onwards and their close resemblance in the level of expression both in the total percentage of cells with positive staining group and in the highly positive staining group strongly supported the possibility of the activation of one component of signal transduction pathway under the influence of the other in MAP kinase cascade of signal transduction due to activated ras. The altered expression PKCα, β and δ might be an indirect influence of other altered gene expression, or it might be an epigenetic change which require further investigation. These alteration in gene expression revealed a common trend of gradual increase in their highly stained positive cells with progressive neoplasia and thus supporting the view that CNCI-PM-20 cell line has achieved its aggressive property with advancement of passage. The differences in the level of expression of the target protein may be attributed to the differences in their gene doses and this variation in the gene doses was probably due to the aneuploidal nature of this particular cell system (submitted for communication). An alternative and equally feasible explanation is that all the transforming events occur during the initial exposure and passages and what happens during the subsequent passages is a gradual enrichment of the cell population with selection for the transformed phenotype. Further investigations are needed to elucidate the molecular basis of gradual acquisition of aggressive properties in this cell system which might throw some light towards our understanding in the mechanism of delayed appearance of tumors in humans/animals after carcinogenic insult.

In a study evidence of gene amplification in *in vitro* growth condition was found to persist even after *in vivo* growth of the cells in mouse skin carcinogenesis model [1], whereas another study reported the retention of characters of malignant cells of human tumor origin after *in vivo* growth of these cells in mouse [7]. Similarly our findings also showed evidence that the pattern of altered gene expression almost remained identical both in *in vivo* and in *in vitro* growth condition in this chemically transformed murine embryonal fibroblast cell line. Thus the possibility of no interference of host physiology and metabolism on the above

characters in *in vivo* growth condition may exist in this particular cell system. The study of the same chemically transformed cell line under both *in vivo* and *in vitro* growth condition may be helpful in understanding the process of cell transformation induced by carcinogens.

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