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Changes in Gene Expressin Accompanying Radiatiom-Induced Transformation of Mouse C3H 10T1/2 Cells

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Abstract Changes in gene expression accompanying radiation-induced transformation of mouse C3H 10T1/2 cells were investigated. The normal parental cells and two clones isolated from X ray-induced type III foci, Tf13 and Tf21 (Terasima et al., 1981), were labeled with [^{35}S]methionine for 24 hr, fractionated into three fractions, that is, nuclear, cytosol and membrane fractions, and then subjected to two-dimensional gel electrophoresis. The autoradiographic patterns of [^{35}S]methionine-labeled proteins from normal cells and radiation-induced transformants were compared. Changes in the synthesis of at least 11 and 9 proteins in the cytosol and membrane fractions, respectively, were observed. Certain changes were commonly detected in both two transformant clones; Increased synthesis of protein spot 35/6.5 (molecular weight in Kd/ isoelectric point) in the cytosol fraction and spots 48/7.0, 48/6.5, and 33/5.5 in the membrane fraction, and decreased synthesis of spots 44/5.0 and 27/6.5 in the cytosol fraction and 41/4.5 in the membrane fraction. On the other hand, any apparent differences could not be detected in the nuclear fraction.

Introduction

Radiation has a potent carcinogenic activity and causes transformation in mammalian cells *in vitro* (Borek & Sachs, 1966; Han & Elkind, 1979; Little, 1979; Terasima et al., 1981; Terzaghi & Little, 1976). In contrast to oncogenic viruses, radiation does not introduce new genetic information into cells. A lot of studies on viral transformation have revealed that changes in gene expression accompany transformational process (Bravo & Celis, 1980; Brzeski & Ege, 1980; Celis et al., 1984; Fransen et al., 1983; Franza & Garrels, 1984; Leavitt & Moyzis, 1978; Radke & Martin, 1979; Strand & August, 1977). Thus, in the case of radiation-induced transformation, there also must be some qualitative and quantitative changes in the expression of some genes which are already present in normal cells. Guerrero et al. (1984) have shown that a point mutation occurred in K-*ras* gene in gamma ray-induced thymic lymphoma *in vivo*. Borek and coworkers (Borek, 1985; Borek et al., 1987) showed that DNAs from hamster embryo cells and mouse C3H 10T1/2 cells transformed *in vitro* by X-irradiation could produce transformed colonies in NIH 3T3, C3H 10T1/2 and Rat-2 cells. They also showed that elevated expression of oncogenes, c-*abl* and c-fms were

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identified in NIH 3T3 transformants containing DNA from X-ray-transformed C3H 10T1/2. Recently, Sawey et al. (1987) reported that activation of both c-K-*ras* and c-*myc* oncogenes were detected in primary rat skin tumors induced by 0.8 MeV of electron radiation. Thus, evidence has been accumulating that radiation-induced transformation is accompanied by changes in expression and/or state of integrity of some genes. However, the molecular mechanism involved in the radiation-induced transformation is not fully elucidated. One approach to searching for gene expression is the analysis of the overall patterns of proteins synthesized in cells by means of high-resolution two dimensional gel electrophoresis (O'Farrell, 1975; O'Farrell & Goodman, 1976). However, there are no reports which analyzed the extent of changes in gene expression by comparing parental cells to clonal transformants induced by radiation. So, we attempted to compare the autoradio-graphic patterns of [^{35}S]methionine-labeled proteins from normal mouse C3H 10T1/2 cells and its X ray-induced transformant clones by the electrophoresis technique.

Materials and Methods

Cell lines

C3H mouse embryo-derived 10T1/2 clone 8 cells (Reznikoff et al., 1973) and two transformant clones, Tf13 and Tf21, used in this study were kindly supplied by Dr. T. Terasima, National Institute of Radiological Science. The transformant clones were separately isolated from the type III foci of C3H 10T1/2 cells induced by X rays (4 Gy) (Terasima et al., 1981). The transformants have been shown to form colonies in soft agar and to induce tumors when injected into immuno-suppressed syngenic mice (Ohtsu et al., 1983; Terasima et al., 1981; Terasima et al., 1983)

Cell growth and labeling conditions

The cells were grown in Eagle's Basal Medium supplemented with 10% heat-inactivated fetal caif serum (FCS), 50 units/ml penicillin, and 50 μg/ml streptomycin. For labeling the cells with [^{35}S]methionine, subconfluent monolayers of cells in 60-mm dishes were incubated in 5 ml of methionine-free medium containing 1% FCS at 37°C for 1 hr. Subsequently, cells were labeled by incubating in fresh methionine-free medium containing 1% FCS supplemented with 200 μCi/ml of [^{35}S]methionine at 37°C for 24 hr.

Fractionation and preparation of cellular proteins for electrophoresis

Labeled cells were fractionated by the procedures described by Shih et al. (1982) with a slight modification. Labeled cells were rinsed and scraped into 1 ml of cold 0.01 M Tris-HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol and then homogenized in a Teflon:glass homogenizer. The nuclear fraction was precipitated by centrifuging the homogenates at 1,000×g for 7 min. The resulting supernatants were subsequently centrifuged at 100,000×g for 30 min, and the supernatants were designated as cytosol fraction and the pellets as membrane fraction.

To avoid the interference by nucleic acids in the nuclear fraction for isoelectric focusing, DNA and RNA were digested by nucleases according to O'Farrell (1975). The nuclear fractions were suspended in 0.1 ml of O'Farrell's sonication buffer

(O'Farrell, 1975) (0.01 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 μg/ml RNase A). After extreme homogenization, DNase I was added to 50 μg/ml and the reaction mixtures were kept on ice for 10 min. 0.3 ml of SDS-sample buffer (0.5% SDS, 9.5 M urea, 5% β-mercaptoethanol and 0.2% Bio-lyte (pH 3–10)) was added to the mixture and incubated at room temperature for 10 min, followed by the addition of 0.6 ml of NP40-sample buffer (2% Nonidet P–40, 9.5 M urea, 5% β-mercaptoethanol and 2% Bio-lyte (pH 3–10)). Proteins in the cytosol fractions were precipitated with saturated $(NH_4)_2SO_4$ and then dissolved in 0.3 ml of SDS-sample buffer. After incubation at room temperature for 10 min, 0.7 ml of NP40-sample buffer was added to the mixtures. The membrane fractions were dissolved in 0.1 ml of 1% Triton X–100, followed by the addition of 0.3 ml of SDS-sample buffer and 0.6 ml of NP40-sample buffer, as described above.

Protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out essentially as described by O'Farrell (1975) and O'Farrell and Goodman (1976). The cellular fractions were subjected to isoelectric focusing in 9 M urea for 5,600 Vhr with 2% ampholytes (Bio-lyte(pH 3–10)), followed by SDS-polyacrylamide gel electrophoresis on 10% slab gels. Each gel contained the samples of 500,000 cpm. After electrophoresis, the gels were fixed with 10% trichloroacetic acid-10% acetic acid-30% methanol for at least 3 hr. Protein spots were visualized by fluorography performed by the procedure described by Chamberlain (1979) in which sodium salicylate was used as a fluor. Then, the gels were dried and exposed to Fuji RX film at $-70^{\circ}C$.

Chemicals

Bio-lyte (40%, pH 3–10) was obtained from Bio-Rad Laboratory (Richmond, CA). Molecular weight marker and pl marker were obtained from Oriental Yeast Co. (Osaka). Acrylamide and N, N'-methylenebisacrylamide was obtained from Nakarai Chemical Co. (Kyoto) and sodium salicylate was the product of Wako Pure Chemicals (Osaka). [^{35}S]methionine (>1000 Ci/mmol) was purchased from New England Nuclear (Boston).

Results

Figugre 1 shows a section of typical, highiy exposed autoradiographs of [^{35}S]methionine-labeled proteins in the cytosol fractions from parental C3H 10T1/2 cells and a transformant clone Tf13, separated in two-dimensional gels. In Tf13 clone (Fig. 1b), it was evident that there was increased synthesis of at least four proteins and decreased synthesis of at least three proteins, compared to parental cells (Fig. 1a). These protein spots are indicated with arrows and arrowheads, respectively, in the figure. The major proteins whose synthesis was greatly modulated in the two transformants, Tf13 and Tf21, are listed in Table I with their isoelecric points (pI) and molecular weights. To assess the reproducibility of the data, several separate experiments were done, and changes listed in the table were common in all experimental

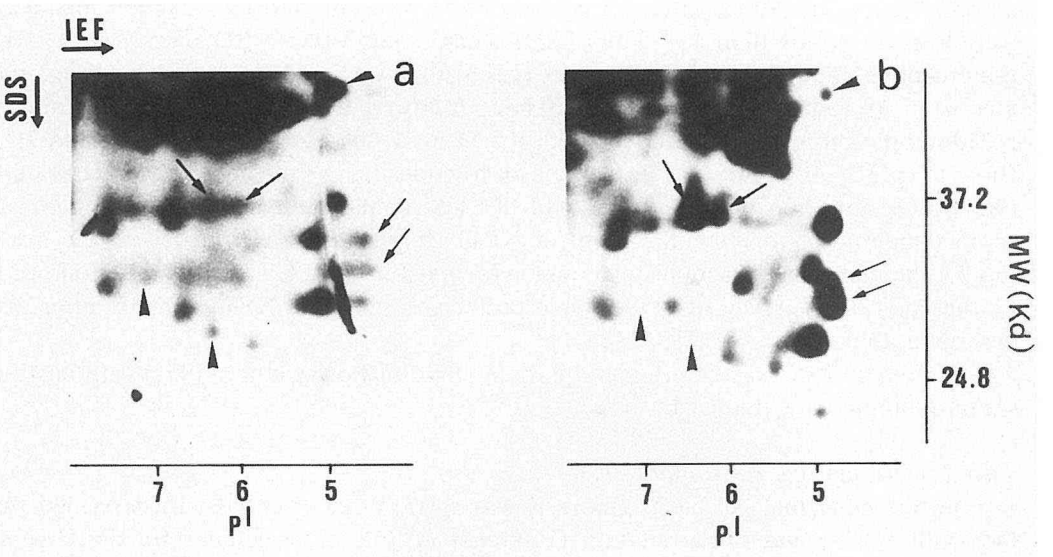

Figure 1. Two-dimensional gel electrophoretic patterns of proteins in the cytosol fractions from normal and X-ray-transformed C3H 10T1/2 cell line.

[^{35}S]methionine-labeled proteins in the cytosol fractions of normal 10T1/2 cells (a) and one X-ray-transformant, Tf13 (b) were prepared and subjected to two-dimensional gel electrophoresis. A section of autoradiogram (corresponding to the pH range 4.5–7.5, and molecular weight range $50–20\times10^3$) is shown. Arrows indicate proteins whose synthesis increased and arrowheads indicate proteins whose synthesis decreased in the transformant. The directions of electrophoresis are shown beside the gel. IEF, isoelectric focusing ; SDS, SDS-polyacrylamide gel electrophoresis.

Table 1. List of proteins in the cytosol fraction whose synthesis is changed in X ray-induced transformant clones of mouse C3H 10T1/2 cells.

Cells	Increased synthesis		Decreased synthesis	
	pI[1)]	M.W.[2)]	pI	M.W.
Tf13	#6.5	35	#5.0	44
	5.0	31	#6.5	27
	4.9	29	7.0	29
	6.1	35		
Tf21	#6.5	35	#5.0	44
	5.0	65	#6.5	27
	5.5	24		
	5.1	26		
	4.6	26		

[1)] Isoelectric point
[2)] Molecular weight (KDa)
Polypeptides whose change of synthesis is commonly detected in both transformants.

sets. Among the protein species listed in Table I, the increased synthesis of spot 35/6.5 (molecular weight in Kd/pI) and decreased synthesis of spots 44/5.0 and 27/6.5 commonly occurred in both Tf13 and Tf21 transformant clones.

[^{35}S]methionine-labeled proteins in the membrane fractions from a transformant clone Tf21, were also compared to those from parental 10T1/2 cells in the two-dimensional gels (Fig. 2). Figure 2b shows a section of autoradiographs and it was

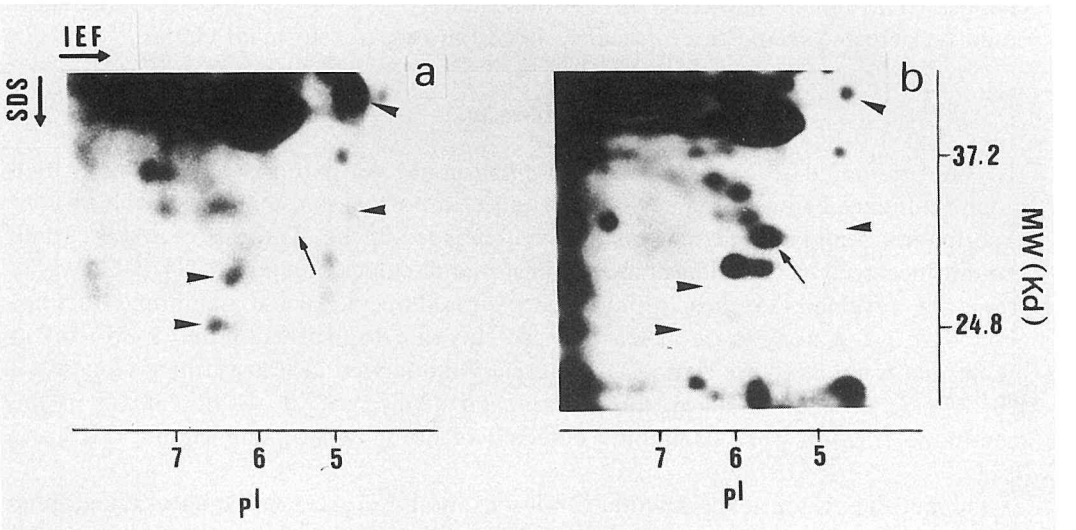

Figure 2. Two-dimensional gel electrophoretic patterns of proteins in the mombrane fractions from normal and X-ray-transformed C3H 10T1/2 cell line.

[^{35}S]methionine-labeled proteins in the mombrane fractions prepared from normal cells (a) and one transformant, Tf21 (b), were subjected to two-dimensional gel electrophoresis. Other details are the same as those described in Figure 1.

Table 2. List of proteins in the membrane fraction whose synthesis is changed in X ray-induced transformant clones of mouse C3H 10T1/2 cells.

Cells	Increased synthesis		Decreased synthesis	
	pI[1)]	M.W.[2)]	pI	M.W.
Tf13	#7.0	48	#4.5	41
	#6.5	48	5.5	66
	#5.5	33		
	7.0	63		
Tf21	#7.0	48	#4.5	41
	#6.5	48	4.7	33
	#5.5	33	6.3	27
			6.5	25

[1)] Isoelectric point
[2)] Molecular weight (KDa)
Polypeptides whose change of synthesis is commonly detected in both transformants.

evident that the synthesis of one protein increased and the synthesis of at least four proteins decreased in Tf21 clone. These protein spots are indicated with arrows and arrowheads, respectively, in the figure and listed Table II with their molecular weights and pIs. It was found that the changes in protein spots 48/7.0, 48/6.5, 33/5.5 and 41/4.5 were common in both Tf13 and Tf21 clones.

The similar comparison was carried out for the autoradiographic patterns of [^{35}S]methionine-labeled proteins in the nuclear fractions. However, any apparent changes could not be detected. In addition, neither new protein spots nor lost spots could be detected in the three fractions from the two transformant clones.

Discussion

We attempted to compare the two-dimensional gel patterns of proteins in two radiation-induced transformant clones to those in normal parental cells. The present experiments demonstrated that quantitative changes in the synthesis of some certain proteins occurred in radiation-induced transformant clones of mouse C3H 10T1/2 cells. It was also revealed that these proteins were located in cytosol and membrane fractions (Figs. 1 & 2). Among these at least 11 proteins in cytosol fraction and 9 proteins in membrane fraction whose synthesis was greatly modulated in transformant clones, 44/5.0, 35/6.5, 27/6.5 in the cytosol fraction and 48/6.5, 48/7.0, 41/4.5, 33/5.5 in the membrane fraction were commonly detected in both transformant clones, Tf13 and Tf21.

Under the present experimental conditions, no differences in the autoradiographic patterns of proteins in the nuclear fraction were found out. These were unexpected results, because earlier studies have detected qualitative and quantitative changes in two-dimensional gel patterns of nuclear proteins in tumor cells isolated from rodents (Takami & Busch, 1979; Takami et al., 1979). One possible explanation is that changes in the synthesis of some nuclear proteins were so small that visual inspection could not detect such minor changes.

It has been suggested that phosphorylations are key molecular modification reactions of cellular proteins associated with regulation of cell growth and transformation (Cohen et al., 1980; Hunter & Cooper, 1981; Hunter & Sefton, 1980; Nishizuka, 1984). We examined the extent of phosphorylation of proteins synthesized in parental 10T1/2 cells and Tf13 and Tf21 transformant clones. We found that the incorporation of [^{32}P]orthophosphate into cellular proteins was much greater in the transformant clones than in the parental cells (data not shown). This indicates that the level of protein phosphorylation is also modulated in radiation-induced transformants as well as gene expression. We are currently examining changes in two-dimensional gel patterns of phosphorylated proteins accompanied by radiation-induced transfomation of mouse 10T1/2 cells.

The detailed nature of these transfomation-sensitive proteins is not understood at present. We compared the characteristics (molecular weight and pI) of these transfomation-sensitive proteins to that of some oncogene products and growth factors whose molecular weight and pI have already been known. Fuhrer et al. (1986) detected the *ras* oncogene products by two-dimentional gel-Western blotting techniques and they found that the isoelectric point and molecular weight of c-H-*ras* oncogene

product were 5.1–5.4 and 21 Kd, respectively. Hence, we examined protein spots present in the molecular weight-pI region, but could not find out any changes. Futhermore, we tried to compare to the characteristics of growth factors and their receptors, but any similarities could not be obtained. Although further studies are needed to understand the biochemical nature of the radiation-induced transformation-sensitive proteins, but it is possible that these transformation-sensitive proteins which were revealed in this study, could play important roles in radiation-induced transformation.

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