AATEX 1, 65－73 (1991)

DETECTION OF NON-MUTAGENIC CARCINOGENS USING CULTURED SYRIAN HAMSTER EMBRYO CELLS

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Summary

The Syrian hamster embryo (SHE) cell transformation assay has a high predictive value (> 90 %) for the detection of carcinogens, including a number of non-mutagenic carcinogens[1]. To validate the assay further, we examined the abilities of 17 chemicals, which were negatived in the *Salmonella* / microsome assay, to induced morphological transformation of SHE cells.

SHE cells (2.5×10^5) in tertiary cultures were plated in 75-㎠ flasks and treated with the chemicals for 48 h. Following trypsinization, the cells were replated on 100-㎜ dishes at 2,000-3,000 cells per dish and allowed to form colonies for 7 additional days. Elevem of the 17 chemicals tested are reported to be carcinogenic in rodents (no data are available for 4 chemicals). Ten of the eleven chemicals induced morphological transformation of SHE cells. SHE cell transformation responses for other chemicals by other investigators also showed a positive transforming activity in agreement with the rodent carcinogenicity data. These results indicate a good correlation besween induction of SHE cell transformation and carcinogenicity, suggesting that the SHE cell assay system could be useful for mechanistic studies and detection of potential carcinogenic activity of non-mutagenic carcinogens. The possible involvement of genetic mechanisms in cell transformation by these chemicals will be discussed.

Key words : cell transformation, non- mutagenic carcinogens, Syrian hamsser embryo cells.

Introduction

The Syrian hamster embryo (SHE) cell transformation assay has been used for mechanistic studies of carcinogenesis and as a screening test for the potetial carcinogenicity of chemicals. The assay has a high predictive value (90.8 %) for the detection of carcinogens, including a number of non-mutagenic carcinogens[1]. To validate the assay further, we examined the abilities of 17 chemicals, which were negative in the *Salmonella* / microsome assay, to induce morphological transformation of SHE cells. In this paper, we report that the SHE cell assay system could be useful for mechanistic studies and detection of potential carcinogenic activity of non-mutagenic carcinogens. The possible involvement of genetic mechanisms in cell transformation by these chemicals will be discussed.

Materials and Methods

Cells, growth medium and chemicals:

SHE cell cultures were established from 13-day-gestation fetuses and grown, as previously described[2-4]. The cell culture medium used was IBR Dulbecco's modified Eagle's reinforced medium (Biolabs, Northbrook, IL), with 0.37 % (w/v) $NaHCO_3$, 10 % fetal bovine serul (FBS) (Gibco, Grand Island, NY), penicillin (100 units /㎖) and streptomycin (100 μg/㎖). Cells were grown in a humidified atmosphere of 12 % CO_2 in air at 37 ℃ and were transferred by gentle trypsinization with 0.1 % trypsin (1 : 250) (Gibco) for 3 min at 37 ℃. 3-Amino-1, 2, 4-triazole (amitrole) (Aldrich Chemical Co., Milwaukee, WI),

asbestos (chrysotile and crocidolite asbdstos) (V. Timbrell, MRC, Great Britain), 2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester (blofibrate) (Sigma Chemical Co., St. Louis, MO), Colcemid (Gibco), diethylstilbestrol (DES) (Sigma), 17β-estradiol (Sigma), eugenol (Wako Pure Chemical Ind. Ltd.,Osaka, Japan), sodium arsenite (Wako Pure Chemical), sodium bisulfate (Sigma), sodium fluoride (Koso Chemical Co., Tokyo, Japan), progesterone (Sigma), testosterone (Sigma), vincristine sulfate (Sigma), [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14, 643) (Sigma), ouabain (Sigma), 6-thioguanine (TG) (Sigma), 5-bromodeoxyuridine (BrdU) (Sigma), hydroxyurea (Sigma) and [^{3}H] thymidine (specific activity: 1.6 TB_q/mmole, Amersham-Searle Corp., Arlington Heights, IL) were obtained from the indicated sources. Benzene, di-(2-ethylhexyl) phthalate and mono (2-ethylhexyl) phthalate (MEHP) were kindly supplied by Dr. M. Shelby, National Toxicology Program, NIEHS, Research Triangle Park, NC.

Cell transformation and gene mutaions:

For cell transformation and gene mutaion assays, cells (2.5×10^5) in tertiary cultures were seeded into 75-㎠ flasks (Falcon Labware. Oxnard, CA) and after overnight incubation, treated with chemicals for 48 h. After the cells were trypsinized, a part of the cell suspension was assayed for morphological transformation, and the remaining cells were subcultured at the density of 4×10^5 cells per 75-㎠ flask for mutation experiments. For morphological transformation, 2,000 cells were plated on 100-㎜ dishes and incubated 7 days for colony formation. The cells were fixed in absolute methanol and stained with a 10% aqueous Giemsa solution. The plating efficiency of untreated cells was between 10 and 15%. The number of surviving colonies and morphologically transformed colonies were scored by previously established criteria[5-8]. For mutation experiments, the cells were grown for an expression time of 4 days and then 10^5 cells were plated on each of 10 dishes (100-㎜ in diameter) with medium containing 3.3 ㎍/㎖ TG or 1.1 mM ouabain[9], and incubated 7 days for colony formation. The mutation frequency was calculated, as described previously[9].

Cell transformation and gene mutations using exogenous metabolic activation system:

Post-mitochondrial supernatant (PMS) was prepared, as described previously[10]. Three male Sprague-Dawley rats (~ 200 g) were pretreated with inducers according to the procedures of Matsushima *et al*[10]. The animals were given intraperitoneal injections of sodium phenobarbital (Daiichi Pure Chemicals Co., Tokyo) at 30 *㎎/㎏* body weight, on day 0 and at 60 ㎎/㎏ on days 1, 2 and 3; 5,6-benzoflavone (Aldrich) at 80 ㎎/㎏ was injected intraperitoneally on day 2. On day 4, the livers of 3 decapitated animals werd excised, washed, and homogenized with a Potter-Elvehjem-type teflon homogenizer in 3 vol. of 0.25 M sucrose at pH 7.4 containing 2 mM $MgCl_2$ and 20 mM HEPES (N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid) (sucrose-HEPES buffer). As described by Kuroki *et al.*[11] PMS fraction was prepared by two successive centrifugations at 9,000 × g for 10 min and 15,000 × g for 20 min. The supernatant was rapidly frozen with dry ice and acetone and kept in liquid nitrogen until use.

Cells (5×10^5) were inoculated into 75-㎠ frasks, incubated overnight and treated with chemicals for 2-3 h in the presence of PMS mixture, consisting of 1.5 ㎖ of the PMS fraction, diluted with sucrose-HEPES buffer to given concentration, 1.5 ml of modified Sorensen phosphate buffer (0.055 M, pH 7.4 containing 0.9% NaCl and 1,6 ㎎ $MgCl_2 \cdot 6H_2O$ per ㎖), 0.5 ㎖ glucose 6-phosphate solution [13 ㎎/㎖ in PBS(+) (phosphate-buffered saline containing Ca^{2+} and Mg^{2+})], 0.5 ㎖ NADPH solution [(6.82 ㎎/㎖ in PBS(+)] and 1.0 ㎖ PBS(+) (pH 7.4). The final concentrations of the cofactors in the reaction mixture were 20 ㎍ moles PO_4^{2-} per ㎖ and 3.1 ㎍ moles Mg^{2+} per ㎖, 5 ㎍ moles glucose 6-phosphate per ㎖ and 0.8 ㎍ moles NADPH per ㎖. After washing 3 times with complete medium and subsequent incubation for 3-4 h, the cells were harvested by trypsinization. Two thousand cells were seeded into 100-㎜ dishes for the morphological transformation assay. The remaining cells were plated in 75-㎠ flasks for mutation assays, as descrived above.

Analysis of chromosome aberrations and chromosome number:

To analyze chromosome aberrations and chromosome number, SHE cells (2.5×10^5 for 48-h treatment group and 5×10^5 cells for 15- and 24-h treatment groups) were seeded into 75-cm^2 flasks. After overnight incubation, chemicals were added to the cultures. Three hours before the end of treatment time, Coledmid was administered at 0.2 μg/ml, and metaphase chromosomes were prepared, as described previously[4, 6]. The aberrations scored were gaps, breaks, exchanges, dicentrics, o-rings and fragmentations. For detemination of chromosome numer, a hundred metaphases per experimental group were scored.

Sister chromatid exchanges:

For analysis of sister chromatid exchanges (SCE), cells (5×10^5) were plated overnight on 100-mm dishes and treated with various concentrations of chemicals for 24 h in the presence of BrdU (10 μg/ml) under dark conditions. Three hours before the end of the treatment, Colcemid was added to give a final concentration of 0.2 μg/ml, and then metaphase chromosomes were prepared, as described above. The differential staining of sister chromatids was performed according to a modification of the fluorescence-plus-Giemsa technique[12]. Thirty second-division metaphases with the diploid number of chromosomes were analyzed for SEC frequency.

Chromosome aberrations and unscheculed DNA synthesis using exogenous metabolic activation system:

For analysis of chromosome aberrations, cells (5×10^5) were plated overnight in 75-cm^2 flasks and treated with various concentrations of chemicals for 2-3 h in the presence of PMS fraction, as described above. After washing 3 times with complete medium, cells were incubated for 17-18 h. Colcemid (0.2 μg/ml) was added so the cultures, which were stbsequently incubated for 3 h.

Detection of unscheduled DNA synthesis:

The procedure for detection of unscheduled DNA synthesis (UDS) has been described previously[13]. Briefly, cells (1×10^5) in logarithmic growth were plated in triplicate on 15-mm-diameter glass coverslips ($\sim$ 2 cm^2) in 16-mm tissue culture cluster dishes (Coster, Cambridge, MA) with complete medium. The cells reached confluence after 24-h incubation. The saturation density was $\sim 7.7\times10^4$ cells/cm^2 and the doubling time was $\sim$ 15 h. The medium of confluent cultures was replaced with medium containing 1% FBS (1% FBS medium) and the cultures incubated for 48 h to reduce DNA synthesis for replication. The cells were then treated with chemicals for 1 h in 1% FBS medium containing 10 mM hydroxyurea (HU) (1% FBS-HU medium).

When exogenous metabolic activation was necessary, the cultures were treated for 2 h with chemicals in the presence of PMS fraction, as described above.

To measure UDS, the cells were washed after treatment with 1 ml of PBS(+) and 1 ml of 1% FBS-HU medium. Following addition of [^{3}H] thymidine (370 KBq/ml) in 1% FBS-HU medium to the cultures, the cells were incubated for 6 h at 37℃. The uptake of [^{3}H] thymidine was stopped and acid-soluble material was removed by washing the coverslips with cold 0.15 M NaCl and then rinsing 3 times in 5% cold trichloroacetic acid over 60 min. The coverslips were put into vials with scintillation fluid to determine the radioactivity in these samples. The background levels of [^{3}H] thymidine incorporation/culturl well in cultures treated with solvent or PMS alone were <1,200 cpm, and these were subtracted from each determination. All experiments were repeated at least twice with similar results. To determine the cytotoxicity of chemicals, the number of cells on coverslips were counted following treatment with the same condition as in the experiments for detection of UDS. No significant difference in the number of cells was found between the control and experimental groups.

Results and Discussion

Ability of 17 chemicals, whico are negative in *Salmonella* mutagenesis, to induce morphological

transformation of cultured Syrian hamster embryo cells was examined. The results are shown in Table 1. Eleven of the 17 chemicals are reported to be carcinogenic in rodents or humans, but no data are available for 4 chemicals. Ten of the eleven chemicals, exception was clofibrate, induced morphological transformation of SHE cells. SHE cell transformation responses for other chemicals by other investigaters are represented in Table 2. All of twelve chemicals, excluding phenobarbital and TCDD, showed a positive transforming activity in agreement with the rodent carcinogenicity date. We also examined the transforming abilities of chemicals which are reported to be non-carcinogenic. All of them were found to be negative in our SHE cell transformation assay (Table 3).

The results indicate a good correlations between induction of SHE cell transformation and carcinogenicity, suggesting that the SHE cell assay system could be useful for mechanistic studies and detection of potential carcinogenic activity of non-mutagenic carcinogens.

We have been using SHE cells for mechanistic

Table 1. Ability of 17 chemicals, which are negative in *Salmonella* mutagenesis, to induce morphological transformation of cultured Syrian hamster embryo cells

Chemicals	*Salmonella* mutagenesis	Carcinogenicity	Morphological transformation
Amitrole (herbicide)	−	+[a]	+
Asbestos (mineral)	−	+	+
Benzene (industrial chemical)	−	+	+
Clofibrate (hypolipidemic drug)	−	+	−/−[e]
Colcemid (mitotic inhibitor)	−	?[b]	+
Di (2-ethylhexyl) phthalate (DEHP) (plasticizer)	−	+	+/++[e]
Diethylstilbestrol (DES) (synthetic estrogen)	−	+	+/++[e]
17β-Estradiol (natural estrogen)	−	+	+
Eugenol (disinfectant)	−	±[c]	+
Mono (2-ethylhexyl) phthalate (MEHP) (plasticizer)	−	?	−/+[e]
Progesterone (progestin)	−	+	+
Na-arsenite (metal)	−	+	+
Na-bisulfite (food additive)	−	?	+
Na-fluoride (cariostatic agent)	−	E[d]	+
Testosterone (androgen)	−	+	+
Vincristine sulfate (mitotic inhpbitor)	−	?	+
Wy-14, 643 (peroxisome proliferator)	−	+	−/+[e]

[a]Positive, [b]no data availabse, [c]not sufficient, [d]equivocal, [e]results from experiments without/with rat liver mitochondrial supernatant.

Table 2. Transforming activity, which has been reported by others, in cultured Syrian hamster embryo cells induced by treatment with chemicals negative in *Salmonella* mutagenesis.

Chemicals	*Salmonella mutagenesis*	*Carcinogenicity*	Morphological transformation	
			Response	*Reference*
5-Azacytidine	−	+	+	*14*
Bis (*p*-dimethylamino)-diphenylmethane	−	+	+	1
Diphanylhydantoin	−	+	+	14
Ethionine	−	+	+	1
Lead acetate	−	+	+	1
Methapyriline-HCl	−	+	+	14
Thioacetamide	−	+	+	1
Thiourea	−	+	+	1
O-Toluidine-HCl	−	+	+	14, 15
Urethane	−	+	+	16
Phenobarbital	−	+	−	1, 15
2, 3, 7, 8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)	−	+	−	17

Table 3. Chemicals which had a negative response in cultured Syrian hamster embryo cell transformation assay

4-Acetylaminofluorene (4-AAF)
Anthracene
Benzalkonium chloride (disinfectant)
Benzethonium chloride (disinfectant)
Benzo [e] pyrene
Chloramphenicol
EDTA
Glutaraldehyde
Lidocaine-HCl (local anesthetic)
Prilocapne-HCl (local anesthdtic)
Tetracyclpne-HCl

studies of carcinogenesis, particularly induced by putative non-mutagenic carcinogens. For the studies, we have assayed SHE cells treated with carcinogens for the following genotoxic endopoints : gene mutations at hypoxanthine phosphoribosyl transferase and / or Na^+ / K^+ ATPase loci, chromosomal mutations, DNA damage and sister chromatid exchages (SCE). Chromosomal mutations were determined from inducibility of structural aberrations and aneuploidy in the cells treated with the chemicals. DNA damage was detected by measuring unscheduled DNA synthesis. The results are summarized in Table Ⅳ. All chemicals, except clofibrate, induced morphological transformation ; some required exogenous metabolic activations. Some chemicals induced gene mutations (e. g., amitrole, bezene, DES with metabolic activation). Most induced chromosome mutations, aneuploidy or aberrations, with two exceptions ··· sodium bisulfite and testosterone.

Amitrole, a widely used harbicide and a thyroid carcinogen, is negative in *Salmonella* muthgenicity assay. However, we have shown that amitrole induced gene mutations and morphological transformation in SHE cells. This suggests that SHE cells are able to metabolize amitrole by an unusual mechanism. Krauss and Eling[28] have demonstrated the conversion of amitrole to mutagenic intermediates by certain

Table 4. Abilities of 17 chemicals, whico are negative in *Salmonella* mutagenesis, to induce cell transformation, gene mutations, chromosomal mutations and SCE in cultured Syrian hamster embryo cells.

Chemicals	Morphological transformatiou	Gene mutations	Unschedulec DNA synthesis	Chromosomal mutations			Reference
				Aberrations	Aneuploidy	SCE	
Amitrole	+	+					18
Asbestos	+	−		±	+		19
Benzene	+	+		−	+	−	20
Clofibrate	−/−[a]			−/−[a]			20
Colcemid	+	−	−	−	+		21
Di (2-ethylhexyl)-phthalate (DEHP)	+/++[a]			−/+[a]			20
Diethylstilbestrol (DES)	+/++[a]	−/+[a]	−/+[a]	−	+		3, 4, 22, 23
17β-estradiol	+	−		−	+		24
Eugenol	+		−/+[a]	+		±[b]	20
Mone (2-ethylhexyl)-Phthalate (MEHP)	−/+[a]			−/+[a]			20
Progesterone	+						20
Na-arsenite	+	−		+	−	+	20
Na-bisulfitd	+	−		−	−	+	25
Na-fluoride	+		+	+		+	14, 26
Testosserone	+	−		−	−	±[b]	20
Vincristine sulfate	+	−	−	−	+		27
Wy-14, 643	−/+[a]			−/+[a]			20

[a]Results from experiments without/with rat liver mitochondrial supernatant.
[b]Inducibility was significant ($p < 0.01$), but less than twice over untreated cultures.

peroxidases, including thyroid peroxidase and prostaglandin synthetase. SHE cells are known to carry out the prostaglandin synthetase-mediated peroxidative metabolism of DES.

DES, 17β-estradiol, Colcemid and vincrissine sulfate induced cell transformation, bus did not induce gene mutations, DNA damage or chromosome aberrations. Numerical chromosome changes, that is, aneuploidy was induced by all these chemicals. When the cells were treated with DES in the presence of exogenous metabolic activation, enhanced cell transformation and induction of ouabain-resistant mutation and unscheduled DNA synthesis were observed. This indicates that two pathways may exist for the induction of cell transformation by DES. One apparently does not involve direct DNA damage, but rather, we have proposed[4], involves disruption of microtubule organization. The other, which requires rat liver post-mitochondrial supernatant-mediated exogenous metabolic activation, is associated with DNA damage and mutagenicity[23]. Since metabolic intermediates of estrogens may be involved in both pathways, we examined whether or not cell transformation, chromosome aberrations and aneuploidy are induced by 17β-estradiol and its metabolites. Treatment with 17β-estradiol for 48 h induced morphological transformation of cells in a does-related fashion. Percentages of heteroploid cells significantly increased by the treatment in the both

ranges of tetraploid and diploid number of chromosomes[24]. 2-Hydroxyestradiol and 4-hyroxyestradiol also induced morphological transformation and numerical chromosome changes. The transformation frequencies induced by treatment with 1 and 3 μg/ml of 4-hydroxyestradiol were slightly higher than those by treatment with the same concentrations of 17 β-estradiol and 2-hydroxyestradiol[29]. Although treatment of SHE cells for 24 h with 17 β-estradiol at the concentrations examined did not induce significant levels of chromosome aberrations, exposure to 2-hydroxyestradiol and 4-hydroxyestradiol at the highest concentrations tested elicited chromosome aberrations of the cells[29]. The results suggest that metabolic intermediates of 17 β-estradiol are involved in each of the two mechanisms for estrogen-induced carcinogenicity.

Colcemid and vincristine sulfate are microtubule disrupting agents which are known to induce aneuploidy and polyploidy in cells in culture. Indeed, the compounds induced morphological and neoplastic transformation, and numerical chromosome changes in SHE cells without measurable gene mutations at HPRT and Na^+/K^+ ATPase loci[21, 27]. This suggests that the induction of aneuploidy and cell transformation is related. Another possible mechanism for the mitotic inhibitor-induced transformation has been reported by Shinohara *et al*[30]. They demonstrated that the compounds induced phosphorylation of microtubule-associated protein 2 kinase, followed by activation of other kinases which initiated DNA synthesis of cells and finally led to the cell division.

Treatment with DEHP or Wy-14, 643 for 2 h with exogenous metabolic activation induced chromosome aberrations in SHE cells. When the cells were treated for 2 h with the compounds in the absence of exogenous metabolic activation, significant levels of chromosome aberrations were not detected in the cells[20]. The results suggest the involvement of metabolic enzymes in liver in SHE cell transformation by DEHP and Wy-14, 643.

Sodium arsenite induced cell transformation, chromosome aberrations and SCE in cultured SHE cells, but did not induce gene mutations, unscheduled DNA synthesis and numerical chromosome changes. Further studies on which DNA damage is involved in cell transformation by sodium arsenite are in progress using alkaline esution assay and ^{32}P-postlabeling assay. As sodium arsenite and sodium arsenate are known as potent enhancers of amplification of the dihydrofolate reductase gene in mouse 3T6 cells[31], we examined whether or not amplification of oncogenes occurred in SHE cells after treatment with sodium arsenite or sodium arsenate, followed by continous cultivation with normal medium. We found sodium arsenite and sodium arsenate induced neoplastic transformation of SHE cells. Moreover, gene amplification occurred at specific oncogenes in some of the transformed cell lines, but was not accompanied by tumorigenicpty of the cells. The tumorigenicity was accompanied by over expression of c-*ras* or c-*myc* gene[20]. Studiez on the role of gene amplification in the process of tumorigenic conversion are in progress.

In summary, the results suggest that SHE cell assay system could be useful for mechanistic studies as well as detection of potential carcinogenic activity of non-genotoxic carcinogens.

RECIEVED, December 12; ACCEPTED, December 28.

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