— Minireview —

In Vitro Assays for the Prediction of Tumorigenic Potential of Non-genotoxic Carcinogens

Kiyomi Ohmori*

Chemistry Division, Kanagawa Prefectural Institute of Public Health, 1–3–1 Shimomachiya, Chigasaki, Kanagawa 253–0087, Japan

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As screening methods to predict carcinogenicity, genotoxicity assays have a major issue in that many carcinogens are negative in such assays. Non-genotoxic mechanisms, which are at least initially independent of direct DNA damage, can play a causal role in carcinogenesis. Also, it is predicted that among these non-genotoxic carcinogens, many will be tumor promoters. There is therefore a need to develop tumor promoter assays, and *in vitro* assays have been developed to detect phenomena such as the inhibition of gap junctional intercellular communication, the promotion or inhibition of cell differentiation, the expression of Epstein-Barr virus early antigen (EBV-EA), and cell transformation assays. However, none of these assays has been adopted in the battery of official safety screening tests for chemicals. One reason is that some methods are not simple for routine screening. Given this lack, we established a novel short-term *in vitro* method to detect the tumor promoting potential of chemicals, the Bhas promotion assay. This is a cell transformation assay using Bhas 42 cells. It has many advantages compared with other focus formation (cell transformation) assays. Transferability and applicability of this assay was confirmed by an inter-laboratory collaborative study. Furthermore, our study has demonstrated that the Bhas promotion assay has practical utility value in monitoring the promoting potential of environmental contaminants.

Key words —— tumorigenic potential, non-genotoxic carcinogen, tumor promoter assay, cell transformation, Bhas 42 cells

INTRODUCTION

A multistage process of carcinogenesis is widely accepted. The two-stage model[1] of initiation and promotion that was proposed for chemical carcinogenesis is considered to be an operational basis for assays to predict chemical carcinogenicity. It is thought that the initiation process includes one or several mutational events, which may occur in certain oncogenes.[2] When initiated cells are subsequently exposed repeatedly to tumor promoters, they deviate from normal growth control to disordered cell proliferation and tumorigenesis. The carcinogenicity of chemicals is determined by lifetime exposure assays using laboratory animals. However, animal experiments are time-consuming and very costly, and also involve ethical issues. It is consequently impracticable to make animal experiments for testing all chemicals. Therefore, as screening methods for prediction of carcinogenicity, various genotoxicity assays (variation carcinogenicity assays) have been devised and are routinely used. The assays adopted by the International Conference of Pharmaceuticals for Human Use (ICH) for prediction of chemical carcinogenicity are the Ames assay, nucleolus assay, and chromosome abnormality assay or mouse lymphoma assay (MLA).[3]

As screening methods to predict carcinogenicity, the above-mentioned genotoxicity assays and variations are performed. Nevertheless, it has become a major issue that there are many carcinogenic substances which are negative in the existing genotoxicity assays. Non-genotoxic mechanisms, which are at least initially independent of direct DNA damage, can play a causal role in carcinogenesis;[4] also, it is predicted that among these non-genotoxic carcinogens, many will be tumor promoters. Therefore, tumor promotion assays as screening methods are needed. Many in vitro assays have been developed. The findings on the biological or biochemical

*To whom correspondence should be addressed: Chemistry Division, Kanagawa Prefectural Institute of Public Health, 1–3–1 Shimomachiya, Chigasaki, Kanagawa 253–0087, Japan. Tel.:+81-0467-83-4400; Fax:+81-0467-83-4457; E-mail: ohmori.n4yf@pref.kanagawa.jp

effects of tumor promoters, such as the inhibition of gap junctional intercellular communication,[5–9] the promotion or inhibition of cell differentiation,[10] the expression of Epstein-Barr virus early antigen (EBV-EA),[11] or the induction of cell transformation,[12–16] were applied to *in vitro* assays for detecting tumor promoters. However, none of the assays has been adopted as a routine method in the battery of official safety screening tests for chemicals. One reason is that some methods are not simple for routine screening. Another reason is that they were not validated as promotion assays for the evaluation of carcinogenesis risk. Given these facts we established a short-term method for the detection of the promoting potential of chemicals by using Bhas 42 cells, the Bhas promotion assay,[17] and carried out an inter-laboratory collaborative study on the Bhas promotion assay among fourteen laboratories in order to validate the transferability and applicability of this assay.[18] Furthermore, the Bhas promotion assay was applied to monitoring the tumor promotion potency of air pollutants. Our study demonstrated that this method is useful in the environmental public health field.[19] In this review, I introduce the usual *in vitro* assays and our novel *in vitro* assay, the Bhas promotion assay, developed to predict the tumorigenic potential of chemicals which cannot be accomplished by genotoxicity testing.

INHIBITION OF INTERCELLULAR COMMUNICATION

Gap junctional intercellular communication is considered to play an important role in the regulation of cellular growth and differentiation. Inhibition of intercellular communication by various chemicals has been shown to be a factor in the tumor promotion phase of carcinogenesis (Yotti *et al.*, 1979;[6] Murray and Fitzgerald, 1979;[5] Umeda *et al.*, 1980[7]) and other chemically-induced diseases. Measurement of gap junctional intercellular communication has been achieved by metabolic cooperation of cells with enzyme deficiencies in certain metabolic pathways[5,6] and by dye transfer following microinjection[8] or scrape-loading.[9] The advantages of these assay methods were: short time frames (within 7 days), little effect of the serum lot on the results, and easy detection techniques. A disadvantage understood is that the toxicity to cell membranes by test compounds can affect cell-cell communication.

Inhibition of Metabolic Cooperation Assay Using Chinese Hamster V79 Cells

This assay method detects the inhibition of intercellular communication between two cell lines by test compounds.[6,7] One cell line used in this assay is wild-type V79 cells which possess hypoxanthine-guanine phosphoribosyl transferase activity (HGPRT+), the other cell line is the 6-thioguanine (6-TG) resistant V79 counterpart which has no HGPRT activity (HGPRT−). HGPRT metabolizes 6-TG to the toxic form, 6-thioguanosine monophosphate. Therefore, HGPRT+ cells cannot survive in a medium containing 6-TG, whereas HGPRT− cells can survive in this medium. When HGPRT+ cells and HGPRT− V79 cells are cocultivated in a medium containing 6-TG, the 6-thioguanosine monophosphate metabolized from 6-TG in HGPRT+ cells passes into HGPRT− cells by cell-cell communication via gap junctions between contacting cells, and HGPRT− cells are killed by 6-thioguanosine monophosphate. A tumor promoter such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) inhibits the communication between the cells, and then HGPRT− cells can survive when co-cultivated with HGPRT+ cells in a medium containing 6-TG. Consequently, resultant colonies of HGPRT− cells are formed in the dishes.

Dye-transfer Assay

In the fluorescent dye-transfer assay, fluorescent dye such as Lucifer Yellow CH is microinjected into a cell after treatment with a test compound, and the extent of dye transferred to neighboring cells through gap junctional intercellular communication is evaluated by a fluorescence microscope. Cell-cell communication among mouse Balb/c 3T3 cells and among Chinese hamster V79 cells was inhibited almost completely by tumor-promoting phorbol esters, but not by nonpromoting derivatives.[8] As a rapid and reliable dye transfer assay, a scrape-loading/dye transfer technique was used instead of microinjection.[9] In this assay, effective blockage of dye transmission was observed in cells pretreated with a tumor promoter, such as TPA, teleocidin, saccharin or mezerein. The scrape-loading/dye transfer assay can be applied to a wide variety of mammalian cells such as Chinese hamster V79 cells, rat liver WB cells, NIH/3T3 cells, calf aorta muscle cells (primary culture) and human foreskin fibroblast MSU-2 cells (primary culture).

EBV-EA INDUCTION

EBV-EA in Raji Cells

EBV-EA in Raji cells was induced by treatment with several tumor promoters, such as TPA and its related congeners, with *n*-butyric acid as a co-inducer.[11, 20] This short-term *in vitro* assay has also been used for the detection of prospective anti-tumor promoters found in medicinal plants, examining their inhibitory effects on the EBV-EA expression induced by TPA. Inhibition of EBV-EA correlated with anti-tumor promoting effects in mouse skin.[21–28]

In the traditional method, Raji cells are cultivated together with test chemicals and *n*-butyric acid for 48 hr. *n*-Butyric acid is used as the EBV-EA co-inducer. After the reaction, the treated cells are smeared on glass slides and stained with EBV-positive sera from nasopharyngeal carcinoma (NPC) patients as the first antibody and then with fluorescein isothiocyanate (FITC)-labeled anti-human IgG as the second antibody. EBV-EA-positive cells are counted using a fluorescence microscope.[11, 20] This method relies on the experience of examiners in judging EBV-EA-positive and EBV-EA-negative cells and is time-consuming.

In order to confirm the applicability of the induction of EBV-EA in Raji cells for the detection of prospective tumor promoters, the microscope detection method was altered to a sensitive quantitative method by using anti-EBV-EA monoclonal antibody, a fluorogenic substrate and a fluorescence microplate reader.[29] In this improved immunofluorometric method for the detection of EBV-EA in Raji cells, TPA was active from 0.1 ng/ml. The method is more sensitive to TPA than the traditional microscope detection method. For the study on the mechanisms of EBV-EA induction, we examined several TPA inhibitors.[35] EBV-EA induction by TPA was decreased to 65 % with 5 μg/ml palmitoyl-DL-carnitine (an inhibitor of protein kinase C), and decreased to 31 % with 10 μg/ml *p*-bromophenacyl-bromide (an inhibitor of phospholipase A_2). These results suggested that EBV-EA induction by TPA in Raji cells is related to the activation of protein kinase C and the inflammatory process. Subsequently 17 chemicals, including tumor promoters and their related compounds, were tested. The positive chemicals were okadaic acid, diethylstilbestrol, progesterone, sodium phenobarbital, aldrin and dieldrin. Lithocholic acid (LCA), testosterone and DDT were equivocal. Eight other chemicals, *viz.*, 17β-estradiol, androsterone, catechol, auramine, acrylamide, sodium fluoride, saccharine sodium salt and phenacetin, did not induce EBV-EA.

CELL TRANSFORMATION

The endpoint of cell transformation assays is the development of transformed phenotypes, for example, loss of anchorage dependency in JB6 cells (the soft agar assay),[12] morphological changes of colonies in Syrian hamster embryo (SHE) cells (the colony assay),[13] or focus formation of transformed cells that lose contact inhibition of cellular growth, *e.g.* Balb/c 3T3 cells,[14, 15] C3H/10T1/2 cells[16] and Bhas 42 cells (the focus formation assay).[30, 31]

Soft Agar Assay Using JB6 Cells

The JB6 cell line was established from newborn Balb/c mouse skin, and JB6 (p+) is a promotion-sensitive clone which retains contact inhibition and anchorage dependency.[12] The cells usually cannot grow in soft agar medium. However, JB6 (p+) cells lose the anchorage dependency after treatment with a tumor promoter, and can grow in the medium to form colonies. In the soft agar assay using JB6 (p+) cells, the cells are cultured in 0.33 % soft agar medium containing the test compound on 0.5 % agar medium for 2 weeks, after which the number of colonies is counted. Colony formation of JB6 (p+) cells induced by phorbol esters was correlated with tumorigenesis in mouse skin but not in other organs. In addition, the soft agar assay using JB6 (p+) cells seems superior for chemicals which produce active oxygen species, such as cisplatin, benzyl peroxide, hydrogen peroxide and xanthine- xanthine oxide.[32]

Colony Assay by SHE Cells

In the SHE cell transformation assay primary cells derived from SHE cells are used, and the colony formation of transformed cells is detected as the endpoint. In the SHE cell assay,[13] the cells at clonal density were treated with a test chemical for 7-days in Dulbecco's modified Eagle's medium at pH 7.1–7.3 containing fetal bovine serum (FBS). Subsequently the colonies are fixed, stained and scored for morphological transformation. The initial standard procedure had several problems, among them low frequencies of morphological transformation following exposure to carcinogens, and difficulties with scoring and identi-

fying colonies of transformed cells. The historical protocol for the SHE cell assay has undergone several modifications, the most extensive of which concern recently introduced changes.[33] In the low pH protocol, Dulbecco's modified Eagle's medium at pH 6.7 for cell culture—originally developed to enhance clonal SHE cell growth—offers substantial benefits.[34, 35] For example, the reduced pH protocol has been shown to result in a 5–10-fold increase in transformation frequencies, a decrease in susceptibility to fluctuations in serum quality, and less ambiguity in the scoring of the transformed phenotype. Nevertheless, the scoring and the identification of transformed cells in this assay are more difficult than other cell transformation assays. In addition, the SHE cell assay is very expensive because of the requirement for primary cells derived from hamster embryos. In 1996, a collaborative study was carried out in which two laboratories evaluated 56 chemicals in the SHE cell assay.[36, 37] A study on the inter-laboratory reproducibility of the SHE cell assay is currently in progress by the European Centre for the Validation of Alternative Methods (ECVAM).

Focus Formation Assay Using BALB/3T3 Cells or C3H/10T1/2 Cells

In a two-stage transformation assay where promoter treatment is performed after initiator treatment, clone A31-1 or A31-1-1 for Balb/c 3T3 cells (fibroblast cell line derived from Balb/c mouse embryo) or clone 8 for C3H/10T1/2 cells (fibroblast cell line derived from C3H mouse embryo) is employed. These cells maintain a feature of contact inhibition of cell proliferation. When cell proliferation leads to cell crowding and saturation in a culture dish, growth comes to a halt in a monolayer, which is a feature of normal cells. However, when the cells lose contact inhibition after treatment with tumor promoters, disordered cell proliferation is induced and the cells tend to pile up in a random criss-cross fashion. Such transformed cells also exhibit a morphological change which shows a distinctive shape (spindle-shape). Tumor promoting potential of chemicals is determined by enumerating the foci of transformed cells. These standard assays take 6 or 8 weeks to perform. In the focus formation assay using Balb/c 3T3 cells, the medium improvement method (addition of 1 % insulin-transferrin-Selenium supplement (ITES) and 2 % bovine serum albumin to Dulbecco's moditied eagle medium; nutriemt mixture F-12, 1 : 1 mixture (DMEM/F12) medium) by Tsuchiya and Umeda (1995) made the assay period shorter from 6 weeks in the former method to 3.5 weeks and made the sensitivity higher than the conventional method.[15]

In the cell transformation assay with Balb/c 3T3 cells, anthralin, dehydroteleocidin B and mezerein, which are mouse-skin tumor promoters, have transformation promoting activity. Catechol, DDT, phenobarbital, lithocholic acid and taurocholic acid enhance Balb/c 3T3 cell transformation at the promotion stage. In the assay with C3H/10T1/2 cells, asbestos, caffeine, cholic acids, cortisone, dexamethasone, 17β-estradiol, diethylstilbestrol, dioxins and formaldehyde induce transformed foci. Saccharin, which is negative in Balb/c 3T3 cells, shows transformation-promoting activity in C3H/10T1/2 cells.[37] Inter-laboratory reproducibility of the cell transformation assays with Balb/c 3T3 cells and C3H/10T1/2 cells has been evaluated and these results have been compared with the SHE cell assay by ECVAM.[37]

Focus Formation Assay Using Bhas 42 Cells

In vitro cell transformation assays using Balb/c 3T3 cells can simulate the process of two-stage carcinogenesis in animals.[14, 15] For the detection of promoting chemicals in the *in vitro* cell transformation assay, the cells treated with an appropriate concentration of an initiating agent are subsequently treated with test chemicals. In this method, treatment with an initiating agent and the subsequent expression period are required before administration of test chemicals. Typically, these assays require more than ten plates of 6 or 10–20 cm dishes for each dose.

In order to improve experimental conditions for the evaluation of chemicals with tumor-promoting potential, a cell line—called Bhas 42—was established from Balb/c 3T3 cells transfected with *v*-Ha*ras* oncogene.[30, 31] According to the original procedure, Bhas 42 cells, co-cultivated with Balb/c 3T3 cells, could develop into transformation foci after treatment with chemicals having promoting potential. Here, treatment with an initiating agent and subsequent cultivation for expression period could be omitted. However, it takes a period of 6 weeks is required for the formation of transformed foci. Recently, Ohmori and colleagues found that by using Bhas 42 cells after advanced sub-culturing and using an enriched basal medium, transformed foci can be efficiently induced in a single culture of the cells by treatment with promoting agents and without the need for co-cultivated Balb/c 3T3 cells.[17]

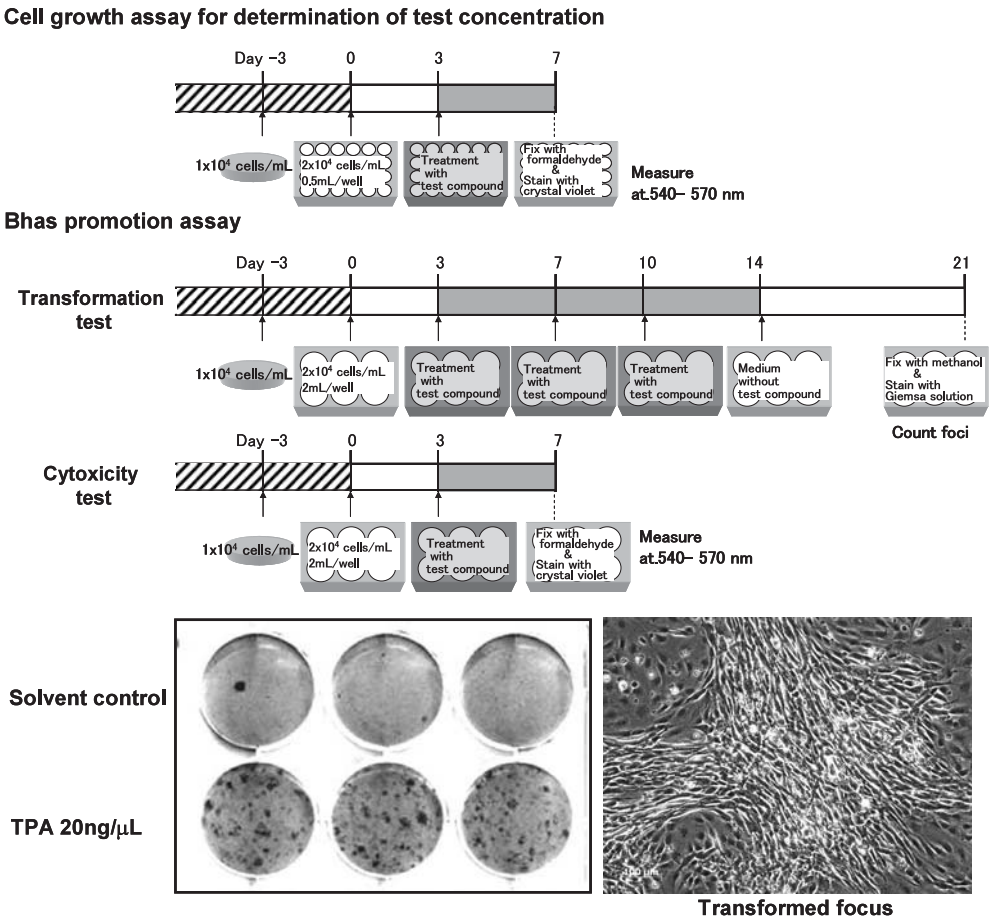

Fig. 1. Protocol of Bhas Promotion Assay
▨; Mother culture (DF5F), □; DF5F, ▩; DF5F+text compound, ↑; medium change.

Furthermore, the period of focus formation can be shortened to 2.5–3 weeks. From these findings, we worked to establish a short-term screening method to detect the promoting potential of non-genotoxic carcinogens and tumor promoters.

The Bhas promotion assay has many advantages compared with other focus formation assays: namely, (1) treatment with an initiating agent and subsequent cultivation for the expression period can be omitted; (2) the experimental period is shortened from 4–6 weeks to 2.5–3 weeks after cell inoculation, and (3) transformation frequency is high, so that only three to six wells of 6-well plates are required for each dose instead of more than ten plates of 6 or 10–20 cm dishes. In order to gain wider awareness of this method, it was necessary to confirm its applicability and transferability between laboratories. We performed an inter-laboratory collaborative study on the Bhas promotion assay. Fourteen laboratories belonging to the Non-genotoxic Carcinogen Study (NGCS) Group in the Environmental Mutagen Society of Japan participated in the project.[18] After confirmation that these laboratories could obtain positive results with two tumor promoters, TPA and LCA, 12 coded chemicals were assayed. Each chemical was tested in four laboratories. For eight chemicals, all four laboratories obtained consistent results, and for two of the other four chemicals, only one of four laboratories showed inconsistent results. Thus the rate of consistency was high. During the study, several issues were raised, each of which was analyzed step-by-step in the inter-laboratory collaborative study, and resulted in protocol revision of the original assay (Fig. 1).

The original Bhas promotion assay protocol[17] was employed, with several modifications as follows, from the results of an inter-laboratory collaborative study.[18] minimum essential medium (MEM) medium supplemented with 10 % FBS was used for stock cell cultivation, while DF5F medium (DMEM/F12 supplemented with 5 % FBS) was

used for transformation experiments and for the mother culture. In addition, it was critically important to use cells at around 60–70 % confluence of the mother culture. A cell suspension of 2×10^4 cells/ml was prepared from mother culture, and 2 ml was distributed into each well of 6-well plates (4×10^4 cells/well). In assaying test chemicals, each dose group consisted of 6 wells. After cultivation for three days, medium was replaced with fresh medium containing the test chemical. The final concentration of vehicle organic solvents in the medium was less than 0.1 % in the case of dimethyl sulfoxide (DMSO) and ethanol, and less than 0.5 % in the case of acetone. The cultures were again provided fresh medium containing test chemicals on Day 7 and Day 10, and then fresh DF5F medium alone on Day 14. On Day 21, cells were fixed with methanol for 10 min and stained with 5 % Giemsa solution for 30 min. From the results of an inter-laboratory collaborative study, the original protocol (2.5 weeks) assay period was extended to three weeks without medium change during the last week in order to make focus counting much easier.

The transformed foci were characterized by the following morphological criteria: deep basophilic staining, dense multi-layering of cells, random orientation of cells at the edge of foci, and more than 50 cells within a focus. Concurrent cell growth assay was performed by preparing additional 6-well plates and treating the cells as in the transformation assay. On Day 7, the cells were fixed and stained as described in the cell growth assay for dose-determination.

The results in the original protocol were evaluated by a *t*-test analysis.[17] Chemicals showing significant increase ($p < 0.05$) of focus number at more than two consecutive concentrations were considered to be positive (+), while those showing a statistically significant effect at only one concentration, even after repeat tests, were judged to be equivocal (±). Negative chemicals were those which induced no statistically significant increase of transformed foci. In the inter-laboratory collaborative study, the statistical analysis and criteria of judgment were investigated by biostatisticians, and the following criteria were set for the evaluation of transformation results:[18] (1) significant increase by one-sided Dunnett test with significance level of 5 % ($p < 0.05$), and (2) more than two-fold increase as compared with the solvent control. Chemicals which satisfied these two criteria were judged positive (+); chemicals which met only the first criterion but not the second one were considered equivocal (±). Negative chemicals were those which induced no statistically significant increase of transformed foci at any concentration.

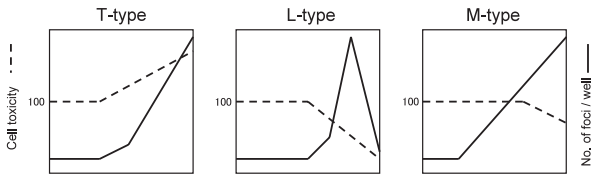

Fig. 2. Three Types of Chemicals Showing Positive Response in Bhas Promotion Assay

From results of the Bhas promotion assay including cell transformation assay and cell toxicity test, it is suggested that there are three different types of chemicals showing positive promoting activity in the assay (Fig. 2). Those designated as T-type induced extreme growth enhancement, and include TPA, mezerein, phorbol 12,13-didecanoate and insulin. LCA and okadaic acid belong to the L-type category in which transformed foci were induced at limited concentrations showing growth-inhibition. In contrast, progesterone, catechol and sodium saccharin (M-type) induced foci at concentrations with little to slight growth inhibition.

The Bhas promotion assay results are summarized together with other information on short-term promotion assays, carcinogenicity in animals and carcinogenicity evaluation by International Agency for Research on Cancer (IARC) (Table 1). The test performance of Bhas 42 cell transformation assay from 63 chemicals which had data of *in vivo* carcinogenicity is as follows (Table 2): 75 % concordance, 77 % sensitivity, 67 % specificity, 91 % positive predictivity, 33 % false positive and 24 % false negative.[38] Concordance in the other cell transformation assays, which were evaluated before being suggested for development into Organisation for Economic Co-operation and Development (OECD) Test Guidelines, were compared with the Bhas 42 cell transformation assay: 68 % for Balb/c 3T3 cell transformation assay, 73 % for C3H/10T1/2 cell transformation assay, and 74 % for SHE transformation assay.[37] The 75 % concordance in the Bhas 42 cell transformation assay is thus the highest among these assays.

As an application of the Bhas promotion assay to environmental toxicology, we measured the tumor promoter potential of airborne particulate matter.[19] The tumor promoting potential

Table 1. Summary of Results in the Short-term Promotion Assays and Carcinogenicity Evaluation from IARC

Chemical	*In vitro*								*In vivo*		IARC
	Bhas promotion assay	Balb 3T3	C3H/10T1/2	SHE	JB6	V79	Raji	Ames	Mouse	Rat	
TPA	+ (i)	+	+	+	+	+	+	−	skin		
PDD	+ (i)	+		+		+		−	skin		
Mezerein	+ (i)	+	+	+	+	+		−	skin		
Phorbol	−				−	−		−			
4α-Phorbol	− (i)							−			
Okadaic acid	+ (i)	+	+	+	±	−	+	−	skin		
Anthralin	± (i)	+		−		±		−	skin		3
Lithocholic acid	+ (i)	+	+			+	±	−	Colon		
op′-DDT	+	+		+		+	±	−		Liver	2B
pp′-DDT	+	+		+		+		−		Liver	2B
Phenobarbital (sodium salt)	±	+/−	+/−			±	+	−		Liver	2B
Progesterone	+ (i)			+/−			+	−	Ovarian, Uterine, Mammary		
17β-Estradiol	− (i)	−	+	+		−	−	−	Mammary, Pituitary, Uterine, Cervical, Vaginal, Testicular, Lymphoid, Bone	Mammary, Pituitary	1
Diethylstilbestrol	− (i)	−	+/−	+		−	+	−		Vagina	1
Dexamethasone	± (i)		+			−		−		Liver	
Arsenic trioxide	+							−			1
Saccharin (sodium salt)	+ (i)	−	+	−	−	±	−	−		Bladder	2B
Catechol	+ (i)	+				±	−	−	Forestomach		2B
Insulin	+ (i)	+			−	−		−			
Teststerone							±	−		Uterine	
Androsterone							−	−			
Aldrin						+	+	−	Liver	Urinary	3
Dieldrin						+	+	−	Liver		3
Auramine							−	+	Liver	Liver	2B
Acrylamide		+					−	−	Lung	Testis, Thyroid	2A
Sodium fluoride				+			−	−			3
Phenacetin							−	+	Urinary tract		2A

(i): result of inter-laboratory collaborative study, +/−: positive and negative

of dichloromethane extracts of suspended particulate matter (SPM), 13 size-fractionated airborne samples—which were collected by an Andersen low pressure impactor—were tested by this assay (Fig. 3). Particles less than 2.5 μm in diameter generally induced more transformed foci per unit mass than particles more than 2.5 μm in diameter. The highest promoting potential per unit mass was observed in the sample less than 0.06 μm in diameter. The promoting potential per unit volume was highest in the three mid-point sequential samples with diameters of 0.52, 0.76 and 1.25 μm in diameter. These results showed that most of the total promoting potential (*ca.* 70 %) was associated with the fine fractions of the particles (< 1.25 μm), suggesting that very fine particles, which are deposited in

Table 2. Performance Characteristics of Cell Transformation Assays

	SHE pH 6.7	SHE > pH 7.0	C3H/10T1/2	BALB/c 3T3	Bhas 42[38)]
Total chemicals	88	204	96	68	63
Concordance (%)	74	85	73	68	75
Sensitivity (%)	66	92	72	75	77
Specificity (%)	85	66	80	53	67
+Predictivity (%)	88	88	95	77	91
−Predictivity (%)	62	75	34	50	40
False+ (%)	15	34	20	47	33
False− (%)	33	8	28	25	24

		In vivo Carcinogenicity	
		Carcinogen	Non-carcinogen
Cell transformation assay	+	*a*	*b*
	−	*c*	*d*

Concordance: % agreement with in vivo experiment $(a + d)/(a + b + c + d) \times 100$, sensitivity: % carcinogens that are positive $a/(a+c) \times 100$, specificity: % non-carcinogens that are negative $d/(b+d) \times 100$, positive predictivity: % positives that are carcinogens $a/(a + b) \times 100$, negative predictivity: % negatives that are non-carcinogens $d/(c + d) \times 100$, false negatives: $c/(a + c) \times 100$, false positives: $b/(b + d) \times 100$.

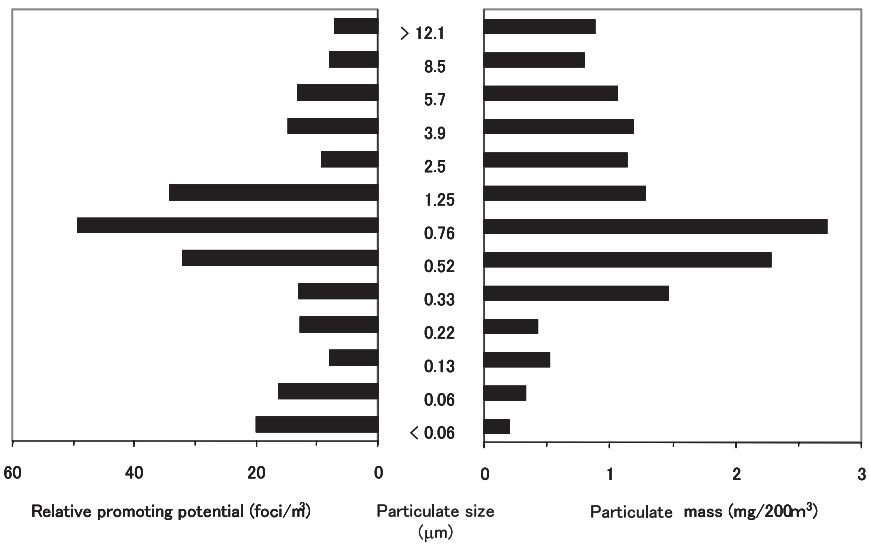

Fig. 3. Promoting Potential of SPM

the lung at high frequency, may carry a higher risk of carcinogenesis than the other particles. Therefore, to monitor the carcinogenic-related activity of air pollutants, not only the mutagenicity assay but also the tumor promotion assay is required.

CONCLUSION

In vitro assay methods have been developed to detect the tumorigenic potential of many carcinogenic substances which are negative in the existing genotoxicity testing: the in inhibition of gap junctional intercellular communication,[5–9)] the promotion or inhibition of cell differentiation,[10)] the expression of EBV-EA,[11)] and the cell transformation assays.[6)] Among these methods, cell transformation assays, which mimic some stage of *in vivo* multistep carcinogenesis, have been proposed to predict the carcinogenic potential of chemicals.

The Bhas promotion assay designed as a cell culture transformation assay at the tumor promotion stage, has many advantages:

1) Treatment with an initiating agent and subsequent cultivation for an expression period can be omitted : a ***simple*** assay

2) The experimental period is shortened from 4–6 weeks to 2.5–3 weeks after cell inoculation : a ***short-term*** assay of focus formation
3) Transformation frequency and specificity are high : a ***sensitive and specific*** assay
4) The applicability and transferability are high : a ***general purpose*** assay
5) Only three to six wells of 6-well plates are required for each dose instead of 6 of 10–20 cm dishes : an ***economical*** assay

Transferability and applicability of this assay were confirmed by an inter-laboratory collaborative study. Furthermore, our study has demonstrated a practical use for the Bhas promotion assay in monitoring the promoting potential of environmental contaminants. The author is currently attempting wider applications of the assay such as for food additives and food contaminants. An understanding of the mechanism of cell transformation in Bhas 42 cells is also being sought by –omics technologies.

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