The induction of transformed foci with or without TPA was examined using various basal media added with 5 or 10% FBS (Fig. 1). The use of 10% FBS produced monolayers a little greater in cell density and induced more spontaneously arising foci than the use of 5% FBS. Among basal media examined, DMEM and DMEM/F12 gave great numbers of transformed foci on treatment with TPA. The foci formed were clearer and larger in DMEM/F12 than in DMEM (Fig. 2). From these results, DMEM/F12 supplemented with 5% FBS (DF5F) was selected as the medium for the subsequent experiments.

The effect of co-cultivation of Bhas 42 cells with BALB/c 3T3 cells was examined. The number of transformed foci of Bhas 42 cells was reduced by the presence of BALB/c 3T3 cells: the greater the number of BALB/c 3T3, the less the number of the foci. The culture of Bhas 42 cells alone resulted in the highest number of transformed foci (data not shown). Therefore, it was concluded that co-cultivation with BALB/c 3T3 cells was unnecessary in this protocol.

In order to determine the optimal time frame of treatment with test chemicals, various treatment periods were tested for TPA (Fig. 3). In this experiment, 2 ml of 2×10^4 Bhas 42 cells/ml (4×10^4 cells per well) were inoculated into each well of six-well plates, and TPA treatment was started from day 3. The number of foci was highest when the medium containing TPA was changed three times, i.e., on days 3, 7 and 10, and with treatment ceasing on day 14.

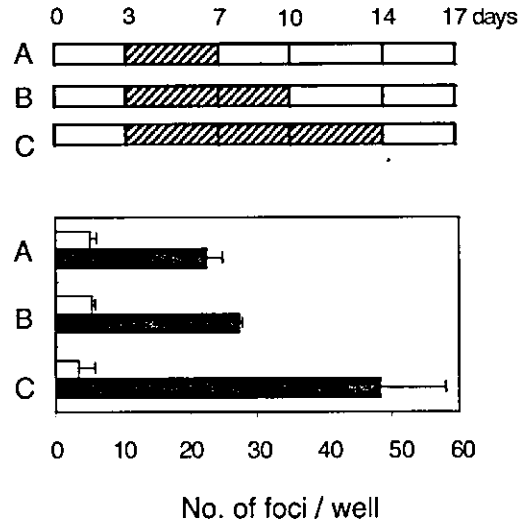

Fig. 3. Effects of period of TPA treatment on the transformation of Bhas 42 cells: (▨) period of TPA treatment; (□) control; (■) TPA (20 ng/ml).

The transformation assay was carried out with Bhas 42 cells inoculated at various cell numbers (Fig. 4). The cells were treated with TPA on days 3, 7 and 10, and fixed on day 17 or 21. When seeded at 4×10^4 cells per well, the cells reached near confluence on day 3. In this condition, the number of transformed foci was highest. The foci obtained from cultures fixed on day 21 were clearer and larger than those fixed on day 17. In addition, the number of foci fixed on day 21 was slightly increased at every inoculum size, but this was not statistically significant in comparison with those fixed on day 17. From these results, an inoculum size of 4×10^4 cells per well of six-well plates and 17 days of culture as the experimental period were adopted. Due to this shorter culture period, it was designed to score foci consisting of 20 or more cells.

3.2. *Variables influencing the formation of transformed foci*

During the course of experiments it was found that the concentration of DMSO used as a solvent affected focus formation. Various solvents were tested. DMSO and ethanol at 0.5% but not 0.1% decreased the number of transformed foci. In contrast, little effect was observed by the addition of 0.5% acetone.

In order to assure the stable supply of Bhas 42 cells, the effect of further passages on transformation was examined using 2 kinds of media, M10F and DF5F. Cells at the 13th passage generation were further cultured for 1 month. The cells before confluence were sub-cultured at a 3–4-day interval. At appropriate intervals, the cells were stocked frozen at −80 °C. Each stock of the cells was thawed and examined for the formation of transformed foci with or without TPA. Fig. 5 reveals that even after a month of culture the number of transformed foci induced by TPA did not change significantly when cultured in M10F. Also, spontaneous formation of foci did not increase after the passages. In contrast, the cells cultured in DF5F formed a gradually increasing number of transformed foci irrespective of the treatment with TPA, suggesting that cultivation in M10F was most appropriate for the proper maintenance of the cells for the assay.

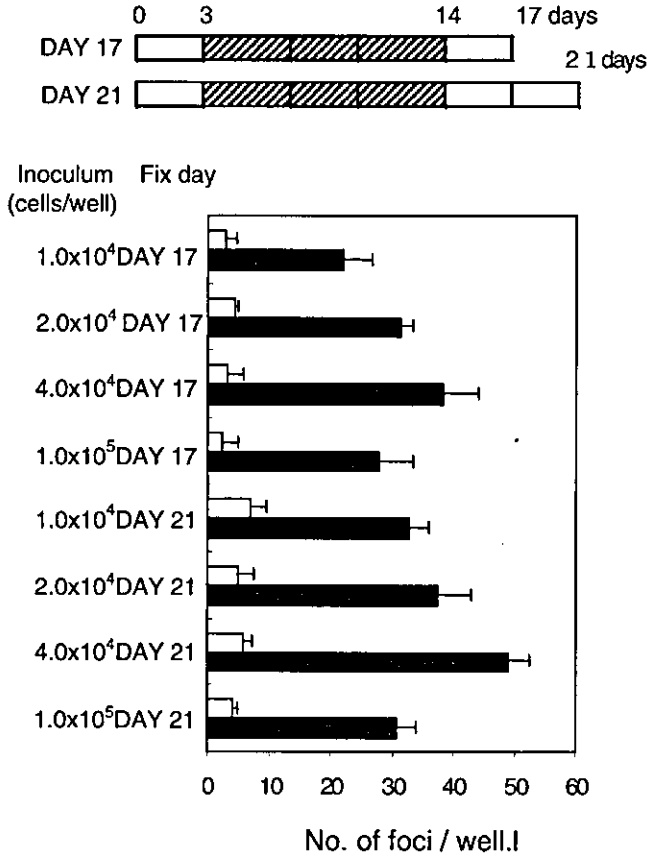

Fig. 4. Effects of inoculum sizes and fixation days on the transformation of Bhas 42 cells: (▨) period of TPA treatment; (□) control; (■) TPA (20 ng/ml).

3.3. Determination of test concentrations for each chemical

From the cytotoxicity tests as described in Materials and Methods, test concentrations of each chemical were determined, covering from 60 to 80% of growth inhibition to no inhibition. Fig. 6a shows the result of the toxicity experiment with TPA. Cell number did not decrease over a large concentration range up to 1 μg/ml, but concentrations inducing transformed foci were far less, even as low as 5–10 ng/ml (Fig. 7a). TPA was an extreme case, and in such a case it was necessary to examine cytotoxicity over a wide range of concentrations. Fig. 6b and c show cytotoxicity with okadaic acid and lithocholic acid at 50 ng/ml and 50 μg/ml, respectively.

3.4. Examination of various chemicals in the transformation protocol

In order to evaluate this cell transformation assay for a variety of chemicals, known tumor promoters together with related and other chemicals were examined (Fig. 7). Dose-response of TPA is shown in Fig. 7a. The number of transformed foci began to increase from 5 ng/ml and reached a plateau at 20–50 ng/ml. PDD and mezerein induced transformed foci from 0.05 ng/ml and reached a maximum level at 0.5 ng/ml (Fig. 7b and c). These chemicals induced transformed foci at concentrations showing overgrowth of the cells. Phorbol, with the basal structure of TPA and PDD but weak tumor promoting activity [17], did not induce transformed foci up to 100 ng/ml (Fig. 7d).

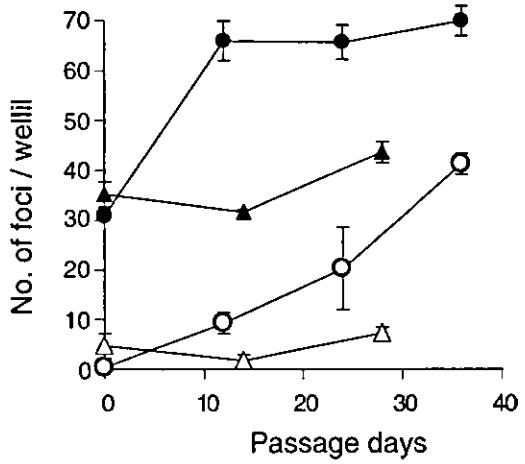

Fig. 5. Effects on transformation frequency of passage generations of Bhas 42 cells cultured in M10F or DF5F: (○) DMEM/F12+5% FBS (DF5F) in the absence of TPA; (△) MEM+10% FBS (M10F) in the absence of TPA; (●) DF5F in the presence of TPA; (▲) M10F in the presence of TPA.

Representatives of other class tumor promoters were examined. Okadaic acid showed growth inhibition at 10 ng/ml and above (Fig. 6b), and induced transformed foci at a narrow concentration range from 5 to 10 ng/ml (Fig. 7e). The highest number of induced foci was relatively low, about 25 foci per well. In the case of anthralin, the highest transformed foci density was 15 foci per well at 1 μg/ml (Fig. 7f). Lithocholic acid induced transformed foci from 1 to 20 μg/ml (about 30 foci per well maximum) (Fig. 7g) and at doses that were marginally cytotoxic (Fig. 6c). Both *o,p′*-DDT and *p,p′*-DDT showed significant increase of foci at 5 and 10 μg/ml (Fig. 7h and i). Sodium phenobarbital induced statistically significant effect only at one concentration, and was judged equivocal (Fig. 7j).

Results examined for other Ames' negative chemicals with reports on carcinogenicity were the followings. Progesterone induced about 20 transformed foci per well at 2 μg/ml (Fig. 7k), but 17β-estradiol was not active in the assay (Fig. 7l). Diethylstilbestrol, a synthetic estrogen, induced statistically significant effect at two non-consecutive concentrations, being judged equivocal (Fig. 7m). Dexamethasone which is a synthetic glucocorticoid, showed significant increase of foci at 12.5 and 25 μg/ml, and was evaluated as positive (Fig. 7n). Arsenic trioxide induced transformed foci dose-dependently in the concentration range 0.02–0.2 μg/ml (Fig. 7o). Arsenic trioxide was not cytotoxic below 0.5 μg/ml (data not shown). Sodium saccharin induced maximum number of transformed foci at 3000 μg/ml (about 30 foci per well) (Fig. 7p). The number of induced transformed foci increased dose-dependently by treatment with catechol at 0.1–2 μg/ml, at up to 25 foci per well (Fig. 7q). Insulin induced about 10 transformed foci per well at 20 and 50 μg/ml, and was evaluated as positive (Fig. 7r).

The average number of foci per well in 18 experiments was 2.9 ± 0.9 in the controls and 37.4 ± 9.9 in cultures treated with 20 ng/ml TPA, demonstrating the stable sensitivity of Bhas 42 cells in the repeated assays.

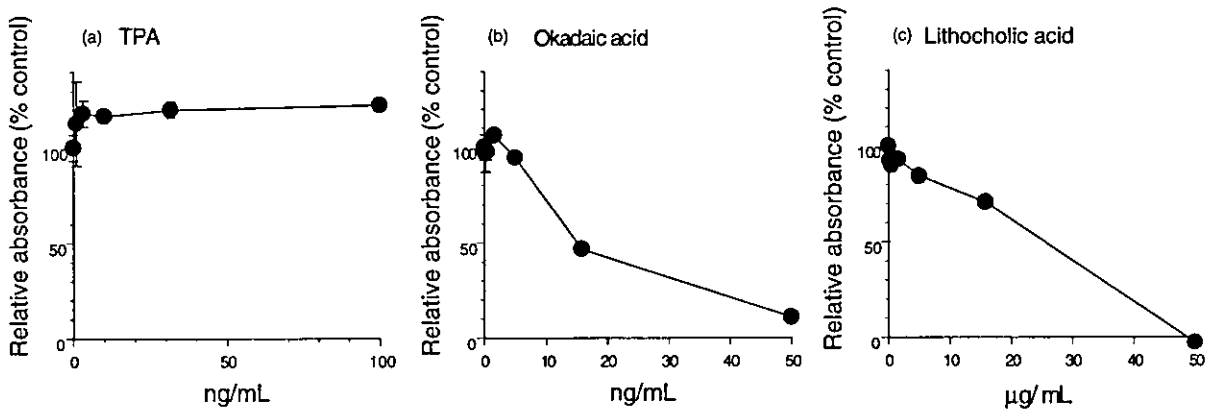

Fig. 6. Cytotoxicity experiments using TPA, okadaic acid and lithocholic acid.

4. Discussion

In most screening tests, chemicals insoluble in water were dissolved in some solvent and diluted with the medium. DMSO is one of the most widely-used solvents. Usually, the final concentration of DMSO was adjusted to be less than 0.5% in the medium. In the current experiments using TPA as a promoting agent, DMSO and ethanol were without effect at 0.1% or less, whereas with acetone up to 0.5% could be used. Kennedy and Symons [18] reported that DMSO at 0.1% or more is capable of suppressing TPA enhancement of radiation-induced transformation by OH free radical scavenging. They also reported that ethanol scavenges OH free radical.

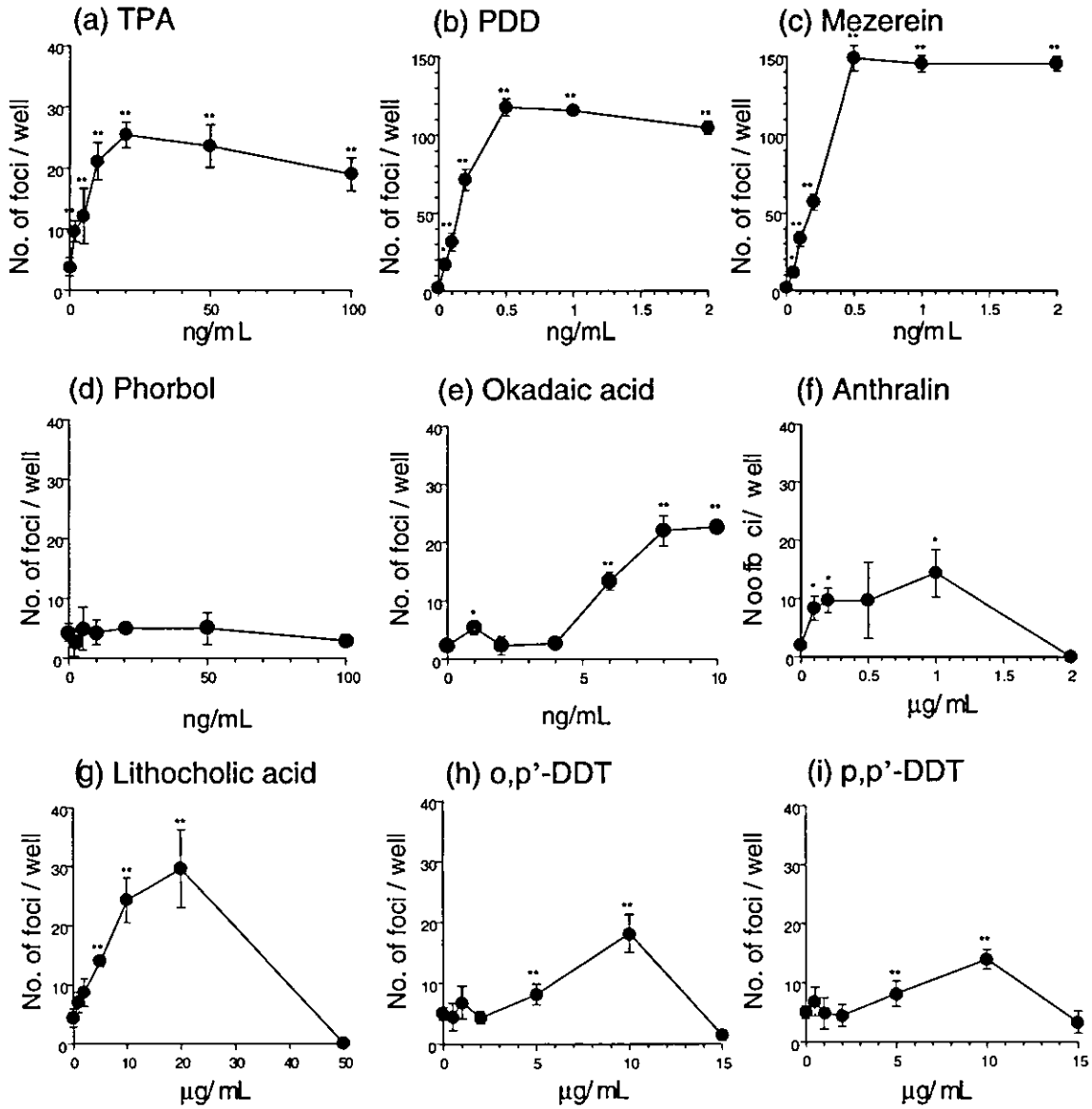

Fig. 7. Transformation experiments using various chemicals: $^{*}P < 0.05$; $^{**}P < 0.01$.

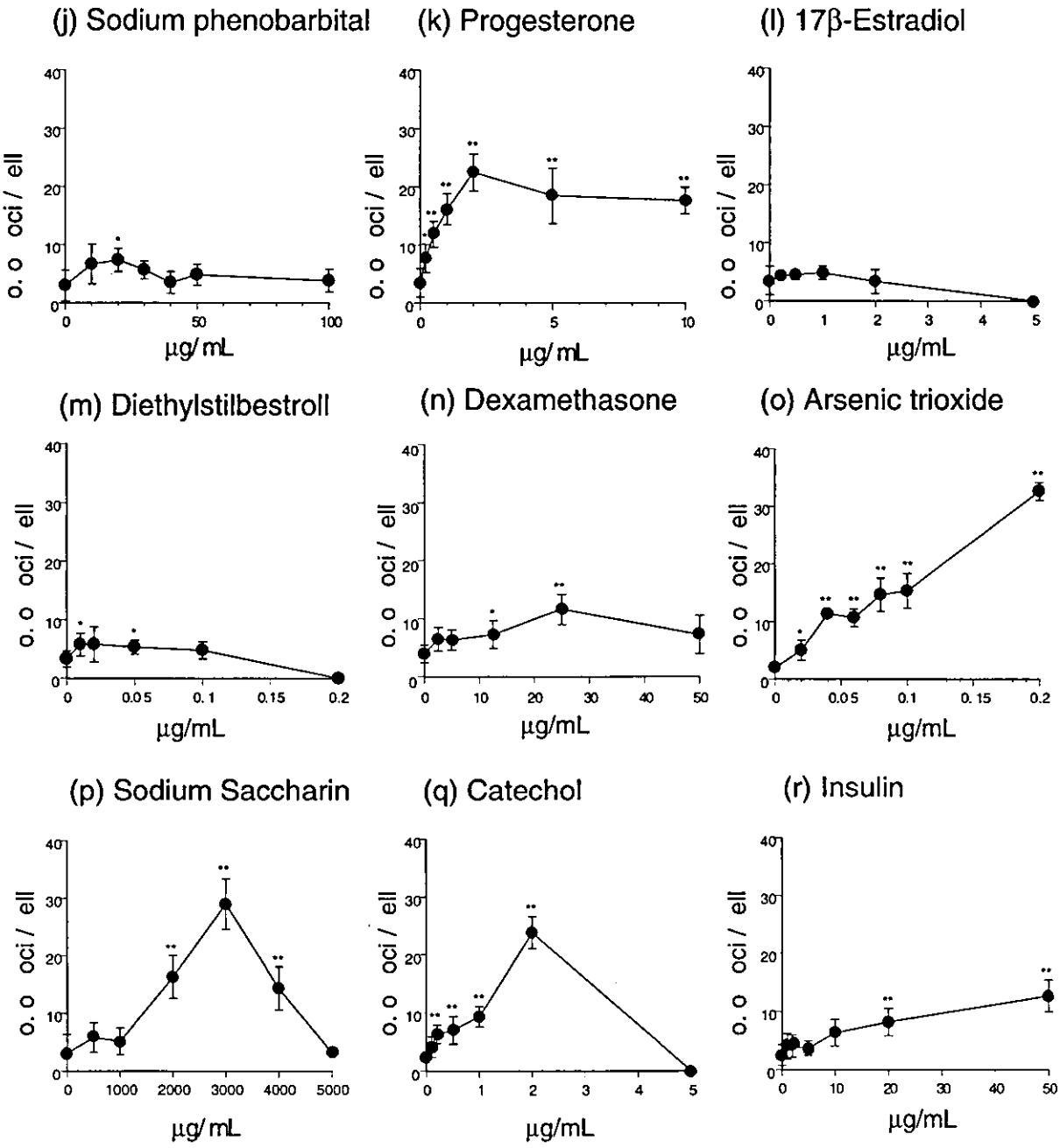

Fig. 7. (*Continued*).

Employing our newly developed promoter assay method, various chemicals were examined (Fig. 7), and the results are summarized together with other information on short-term tests and animal carcinogenicity (Table 1). Typical tumor promoters such as TPA, PDD, mezerein, okadaic acid, anthralina and lithocholic acid showed positive results. It is noteworthy that PDD and mezerein were extremely

Table 1
Summary of results in the Bhas 42 cell transformation assay together with those in other short-term assays [12,16,31] and carcinogenicity evaluation from IARC

Chemical	Bhas assay[a]	Bhas original	Balb 3T3	C3H10T1/2	SHE	JB6	V79	Raji	Ames	IARC
TPA	+	+	+	+	+	+	+	+	−	
PDD	+	+	+		+		+		−	
Mezerein	+	+	+	+	+	+	+		−	
Phorbol	−					−	−		−	
Okadaic acid	+		+	+	+	±	−	+	−	
Anthralin	+		+		−		±		−	3
Lithocholic acid	+		+	+			+	±	−	
o,p'-DDT	+	+	+		+		+	±	−	2B
p,p'-DDT	+	+	+		+		+		−	2B
Phenobarbital (sodium salt)	±	+	±	±			±	+	−	2B
Progesterone	+				±			+	−	
17β-Estradiol	−		−	+	+		−	−	−	1
Diethylstilbestrol	±		−	±	+		−	+	−	1
Dexamethasone	+			+			−		−	
Arsenic trioxide	+								−	1
Saccharin (sodium salt)	+	−	−	+	−	−	±	−	−	2B
Catechol	+		+				±	−	−	2B
Insulin	+	±	+			−	−		−	

[a] +: positive, +: equivocal, −: negative.

potent in inducing transformation foci in this assay. High sensitivity to mezerein was reported by Tsang et al. [19] using a bovine-papilloma DNA-carrying C3H10T1/2 cell line. Stability in the medium or other factors may influence the sensitivity difference of these chemicals among various assays.

Both DDT's [20] and phenobarbital [21,22] are typical tumor promoters of the rodent liver. The present results showed that DDT's were positive and phenobarbital was equivocal. This may suggest that these compounds have different mechanism of promotion action. Progesterone [23] showed a positive result. In contrast, 17β-estradiol [24] and diethylstilbestrol [23] showed negative or equivocal results. These female sex hormones show their activity through their hormone receptors and carcinogenicity to female organs [25]. These results suggest that this assay has difficulties in detecting some specific types of tumor promoters.

Arsenic trioxide [26], dexamethasone [27], saccharin [22], catechol [28] and insulin [29] are related to promotion effect without genotoxicity. In the present study these chemicals gave positive results. More investigations on many other chemicals are required in order to evaluate this method as a predictor of tumor-promoting potential for a wide variety of chemicals.

Here we have used v-Ha-ras-transfected BALB/c 3T3 cells. Similarly, Kowalski et al. [30] demonstrated that an assay employing a bovine-papillomavirus DNA-carrying C3H10T1/2 cell line could well predict carcinogens, promoters and non-carcinogens. Our present study was focused on promoters among non-genotoxic carcinogens, and the method is more simple and economical than the preceding transformation experiments using BALB/c 3T3 cells. Further studies with more chemicals are necessary to establish the reproducibility, reliability and relevancy of the method. In time, this test method will be evaluated for its utility in predicting the tumor promoting potential of chemicals.

Acknowledgements

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