than the control subjects. Thus, the patient's ATL cells were not functional in the cytokine production. In our patient, $CD8^+$ T cells infiltrated in a lichenoid fashion.

Cytotoxic $CD8^+$ T cells are thought to be activated with evolution of HTLV-I infection.[6] It is possible that HTLV-I–induced activation of $CD8^+$ T cells attack epidermal cells on sun exposure. In this scenario, UV irradiation may stimulate keratinocytes to express autoantigen or autoantigens for $CD8^+$ cells. As demonstrated in most ATL cases,[7] the tumor cells in our patients expressed Th2 chemokine receptor CCR4. HTLV-1–infected $CCR4^+$ T cells have growth advantages by deviating host immune response to Th2,[7] because Th2 cytokines suppress $CD8^+$ tumoricidal T cells. In this case, however, the production of Th2 cytokines by HTLV-1–infected T cells was greatly reduced, and moreover, normal T cells scarcely existed. This may allow $CD8^+$ T cells to be activated, resulting in the development of the skin eruption. Recently, CAD has been reported in patients with HIV infection.[8-10] In these patients, the abnormal response to UVB radiation and lichenoid tissue reaction seem to be hallmarks of the advanced disease.[11] We note striking similarities in photosensitivity dermatitis between patients with HIV infections and this case. In our patient, $CD4^+$ tumor cells were functionally impaired in the production of cytokines, and normal $CD4^+$ T cells were very low in number. This immunologic state is virtually the same as that seen in AIDS. Our findings may provide a new clue to elucidate the pathogenesis of CAD from the view of immune dysfunction.

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Regular Paper

Analysis of 8-Hydroxyguanine (8-OH-Gua) Released from DNA by the Formamidopyrimidine DNA Glycosylase (Fpg) Protein: A Reliable Method to Estimate Cellular Oxidative Stress

Hiroshi ORIMO[1,4], Nan MEI[2], Serge BOITEUX[3], Yoshiki TOKURA[4] and Hiroshi KASAI[1]*

8-OH-Gua background level/Fpg protein/γ-irradiation.

To improve the analyses of a form of oxidative DNA damage, 8-hydroxyguanine (8-OH-Gua), we treated isolated DNA with formamidopyrimidine DNA glycosylase (Fpg) and analyzed the released 8-OH-Gua by using a high-performance liquid chromatography system equipped with an electrochemical detector (HPLC-ECD). The human lung carcinoma cells (A549) and human keratinocyte (HaCaT) were irradiated with γ-rays. After the isolated DNA was treated with the Fpg protein, we analyzed the released 8-OH-Gua by using an HPLC-ECD. With this method, the background level of 8-OH-Gua in DNA from human lung carcinoma cells was determined to be 3.4 residues per 10^7 guanine (Gua). A similar background level of 8-OH-Gua (3.1 residues per 10^7 Gua) was also detected in human keratinocyte DNA with this method. These background 8-OH-Gua levels in cellular DNA are comparable to that obtained previously by an analysis of 8-OH-dGMP after nuclease P1 digestion of cellular DNA (4.3 residues per 10^7 dCMP). A dose-dependent increase of 8-OH-Gua ($0.17/10^7$ Gua/Gy) was observed after cells were irradiated with γ-rays. Twenty hours after γ-irradiation with 60 Gy, 75% of the 8-OH-Gua produced in keratinocyte DNA was repaired. With our new analysis method, it is possible to detect the small changes in the 8-OH-Gua levels in cellular DNA induced by various environmental factors.

INTRODUCTION

The formation of 8-hydroxyguanine (8-OH-Gua) in DNA by reactive oxygen species (ROS) was first reported in 1984.[1)] The 8-OH-Gua is a major product of DNA damage induced by the reactions of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), singlet oxygen, and hydroxyl radicals (· OH).[2)] It is also called 7,8-dihydro-8-oxoguanine (8-oxo-Gua).[1)] It is used as a biomarker of oxidative DNA damage[3–4)] and causes mainly GC to TA transversions in mammalian cells.[5)] Floyd *et al.*[6)] introduced a highly sensitive analytical method for 8-OH-Gua, which uses high-performance liquid chromatography (HPLC) coupled to an electrochemical detector (ECD). DNA isolation methods have also improved the analysis, and the modified chaotropic sodium iodide (NaI) technique yields the lowest and least variable 8-OH-Gua values.[7,8)]

This report describes an assay to detect 8-OH-Gua after cellular DNA is treated with the formamidopyrimidine DNA glycosylase (Fpg) enzyme following DNA extraction.[9–11)] In this assay, we used the biological function of the Fpg protein to remove the 8-OH-Gua that is formed in ds-DNA.[9–11)] In the present study, two different cell lines, A549 (lung carcinoma) and HaCaT (keratinocyte), were used, since these cell lines from an internal organ and skin may have different defense abilities against ROS and different repair responses. This method reduced the background level of 8-OH-Gua in DNA and improved the reliability once the increase in oxidative DNA damage in these γ-irradiated human cells was measured.

MATERIALS AND METHODS

Cells and culture conditions

Human alveolar epithelial cells (A549), originally derived from an individual with alveolar cell carcinoma, were pur-

*Corresponding auther: Phone: +81-93-691-7469,
Fax: +81-93-601-2199.
E-mail: h-kasai@med.uoeh-u.ac.jp

[1]Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu, 807-8555 Japan.

[2]Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, FDA, Jefferson, AR 72079, U.S.A.

[3]Commissariat àl'Energie Atomique (CEA), Direction des Sciences du Vivant (DSV), Département de Radiobiologie et Radiopathologie, Unité Mixte de Recherche (UMR) 217 CNRS-CEA Radiobiologie Moléculaire et Cellulaire, 92265 Fontenay aux Roses, France.

[4]Department of Dermatology, University of Occupational and Environmental Health, Kitakyushu, 807-8555 Japan.

chased from the American Type Culture Collection (Rockville, MD, USA). This cell line was cultured in Eagle's Minimum Essential Medium with kanamycin (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal bovine serum, L-glutamine (2 mM), and nonessential amino acids (0.1 mM), and was maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. Unless otherwise noted, all culture supplies were purchased from Gibco-Invitrogen (Carlsbad, CA, USA).

The human keratinocyte (HaCaT) cell line[12] was cultured in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal bovine serum, L-glutamine (2 mM), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and sodium pyruvate (1 mM) and was maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air.

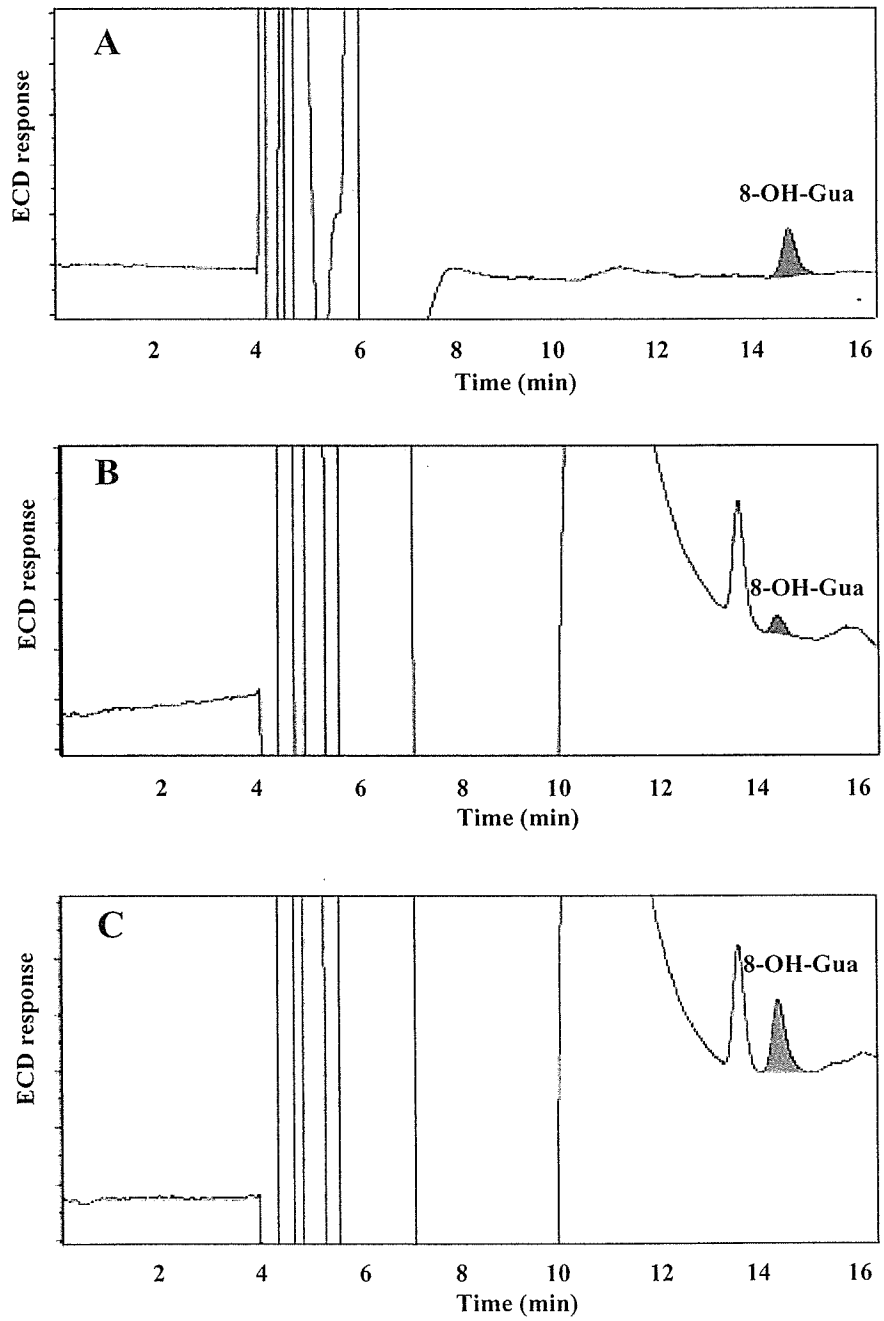

Fig. 1. Typical example of 8-OH-Gua analysis. DNA isolated from A549 cells was treated with the Fpg protein, as described in the Materials and Methods section, and 70 μl portions of the samples were analyzed by an HPLC-ECD. (A) Authentic 8-OH-Gua (5.6 pg). (B) DNA from unirradiated cells. (C) DNA from 80 Gy γ-irradiated cells.

Irradiation of cells with γ-rays

The cells were seeded into 100 mm diameter tissue culture dishes and allowed to attach for a period of 16–24 hours at 37°C. The cells were then irradiated on ice with a gamma cell irradiator (GE40E; Nordion, Ottawa, Canada) at varying doses (dose rate, 0.96 Gy/min). Control cells were not irradiated. After irradiation, the cells were harvested, washed with ice-cold phosphate-buffered saline, and immediately processed to a cell viability test and DNA isolation. Cell viability was determined by a trypan blue dye-exclusion test (0.4%) (GIBCO-BRL, Grand Island, NY, USA).[13]

Determination of 8-OH-Gua in cellular DNA

Cellular DNA was isolated by using a DNA extractor WB kit (Wako, Osaka, Japan).[7,14] Desferal (deferoxamine mesylate; Sigma, St. Louis, MO, USA) was added to the lysis solution (1 mM) to prevent DNA oxidation.[8] The isolated DNA (50–150 μg) was dissolved in 200 μl distilled water, and its concentration was measured with a UV spectrophotometer (JASCO V-520). The DNA solution (150 μl) was mixed with 15 μl of buffer (0.5 M Tris-HCl, 0.5 M KCl, and 20 mM EDTA; pH 8.0) and bovine serum albumin (final concentration, 90 μg/ml), and it was treated with the Fpg protein (10 μg /sample) at 37°C for 20 min.[15] This solution was filtered with an Ultrafree-Probind filter (Millipore, Bedford, MA, USA), and a 70 μl aliquot of the sample was injected into an HPLC column (YMC-Pack ODS-AM, 5 μm, 4.6 × 300 mm; temperature, 25°C; flow rate, 0.7 ml/min) equipped with an ECD (Coulochem II; ESA Inc., USA; electrode 1, 150 mV; electrode 2, 300 mV; guard cell, 350 mV). The mobile phase consisted of 10 mM sodium dihydrogenphosphate dihydrate containing 3% methanol. As the standard sample, 80 μl of an 8-OH-Gua solution (70 pg/ml) was injected. To obtain the standard solution, 8-OH-Gua (2 mg) was completely mixed with 1 liter of deionized water for 30 min at room temperature, then centrifuged (at 800 *g*, 5 min). The concentrations of 8-OH-Gua were calculated based on its UV absorption extinction coefficient value.[16] The digested DNA and the standard samples showed 8-OH-Gua peaks within the limits of detection. The 8-OH-Gua level in the DNA was expressed as the number of 8-OH-Gua per 10^7 Gua, by assuming that 20 O.D. units of DNA is 1 mg[17] and that the GC content of human DNA is 41%.[18]

Statistical analysis

Analyses were performed by use of the StatView-J 5.0 program (Berkeley, CA, USA). All data were expressed as the mean ± standard deviation (SD) from 3–8 independent measurements. Statistical significance was determined by the one-way analysis of variance (ANOVA), followed by the Scheffe test, using $P < 0.05$ as the level of significance.

RESULTS

Analysis of 8-OH-Gua in γ-irradiated A549 cells

We first optimized the conditions for the release of 8-OH-Gua from DNA by Fpg treatment. DNA samples from A549 cells were incubated with various amounts of the Fpg protein for different times. The amount of 8-OH-Gua released from DNA reached a plateau above 5 μg/tube Fpg concentration (20 min incubation time). Time-course experiments showed that the release of 8-OH-Gua became a plateau after a 20 min incubation (with 10 μg Fpg). Therefore we chose assay conditions of 10 μg/tube Fpg concentration and 20 min incubation time. A typical example of 8-OH-Gua analysis is shown in Fig. 1. The detection limit of 8-OH-Gua was about 0.5 pg. In the DNA of untreated A549 cells, the 8-OH-Gua level was measured as 3.35 ± 1.67 (SD) per 10^7 Gua (Fig. 2). Irradiation by γ-rays (20–80 Gy) induced the formation of 8-OH-Gua in a dose-dependent manner, and its yield was estimated to be approximately 0.17 residues per 10^7 Gua per Gy. Immediately after the 80 Gy γ-irradiation, the cell viability was virtually the same as that of the control, as monitored by trypan blue staining.

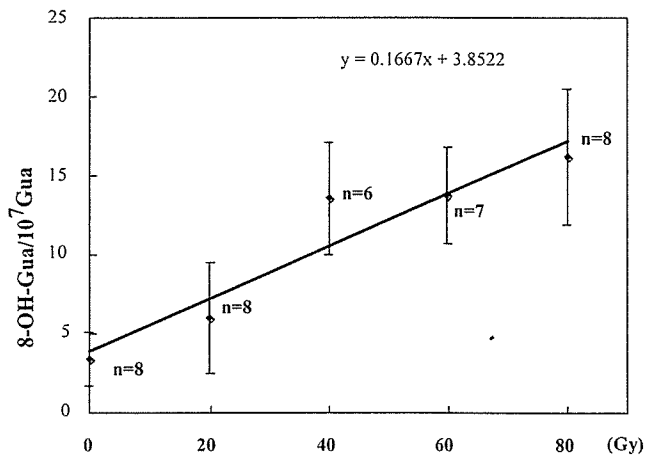

Fig. 2. Effect of γ-irradiation on the 8-OH-Gua formation in A549 cells. A549 cells were exposed to γ-irradiation at doses of 20–80 Gy. The 8-OH-Gua levels in the isolated DNA were determined as described. The results represent the means ± SD of 6–8 experiments.
Correlation (y = 0.1667x + 3.8522) *$P < 0.0001$

Analysis of 8-OH-Gua in γ-irradiated HaCaT cells

We also tried this new approach for 8-OH-Gua analysis in keratinocyte (HaCaT cells) DNA. In the DNA of untreated HaCaT cells, the 8-OH-Gua level was measured as 3.14 ± 1.94 (SD) per 10^7 Gua (Fig. 3). The formation of 8-OH-Gua was dependent on the γ-ray dose, and the yield was 0.18 residues per 10^7 Gua per Gy. Immediately after the γ-irradiation with 80 Gy, the cell viability was unchanged compared to

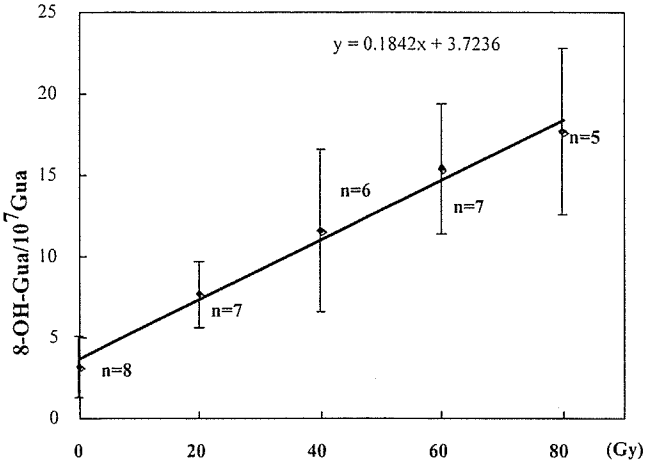

Fig. 3. Effect of γ-irradiation on 8-OH-Gua formation in HaCaT cells. HaCaT cells were exposed to γ-irradiation at doses of 20–80 Gy. The results represent the means ± SD of 5–8 experiments. Correlation (y = 0.1842x + 3.7236) $^*P < 0.0001$.

that of the control, as assessed by trypan blue dye-exclusion test.

8-OH-Gua repair in HaCaT cell DNA after γ-irradiation

We also compared the 8-OH-Gua levels in keratinocyte (HaCaT cells) DNA immediately after and 20 hours after 60 Gy γ-irradiation (Fig. 4). We found that 75% of the 8-OH-Gua formed by the γ-irradiation was repaired during the 20 hour period. Immediately after the 60 Gy γ-irradiation, the cell viability was similar to that of the control. Therefore the level of 8-OH-Gua determined by this analysis may actually be produced in living cells.

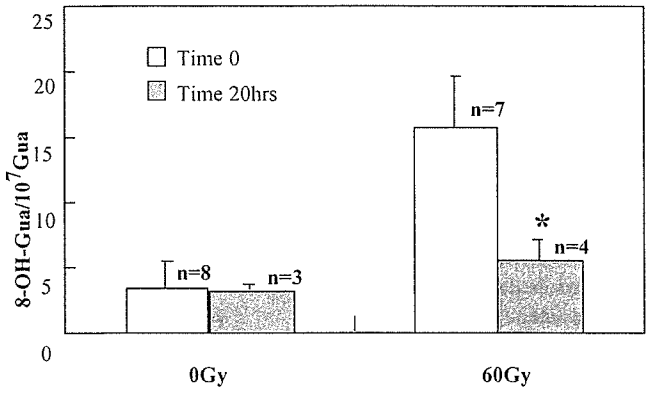

Fig. 4. The 8-OH-Gua levels in HaCaT cells immediately after and 20 hours after 60 Gy γ-irradiation (right figure). The results represent the means ± SD of 3–8 experiments. Results without γ-irradiation (0 Gy) are shown on the left.
$^*P = 0.001$ (significantly lower than the group analyzed immediately after 60 Gy γ-irradiation).

DISCUSSION

Several techniques have been developed to detect 8-OH-Gua, a type of oxidative DNA damage. The measured background levels of 8-OH-Gua differ, depending on the DNA isolation technique and the 8-OH-Gua analysis method. To measure the steady-state level of DNA oxidation, HPLC-ECD is particularly useful with its selectivity, sensitivity, and ease of quantification. During the past two decades, with improved DNA isolation techniques and enhanced HPLC-ECD sensitivity, the assayed background levels of 8-OH-Gua have decreased considerably.[7] Reliable and reproducible data with low background 8-OH-Gua levels (4/10^7 Gua) have been obtained by analyzing 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OH-dG) after digesting DNA prepared by an improved method, using a commercially available kit, that employs an iron chelator, desferal, in the lysis step.[8] In this study we analyzed the 8-OH-Gua base by HPLC-ECD after the DNA was treated with the Fpg protein. This method was first described by Loft and collaborators.[15] They analyzed 8-nitroguanine and 8-OH-Gua in DNA after treatment with NO-generating agents, mainly in vitro, but detailed data on the background level of 8-OH-Gua in cellular DNA were not reported. Beckman *et al.* also used the same method to detect an increase of 8-OH-Gua in the DNA of H_2O_2 treated cells.[19] Karakaya *et al.*[20] detected 8-OH-Gua and many oxidized bases with a gas chromatography/isotope-dilution mass spectrometry (GC/IDMS) method, after γ-irradiated DNA was treated with the Fpg protein. In the present study, we confirmed that the background level of 8-OH-Gua in cellular DNA is 3–4 residues per 10^7 Gua. This baseline 8-OH-Gua value closely resembles that obtained by our previous method, which analyzed 8-hydroxydeoxyguanosine 5'-monophosphate (8-OH-dGMP) after the cellular DNA was digested by nuclease P1 only (4.3 8-OH-dGMP/10^7 deoxycytidine monophosphate [dCMP]).[21] We also detected a similar background level of 8-OH-Gua in the human keratinocyte DNA. In human keratinocytes, the 8-OH-Gua levels have never been measured with this level of precision. It was recently reported that relatively low doses of ultraviolet-B (62.5–500 mJ per cm^2) caused dose-dependent increases in 8-OH-dG.[22] Furthermore, that study found that unirradiated, normal, human epidermal keratinocyte DNA contained 1.49 (± 0.11) 8-OH-dG residues per 10^6 dG. In our study, the amount of 8-OH-Gua in unirradiated cell DNA was much smaller, about 1/5 of their level, though it is possible that different keratinocyte cell lines have distinct background 8-OH-Gua levels.

The Fpg protein creates single-strand breaks in the DNA at the sites of altered purines, including 8-OH-Gua. The comet assay (single cell alkaline gel electrophoresis) measures DNA breaks, which form a comet-like image in which the ratio of DNA in the tail reflects the break frequency. The

ESCODD (European Standards Committee on Oxidative DNA Damage)[23] reported that the background level of 8-OH-Gua in HeLa cell DNA is 0.53 per 10^6 guanines, based on the comet assay after Fpg treatment. Our method produced a lower background level than that of the comet assay, which is known as a highly sensitive method to measure DNA damage. They reported that the bulk of the Fpg-sensitive sites generated by a photoexcited photosensitizer (Ro 19-8022) was quite likely to be 8-OH-Gua lesions, but 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde), which are probably present at lower levels, might also be recognized by the Fpg protein.[23] The difference in the background levels between our analysis and the comet assay may correspond to the amounts of FapyGua and FapyAde and other unknown oxidized products, though it may also be a consequence of using different cell lines.

With our new analysis method, it is possible to detect the small changes in the 8-OH-Gua levels in cellular DNA induced by various environmental factors, such as ionizing radiation, UV light, and chemicals. Because of the low background, we could accurately analyze the reduction of 8-OH-Gua during the 20 hours after γ-irradiation (Fig. 4). Enhanced measurement precision may also contribute to the elucidation of DNA repair mechanisms. Further efforts to improve DNA isolation and analysis methods for 8-OH-Gua assays should be continued.

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cell activation.

The functions of LC are modified or abrogated by various stimuli. Among them, ultraviolet (UV) light is a well studied modality, which alters the antigen-presenting ability of LC. Therefore, LC has been a target in investigation of UVB-induced immunosuppression or photoimmunology.

3. Keratinocytes

More than 90% of cells in the epidermis are keratinocytes, and this epitherlial cell had been considered for long time as merely cornified, barrier-structuring cells. In early 1980's, however, keratinocytes were found to produce IL-1α. This put keratinocytes forward as immunocompetent cells and triggered discoveries of various cytokines and chamokines, such as TNF-α, GM-CSF, IL-6, IL-8, IL-10 (mice), RANTES, IP-10, Mig, I-TACK, TARC, MDC, C-TACK and etc. These molecules deeply participate in inflammation and T cell infiltration in the skin.

In another line of study, it was found that keratinocytes express MHC class II molecules, i.e. antigen-presenting molecules. Upon bearing MHC class II, keratinocytes are capable of presenting superantigens but not other antigens. Therefore, keratinocytes are a non-professional antigen-presenting cell. In the case of hapten-presentation, even immunological tolerance may be induced by class II+ keratinocytes.

Furthermore, keratinocytes express ICAM-1 or CD54 molecules when stimulated with interferon-γ. Since ICAM-1 is an adhesion molecule that binds to LFA-1 on T cells, this finding further provides evidence for immunocompetency of keratinocytes. It should be strengthened that interferon-γ released by T cells stimulates keratinocytes to express both MHC class II and CD54. Therefore, keratinocytes and T cells are mutually stimulated to evoke immunogical or inflammatory conditions.

4. Photoimmunology, photodermatology, and immunodermatology

The skin milieu is the primary target of UV light. Thus, there are close relationship among dermatology, photobiology, and immunology. When investigators in these fields study the biological effects of UV, they may need to understand the skin immune system so that accumulation of basic knowledge effectively solves clinical issues.

Skin as an immunological organ

Yoshiki Tokura, M.D.
Department of Dermatology
University of Occupational and Environmental Health

1. Skin-associated lymphoid tissue

Since skin is an organ continuously exposed to the external stimuli, one can easily estimate the skin to be an immunologic organ. This led to the concept of skin-associated lymphoid tissue (SALT). Such organs that contact the external world include gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT) as well as SALT.

The constituents of SALT include Langerhans cells, keratinocytes, skin-recruiting T cells, vascular endothelial cells, and additionally dendritic epidermal $\gamma\delta$ T cells or DETC in mice. These cells are orchestrated to give a well-organized immune system specialized for the primary defense system.

2. Langerhans cells (LC)

Langerhans cells are bone-marrow-derived hematologic cells with a professional antigen-presenting cell ability. This dendritic cell resides in the epidermis of the skin and surrounded by keratinocytes. Thus, LC live harmoniously with the epithelial cells. The adhesion of LC to keratinocytes is performed via E-cadherin expressed on both cells. Upon external stimuli, keratinocytes produce IL-1α and TNFα, which reduces the expression of E-cadherin on LC, allowing LC to migrate to draining lymph nodes.

As antigen-presenting cells, LC present conventional protein antigens (peptides), superantigens, and haptens to T cells. This presentation is carried out with major histocompatibility complex (MHC) that binds to antigens. T cell receptors on T cells recognize antigens in the context of MHC. In addition to MHC molecules, costimulatory molecules on LC, such as CD80, CD86, CD40 and CD54, play an important role for T

Reprint requests to: Yoshiki Tokura, MD, Department of Dermatology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

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An assay method for the prediction of tumor promoting potential of chemicals by the use of Bhas 42 cells

Kiyomi Ohmori [a,*], Kiyoshi Sasaki [b], Shin Asada [b], Noriho Tanaka [b], Makoto Umeda [b]

[a] *Chemistry Division, Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa 253-0087, Japan*
[b] *Department of Cell Biology, Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan*

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Abstract

It has become an important task to develop a simple in vitro method for the detection of non-genotoxic carcinogens, among which tumor promoters are included. Bhas 42 cells are v-Ha-ras-transfected BALB/c 3T3 cells and are regarded as initiated cells in the 2-stage transformation paradigm. We designed a method for detecting tumor promoters by the use of Bhas 42 cells at advanced passage generation. In this method, the cells are cultured in six-well plates for 17 days during which test chemicals are added in the medium for 11 days from days 3 to 14. The end-point of the assay is the induction of transformed foci. When the tumor promoter TPA was used, a significant number of transformed foci were induced concentration-dependently, whereas only a few foci were observed in control cultures. When various chemicals were examined by the method, a reasonable correlation was observed with the reported tumor-promoting ability in animal experiments. We propose that the Bhas 42 cell transformation method is practical and useful for the detection of tumor promoters.

Keywords: Tumor promoter; Transformation; Bhas 42 cell; BALB/c 3T3 cell; v-Ha-ras

1. Introduction

Non-genotoxic carcinogens have posed a major problem in the toxicity screening of chemicals [1]; that is, a considerable number of non-genotoxic chemicals have been shown to be carcinogenic in long-term animal experiments. Therefore, adoption of some screening tests related to carcinogenicity other than the genotoxicity screening is necessary before contemplating long-term animal cancer bioassays.

Carcinogenesis is known to be a multi-step process, involving at least initiation, promotion and progression [2]. Initiators induce changes in DNA and can be detected by various genotoxicity screening tests. Meanwhile, promoters, by the repeated application on initiated cells, can cause development of tumors. These chemicals can be considered to be one kind of non-genotoxic carcinogens, and several screening methods for the detection of promoters have been proposed; for example, in vitro cell transformation [3–6], inhibition of metabolic cooperation

* Corresponding author. Tel.: +81-467-83-4400; fax: +81-467-83-4457.
E-mail address: ohmori.n4yf@pref.kanagawa.jp (K. Ohmori).

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through gap-junctional intercellular communication [7–9], promotion or inhibition of cell differentiation [10], expression of Epstein–Barr virus early antigen [11,12], and in vivo cell-proliferation (in vivo RDS test) [13]. However, none of these methods is yet included in the battery of regular safety screening tests for chemicals. One reason why they are not adopted for the regulatory screening tests is that some of these methods are not simple enough for routine screening.

In vitro cell transformation tests using BALB/c 3T3 cells or C3H10T1/2 cells can simulate the process of animal two-stage carcinogenesis [14]. For the detection of promoting chemicals in the in vitro cell transformation test, 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 subsequent expression period are required before administration of test chemicals. Typically, these assays require 4–8 weeks to complete.

In order to improve experimental conditions for the examination of chemicals with tumor-promoting potential, Sasaki et al. [15,16] worked with a cell line, named Bhas 42, which was established from BALB/c 3T3 cells transfected with v-Ha-ras oncogene. According to their 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 for the formation of transformed foci.

Recently, we found that 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. 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 for the detection of promoting potential of chemicals.

The aim of this report is to describe a screening method for tumor promoters using Bhas 42 cells and to present test results for a range of chemicals.

2. Materials and methods

2.1. Media, cells and culture conditions

MEM, DMEM, RPMI 1640 and F12 media were obtained from Nissui Pharmaceutical Co., Tokyo, Japan. BME and DMEM/F12 were the products of GIBCO Laboratories, Grand Island, NY, USA. ITES, a mixture of insulin, transferrin, ethanolamine and sodium selenite, was obtained from Wako Pure Chemical Industries, Osaka, Japan. Fetal bovine serum (FBS) was purchased from Moregate, Australia.

Bhas 42 cells and BALB/c 3T3 A31-1-1 cells were routinely cultured in a medium consisting of MEM supplemented with 10% FBS (M10F), at 37 °C in an atmosphere of 5% CO_2 and 95% air. The cells were sub-cultured before confluence by the use of trypsin (Wako Pure Chemical Industries). Bhas 42 cells at passage generations between 12 and 20 were used in the present experiments.

Plastic culture dishes and plates were either products of Sumitomo Bakelite, Tokyo, Japan, or those of Costar, Corning Incorporated, Corning, NY, USA.

2.2. Chemicals

12-*O*-Tetradecanoylphorbol-13-acetate (TPA), okadaic acid, lithocholic acid, sodium phenobarbital, arsenic trioxide, sodium saccharin, catechol, acetone and ethanol were purchased from Wako Pure Chemical Industries. Phorbol 12,13-didecanoate (PDD), mezerein, phorbol, anthralin, progesterone, 17β-estradiol, dexamethasone, insulin and dimethyl sulfoxide (DMSO) were obtained from Sigma, St. Louis, MO, USA. *o,p′*-DDT and *p,p′*-DDT were obtained from GL Science, Tokyo, Japan. Diethylstilbestrol was the product of Tokyo Kasei Kogyo, Tokyo, Japan.

TPA was dissolved in DMSO at 1 mg/ml; stock aliquots were stored in a deep freezer. An aliquot was used in each experiment. Arsenic trioxide was dissolved in 0.1 mol/l sodium hydroxide solution and insulin was dissolved in 0.1 mol/l hydrochloric acid. The solutions had no effect on pH of the culture medium when diluted more than 1000 times. Other chemicals were dissolved in DMSO or directly in the culture medium.

2.3. Cytotoxicity test

A cell suspension of Bhas 42 cells in DMEM/F12+5% FBS (DF5F) at 2×10^4 cells/ml was distributed into each well of 24-well plates at 0.5 ml amounts (1×10^4 cells per well) and cultured. This cell number is almost equal to the 2 ml used per well of six-well plates. On day 3, medium in each well was changed with the medium containing test chemical. Three wells were used for one concentration. On day 7, the cells were fixed with 3.7% formaldehyde for 30 min, washed with water, and stained with 1% crystal violet (CV) in water for 30 min. After thorough rinsing with water, the plates were dried. Crystal violet from stained cells in each well was extracted with 0.5 ml of a solution consisting of 0.9% trisodium citrate dihydrate, 0.02 mol/l hydrochloric acid, and 50% ethanol. The optical density of the extracts was measured at 540–570 nm.

2.4. Bhas 42 transformation test

In the present experiments, several variables were examined, and the finally adopted protocol was as follows; a cell suspension of Bhas 42 cells was prepared in DF5F medium at 2×10^4 cells/ml, and distributed into each well of six-well plates at 2 ml amounts (4×10^4 cells per well). After cultivation for 3 days, medium was replaced with fresh medium containing test chemical (for the preparation of test chemicals, attention should be paid to ensuring the final concentrations of solvents is less than 0.1% in the case of DMSO and ethanol, and 0.5% in the case of acetone). The culture received medium containing test chemical on days 7 and 10, and then fresh DF5F medium on day 14. On day 17, the cells were fixed with methanol for 30 min and stained with 2.5% Giemsa solution for 30 min.

Transformed foci were characterized by the following morphological criteria: deep basophilic staining and dense multi-layering of cells; random orientation of cells at the edge of foci; more than 20 cells within a focus.

2.5. Statistical analysis and criteria of judgment

For evaluating the results, t-test analysis was performed. Chemicals showing significant increase ($P < 0.05$) of focus number at more than two consecutive concentrations were considered to be positive (+). Chemicals which showed 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.

3. Results

3.1. Fundamental conditions for efficient induction of transformed foci

When the protocol of the original transformation procedure using Bhas 42 cells [15,16] at the 13th passage generation was followed, no transformed foci appeared after treatment with TPA. However, Bhas 42 cells at the 13th passage generation alone easily grew into a monolayer which contained only several spontaneously transformed foci. In addition, the number of transformed foci significantly increased by treatment with TPA. From these observations, the possibility of developing a short-term cell transformation assay for

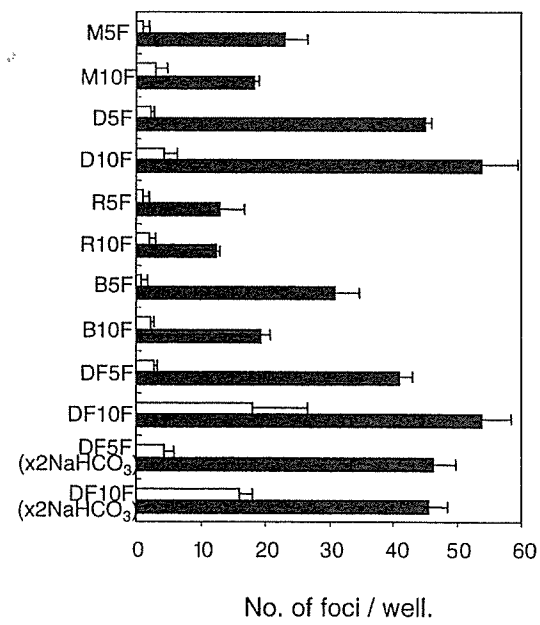

Fig. 1. Effects of various basal media and FBS concentrations on the transformation of Bhas 42 cells: (□), control; (■), TPA (20 ng/ml); M5F, MEM + 5% FBS; M10F, MEM + 10% FBS; D5F, DMEM + 5% FBS; D10F, DMEM + 10% FBS; R5F, RPMI1640 + 5% FBS; R10F, RPMI1640 + 10% FBS; B5F, BME+5% FBS; B10F, BME+10% FBS; DF5F, DMEM/F12+5% FBS; DF10F, DMEM/F12 + 10% FBS.

the detection of promotion potential of chemicals using a single culture of Bhas 42 cells at advanced passage generations was explored.

In order to establish a new transformation protocol using Bhas 42 cells, several variables which would influence the formation of transformed foci were examined. Essentially, the second stage of the two-stage transformation protocol using BALB/c 3T3 cells [6] was emulated. Bhas 42 cells near confluence were treated with TPA for 11 days and further cultured 3 or 7 days in fresh medium. The endpoint was the formation of transformed foci.

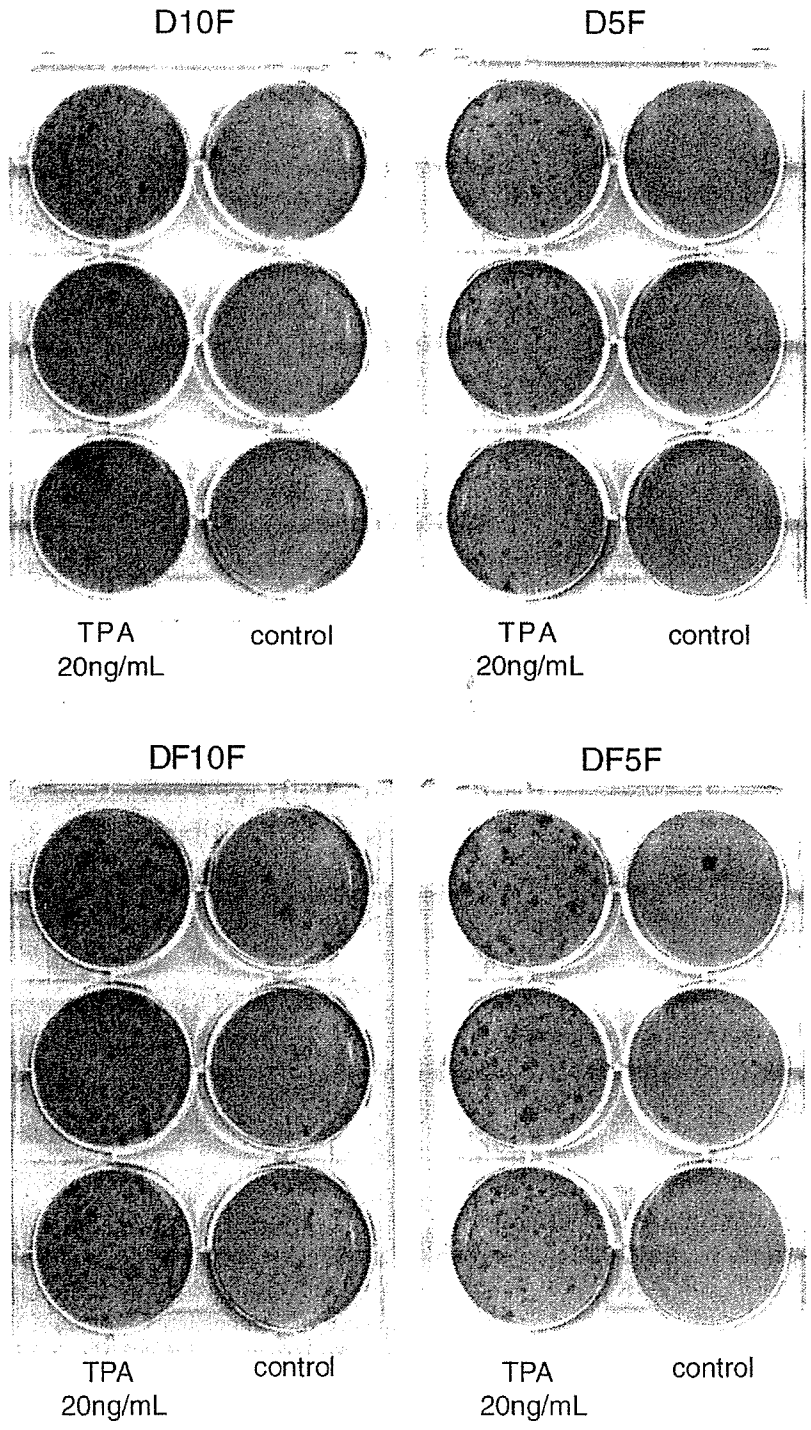

Fig. 2. Representative plates showing formed foci of Bhas 42 cells cultured in different media with or without TPA.

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.

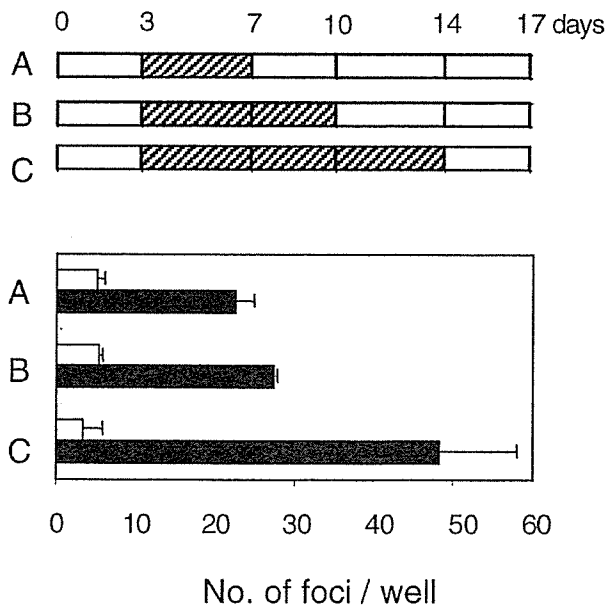

Fig. 3. Effects of period of TPA treatment on the transformation of Bhas 42 cells: (▨) period of TPA treatment; (□) control; (■) TPA (20 ng/ml).

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.

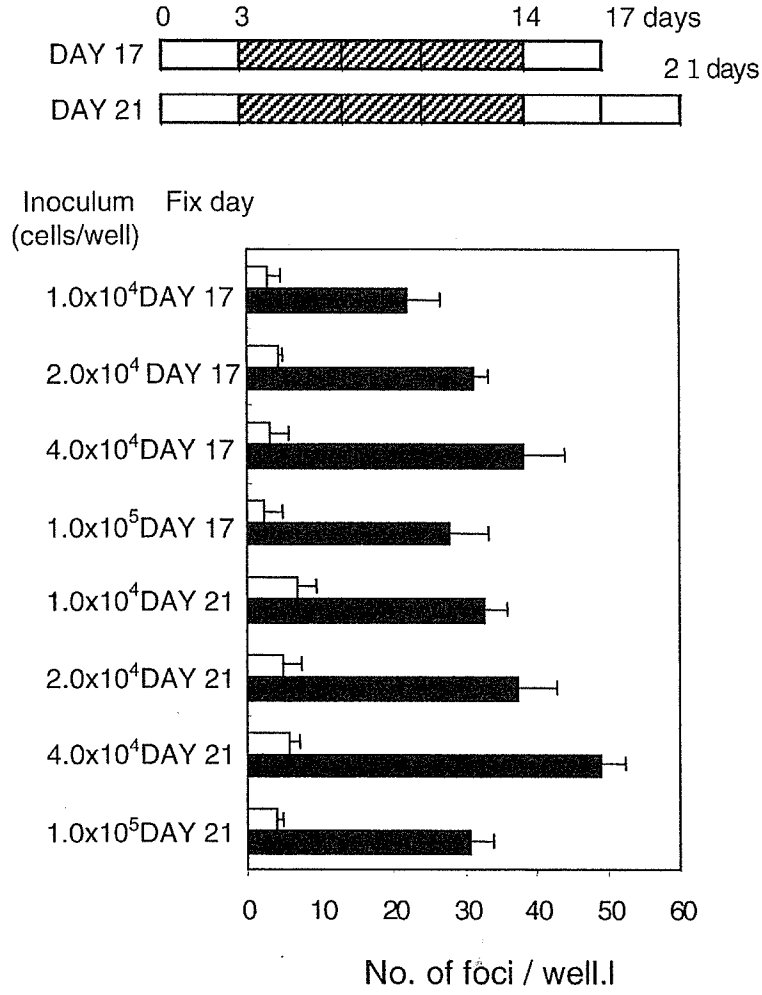

Fig. 4. Effects of inoculum sizes and fixation days on the transformation of Bhas 42 cells: (▨) period of TPA treatment; (□) control; (■) TPA (20 ng/ml).

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).

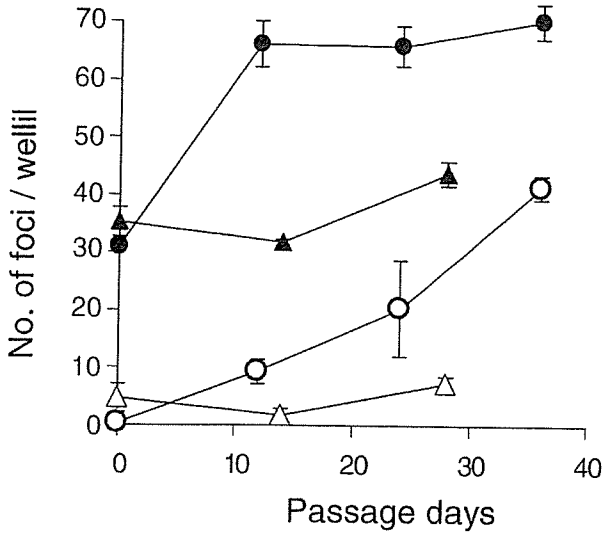

Fig. 5. Effects on transformation frequency of passage generations of Bhas 42 cells cultured in M10F or DF5F: (○) DMEM/F12+5% FBS (DF5F) in the absence of TPA; (△) MEM+10% FBS (M10F) in the absence of TPA; (●) DF5F in the presence of TPA; (▲) M10F in the presence of TPA.

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.

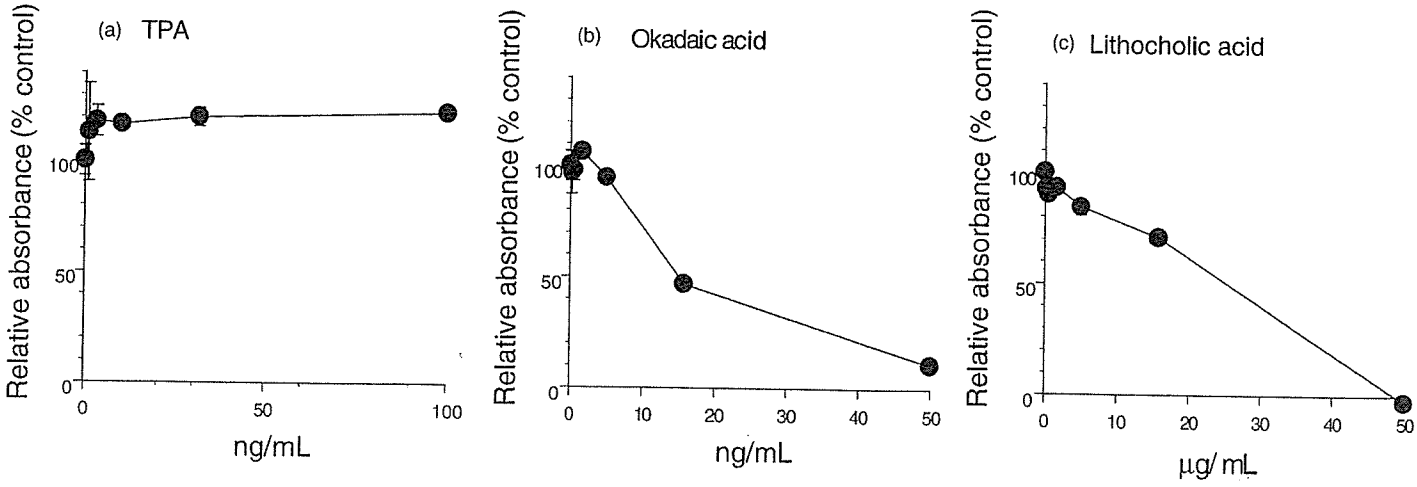

Fig. 6. Cytotoxicity experiments using TPA, okadaic acid and lithocholic acid.

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.

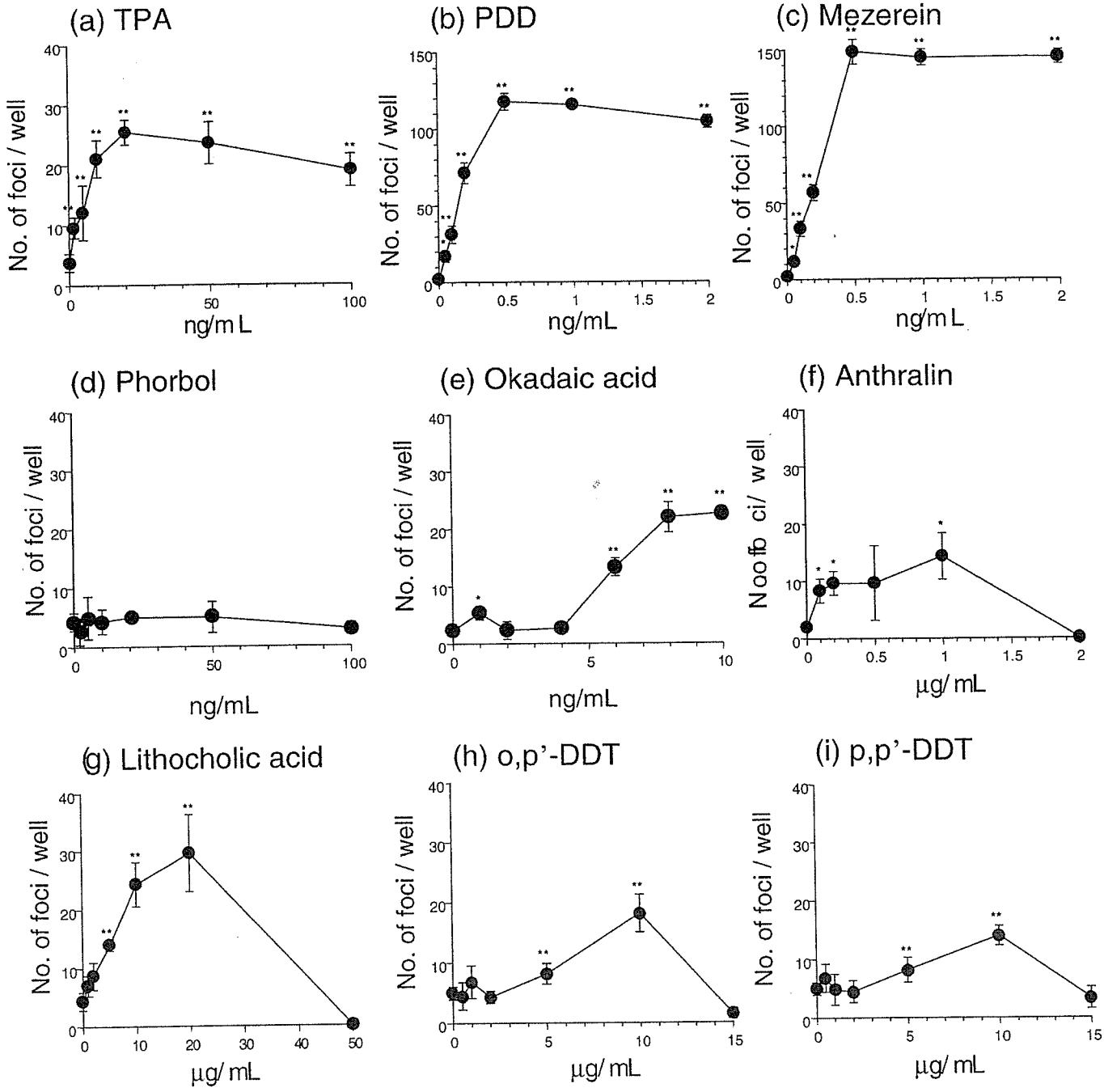

Fig. 7. Transformation experiments using various chemicals: $^{*}P < 0.05$; $^{**}P < 0.01$.

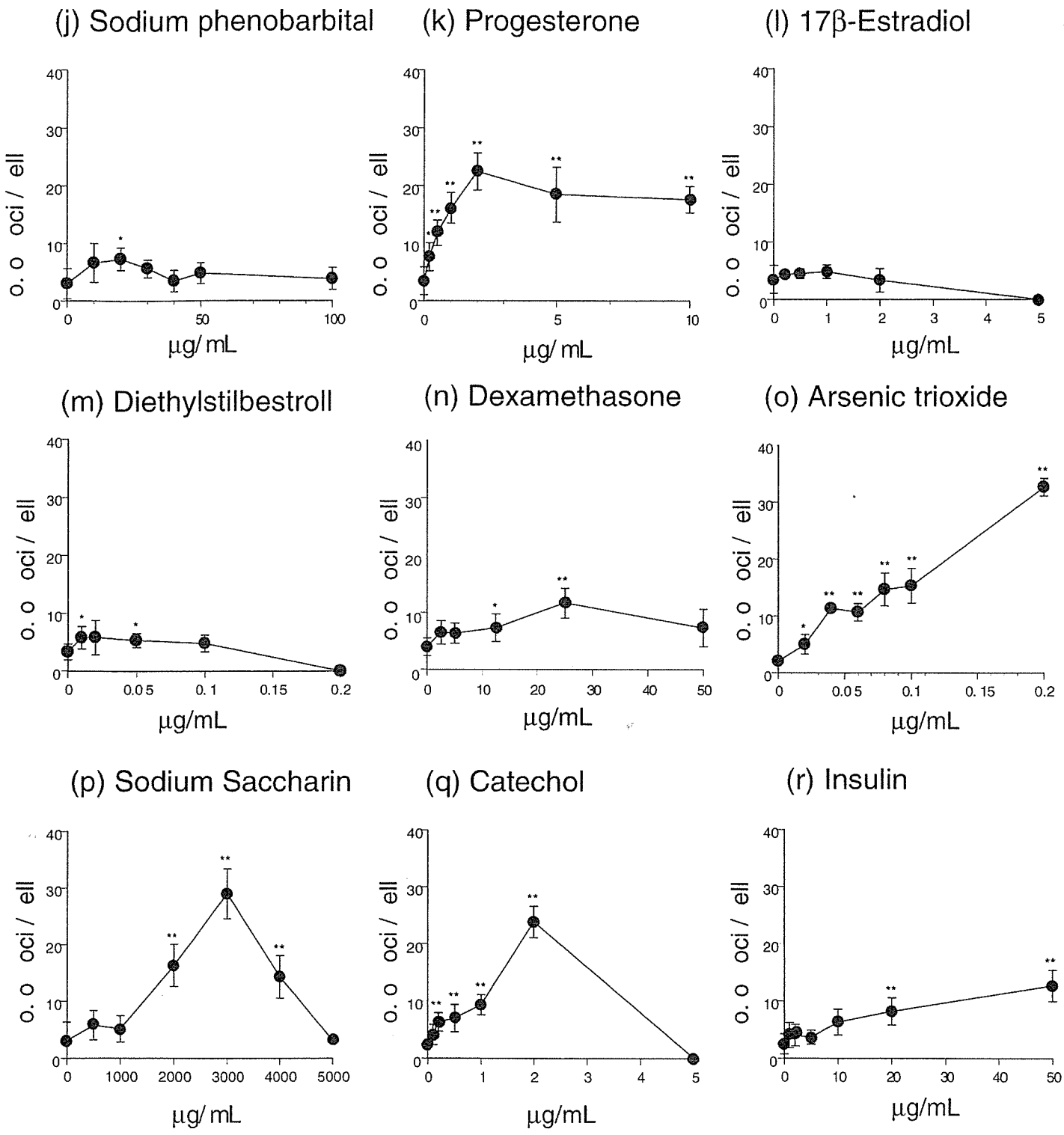

Fig. 7. (*Continued*).

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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