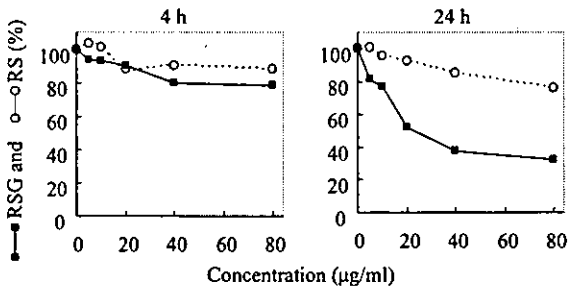

Fig. 1. Cytotoxic responses represented by RS and relative cell growth (RSG) of TK6 cells treated with MCLR for 4 or 24 h.

3. Results

3.1. Cytotoxic response to MCLR

TK6 cells were exposed to various doses of MCLR for 4 or 24 h. Fig. 1 shows cytotoxic responses; relative survival (RS) and relative suspension growth (RSG), which is relative cell growth during 72 h after exposure. Exposure to MCLR for 4 h did significantly affect RS or RSG. Exposure for 24 h, however, decreased RSG concentration-dependently, but did not significantly alter RS.

3.2. Genotoxic responses to MCLR

Exposure to MCLR for 24 h induced both MN and *TK* mutation in a concentration-dependent manner (Fig. 2). The maximum induction of MN and *TK* mutations were 4.8- and 5.1-times the control values. Two distinct phenotypic classes of *TK* mutants were generated. Normally growing (NG) mutants grew at the same rate as the wild type cells (doubling time 13–17 h), and slowly growing (SG) mutants grew at a slower rate (doubling time >21 h). NG mutants result mainly from intragenic mutations, while SG mutants result from gross genetic changes beyond the *TK* gene. The proportion of SG mutants increased in MCLR induced mutants, suggesting that MCLR was clastogenic.

3.3. Molecular analysis of TK mutants

Spontaneously arising and MCLR-induced *TK* mutants were isolated independently. The MCLR-induced

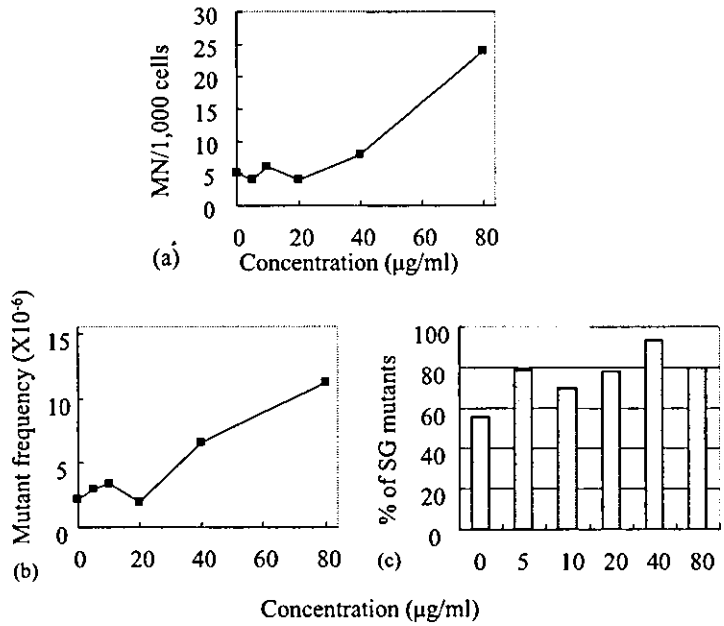

Fig. 2. MN induction (a), mutation frequency at *TK* locus (b), and percentage of slowly growing (SG) mutants (c) among TK-deficient mutants treated with MCLR for 24 h.

Table 1
Cytotoxic and mutational response to MCLR[a] and LOH analysis of TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at *TK* gene			
	RSG (%)	MF ($\times 10^{-6}$)	% SG	No. analyzed	None LOH	Hemizygous LOH	Homozygous LOH
Spontaneous	100	2.19	56	56			
NG mutants				19	14 (74)	3 (16)	2 (11)
SG mutants				37	0 (0)	9 (24)	28 (76)
MCLR-induced	32.6	11.2	80	36			
NG mutants				9	4 (44)	5 (56)	0 (0)
SG mutants				27	0 (0)	10 (37)	17 (63)

[a] 80 μg/ml for 24 h.

mutants were produced by the treatment at 80 μg/ml for 24 h. The cytotoxicity (RSG), mutation frequency, and proportion of SG mutants by the treatment are shown in Table 1. We used PCR-based LOH analysis of genomic DNA from *TK* mutants to classify the mutants into 3 types; Non-LOH, hemizygous LOH, and homozygous LOH. We analyzed 58 spontaneous and 36 MCLR induced *TK* mutants, including NG and SG type (Table 1). Every SG mutant was a result of LOH regardless of the treatment, suggesting that SG mutants were always associated with gross genetic changes. Among the MCLR-induced mutants, 56% of NG mutants and 100% of SG mutants exhibited LOH. Every LOH in the NG mutants was hemizygous, and 63% of LOH in the SG mutants was homozygous. This is in contrast to spontaneous *TK* mutants, where the majority of spontaneous NG and SG mutants were non-LOH (74%) and homozygous LOH (76%), respectively. Fig. 3 shows the spectra of spontaneous and MCLR-induced *TK* mutants in TK6 cells, which were adjusted by considering % SG mutants. These data clearly indicate that MCLR induced LOH, but not point mutation or other small genetic changes.

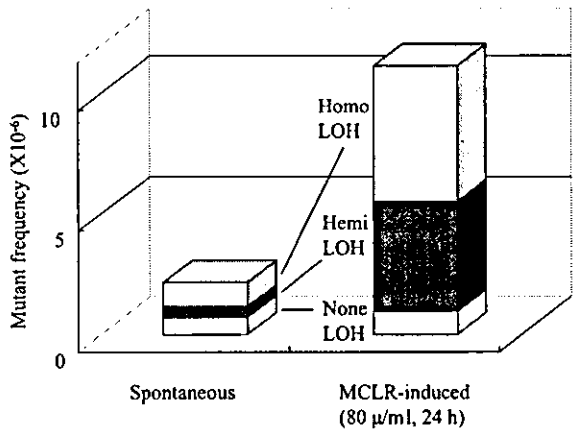

Fig. 3. Frequency and spectra of *TK* mutations in spontaneous and MCLR (80 μg/ml, 24 h) induced *TK* mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of NG and SG mutants and the result of molecular analysis (Table 1).

4. Discussion

Although MCLR causes severe hepatoxoxicity in mammals [9–12], its genotoxicity and carcinogenicity are inconclusive. Ding et al. [13] reported that microcystic cyanobacteria extract (MCE) significantly induced mutations in the Ames assay regardless of metabolic activation, although pure MCLR did not. Tsuji et al. [22,23] also failed to demonstrate MCLR genotoxicity in the Ames assay. On the other hand, MCLR has some genotoxic effects in mammalian cells. Ding et al [13] observed DNA damage in primary rat hepatocytes in comet assay, and Rao and Bhattacharya [14] found that MCLR could induce DNA fragmentation and strand breaks in mouse liver in vivo. Two studies reported the induction of chromosome aberrations and gene mutations in mammalian cells [15,24].

Our present study clearly demonstrated the in vitro genotoxicity of MCLR, which induced MN formation as well as gene mutations in human cells. A 24 h treatment was required, however, to express the effects. Although MCLR is toxic and highly lethal to

mice in vivo, it seems less toxic in vitro, particularly in non-liver cells. That may be because cyclic heptapeptide microcystines do not generally penetrate most cells including bacteria, and a specific transport system may be required [25]. We used 2 parameters to estimate MCLR cytotoxicty—RS and RSG. RS is relative plating efficiency just after exposure, while RSG is relative cell growth for the 3 days following exposure. RSG exhibited stronger response than RS, suggesting that MCLR has an inhibitory effect on cell growth [26]. Because the cytotoxicity was not severe, the genotoxic responses to MCLR must have been due to physiological effects. In the *TK* gene mutation assay, MCLR elevated not only the frequency of mutants, but also the fraction of SG mutants, suggesting that MCLR induced predominantly gross structural changes, such as large deletions, recombinations, and rearrangements.

Molecular analysis strongly supported this hypothesis. Most of the *TK* mutants induced by MCLR were the result of LOH, while the fraction of non-LOH mutants hardly changed (Fig. 3). LOH is an important genetic event in tumorigenesis and is frequently observed in a variety of human tumors. The two major mechanisms for generating LOH are deletion (hemizygous LOH) and inter-allelic recombination (homozygous LOH) [18,19]. Both mechanisms involve the repair of chromosomal double strand breaks (DSBs), either non-homologous end-joining and homologous recombination (HR), although their regulation and role have not been clarified [27]. Other mechanisms may be involved, too, including illegitimate recombination and mitotic non-disjunction [26]. DSB-inducing agents, such as ionizing irradiations, effectively produce LOH mutations through the repair pathways [17,18]. MCLR clastogenic activity may also involve DSBs. Honma and Little [28] demonstrated that 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), which is the most active tumor promoter known, preferably induces homozygous LOH through HR. MCLR also has tumor promoting activity; like the tumor promoter Okadaic acid, it inhibits protein phosphatase types 1 and 2A [29]. A cyanobacterial toxin, nodularin, which also inhibits protein phosphatases 1 and 2A with the same potency as does MCLR has been recognized as rat liver carcinogen rather than a tumor promoter [30]. The genotoxicity of nodularin, however, has not been clear. Matsushima et al. [31] demonstrated that MCLR promotes rat liver cancer initiated with diethyl-nitrosamine. The tumor promoting activity of MCE has been also shown in a two-stage transformation assay in vitro using Syrian hamster embryonic cells [32]. The induction of LOH by MCLR through recombination may be associated with its tumor promoting activity. It is reported that Okadaic acid induces minisatellite mutation in NIH3T3 cells probably through recombination events [33]. The potent hepatocarcinogen aflatoxin B1 also preferably induces LOH through HR in TK6 cells and mouse lymphoma L5178Y cells [34,35].

In conclusion, MCLR was clastogenic in human cells in the present study. It induced LOH, but not point mutations. The genotoxic activity may have been associated with the inductions of DSBs and/or its promoting activity. The association between a high incidence of primary liver cancer and drinking of pond and ditch water polluted by high level of cyanobacteria producing MCLR [3,36,37] suggests that liver is a target organ for MCLR carcinogenisity. Further studies using liver cells and tissues are required to clarify the mechanisms of MCLR genotoxicity in the liver.

Acknowledgements

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Simultaneous HPLC analysis of 8-hydroxydeoxyguanosine and 7-methylguanine in urine from humans and rodents

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Abstract

With a recently developed high-performance liquid chromatography (HPLC) method based on anion exchange chromatography, precise fraction collection, and reversed-phase chromatography, the oxidative DNA damage marker 8-hydroxydeoxyguanosine (8-OH-dG) was measured in human urine samples. The HPLC analysis was further modified to measure 8-OH-dG in rat and mouse urine samples. In addition, the urinary RNA degradation product 7-methylguanine (m^7Gua) was analyzed simultaneously. The correlation coefficient (r) for the correlation between urinary creatinine and m^7Gua was 0.9 for rats and 0.8 for humans and mice. Levels of 8-OH-dG in relation to urinary creatinine were compared and found to be similar for humans and rats and twice as high for mice. Urinary levels of m^7Gua, as normalized to creatinine, were several-fold higher in rodents as compared with human levels, thereby correlating with the higher resting metabolic rate of rodents. The presented results show that 8-OH-dG and m^7Gua can be analyzed simultaneously and reliably in urine from humans and rodents. In addition, m^7Gua may be used as a reliable marker instead of creatinine for the normalization of 8-OH-dG in urine from rats and mice and also may be used in addition to normalization with creatinine in measurements of 8-OH-dG in human urine samples.

Keywords: 7-Methylguanine; 8-Hydroxydeoxyguanosine; Oxidative DNA damage

Oxidative DNA damage occurs intracellularly in response to the endogenous formation of oxygen radicals and as a result of attacks from exogenous sources such as ionizing radiation and certain mutagenic compounds [1]. The types of damage produced may be strand breaks in DNA or different types of base damage such as 8-hydroxydeoxyguanosine (8-OH-dG)[1] [2]. This potentially mutagenic product is repaired by the process of base and nucleotide excision, released from the cell, and eventually excreted through the urine [3]. It has been estimated that several hundred molecules of 8-OH-dG/cell/day are formed, as well as similar levels of the base 8-OH-guanine, as measured in excreted urine [1,4].

Recently, the reliability and speed of 8-OH-dG analysis in human urine have been further improved using an automated high-performance liquid chromatography system coupled to an electrochemical detector (HPLC-EC) [5]. This system is based on anion exchange chromatography in the first chromatography step (HPLC-1), precise fraction collection, and reversed-phase chromatography in the second chromatography step (HPLC-2). There is no need to prepurify the urinary samples; thus, the sensitivity and reproducibility are enhanced. Currently, this new system is being used to measure the human urinary excretion of 8-OH-dG in response to

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[1] *Abbreviations used:* 8-OH-dG, 8-hydroxydeoxyguanosine; HPLC-EC, high-performance liquid chromatography system coupled to an electrochemical detector; m^7Gua, 7-methylguanine; 8-OH-G, 8-hydroxyguanosine; RMR, resting metabolic rate.

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various lifestyle factors, such as diet and smoking, and exposure to toxic agents, such as mercury and polycyclic aromatic hydrocarbons.

In this article, we present a modification of this method to measure the urinary excretion of 8-OH-dG from rats and mice. In addition, with anion exchange chromatography (HPLC-1), we identified the human and rodent urinary RNA degradation product 7-methylguanine (m^7Gua). This urinary product was found to be a suitable marker for the normalization of 8-OH-dG values between samples from different individuals or between different collection times. Currently, the metabolic product creatinine, from skeletal muscle metabolism [6,7], is measured to normalize urinary 8-OH-dG values. However, creatinine values may differ between individuals due to age and sex differences in the ratio between skeletal muscle and total body lean mass [8], and they are also affected by exercise and diet [9–12]. The degradation and urinary excretion of specific RNA metabolites, such as 5,6-dihydrouridine, pseudouridine, m^7Gua, and N2-dimethylguanosine, have been shown to correlate with the resting metabolic rate (RMR) in humans and rats [8,13,14]. Topp et al. [15] suggested that the excretion values of 8-OH-dG should be normalized to the metabolic rate to account for the effects of prooxidants, antioxidants, and individual differences in DNA repair [15]. Thus, we evaluated the normalization of urinary 8-OH-dG to the urinary m^7Gua, as well as to creatinine, in humans, rats, and mice. The benefits of this analysis would be that a single urine sample may be analyzed for both 8-OH-dG and m^7Gua in the same sample run, thereby eliminating the need for creatinine measurements with variations due to different amounts and aliquots of the sample analyzed. In addition, a sample may be assayed for 8-OH-dG and m^7Gua rapidly, that is, in approximately 1 h. Urinary levels of m^7Gua increase in response to methylating agents such as those from tobacco smoke [16]. Thus, measuring urinary m^7Gua would also be useful for detecting changes in DNA and RNA methylation levels due to exposure to exogenous methylating agents such as *N*-nitroso compounds or to the endogenous *S*-adenosylmethionine activity [17].

For studies of the various effects of agents or conditions on homogeneous groups, such as rats and mice, normalizing urinary 8-OH-dG to urinary m^7Gua would be expected to be a more rapid and comparably accurate method than would normalizing levels to creatinine.

Materials and methods

Materials

The 8-OH-dG and m^7Gua used for standards were obtained from Sigma Chemical (USA). 8-Hydroxyguanosine (8-OH-G), used as a marker for fraction collection, was prepared as described previously [5,18]. The anion exchange resin MCI GEL CA08F (7 μm, Cl^- form) was purchased from Mitsubishi Chemical (Japan) and was prepared as described previously [5] before it was manually packed in a guard (1.5 × 50-mm) column and a main (1.5 × 150-mm) column for use in HPLC-1. The reversed-phase column (Capcell Pak C18, 5 μm, 4.6 × 250 mm) used in HPLC-2 for the analysis of the 8-OH-dG fraction was purchased from Shiseido (Japan). The same type of reversed-phase column was used for the separation of m^7Gua and its subsequent detection with a photo diode array UV detector. HPLC-grade methanol and acetonitrile were purchased from Wako Pure Chemical (Japan) and Kanto Chemical (Japan), respectively.

Collection of urine samples

From a stock of frozen (−80 °C) human urine samples from a previous study [19], a subset of 44 urine samples from nonsmokers was used in the current study. Male Wistar rat urine samples were collected individually from 36 rats, ages 10–11 weeks, during a period of 24 h by spontaneous excretion in metabolic cages. Female C3H/He mouse urine samples were collected individually from 22 mice, ages 13–24 weeks, during a period of 4 or 24 h by spontaneous excretion in metabolic cages. Rats and mice were furnished with a standard diet and drinking water ad libitum. At the end of the collection period, the urine from rats or mice was transferred to Eppendorf tubes and frozen (−80 °C).

Measurement of urinary creatinine levels

Creatinine in urine samples (1 ml) from humans and rats was measured by a commercial laboratory (BML, Japan) using a colorimetric method. Because the amount of available urine from the mice was small (500–1000 μl), samples were diluted with an equal amount of water before the creatinine analysis by the commercial laboratory. A few mouse urine samples that were very small (100 μl) were measured for creatinine with a colorimetric assay kit (Jaffe's method) at our university.

Analyses of 8-OH-dG and m^7Gua in human urine

Human urine samples were defrosted and mixed with an equal volume of a 4% acetonitrile solution containing the ribonucleoside marker 8-OH-G (120 μg/ml), 130 mM NaOAc, and 0.6 mM H_2SO_4 [5]. The Eppendorf tubes containing this solution were then stored at 5 °C for a minimum of 4 h before they were centrifuged at 13,000 rpm for 5 min. Samples were transferred to plastic HPLC injector vials, and 20-μl aliquots were analyzed for 8-OH-dG by the use of an automated HPLC system,

as described in detail previously [5]. In essence, the system was composed of a sampling injector (Gilson 231XL), a pump (Shiseido Nanospace SI-2) for the anion exchange guard and main column in HPLC-1 (the flow rate was 37 μl/min and the column oven was set at 65 °C), a UV detector (Toso UV-8020 with a micro cell), a second pump (Shimadzu LC-10AD) for the analysis of the 8-OH-dG fraction with a reversed-phase column in HPLC-2 (the flow rate was 1 ml/min and the column oven was set at 40 °C) connected with an EC detector (ESA Coulochem II), and two switch valves. A third pump (Shiseido Nanospace SI-2) was used to back-wash the guard column (flow rate 37 μl/min) for 32 min after valve switching at approximately 13 min after each sample injection. For HPLC-1, the solvent was composed of 2% acetonitrile in 0.3 mM sulfuric acid. For HPLC-2, the solvent was composed of 10 mM phosphate buffer, pH 6.7, 5% methanol, and an antiseptic Reagent MB (100 μl/L), and it was recycled for a time period of 1 week. The guard column was back-washed with 0.5 M ammonium sulfate:acetonitrile (7:3 v/v). For the detection of the m^7Gua peak in HPLC-1, the UV detector was set to 305 nm instead of 254 nm, as described previously [5], to minimize interference from neighboring peaks. The 8-OH-G marker peak used for automatic peak detection [5] of 8-OH-dG was detected at 305 nm. After automatic peak detection at two-thirds of the height of the marker peak, the 8-OH-dG fraction was precisely collected by valve switching, injected on HPLC-2, and detected by a Coulochem II EC detector (ESA) with a guard cell (5020) and an analytical cell (5011). The applied potentials were as follows: guard cell = 400 mV, E1 = 280 mV, and E2 = 350 mV. The total time between the analyses of consecutive samples was 60 min. The automatic peak detection was controlled by software from Gilson, and the chromatograms were recorded (PowerChrom EiCOM EPC-300 Data Processor) and integrated with computer software (PowerChrom 2.1).

Analyses of 8-OH-dG and m^7Gua in rodent urine

Urine samples from rats and mice were prepared in the same way as the human samples and were stored at 5 °C overnight before they were centrifuged at 13,000 rpm for 5 min. Samples (20 μl) were analyzed for 8-OH-dG by the use of a similar automated HPLC system as described for the human samples above. The system was composed of a sampling injector (ESA 542), a pump (ESA 582) for the anion exchange guard and main column in HPLC-1 (the flow rate was 45 μl/min and the column oven was set at 65 °C), an experimental model of a UV detector (FLOM, Japan) with integrated hardware peak recognition set at a single wavelength (254 nm), an additional UV detector (Tosoh UV-8020 with micro cell) for the detection of m^7Gua at 305 nm, a second pump (ESA 542) for the reversed-phase HPLC-2 column (for rat urine analysis, the flow rate was 0.67 ml/min and the column oven was set at 48 °C; for mouse urine analysis, the flow rate was 0.33 ml/min and the column oven was set at 60 °C) connected with an EC detector (ESA Coulochem III), and two switch valves. A third pump (ESA 582) was used to back-wash the guard column (the flow rate was 45 μl/min) for 32 min after valve switching at approximately 13 min after each sample injection. The solvents used for the HPLC-1 and guard columns were the same as those described above for the analysis of human urine. The solvent used in HPLC-2 was composed of 10 mM phosphate buffer, pH 6.0, 2% methanol, and an antiseptic Reagent MB (100 μl/L), and it was recycled for a time period of 1 week. After automatic peak detection at one-half the height of the marker peak, the 8-OH-dG fraction was collected and then injected on HPLC-2, for detection by a Coulochem III EC detector (ESA) with a guard cell (5020) and an analytical cell (5011). The applied potentials were as follows: guard cell = 350 mV, E1 = 170 mV, and E2 = 300 mV. The total time between the analyses of consecutive samples was 80 min. Chromatograms were recorded (Dionex UCI-100) and integrated with computer software (Chromeleon 6.30).

Verification of urinary 8-OH-dG and m^7Gua with standards

The specificity of the 8-OH-dG peak in HPLC-2 for the human, rat, and mouse samples was verified using three different methods. The first test was to calculate the peak ratio between the lower potential setting on the EC detector (E1 = 170 mV) and the higher setting (E2 = 300 mV) for a random sample of mouse urine (prepared as described above) using two different flow rate and column temperature conditions in HPLC-2 (0.67 ml/min and 48 °C or 0.33 ml/min and 60 °C). The calculated ratios were compared with that of an 8-OH-dG standard analyzed using the same conditions. In the second test, three random urine samples from human, rat, and mouse, respectively, were pooled, prepared as described above, and then spiked with known concentrations of an 8-OH-dG standard (0, 1, 2.5, and 4 ng/ml). For the spiked samples, the recovery of 8-OH-dG in HPLC-2 was calculated. The applied potentials on the EC detector were as follows: guard cell = 420 mV, E1 = 200 mV, and E2 = 370 mV. Finally, in the pooled samples from human, rat, and mouse, the specificity of the 8-OH-dG peak detected in HPLC-2 was verified with that of an 8-OH-dG standard by comparing the electrochemical voltammograms. A pooled urine sample or an 8-OH-dG standard (5 ng/ml) was analyzed at the following applied potentials on the EC detector: guard cell = 450 mV, E1 = 0 mV, and E2 = 175–400 mV. For these tests, the HPLC equipment described above for the analysis of rodent urine was used. The solvent for

HPLC-2 was that used for the analyses of human and rodent urine. The peak detection for automatic fraction collection in HPLC-1 was set at one-half the height of the marker peak (254 nm). The volume of the injected sample or standard was always 20 μl (after mixing with an equal volume of a 4% acetonitrile solution containing the ribonucleoside marker). Settings for the applied potentials on the Coulochem III EC detector were adjusted according to the sensitivity of each different analytical cell (5011) used. The concentration of the 8-OH-dG standard was determined using a molar absorption extinction coefficient (ε) of 12,300 $M^{-1} cm^{-1}$ at 245 nm in H_2O [20].

The m^7Gua was identified from rat urine after repeated collections of 20-μl fractions of the eluted peak at 12 min. From these pooled fractions, a 150-μl aliquot was injected on an HPLC system connected with a photo diode array UV detector (Hewlett–Packard, series 1100), and after separation with a reversed-phase column (the mobile phase used was a gradient of 0.1–20% acetonitrile) the absorbance spectrum for the eluted peak at 12 min was recorded and compared with that of a 150-μl aliquot of the m^7Gua standard (28.4 mg/L). The concentration of the m^7Gua standard was determined using a molar absorption extinction coefficient of 7300 $M^{-1} cm^{-1}$ at 283 nm in 100 mM sodium phosphate, pH 7.0.

Summary of various electrode settings used

The settings for the applied potentials on the Coulochem II/III EC detectors had to be manually adjusted for each different analytical cell (5011) used in HPLC-2. This is due to the inherent different sensitivity of each analytical cell. The guard cell (5020), used to remove traces of impurities from the recycled solvent, was always set at a 50-mV higher setting than the highest setting used for E2 of the analytical cell. Thus, for measurement of human urine samples, the applied potentials of the specific analytical cell used were E1 = 280 mV and E2 = 350 mV (guard cell = 400 mV). For rodent urine samples, another analytical cell was used with the applied potentials of E1 = 170 mV and E2 = 300 mV (guard cell = 350 mV). Because of deterioration of the previously used analytical cells, a new analytical cell was used with human and rodent urine to obtain electrochemical voltammograms. In this case, the analytical cell was set to E1 = 0 mV and E2 = 175, 200, 225, 250, 275, 300, 325, 350, 375, and 400 mV (guard cell = 450 mV). After the same analytical cell was washed with acetone, it was used for the analysis of urine samples spiked with known concentrations of an 8-OH-dG standard. For that purpose, the analytical cell was set to E1 = 200 mV and E2 = 370 mV (guard cell = 420 mV). With the use of an in-line graphite filter (ESA) before the analytical cell, the cell may be used for several months without washing or replacement.

Calculation of results and statistics

The HPLC chromatograms for 8-OH-dG and m^7Gua were recorded and integrated with a computer, as specified above, and were quantified by comparing the peak areas with those obtained from external standards analyzed on a daily basis. The yields were recalculated to 8-OH-dG/creatinine (μg/g creatinine) and nanograms of 8-OH-dG collected during 24 h normalized to body weight (ng/24 h/kg BW). Yields of m^7Gua are presented after normalization to creatinine (mg/g creatinine) or as micrograms of m^7Gua collected during 24 h normalized to body weight (μg/24 h/kg BW). To calculate the correlation between creatinine and m^7Gua, yields were recalculated to grams per liter of urine (g/L).

To test for significant differences between the mean values, the Student's t test was used. Results were considered as significantly different at $P < 0.05$.

Results

Analysis of 8-OH-dG in human and rodent urine

In addition to the electrochemical analysis of human urinary 8-OH-dG after automatic peak recognition and fraction collection [5], we detected 8-OH-dG in rat and mouse urine. In HPLC-2, at a flow rate of 1 ml/min (eluent pH 6.7 and 5% methanol) and with the column oven set at 40 °C, the human urinary 8-OH-dG peak eluted at around 20 min (Fig. 1B). With the flow rate changed to 0.67 ml/min (eluent pH 6.0 and 2% methanol) and the column oven set at 48 °C, the rat urinary 8-OH-dG peak eluted at around 37 min (Fig. 2B). For the detection of mouse urinary 8-OH-dG, the flow rate was set to 0.33 ml/min (eluent pH 6.0 and 2% methanol) and the column oven was set at 60 °C. Under these conditions, all interfering peaks were separated and the mouse urinary 8-OH-dG peak eluted at around 39 min (Fig. 3B). As shown in Figs. 1B, 2B, and 3B, the 8-OH-dG peaks are completely separated from the neighboring peaks. The peak purity was further verified by calculating the ratio between the E1 peak areas (lower lines in Figs. 1B, 2B, and 3B) and the E2 peak areas (upper lines in those figures). These peak ratios were equal to those of the analyzed 8-OH-dG standards, thereby confirming the peak purity for 8-OH-dG in each individual sample analyzed (chromatograms are not shown for the 8-OH-dG standards).

Evaluation of the 8-OH-dG detection reliability in human and rodent urine

It is important to confirm that the 8-OH-dG peak in HPLC-2 is not contaminated by impurities. In Fig. 4B, we show an example of the detection of a falsely positive

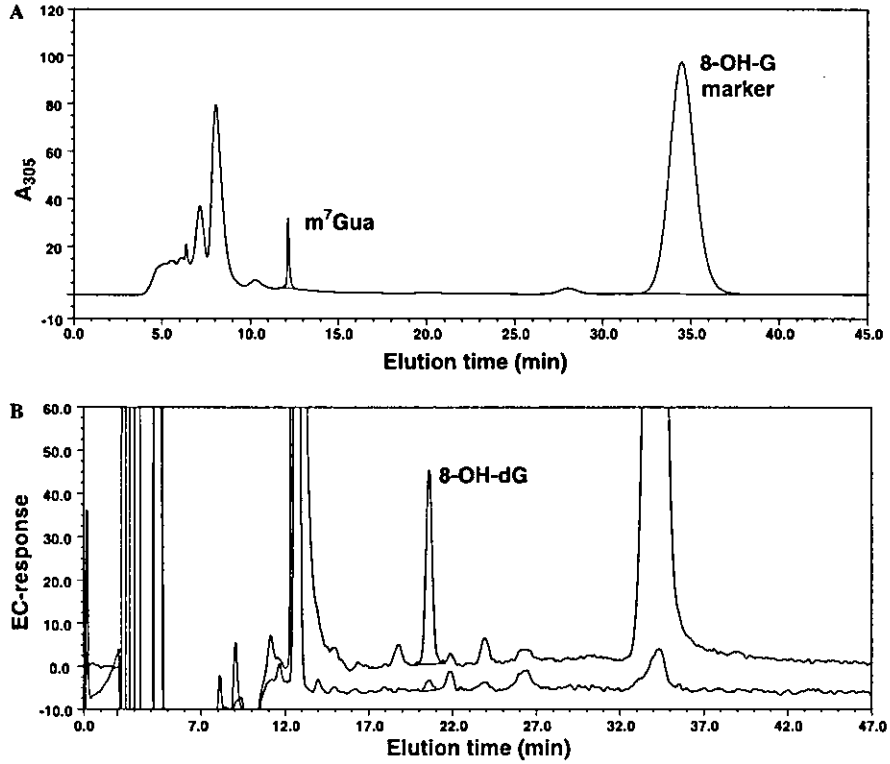

Fig. 1. Chromatograms of a human urine sample (male nonsmoker): (A) HPLC-1 UV detection of m^7Gua and 8-OH-G marker peaks and (B) HPLC-2 electrochemical detection of 8-OH-dG at applied potentials of E1 = 280 mV (lower line) and E2 = 350 mV (upper line).

8-OH-dG peak. A random sample of mouse urine was analyzed using two different flow rate and column temperature conditions in HPLC-2. In the first chromatogram (Fig. 4A), where the flow rate was 0.67 ml/min and the column temperature was 48 °C, the 8-OH-dG peak detected at an applied potential of E1 = 170 mV (lower line in Fig. 4A) had a peak area that was 55% of the peak area detected at an applied potential of E2 = 300 mV (upper line in Fig. 4A). However, using the same conditions, the mean peak area of the 8-OH-dG standards ($n = 4$), as detected at E1, was 22% of the peak area detected at E2 (chromatograms not shown). This means that the mouse sample peak area, as detected at E1 (lower line in Fig. 4A), was more than twice as high as expected and, thus, would be classified as a false positive due to the presence of contamination. Changing the conditions for HPLC-2, to a flow rate of 0.33 ml/min and a column temperature of 60 °C, resolved the interfering peak/peaks in the same mouse urine sample (Fig. 4B). The 8-OH-dG peak detected at E1 (lower line in Fig. 4B) had a peak area that was 26% of the peak area detected at E2 (upper line in Fig. 4B). Under these conditions, the mean peak area of the 8-OH-dG standards ($n = 4$), as detected at E1, was 24% of the peak area detected at E2 (chromatograms not shown). This means that the ratio between the E1 and E2 peak areas (cf. lower and upper lines in Fig. 4B) was nearly equal to the expected ratio. Thus, each urine sample analyzed can be tested for a false 8-OH-dG positive if an interfering compound has significantly different electrochemical properties than does genuine 8-OH-dG. To check for urine sample matrix effects on the 8-OH-dG detection, pooled urine samples from human, rat, and mouse were spiked with 0, 1, 2.5, and 4 ng/ml of an 8-OH-dG standard (Table 1). All spiked samples had 8-OH-dG recoveries of nearly 100% and showed a linear increase in the amount of 8-OH-dG detected (Table 1). Thus, we concluded that the detected 8-OH-dG peak was not affected by any urine sample matrix effects. Also, the linear increase in the amount of 8-OH-dG detected in the spiked urine samples shows that there is no binding of the added 8-OH-dG to the vial surface or coprecipitation of 8-OH-dG with urine components. In Fig. 5, unspiked pooled urine from humans (lower line) can be compared with the same urine that was spiked with 4 ng/ml of an 8-OH-dG standard (upper line). As expected, the peak area increased after spiking the sample (cf. lower and upper lines in Fig. 5), corresponding to an increase in the amount of 8-OH-dG from 7.2 ng/ml (unspiked sample) to 11.2 ng/ml (spiked sample). The urine samples and standards (5 ng/ml) were also analyzed at different potentials set on the electrochemical detector to obtain voltammograms. Figs. 6–8 show that

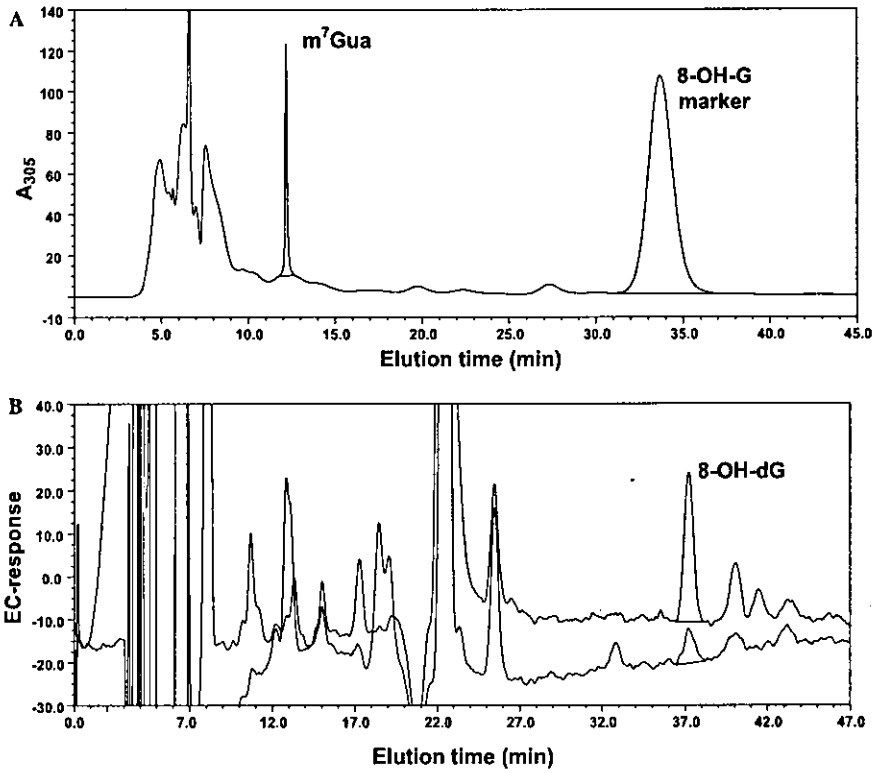

Fig. 2. Chromatograms of a rat urine sample (male Wistar rat): (A) HPLC-1 UV detection of m^7Gua and 8-OH-G marker peaks and (B) HPLC-2 electrochemical detection of 8-OH-dG at applied potentials of E1 = 170 mV (lower line) and E2 = 300 mV (upper line).

the voltammograms of 8-OH-dG from the pooled urine samples from humans, rats, and mice, respectively, conform well with the voltammograms of the corresponding 8-OH-dG standards under the different conditions used (see Materials and methods).

Analysis of m^7Gua in human and rodent urine

In addition to the detection of urinary 8-OH-dG, a sharp peak appeared at 12 min in every HPLC-1 chromatogram (Figs. 1A, 2A, and 3A). Based on its UV spectrum (see Materials and methods), it was identified as m^7Gua, an RNA degradation product. The absorbance spectrum of m^7Gua in rat urine is identical to that of a pure m^7Gua standard (cf. Figs. 9A and B). For the detection of m^7Gua, a 305-nm wavelength was used to reduce the interference from the neighboring peaks in human or mouse urine. For rat urine, it is also possible to use a 254-nm wavelength for the detection of m^7Gua (chromatogram not shown).

Correlation of urinary levels of m^7Gua with creatinine

The linear regression lines for the correlations between the concentrations of urinary creatinine and m^7Gua for humans, rats, and mice are presented in Figs. 10–12, respectively.

For the human data from 44 male nonsmokers, the linear relationship for the concentrations [g/L] of urinary creatinine and m^7Gua is $[m^7Gua] = 0.0067x[\text{creatinine}] + 0.0023$ and the correlation coefficient (r) is 0.79 ($r^2 = 0.62$) (Fig. 10). For the data from 36 rats, the linear relationship is $[m^7Gua] = 0.0302x\ [\text{creatinine}] + 0.0026$ with a good correlation coefficient (r) equal to 0.90 ($r^2 = 0.81$) (Fig. 11). For the data from 22 mice, the linear relationship is $[m^7Gua] = 0.0284x[\text{creatinine}] + 0.0065$ with a correlation coefficient equal to 0.77 ($r^2 = 0.59$) (Fig. 12).

The levels of urinary m^7Gua are also presented as milligrams per gram creatinine (Table 2). All of these levels are significantly different ($P < 0.01$) among humans, rats, and mice. The level of human m^7Gua is four times lower than that of rats and five times lower than that of mice (Table 2). The level in mice is similar to that in rats (16% higher in mice). For a convenient comparison with the values found in the literature, the yields of m^7Gua from a subset of the samples from rats ($n = 10$) and mice ($n = 10$) were also calculated as micrograms of m^7Gua collected during 24 h normalized to body weight (μg/24 h/kg BW) (Table 2).

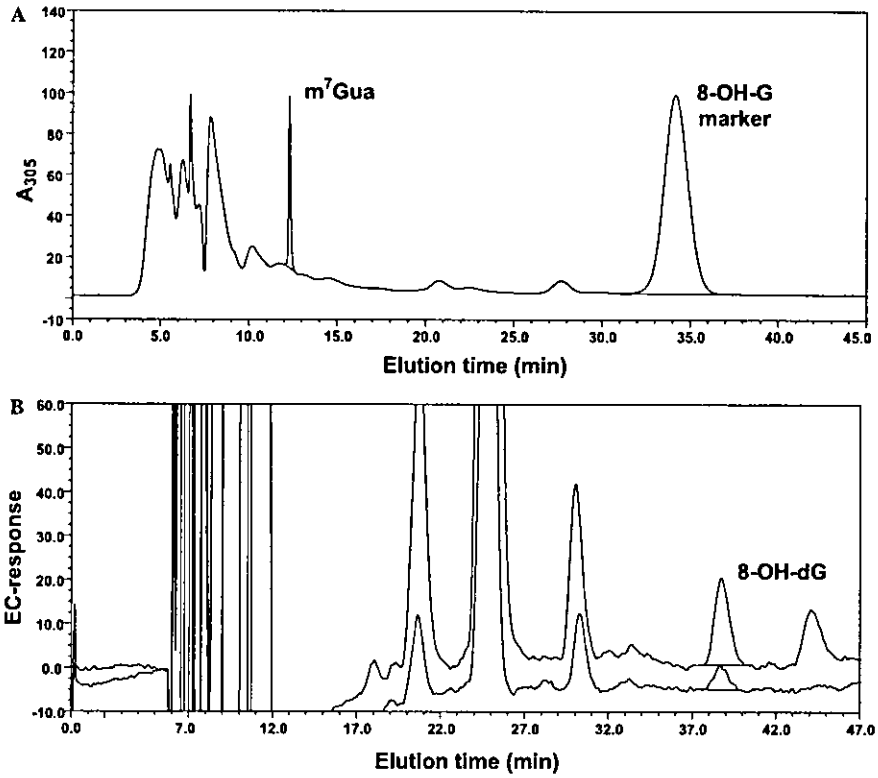

Fig. 3. Chromatograms of a mouse urine sample (female C3H/He mouse): (A) HPLC-1 UV detection of m^7Gua and 8-OH-G marker peaks and (B) HPLC-2 electrochemical detection of 8-OH-dG at applied potentials of E1 = 170 mV (lower line) and E2 = 300 mV (upper line).

8-OH-dG levels in human and rodent urine

The levels of 8-OH-dG normalized to creatinine (μg/g creatinine) all are significantly different ($P < 0.05$) among humans, rats, and mice (Table 2). The level of human 8-OH-dG is similar (15% higher) to that of rats. The ratios of 8-OH-dG/creatinine in mice are approximately twice as high as those in humans and rats. For comparison with other published values, the yields of 8-OH-dG from a subset of the samples from rats ($n = 10$) and mice ($n = 10$) were also calculated as nanograms of 8-OH-dG collected during 24 h normalized to body weight (ng/24 h/kg BW) (Table 2).

Discussion

The measurement of urinary 8-OH-dG is difficult due to the large amounts of other compounds present. Also, the amount of urine excreted, and thus the concentration of 8-OH-dG, differs among sampling times and individuals. Thus, a reliable method to distinguish only 8-OH-dG among all of the other compounds is needed. At the same time, the level of 8-OH-dG must be corrected for the degree of dilution between different samples. This correction is usually made by normalizing the 8-OH-dG values from a 24-h urine collection to the body weight or by normalizing the 8-OH-dG values to the amount of urinary creatinine. A new method based on HPLC-EC was developed to improve the speed and reliability of the urinary 8-OH-dG measurements [5]. The main features are the use of an 8-OH-G marker that is added to the urine sample, anion exchange chromatography (HPLC-1), and automatic peak detection of the marker peak to precisely collect the subsequent fraction of 8-OH-dG [5]. The 8-OH-dG is then detected with an electrochemical detector after reversed-phase chromatography in HPLC-2 [5]. The main advantages are that the fraction collected is not sensitive to daily variations in elution time and that the collection of neighboring interfering peaks is avoided [5]. For human samples, this system is currently used routinely to detect 8-OH-dG. However, urine samples from animal experiments (rats and mice) could not be analyzed reliably using the same conditions as for the detection of human 8-OH-dG in HPLC-2 (see Materials and methods). This is probably due to differences in the compositions and concentrations of the compounds found in urine samples from different species. Also, regarding the collection of urine from animals in metabolic cages, some contamination would be expected from feces and food [21]. Thus, keeping the conditions for the first anion exchange column constant,

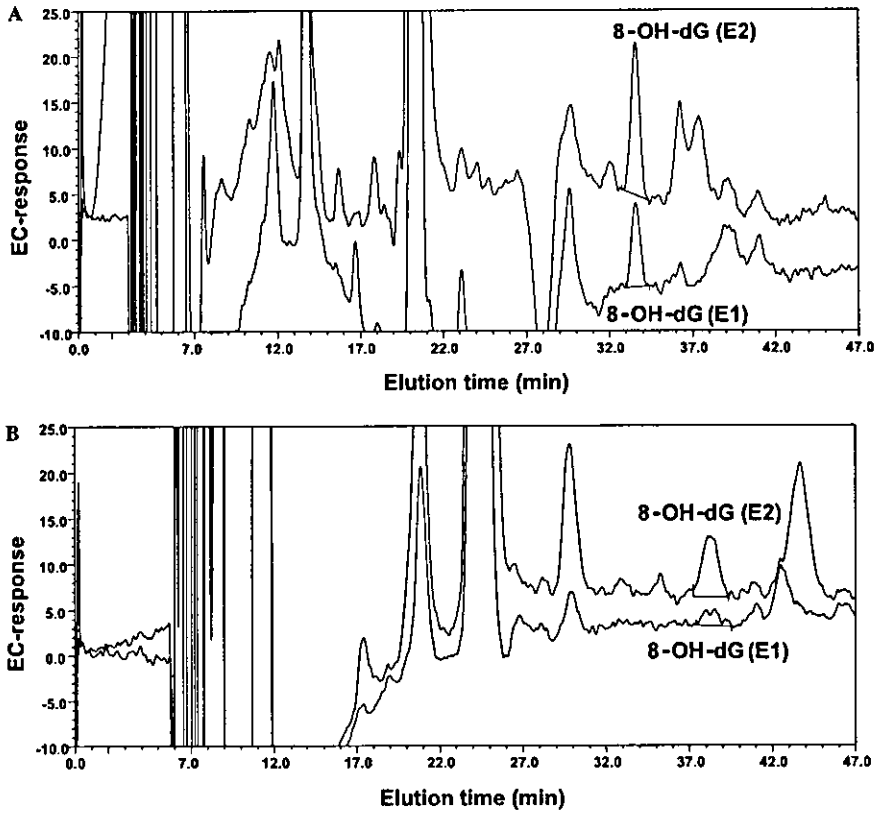

Fig. 4. Chromatograms of a mouse urine sample (female C3H/He mouse) showing the detection of false positives for 8-OH-dG. (A) HPLC-2 electrochemical detection of 8-OH-dG using the same conditions as in the rat urine analysis (the flow rate was 0.67 ml/min and the column oven was set at 48 °C) was unfavorable due to contamination of the 8-OH-dG peak detected at an applied potential of E2 = 300 mV (upper line), as seen from the large peak area ratio (55%) between the peak area at an applied potential of E1 = 170 mV (lower line) and the peak area of E2 (upper line). (B) Changing the HPLC-2 conditions for electrochemical detection of 8-OH-dG to resolve the interfering peaks in mouse urine (the flow rate was 0.33 ml/min and the column oven was set at 60 °C) decreased the ratio (24%) between the peak area at an applied potential of E1 = 170 mV (lower line) and the peak area at an applied potential of E2 = 300 mV (upper line). The decreased peak area ratio for E1/E2 in B, as compared with that in A, shows that the contaminating compounds have been removed from the 8-OH-dG peak by changing the HPLC conditions.

Table 1
Recovery of 8-OH-dG from spiked urine of humans, rats, and mice

Spike	Urine from humans[a]		Urine from rats[b]		Urine from mice[c]	
Added 8-OH-dG (ng/ml)	Detected 8-OH-dG (ng/ml)	Recovery 8-OH-dG (%)	Detected 8-OH-dG (ng/ml)	Recovery 8-OH-dG (%)	Detected 8-OH-dG (ng/ml)	Recovery 8-OH-dG (%)
0.0	7.4 ± 0.2		5.7 ± 0.2		4.9 ± 0.2	
1.0	8.5 ± 0.2	108 ± 10	6.8 ± 0.3	101 ± 23	5.9 ± 0.3	108 ± 14
2.5	10.0 ± 0.2	102 ± 2	8.4 ± 0.2	105 ± 11	7.6 ± 0.1	111 ± 7
4.0	11.5 ± 0.4	102 ± 8	10.0 ± 0.3	106 ± 7	9.1 ± 0.1	106 ± 6

Note. Mean values ± standard deviations are presented.

[a] Each value is based on four repeated analyses of pooled urine samples from humans ($n = 3$). The linear correlation between added and detected 8-OH-dG in human urine is $r = 0.99$.

[b] Each value is based on three repeated analyses of pooled urine samples from rats ($n = 3$). The linear correlation between added and detected 8-OH-dG in rat urine is $r = 0.99$.

[c] Each value is based on three repeated analyses of pooled urine samples from mice ($n = 3$). The linear correlation between added and detected 8-OH-dG in mouse urine is $r = 0.99$.

we modified some conditions for the reversed-phase column in HPLC-2 by reducing the mobile phase pH and the methanol content, reducing the flow rate, and increasing the column temperature (see Materials and methods). However, measuring rat samples typically means that the anion exchange column in HPLC-1 lasts

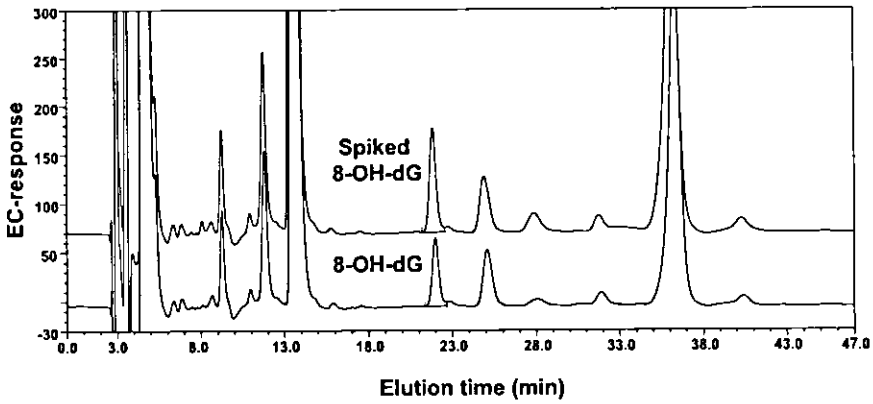

Fig. 5. Chromatograms of pooled urine from three humans (two male nonsmokers and one female nonsmoker), unspiked or spiked with the 8-OH-dG standard: (lower line) HPLC-2 electrochemical detection (E2) of 8-OH-dG from pooled urine with a peak area corresponding to 7.2 ng/ml of 8-OH-dG and (upper line) pooled urine spiked with 4.0 ng/ml of the 8-OH-dG standard with a peak area corresponding to 11.2 ng/ml of 8-OH-dG. Applied potentials were E1 = 200 mV and E2 = 370 mV.

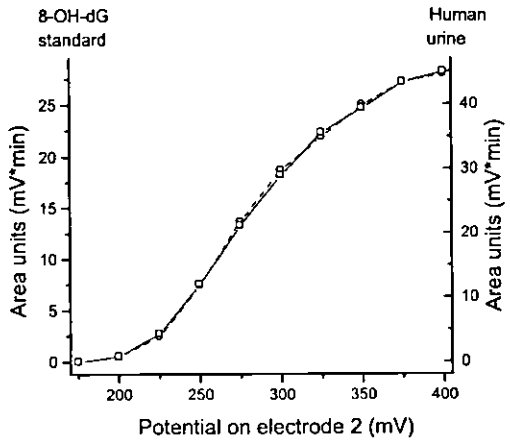

Fig. 6. Voltammograms of (□) 8-OH-dG standard and (○) pooled urine from humans at applied potentials on electrode 2 (E2) of 175–400 mV (E1 = 0 mV and guard cell = 450 mV).

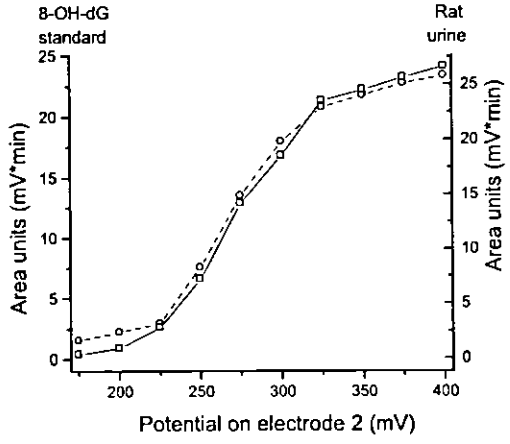

Fig. 7. Voltammograms of (□) 8-OH-dG standard and (○) pooled urine from rats at applied potentials on electrode 2 (E2) of 175–400 mV (E1 = 0 mV and guard cell = 450 mV).

for a shorter time and also needs the column filter replaced due to a pressure increase after 100–200 samples. When analyzing mouse urine, the filter might need to be replaced after every 10–20 samples injected. Also, the reversed-phase column in HPLC-2 needs to be washed more frequently with methanol and might last a shorter time due to the increased column oven temperatures used. In comparison, up to 1000 human urine samples can be analyzed continuously without any replacements [5]. Currently, these modified system settings allow the reliable and continuous automated analysis of 10–200 mouse or rat samples.

The human whole-body lean mass degradation rates of mRNA, tRNA, and rRNA all have been correlated with their specific degradation products in urine and with the RMR [8]. For example, the urinary 5,6-dihydrouridine levels were 4.7 times higher in rats than in humans, reflecting the difference between their RMRs [13]. Another such product is m^7Gua, which is derived from all three RNA classes [8]. Thus, it would be expected that the amount of m^7Gua detected in urine would reflect the RMR and be a suitable marker for the normalization of urinary 8-OH-dG. No significant variation in the urinary excretion of m^7Gua was found in terms of different types of diet [8]. However, some variations in the expected amount of human m^7Gua between individuals would be expected due to the fact that approximately 30–40% of the m^7Gua is converted to 8-hydroxy-7-methylguanine by human xanthine oxidase [8,22]. We may also find some deviations in the amounts of excreted m^7Gua due to exposure to methylating agents such as *N*-nitroso compounds from tobacco smoke and the endogenous *S*-adenosylmethionine activity [16,17].

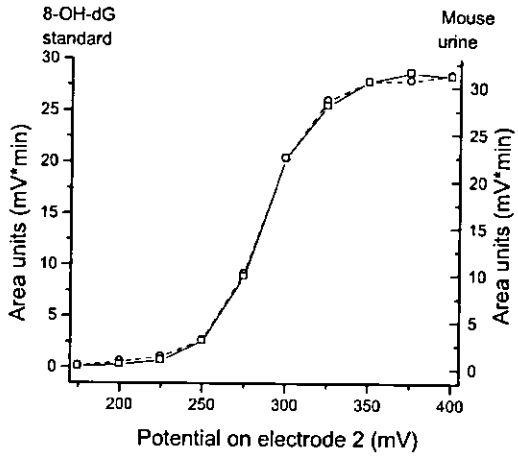

Fig. 8. Voltammograms of (□) 8-OH-dG standard and (○) pooled urine from mice at applied potentials on electrode 2 (E2) of 175–400 mV (E1 = 0 mV and guard cell = 450 mV).

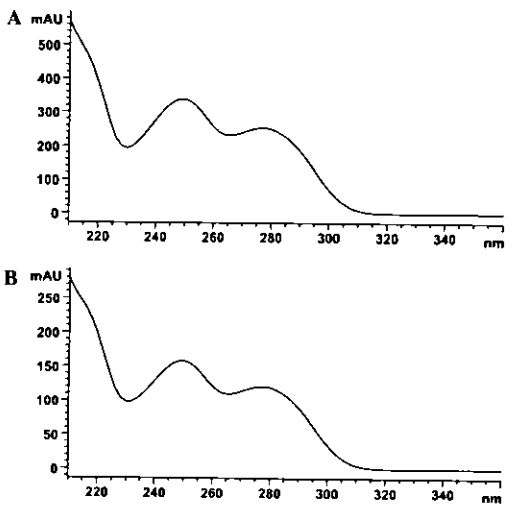

Fig. 9. UV absorbance spectrum of (A) m^7Gua standard and (B) a rat urine HPLC-1 fraction (0.5 min) eluted at 12 min.

In this article, we have presented a good correlation between urinary creatinine and m^7Gua in humans and those in mice ($r = 0.80$) and rats ($r = 0.90$) (Figs. 10–12). For human samples, the correlation was lower (Fig. 10), probably due to larger variations in the excreted creatinine and m^7Gua in the human population. For the mouse samples, the lower correlation would be explained by the small deviations in the m^7Gua and creatinine content among the individual urine samples collected in this study (Fig. 12). The urinary level of human m^7Gua presented in Table 2, 8.6 mg/g creatinine, correlates well with the previously published value of 7.0 mg/g creatinine (as recalculated from 4.80 nmol/μmol creatinine) [23]. For rats, the value for m^7Gua excreted during 24 h and normalized to body weight, 1100 μg/24 h/kg

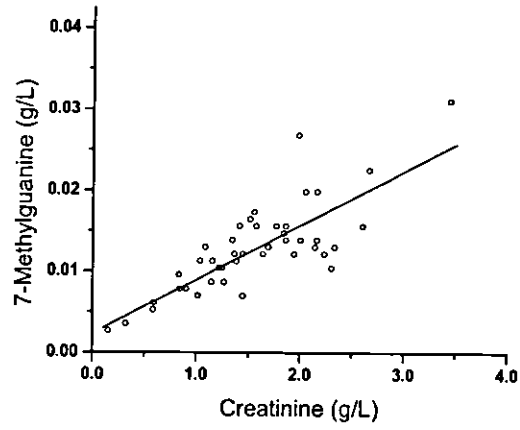

Fig. 10. Regression line for the correlation ($r = 0.79$) between human urinary creatinine and m^7Gua content. Urinary samples were measured in 44 individuals.

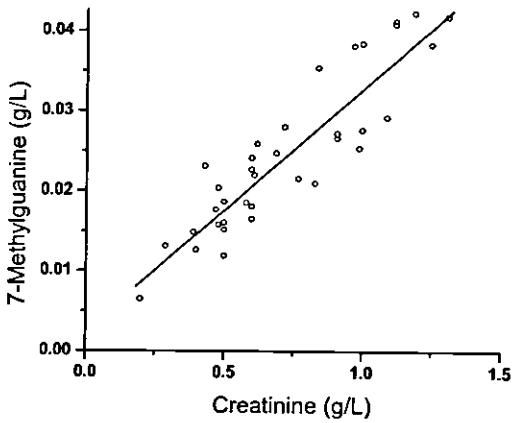

Fig. 11. Regression line for the correlation ($r = 0.90$) between rat urinary creatinine and m^7Gua content. Urinary samples were measured in 36 individual rats.

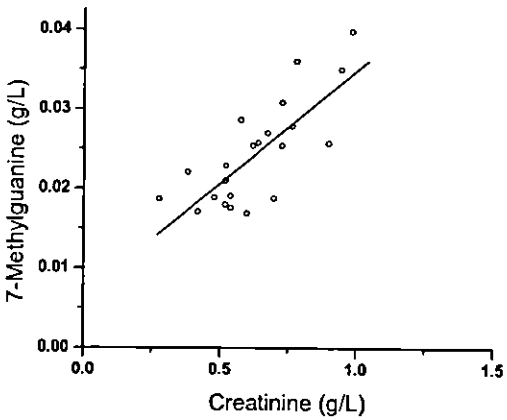

Fig. 12. Regression line for the correlation ($r = 0.77$) between mouse urinary creatinine and m^7Gua content. Urinary samples were measured in 22 individual mice.

Table 2
Urinary excretion of 8-OH-dG and m^7Gua in humans and rodents

Species	8-OH-dG		m^7Gua	
	Creatinine[a] (μg/g)	BW[b] (ng/24 h/kg)	Creatinine[a] (mg/g)	BW[b] (μg/24 h/kg)
Human	4.2 ± 1.2		8.6 ± 2.3	
Rat	3.7 ± 0.6	120 ± 39	34.3 ± 6.4	1100 ± 300
Mouse	8.2 ± 1.2	159 ± 55	39.9 ± 9.2	720 ± 110

Note. Mean values ± standard deviations are presented.

[a] Values are based on urine samples from humans ($n = 44$), rats ($n = 36$), and mice ($n = 22$). Mean values of 8-OH-dG, as normalized to creatinine, are significantly different between humans and rats ($0.01 < P < 0.05$), between humans and mice ($P < 0.001$), and between rats and mice ($P < 0.001$). Mean values of m^7Gua, as normalized to creatinine, are significantly different between humans and rats ($P < 0.001$), between humans and mice ($P < 0.001$), and between rats and mice ($0.001 < P < 0.01$).

[b] Values are based on urine samples from rats ($n = 10$) and mice ($n = 10$). Urine was collected during 24 h, and the average volumes were 13.7 ml/rat and 0.9 ml/mouse. The average weights of the animals were 363 g/rat and 23.6 g/mouse.

BW, is only 11% higher than the previously published value of 983 μg/24 h/kg BW for female LAC:P rats (recalculated from 172 μg/24 h and an average BW of 175 g/rat) [24]. For mice, assuming similar weights of animals, our value for m^7Gua excreted during 24 h, 16.9 μg/24 h (recalculated from 720.0 μg/24 h/kg BW), is within the range of the previously presented value, 12.6 μg/24 h, for 17-week-old male C57Bl/J6 mice (recalculated from 76.0 nmol/24 h) [25] (Table 2).

The mean level of human 8-OH-dG in Table 2, 4.2 μg/g creatinine, correlates well with our findings using a previous version of our HPLC-EC method (4.1 μg/g creatinine) [19] and data from others using the HPLC-EC or GC-MS methods (3.3–4.0 μg/g creatinine) [21,26]. For rats, our value of 3.7 μg 8-OH-dG/g creatinine is approximately half of the previously presented value (7.8 μg/g creatinine) [21,27]. However, a recently published value for the excretion of urinary 8-OH-dG in male Wistar rats is 94.4 μg/24 h/kg BW (recalculated from 333.2 pmol/24 h/kg BW) [28], which is comparable to our value of 120 μg/24 h/kg BW. Differences in the amounts of urinary 8-OH-dG could be due to various factors such as contamination of urine with feces and food, differences in rat strains used, and ages of rats. In the same report [28], the values expressed as picomoles of 8-OH-dG excreted in urine per day and kilograms body weight were similar for humans (281.7 ± 179.1) and rats (333.2 ± 47.4), a comparison in agreement with our values for 8-OH-dG/creatinine, where the human levels were only 15% higher than those in rats. In addition, in this report [28], the RMR difference was calculated as 4.2 times higher for rats (420 kJ/day/kg BW) than for humans (100 kJ/day/kg BW), similar to the ratio of 3.4 calculated previously (humans = 107 and rats = 364 kJ/day/kg BW) [29]. Interestingly, this is in good agreement with our value for human m^7Gua as normalized to creatinine, which is four times lower than that for rats. Thus, for the comparison between humans and rats, the urinary m^7Gua content agrees relatively well with the RMR level, although no correlation exists between RMR and urinary 8-OH-dG/creatinine (Table 2) or between RMR and 8-OH-dG excreted in urine per day and kilograms body weight [28]. For mice, the level of urinary 8-OH-dG (8.2 μg/g creatinine) (Table 2) is close to the previously estimated value of 6.9 μg/g creatinine [21,30]. This means that the level of 8-OH-dG excreted per gram of creatinine is 2.2 times higher in mice than in rats, which correlates well with the twofold higher RMR for mice than in rats (mice = 760 and rats = 364 kJ/day/kg BW) [29]. However, the amount of m^7Gua excreted in urine, when normalized to creatinine, is only 16% higher in mice than in rats (Table 2). Thus, RMR and the amount of excreted m^7Gua might not show a linear relationship among the various species studied. Also, the relatively large differences in RMR among humans, rats, and mice do not correlate with the similar differences among the levels of excreted 8-OH-dG/creatinine. Thus, when considering the RMR differences, the repair capacity for 8-OH-dG is similar in rats and mice and also is considerably lower than the repair capacity for 8-OH-dG in humans.

The presented results show that urinary 8-OH-dG and m^7Gua in humans, rats, and mice can be analyzed rapidly and reliably in the same sample run. For rats and mice, m^7Gua may be used as a reliable marker instead of creatinine for the normalization of 8-OH-dG content in urine. In human urine samples, m^7Gua may be analyzed in addition to 8-OH-dG normalized with creatinine.

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

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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

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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

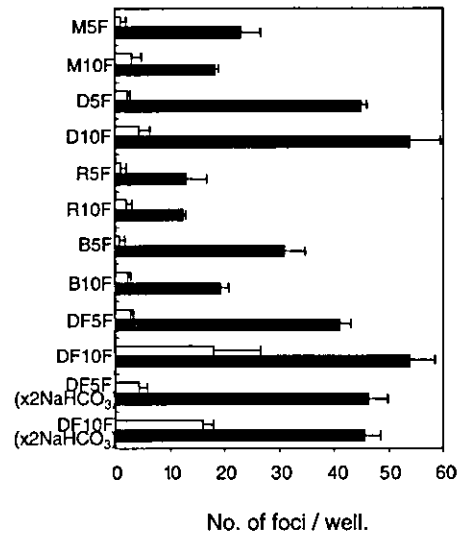

Fig. 1. Effects of various basal media and FBS concentrations on the transformation of Bhas 42 cells: (□), control; (■), TPA (20 ng/ml); M5F, MEM + 5% FBS; M10F, MEM + 10% FBS; D5F, DMEM + 5% FBS; D10F, DMEM + 10% FBS; R5F, RPMI1640 + 5% FBS; R10F, RPMI1640 + 10% FBS; B5F, BME+5% FBS; B10F, BME+10% FBS;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

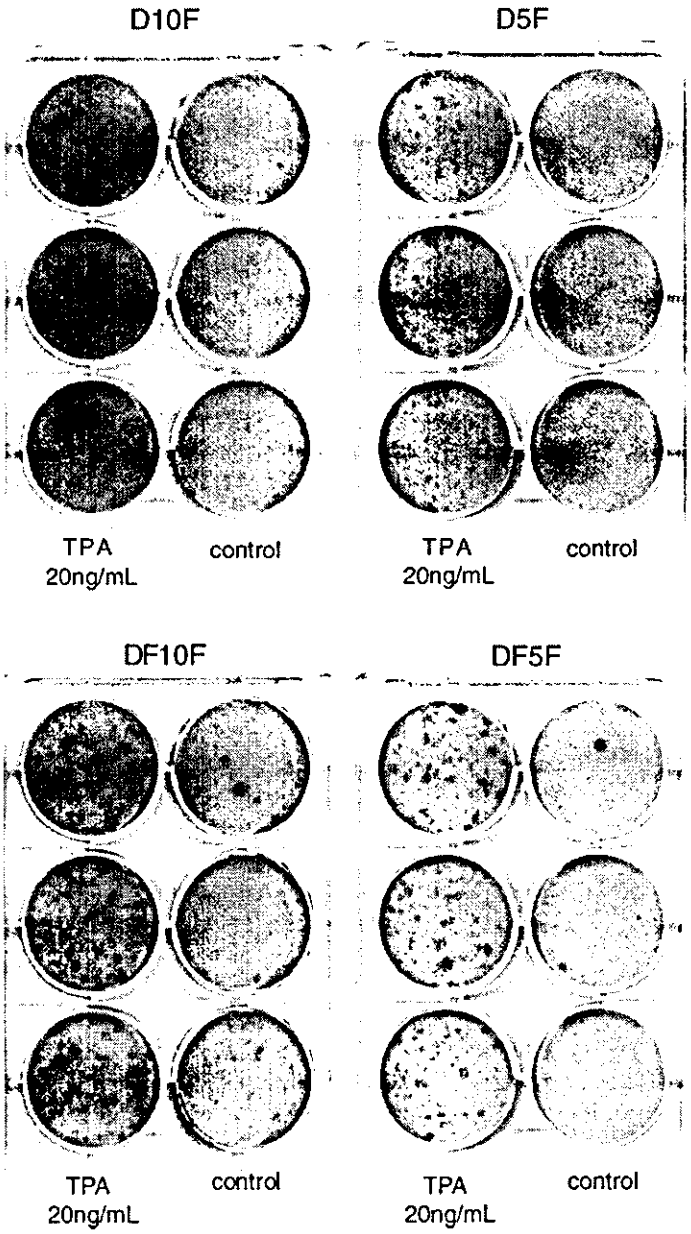

Fig. 2. Representative plates showing formed foci of Bhas 42 cells cultured in different media with or without TPA.