Review

Attempts at Organ-specific In Vivo Short-term Tests for Environmental Mutagens and Carcinogens in Rodent Liver and Stomach

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(Received December 27, 2012; Revised April 2, 2013; Accepted April 3, 2013)

The primary motivation for conducting short-term tests for environmental mutagens and carcinogens has been to predict mutagens and/or carcinogens and to assess any associated risks. Organ-specific in vivo short-term tests in rodents are valuable because chemical carcinogenesis is generally organ-specific. I have attempted to develop various organ-specific in vivo short-term tests mainly in rodent liver and stomach. Recently, our collaborative study group, Toxicogenomics/Japanese Environmental Mutagen Society · Mammalian Mutagenicity Study Group (JEMS · MMS), attempted to use gene expression profiling in in vivo short-term tests, conducted DNA microarrays to extract candidate marker genes, and later shifted to quantitative real-time PCR (qPCR) to profile the expression of selected genes. We successfully discriminated 8 genotoxic hepatocarcinogens from 4 non-genotoxic hepatocarcinogens by statistical analysis using principal component analysis (PCA) based on the gene expression profiles for 12 genes (Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Gdf15, Lrp1, Mbd1, Phlda3, Plk2, and Tubb2c) in mouse liver at 4 and 48 h following a single intraperitoneal administration of chemicals as determined by qPCR. More recently, we successfully performed a similar study in rat liver. Previously, my collaborators and I developed various organ-specific in vivo short-term test methods, including UDS (unscheduled DNA synthesis); RDS (replicative DNA synthesis) using a liquid scintillation counter in rat glandular stomach, forestomach, colon, and liver and hairless mouse epidermis; DNA single-strand scission (DSS); and ornithine decarboxylase assay (ODC) in rat glandular stomach on 62 compounds. Developing short-term tests that are helpful for the risk assessment of human mutagens and carcinogens would contribute to the development of ideal prediction methods.

Key words: organ-specific in vivo short-term tests, toxicogenomics, gene expression profiling, quantitative real-time PCR, principal component analysis, UDS, RDS, DNA single strand scission, ornithine decarboxylase

Introduction

Mahadevan et al. suggested that “driven by regulatory concerns, the primary reason for conducting in vitro and in vivo genotoxicity analyses has been to try to predict which molecules are likely to be rodent and/or human carcinogens” (1). Additionally, Bucher and Portier suggested that “as our collective understanding of carcinogenesis advances, toxicologists and regulatory scientists will at some point begin to rely on mechanism-based biological observations rather than the 2-year rodent bioassay to predict human cancer hazards” (2).

According to Waters et al., although the number of presumed non-genotoxic rodent carcinogens has dramatically increased over the past 2 decades, ~90% of the known, probable and possible human carcinogens classified by the International Agency for Research on Cancer are detected in conventional short-term tests for genotoxicity and can induce tumors at multiple sites in rodents (3). New and innovative assays to characterize the key steps in toxicity pathways, including genotoxic and nongenotoxic mechanisms for carcinogenesis, are waiting to be developed.

In the 1980s, my collaborators and I began investigating organ-specific in vivo short-term tests for environmental mutagens and carcinogens using simple methods to detect DNA damage, DNA repair, and cell proliferation, including UDS (unscheduled DNA synthesis), DSS (DNA single-strand scission), RDS (replicative DNA synthesis), and ODC (ornithine decarboxylase assay), to identify any hazards. In the 2000s, our collaborative study group, Toxicogenomics/Japanese Environmental Mutagen Society · Mammalian Mutagenicity Study Group (JEMS · MMS), focused on gene expression profiling to investigate the mode of action of particular
chemicals on gene expression.

Here, I review our studies on gene expression profiling in rodent liver induced by hepatocarcinogens and various organ-specific in vivo short-term tests in glandular stomach and additional organs.

An Attempt at Using Gene Expression Profiling for an Organ-specific In Vivo Short-term Test

Discrimination of genotoxic and non-genotoxic mouse hepatocarcinogens as analyzed by qPCR (quantitative real time polymerase chain reaction) and PCA (principal component analysis): It is hypothesized that genotoxic and non-genotoxic carcinogens induce distinct gene expression profiles, which consequently may be used for a mechanism-based classification of unknown compounds as either genotoxic or non-genotoxic carcinogens (4). DNA microarray analysis is a powerful technique used for characterizing gene expression on a genome scale (5), although issues of reliability, reproducibility, and the correlation of data produced across different DNA microarrays are still being addressed (6). qPCR is generally considered the “gold-standard” assay for measuring gene expression and is often used to confirm DNA microarray data (7). qPCR is the most sensitive technique used for the detection and quantification of mRNA targets (8). Mahadevan et al. suggested that it is possible to screen for carcinogenicity and discern the potential mode of action (MOA) of a chemical based on analysis of gene expression using toxicogenomics methods, with the potential for screening putative MOA by qPCR (1). However, the published studies using DNA microarray or qPCR on in vivo rodent livers with hepatocarcinogens are limited.

Toxicogenomics/JEMS·MMS attempted to use gene expression profiling as an in vivo short-term test to identify a mechanism-based classification of unknown compounds. In addition, we studied hepatocarcinogens, as the liver is the major target organ for experimental chemical carcinogenesis in rodents. We recently successfully discriminated 8 mouse genotoxic hepatocarcinogens from 4 non-genotoxic hepatocarcinogens through selected gene expression profiling in mouse liver based on qPCR and statistical analysis using PCA (9). The total RNA from an individual liver from groups of 5 male 9-week-old B6C3F1 mice was reverse-transcribed to cDNA, and the amount of each gene was quantified by qPCR at 4 and 48 h following a single intraperitoneal administration of chemicals. It was speculated that DNA damage would occur 4 h post-hepatocarcinogen administration in the liver, as determined by in vivo/in vitro UDS assay (10–12) and DNA adduct analysis (13), and that the 48-h time point would represent the period of RDS testing (10–12, 14). Intraperitoneal injection or oral administration was usually used in genotoxicity tests in rodent liver (15,16). We quantified 35 genes selected from our previous DNA microarray and qPCR studies (17,18) using 12 different chemicals with various chemical properties: 8 genotoxic hepatocarcinogens [2-acetylaminofluorene (2AAF), 2,4-diaminotoluene (DAT), diisopropanolnitrosamine (DIPN), 4-dimethylaminoazobenzene (DAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK), N-nitrosomorpholine (NNM), quinoline (QN), and urethane (URE)] and 4 non-genotoxic hepatocarcinogens [1,4-dichlorobenzene (DCB), dichlorodiphenyltrichloroethane (DDT), di(2-ethylhexyl)phthalate (DEHP), and furan (FUR)] (9). All of the genes except Gapdh, a housekeeping gene, exhibited significant changes in their gene expression ratios (experimental group/control group) according to statistical analysis using Dunnett’s test, at least once, at 4 h and/or 48 h. Gapdh was used to normalize the gene expression ratio, as this gene did not show changes in expression. Different sets of 17 genes at 4 h and 19 genes at 48 h showed statistically significant differences between the genotoxic and non-genotoxic hepatocarcinogens as determined by Welch’s t-test. However, no single gene completely discriminated the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens.

Finally, we successfully discriminated the genotoxic from the non-genotoxic hepatocarcinogens by statistical analysis using PCA on the gene expression profiles. PCA on the gene expression profile of 7 genes (Btg2, Ccnf, Ccn1, Lrp1, Mbd1, Phlda3, and Tubb2c) at 4 h (Fig. 1-A) and 12 genes (Aen, Bax, Btg2, Ccnf, Ccn1, Cdkn1a, Gdf15, Lrp1, Mbd1, Phlda3, Plk2, and Tubb2c) at 48 h (Fig. 1-B) yielded an optimal separation between the genotoxic and the non-genotoxic hepatocarcinogens and successfully discriminated the genotoxic from the non-genotoxic hepatocarcinogens by the first principal component (PC1). The changes appeared to be greater at 4 h than at 48 h. Therefore, we used the 4-h time point for the first experiment. Statistical analysis by PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called “principal components”.

Seven major biological processes were extracted by gene ontology analysis (http://www.geneontology.org/): apoptosis, the cell cycle, cell proliferation, DNA damage, DNA repair, oncogenesis, and tumor suppression. The major, biologically relevant gene pathway identified through this analysis was the DNA damage response pathway, which results from signal transduction through a p53-class mediator leading to the induc-
Fig. 1. PCA of the gene expression levels between genotoxic and non-genotoxic mouse hepatocarcinogens as quantified by qPCR. The mean values of triplicate qPCR assays for each chemical were statistically analyzed using PCA programs in GeneSpringGX11.0.1. The results of the PCA are shown in the two-dimensional contribution scores for component numbers 1 and 2 (PC1 and PC2). The contribution scores were produced by conversion from each eigenvector value. A: 4 h with 7 genes (Btg2, Ccnf, Ccnf1, Lrp1, Mbd1, Phlda3 and Tubb2c); B: 48 h with 12 genes (Aen, Bax, Btg2, Ccnf, Ccnf1, Cdkn1a, Gdf15, Lrp1, Mbd1, Phlda3, Plk2 and Tubb2c). Genotoxic hepatocarcinogens (■, DIPN: diisopropanol-nitrosamine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane, NNM: N-nitrosomorpholine, QN: quinoline, DAT: 2,4-diaminotoluene, DAB: 4-dimethylaminoazobenzene, 2AAF: 2-acetylaminofluorene, URE: urethane) and non-genotoxic hepatocarcinogens (□, FUR: furan, DDT: dichlorodiphenyltrichloroethane, DEHP: di(2-ethylhexyl)phthalate, DCB: 1,4-dichlorobenzene). Dashed line is added between genotoxic and non-genotoxic hepatocarcinogens. (9)

Fig. 2. The gene networks and pathways of 19 mouse genes as determined by qPCR. The network was combined from the results of IPA, GeneSpring software and references from PubMed. The 9 genes indicated by “*” mark genes that significantly contributed to the discrimination of the genotoxic from the non-genotoxic mouse hepatocarcinogens by PCA. (9)

Organ-specific In Vivo Short-term Tests

A

B

PC2 (14.2%)

PC1 (71.5%)

expression of apoptosis. Eight genes (Aen, Bax, Btg2, Ccnf1, Cdkn1a, Gdf15, Phlda3, and Plk2) that are directly associated with Trp53 contributed to the PCA (Fig. 2). The current findings demonstrate a successful discrimination between genotoxic and non-genotoxic hepatocarcinogens, using qPCR and PCA, on 12 genes associated with a Trp53-mediated signaling pathway for DNA damage response at 4 and 48 h after a single administration of chemicals. Regarding the expression of Trp53 itself, a statistically significant increase (less than twofold) with only the NNK injection at the 48-h time point was observed, although the basal expression of Trp53 in the control animal may already have been sufficient for DNA damage under the present experimental conditions (9). Little is known about the acute expression changes of Trp53 in rodent liver after exposure to hepatocarcinogens; only a few reports have suggested the activation of a Trp53-mediated signaling pathway following the administration of hepatocarcinogens (19).

Seven genotoxic hepatocarcinogens were positive using the standard Ames test [2AAF (20), DAB (21), DAT (22), DIPN (23), NNK (24), NNM (22), and QN (25)]. URE was negative using the standard Ames test but positive under special conditions (26). Seven genotoxic hepatocarcinogens were positive in the liver in transgenic mouse studies [2AAF (27); DAT (28); DIPN: Takayoshi Suzuki, unpublished data; NNK (29); NNM: Takayoshi Suzuki, unpublished data; QN (30); and URE (31)]. DAB was positive in the mouse liver using the Comet assay (32). Four non-genotoxic hepatocarcinogens were negative using both the Ames test [DCB (33), DDT (34), DEHP (35) and FUR (36)] and in vivo
genotoxicity tests [DCB (37), DDT (38), DEHP (39), and FUR (40)].

In future studies, two recently described, advanced techniques, high-density real-time PCR (41) and ion torrent semiconductor chip based sequencing (42), would increase the efficiency of examining gene expression profiles; however, we have performed a large number of qPCR experiments manually. In the present study, we simply discriminated genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens. However, mechanism-based tests to predict human non-genotoxic carcinogens will be required in a different way; non-genotoxic carcinogens are designated as substances that induce cancer through the indirect stimulation of hyperplastic responses, without altering DNA, chromosome number, or structure. A wide variety of mechanisms of cancer induction, including receptor-mediated endocrine modulation, non-receptor mediated endocrine modulation, tumor promotion, inducers of tissue-specific toxicity and inflammatory responses, immunosuppressants, and gap junction intercellular communication inhibitors, are proposed for non-genotoxic carcinogens (43).

**Dose-dependent alterations in gene expression in mouse liver by diethylnitrosamine (DEN) and ethyl-nitrosourea (ENU) as determined by qPCR:** Previously, we examined the dose-dependency of changes in gene expression for 51 genes in mouse liver treated with 4 doses of 2 N-nitroso genotoxic hepatocarcinogens, DEN and ENU, by qPCR (18). Fifty-one genes were selected from our previous DNA microarray studies (17). DEN (3, 9, 27, and 80 mg/kg bw, 1/40–1/2 of 50% lethal dose (LD50)) or ENU (6, 17, 50, and 150 mg/kg bw, 1/80–1/3 of LD50) was injected intraperitoneally into groups of 5 male 9-week-old B6C3F1 mice, and the livers were dissected after 4 h and 28 days after a single intraperitoneal administration of the chemical. The changes in gene expression at 4 h were the most remarkable. We examined ethanol as a non-genotoxic non-hepatocarcinogen because it is not a bacterial or mammalian cell mutagen, is generally negative in in vivo genotoxicity tests (44), and has not generated distinct carcinogenicity data in rodent liver cancer, although there is some inadequate evidence for the carcinogenicity of ethanol and alcoholic beverages in experimental animals (45).

**Differential gene expression profiling between genotoxic and non-genotoxic rat hepatocarcinogens as analyzed by qPCR and PCA:** More recently, we applied our mouse candidate marker genes (9,17,18) to rat hepatocarcinogens in an established rat liver genotoxicity test system (46). We evaluated the gene expression profiles in rat liver treated with 4 chemicals [DEN, 2,6-dinitrotoluene (DNT), DEHP, and phenacetin (PNT)] that were previously examined using the liver micronucleus assay by the CSGMT/JEMS-MMS collaborative study group (47,48).

qPCR analysis of 33 genes was conducted on liver samples from groups of 4 male 4-week-old F344 rats at 4 and 48 h after a single oral administration of chemicals [2 genotoxic hepatocarcinogens, DEN (12.5, 25 and 50 mg/kg bw) and DNT (125 and 250 mg/kg bw); a non-genotoxic hepatocarcinogen, DEHP (1,000 and 2,000 mg/kg bw); and a non-genotoxic non-hepatocarcinogen, PNT (500 and 1,000 mg/kg bw)]. All 32 genes, with the exception of Gapdh, exhibited statistically significant changes in gene expression as calculated using Williams’ test (at least once), at 4 h and/or 48 h. Changes in gene expression were generally greater at 4 h than at 48 h. Furthermore, at 4 h, a statistically significant difference was observed by Dunnett’s test between genotoxic hepatocarcinogens (DEN and DNT).
and the non-genotoxic hepatocarcinogen (DEHP) in 19 genes (Aen, Btg2, Ccnf, Ccnf1, Ddit4l, Gadd45g, Gdf15, Hspb1, Jun, Lpp, Myc, Net1, Phlda3, Pik2, Pml, Pmm1, Rcan1, Tnf, and Tubb2c) and between genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen (PNT) in 18 genes (Aen, Bax, Ccnf, Ccnf1, Ddit4l, Ephx1, Gdf15, Hspb1, Jun, Myc, Net1, Phlda3, Pik2, Pml, Pmm1, Rcan1, Tnf, and Tubb2c). At 48 h, a statistically significant difference was observed by Dunnett’s test between genotoxic hepatocarcinogens (DEN and DNT) and the non-genotoxic hepatocarcinogen (PNT) in 18 genes (Aen, Bax, Ccnf, Ccnf1, Ddit4l, Ephx1, Gdf15, Hspb1, Jun, Myc, Net1, Phlda3, Pik2, Pml, Pmm1, Rcan1, Tnf, and Tubb2c) and between genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen (PNT) in 8 genes (Ccnf1, Cdkn1a, Cyp4a1, Gdf15, Igfbp1, Mdm2, Myc, Phlda3, Pml, Pmm1, and Tubb2c). However, no single gene completely discriminated genotoxic hepatocarcinogens from non-genotoxic carcinogens and/or the non-genotoxic non-hepatocarcinogen.

At 4 h, DEN and DNT produced a distinct dose-dependent increase in 10 genes (Aen, Btg2, Ccnf, Ccnf1, Cdkn1a, Ddit4l, Gdf15, Jun, Phlda3, Rcan1, and Tubb2c), with the exception of Aen under DNT. At 48 h, DEN and DNT produced dose-dependent increases in Ccnf1, Cdkn1a, and Phlda3. However, DEHP and PNT did not necessarily cause dose-dependent increases in these 10 genes at 4 and 48 h. Furthermore, a statistically significant difference was observed using Dunnett’s test between genotoxic hepatocarcinogens and one non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT) for 9 of the genes, with the exception of Cdkn1a at 4 h and Aen, Ccnf1, Cdkn1a, Gdf15, Phlda3, and Tubb2c at 48 h.

Statistical analysis using PCA clearly differentiated the gene expression profiles between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen. The PCA of all 32 genes were able to differentiate genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen at 4 and 48 h. Furthermore, we selected specific genes to obtain an optimal separation between genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen using PCA. PCA on 16 genes (Ccnf, Ccnf1, Cyp4a1, Ddit4l, Egfr, Gadd45g, Gdf15,
Attempts at Organ-specific In Vivo Short-term Assays for the Potential Tumor-Initiating and -Promoting Activities of Chemicals in the Glandular Stomach of Fischer Rats

Cancers of the stomach are the second leading causes of human cancer mortality in the world; however, only a few glandular stomach carcinogens have been identified in experimental animal carcinogenesis (55). Prior to 1999, stomach cancer was the major cause of cancer-related death in Japan. In the 1960s, Sugimura and Fujimura successfully induced tumor production in rat glandular stomach mucosa using a mutagen, N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) (56). Organ-specific in vivo short-term tests for stomach carcinogens were not reported in the early 1980s. Therefore, my collaborators and I developed the potential tumor-initiating and -promoting activities of compounds in rat glandular stomach mucosa by measuring DNA damage and repair and cell proliferation to identify associated hazards. We studied potential tumor-initiating activity by measuring the induction of UDS and DSS and tumor-promoting activity by measuring the induction of RDS and ODC in the rat glandular stomach mucosa of male Fischer (F344) rats after oral administration. We reported our results on 54 compounds and summarized these results in our review (57, 58).

UDS and RDS are commonly measured by autoradiography, but we measured them using a liquid scintillation counter. We believed that using a liquid scintillation counter would eliminate the subjective counting of [3H]dThd incorporation into cells (59). Initially, we compared these two approaches by quantifying the DNA synthesis in the pyloric mucosa of individual rats in the presence and absence of hydroxyurea (HU, an inhibitor of RDS) simultaneously. We confirmed that the liquid scintillation counter method was useful in examining UDS and RDS in rat glandular stomach mucosa (Fig. 4 and Table 1) (60).

As stomach cancer is often induced in the pyloric mucosa of the glandular stomach mucosa in humans and rodents, we mainly studied the pyloric mucosa. The glandular stomach mucosa is composed of fundic mucosa and pyloric mucosa. The pyloric mucosa is in turn mainly composed of surface mucous cells and pyloric gland cells. The fundic mucosa is mainly composed of surface mucous cells, chief cells, parietal cells, and mucous neck cells.

**Results of UDS in rat glandular stomach:** Four types of responses were observed after the oral administration of compounds (61). Type 1: the induction of UDS but not the stimulation of RDS was observed at 1–2 h and strong enhancement of RDS was observed at 16 h (demonstrated by 4-nitroquinoline 1-oxide (4NQO), a genotoxic glandular stomach carcinogen). Type 2: the induction of UDS and some stimulation of
Fig. 4. UDS in rat stomach pyloric mucosa induced by oral administration of MNNG (100 mg/kg body weight) (Autoradiography). (60)

![UDS](image)

**Table 1. Effects of HU on UDS induced in the rat stomach pyloric mucosa by MNNG**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>MNNG mg/kg bwM</th>
<th>HU mM</th>
<th>Analytical method</th>
<th>Liquid scintillation counter method[^a^]</th>
<th>Autoradiography[^a^]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
<td>4,450 ± 2,370 ([H]dThd dpm/μg DNA[^*^])</td>
<td>0.68 ± 1.06</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>10</td>
<td></td>
<td>271 ± 96</td>
<td>0.54 ± 0.50</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>0</td>
<td></td>
<td>2,640 ± 644</td>
<td>8.50 ± 6.84</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>10</td>
<td></td>
<td>1,925 ± 459</td>
<td>8.47 ± 6.79</td>
</tr>
</tbody>
</table>

[^a^]: Averages and standard deviations of values in single assays on 5 rats.

[^*^]: Averages and standard deviation of counts on the totals of 500 nuclei in 5 rats. Grains on S-phase cells were not counted. The value for group 4 was significantly higher than that for group 2 by Student’s t-test (p < 0.01).

RDS were observed at 1–2 h and strong enhancement of RDS was observed at 16 h (demonstrated by MNNG, a genotoxic glandular stomach carcinogen). Type 3: the induction of UDS was not observed, but strong enhancement of RDS was observed at 1–2 to 16 h (demonstrated by NaCl, a glandular stomach tumor promoter). Type 4: the induction of neither UDS nor RDS was observed at 1 to 16 h (demonstrated by dimethylnitrosamine (DMN), a liver carcinogen).

Six genotoxic known glandular stomach carcinogens [N-ethyl-N’-nitro-N-nitosoguanidine (ENNG) (60), MNNG (60,61), 1-methyl-1-nitrosourea (MNU) (62), N-nitroso-N-methylethane (NMUT) (60), 4NQO (60,61), and N-propyl-N’-nitro-N-nitosoguanidine (PNNG) (60)] all induced UDS dose-dependently 2 h after administration (57). In contrast, 5 liver carcinogens [2AAF (60), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) (60), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) (60), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (59), and DMN (60,61)], a lung and kidney carcinogen [ethyl methanesulfonate (EMS) (59)], a forestomach carcinoma [aristolochic acid (59)], and a glandular stomach tumor promoter [NaCl (61)] showed negative results. Two known non-genotoxic glandular stomach carcinogens [catechol (63) and 4-methylcatechol (64)] and their analogue, methylhydroquinone (64) showed negative results. Additionally, two direct-acting mutagens [3-diazo-N-nitrosobenzenethan (65) and hickory smoke condensate with nitrite (66)] induced UDS dose-dependently 2 h after administration (57). Six chemicals showed equivocal results: 5 direct-acting mutagens [2-chloro-4-(methylthio)butanoic acid (67), glyoxal (68), diacetyl (68), methylglyoxal (69), and 1-nitrosoimidazole-3-acetonitrile (NIAN) (70)] and an anti-ulcer drug, omeprazole [6-mercapto-2-[(4-mercapto-3,5-dimethyl-2-pyridyl)methyl]sulfanyl]-1H-benimidazole (71). Three direct-acting mutagenic compounds [2-hydroxy-3-nitroso-α-carboline (59), nitrosated cimetidine (59), and broiled fish extract (59)], hickory smoke condensate without NaNO2 (66), and glycocholate (72) showed negative results.

We tested 30 compounds using the UDS method. Eight compounds were positive, including 6 genotoxic glandular stomach carcinogens; 16 compounds were negative, including 5 liver carcinogens, a lung and kidney carcinogen, a forestomach carcinogen, a glandular stomach tumor promoter, and 2 non-genotoxic glandular stomach carcinogens; and 6 compounds were equivocal. The organ specificity of UDS induction in the glandular stomach, forestomach, colon, liver, and epidermis was high as shown in Table 2 (partly in 57).

**Results of DSS in rat glandular stomach:** Figure 5 shows the elution patterns of DNA from a filter. MNNG, a glandular stomach carcinogen, at doses of 1–100 mg/kg body weight, induced DSS dose-dependently 2 h after oral administration (73). The elution rate constant was calculated from the slope of the elution curve. Figure 5 shows the time dependency of DSS induction, with repair expressed as an elution rate constant with MNNG at a dose of 10 mg/kg body weight. The induction of DSS was maximal 2 h after oral administration of MNNG (61) and 4NQO (61).

Six genotoxic glandular stomach carcinogens [ENNG (74), MNNG (73), MNU (62), NMUT (74), 4NQO (74), and PNNG (74)] all showed dose-dependent induction of DSS 2 h after administration (57). In contrast, 2 liver...
Table 2. Organ specificity of UDS induction and carcinogenicity in rat and mouse

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Glandular stomach</th>
<th>Forestomach</th>
<th>Colon</th>
<th>Liver</th>
<th>Epidermis</th>
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<td>+/+* (58)</td>
<td>+/+ (89)</td>
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<tr>
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</tr>
<tr>
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<td>+/+* (58)</td>
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<tr>
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<td>-/- (58)</td>
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<tr>
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<tr>
<td>DMBA</td>
<td>+/+ (89)</td>
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+, positive; -, negative; ±, equivocal, blank column, not examined, *, colon instillation, **, non-genotoxic hepatocarcinogen.

MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; ENNG, N-ethyl-N'-nitro-N-nitrosoguanidine; PNNG, N-propyl-N'-nitro-N-nitrosoguanidine; MNU, 1-methyl-1-nitrosourea; NMUT, N-nitroso-N-methylurethane; 2AAF, 2-acetylaminofluorene; DMN, dimethylnitrosamine; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; AA, aristrochic acid; EMS, ethyl methanesulfonate; NCM, nitrosated cimetidine; MAMAc, methylazoxymethanol acetate; DPB, diepoxybutane; DMBA, 7,12-dimethylbenzo[a]pyrene; B[α]P, benzo[a]pyrene; EPD, 1,2-epoxydodecane; 4AAF, 4-acetylaminofluorene; P, pyrene.

Fig. 5. (A) Alkaline elution patterns of DNA from rat stomach pyloric mucosa 2 h after treatment with MNNG or NaCl. Doses of MNNG (mg/kg body weight): 0 (H₂O), ○; 10, □; 100, ; NaCl, (1.5 g/kg) (73). (B) Time dependence of induction of DNA single strand scission by oral MNNG at 10 mg/kg body weight. Values at 2 and 4 h differ significantly from the 0 time value (P<0.01) (61).
carcinogens [2AAF (73) and DMN (61)], 2 non-genotoxic glandular stomach carcinogens [catechol (63) and 4-methylcatechol (64)], and a glandular stomach tumor promoter (NaCl) (73,75) showed negative results. Additionally, 5 direct-acting mutagenic compounds [3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) (76), glyoxal (73), NIAN (70), nitrosated Oroxylum indicum Vent (OiV) (77), and hickory smoke condensate without NaNO2 (66)] showed the dose-dependent induction of DSS 2 h after administration (57). Methylhydroquinone showed positive results (64). Hickory smoke condensate with NaNO2 (66) and loxtidine [1-methyl-5-{3-[3-(piperidin-1-ylmethyl)phenoxy]propyl} amino]-1H-1,2,4-triazol-3-yl]methanol (74) showed negative results. Omeprazole showed an equivocal result (74).

We examined 20 compounds by the DSS assay. Twelve compounds were positive, including 6 genotoxic glandular stomach carcinogens, 7 compounds were negative, including 2 liver carcinogens, 2 non-genotoxic glandular stomach carcinogens, and 1 glandular stomach tumor promoter and 1 compound was equivocal.

Results of RDS in the Rat Glandular Stomach: Figure 6 shows the time-dependency of the increase in RDS induced by the glandular stomach carcinogen MNNG and glandular stomach tumor promoter NaCl as determined by both the liquid scintillation counter method and autoradiography (78). Maximum responses occurred 16–24 h after administration.

Six genotoxic glandular stomach carcinogens [ENNG (79), MNNG (79), MNU (62), NMUT (79), 4NQO (79), and PNNG (79)], 2 non-genotoxic glandular stomach carcinogens [catechol (63) and 4-methylcatechol (64)] and 5 glandular stomach tumor promoters [formaldehyde (80), glyoxal (73), K2S2O5 (81), NaCl (78), and taurocholate (72)] all induced dose-dependent increases in RDS after oral administration (57). In contrast, 3 liver carcinogens [2AAF, DMN, and Trp-P-2] (79) and the stomach irritant ethyl alcohol (79) showed negative results. Additionally, 7 direct-acting mutagens [diacetyl (68), methylglyoxal (69), MX (76), nitrosated OiV (77), NIAN (70), 3-diazo-N-nitrosobismuthan (65), and hickory smoke condensate with or without NaNO2 (66)] induced dose-dependent increases in RDS after oral administration (57). The salts [sodium acetate (81), KCl (81), CaCl2 (81), and glycocholic acid sodium salt (72)] and a forestomach carcinogen aristrochic acid (59) showed positive results. OiV (77) and methylhydroquinone (64) showed equivocal results.

We examined 32 compounds by the RDS method. We obtained 26 positive results, including 6 genotoxic glandular stomach carcinogens, 2 non-genotoxic glandular stomach carcinogens, and 5 glandular stomach tumor promoters; 4 negative results, including 3 liver carcinogens; and 2 equivocal results.

Results of ODC in the rat glandular stomach: ODC activity catalyzes the production of putrescine in the first rate-limiting step of polyamine biosynthesis. The diamine putrescine and the polyamines spermidine and spermine are cationic molecules found in all eukaryotic cells. Polyamine content is tightly regulated
through biosynthesis, catabolism, uptake, and efflux mechanisms to maintain optimal levels that are required for cellular events such as DNA replication, gene transcription, mRNA translation and ion channel function. Excess polyamine accumulation is linked to neoplastic growth (82). We assayed ODC activity as a measure of potential tumor promoting activity.

The time-dependency of ODC activity induction in the pyloric mucosa by taurocholate, NaCl, catechol, K₂S₂O₅, formaldehyde and glyoxal was transient and reached a maximum after 4 h by taurocholate (72), 6–9 h by NaCl (78) and catechol (63), and 16 h by glyoxal (68), formaldehyde (80) and K₂S₂O₅ (81). ODC activity returned to the control level within 48 h after administration of these compounds (57).

Six genotoxic glandular stomach carcinogens [ENNG (79), MMNG (79), MNU (62), NMUT (79), 4NQO (79), and PNNG (79)], 2 non-genotoxic glandular stomach carcinogens [catechol (63) and 4-methylcatechol (64)], 5 glandular stomach tumor promoters [formaldehyde (80), glyoxal (68), K₂S₂O₅ (81), NaCl (78), and taurocholate (72)] all showed dose-dependent induction of ODC activity in rat stomach pyloric mucosa after oral administration (57). However, 3 liver carcinogens [2AAF, DMN, and Trp-P-2] (79) showed negative results (57). Additionally, 7 direct-acting mutagenic compounds [diacetyl (68), 3-diazo-N-nitrosobenzenethiol (65), hickory smoke condensate without NaNO₂ (66), methylglyoxal (68), MX (76), nitrosated OiV (77), and NIAN (70)], hickory smoke condensate with NaNO₂ (66), 9 different salts [CaCl₂, KCl, K₂SO₃, Na₂CO₃, (NH₄)₂SO₄, sodium acetate, sodium L-ascorbate, sodium L-glutamate, and sodium sorbate] (81), 6 bile acids [glycochenodeoxycholate, glycocholic acid free acid, glycocholic acid sodium salt, glycotocholate, taurochenodeoxycholate, and taurodeoxycholate] (72), and omeprazole (71) showed dose-dependent induction of ODC activity in rat stomach pyloric mucosa after oral administration (57). Methylhydroquinone showed an equivocal result (64). MgSO₄ saccharose (81), taurocholate (72), and OiV fraction (77) showed negative results (57).

We observed 37 positive results, including 6 genotoxic glandular stomach carcinogens, 2 non-genotoxic glandular stomach carcinogens, and 5 glandular stomach tumor promoters; 7 negative results, including 3 liver carcinogens; and 1 equivocal result.

**Predictions regarding Glyoxal and NIAN:** In our test, glyoxal induced DSS, RDS, and ODC in rat stomach pyloric mucosa (73). Induction of UDS by glyoxal was equivocal (73). Glyoxal is a mutagenic dicarbonyl compound found in heated foods (83). Later, it was reported that glyoxal promoted the incidence of adenocarcinomas in rat stomach pyloric mucosa pretreated with MNNG and NaCl (84).

Additionally, our analysis demonstrated that NIAN induced DSS, RDS, and ODC in rat stomach pyloric mucosa (70). Induction of UDS by NIAN was equivocal (70). The precursor of NIAN, indole-3-acetonitrile, was present in Chinese cabbage (85). It was recently reported that NIAN induced glandular stomach cancers in *Helicobacter pylori*-infected Mongolian gerbils (86).

**In Vivo Short-term Tests in the Rat liver, Fore-stomach, Colon and in the Hairless Mouse Epidermis as Determined by UDS and RDS Using a Liquid Scintillation Counter**

We studied the UDS and RDS methods in the rat liver (10–12). We quantified DNA synthesis in hepatocytes isolated from liver after chemical administration in the presence and absence of HU, an inhibitor of RDS, simultaneously. We compared autoradiography method and the liquid scintillation counter method and confirmed that liquid scintillation counter method was useful in examining UDS and RDS in the rat liver (12,87). Two genotoxic liver carcinogens, 2AAF and DMN, showed induction of UDS dose-dependently 2 h after administration (10). However, the non-genotoxic hepatocarcinogen CCl₄ showed a negative result in the induction of UDS but a positive result in the induction of RDS (12).

We studied the UDS method in the rat forestomach and colon. Three forestomach carcinogens, MNNG, MNU, and NMUT induced UDS in forestomach stratified squamous epithelium but forestomach carcinogen benzo[a]pyrene showed an equivocal result and non-carcinogens 4-acetylaminofluorene and pyrene exhibited a negative result (58,88). Three colon carcinogens, MNNG, MNU, and methylazoxymethanol acetate, induced UDS in colon mucosa but non-colon carcinogens 4NQO and 2AAF did not (58).

We studied the UDS method in the hairless mouse epidermis (89–92). Five genotoxic skin carcinogens, benzo[a]pyrene, 1,2-dimethylbenzo[a]pyrene, MNNG, and 4NQO showed induction of UDS dose-dependently 2 h after treatment and a negative control, 1,2-epoxydodecane showed negative UDS (89). UDS was induced in hairless mouse epidermis by ultraviolet light (90,91) as well as by 8-methoxypsoralen plus ultraviolet A (92).

**Chromosomal Aberrations, Micronuclei, and Sister-chromatid Exchanges in the Rat Liver**

Chromosomal aberrations, micronuclei, and sister-chromatid exchanges were induced in rat liver with genotoxic hepatocarcinogens 2AAF and DMN but not with a non-genotoxic hepatocarcinogen CCl₄ (93,94). Heterocyclic amines [Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, IQ, nitro-IQ, and MeIQx] induced chromosomal aberrations and sister-chromatid exchanges (94). We
performed the experiments without partial hepatectomy and mitogens treatment. Recently, a similar method was used in micronucleus test in young rat liver (47,48).

**Conclusion**

The prediction of carcinogenicity of environmental chemicals is essential for human health. Five decades of experimental animal carcinogenicity studies, 4 decades of short-term test studies for mutagens and carcinogens, and recent progress in statistical analysis have not yet yielded ideal methods for the prediction of human cancer hazards and risks. We are still in the incomplete stages of such a development. I have attempted to develop various organ-specific in vivo short-term tests, mainly in the liver and stomach (Table 3).

During the past decade, our study group Toxicogenomics/JEMS-MMS attempted to use gene expression profiling in the rodent liver for in vivo short-term tests to identify mutagens and carcinogens to study the mode of action of particular chemicals. We successfully discriminated genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens in the mouse and rat liver by statistical analysis using PCA based on gene expression profiles as determined by qPCR. I expect that toxicogenomics methods would promote the investigation of the mode of action of particular chemicals for carcinogenicity in future. At present Comet assay (95), UDS (87) and micronucleus tests (47,48) are often used for in vivo short-term liver tests and are rather simple procedures; however, they simply detect DNA damage but not reveal the mode of action of particular chemicals.

Previously, our group developed UDS, RDS, DSS, and ODC, mainly in the glandular stomach and addi-

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**Acknowledgements:** For the toxicogenomics study, I would like to thank many collaborators in the Toxicogenomics/JEMS-MMS collaborative study group, especially Dr. Takayoshi Suzuki (National Institute of
Health Sciences), Dr. Madoka Nakajima (Public Interest Incorporated Foundation Biosafety Research Center) and Dr. Shuichi Hamada (Mitsubishi Chemical Medience Corporation). I would like to thank Dr. Hideo Tashiro (RIKEN), Drs. Tomoko Tashiro and Takashi Watanabe (Aoyama Gakuin University) and Dr. Yoshiyuki Sakaki (Institute of Medical Science, University of Tokyo). For the previous organ-specific in vivo short-term tests, I would like to thank many collaborators including Dr. Shigeki Sawada (Eisai Co. Ltd.) and Dr. Masaaki Mori (Shiseido Co., Ltd.) and greatly appreciate Drs. Taijiro Matsushima and Takashi Sugimura (Institute of Medical Science, University of Tokyo). This work was partly supported by the Special Coordination Funds for Promoting Science and Technology (C. Furihata), KAKENHI (18210047) (C. Furihata, T. Watanabe and T. Suzuki) and a Grant-in-aid for the Private School High-tech Research Center Program (C. Furihata), The Ministry of Education, Culture, Sports, Science and Technology, Japan and by Grants-in-Aid for Cancer Research (C. Furihata) from the Ministry of Health and Welfare of Japan and a grant (C. Furihata) from the Japanese Society for Promotion of Cancer Research.

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