Assessment of the genotoxic potential of indirect chemical mutagens in HepaRG cells by the comet and the cytokinesis-block micronucleus assays

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Many chemical carcinogens require metabolic activation to form genotoxic compounds in human. Standard in vitro genotoxicity assays performed with activation systems, such as rat liver S9, are recognised to lead to a high number of false positives. The aim of this study was to evaluate the suitability of differentiated human hepatoma HepaRG cells as an in vitro model system for the detection of DNA damage induced by promutagens using the comet and the cytokinesis-block micronucleus assays. Several promutagens were tested, including aflatoxin B1 (AFB1), benzo[a]pyrene (B[a]P), acrylamide, N-nitrosodimethylamine (NDMA), cyclophosphamide (CPA), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). Cytotoxicity of these compounds was assessed by measuring lactate dehydrogenase leakage. A 24 h exposure was generally needed to obtain an obvious positive response in differentiated HepaRG cells in the comet and in the cytokinesis-block micronucleus assays. Comet formation was observed with all compounds except IQ. B[a]P, CPA and AFB1 showed a dose-dependent increase in micronucleated cells, whereas no increase was observed with PhIP, IQ and acrylamide. These preliminary data on genotoxicity in differentiated HepaRG cells are promising but more chemicals must be tested to determine the ability of HepaRG cells to assess genotoxicity of chemicals in humans.

Introduction

Many mutagenic chemicals require a bioactivation step to become genotoxic. The most commonly used in vitro genotoxicity assays are the Ames test, the mouse lymphoma assay, the in vitro micronucleus and the chromosome aberration tests, which are performed on models with restricted metabolic activity (1,2). This lack of metabolic activity is counteracted by adding Aroclor 1254-induced rat liver S9 fractions. However, it is widely recognised that the specificity (percentage of non-carcinogens found negative) of these in vitro mammalian assays is very poor (<45%) (1). There are several limitations for using rodent exogenous activation systems to identify human hazards. Indeed, species differences between humans and rats have been reported in their liver metabolic capacities. In the rat S9 fractions, the CYP1A and CYP2B subfamilies are over-represented, whereas the catalytic activity of the phase II enzymes relies mostly on the addition of exogenous cofactors (2–4). Another reason for the low specificity of the in vitro mammalian cell assays is that the genotoxicity of chemicals is currently evaluated at high concentrations according to the regulatory guidelines (5). Obviously, there is a general agreement at the moment that the cell systems which maintain a high capacity of phase I and phase II metabolisms and are DNA-repair proficient offer the best hope of reduced false positives (5). When compared with rodent carcinogenicity, the unacceptably high rate of occurrence of false-positive results obtained with mammalian cells routinely employed in in vitro standard genotoxicity tests is expected to be greatly reduced by the use of metabolically competent human liver cells (6). Primary hepatocyte cultures are acknowledged to be the most pertinent model system for xenobiotic metabolism and toxicity studies, but they exhibit early phenotypic alterations with only a few days survival in standard culture conditions. In addition, the availability of human hepatocytes is in short supply and unpredictable, and they are characterised by a wide interindividual functional variability (7). Primary rat hepatocyte cultures also express xenobiotic drug metabolising enzymes for a short period of cultivation (8).

Human hepatoma cell lines are an attractive alternative for predicting human toxicity (7). Although they usually express only low, if any, levels of drug metabolising enzymes and, consequently, are inadequate as a surrogate for primary hepatocytes, some of them, especially HepG2 cells, are often used in genetic toxicity (6,9,10). Subclones of HepG2 cells have been established, which exhibit different levels of phase I enzyme activities (11,12). Another in vitro strategy for studying indirect carcinogens is the use of genetically engineered cell lines overexpressing one or more specific phase I enzymes. However, such cell lines are more appropriate for mechanistic than for screening studies (2,13).

Recently, a new hepatoma cell line, named HepaRG, was isolated from a differentiated liver tumour (14); it exhibits limited karyotypic alterations and has the property of transdifferentiation (15). Differentiated HepaRG cells express several cytochromes P450, phase II enzymes, transporters and nuclear factors, including the constitutively activated receptor, at levels close to those found in primary human hepatocyte cultures (16,17). As a proof of concept, we demonstrated a direct relationship between DNA damage caused by the promutagen aflatoxin B1 (AFB1) in HepaRG cells and their xenobiotic metabolic capacity using the comet assay (18). In the present study, compounds with various metabolic activation pathways were tested: AFB1, benzo[a]-pyrene (B[a]P), acrylamide, N-nitrosodimethylamine (NDMA),
cyclophosphamide (CPA), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). Two direct mutagens, methylmethanesulfonate (MMS) and glycidamide were also tested. The DNA damage was evaluated by the comet and the cytokinesis-block micronucleus assays.

Materials and methods

Chemicals

AFB1, acrylamide, B[a]P, CPA, NDMA, pyrene, dimethylsulfoxide (DMSO), phalloidin-fluorescein isothiocyanate (FITC), cytochalasin-B (cyt-B), propidium iodide, 4′,6-diamidino-2-phenylindole (DAPI) and insulin were purchased from Sigma (St Quentin-Fallavier, France). PhIP and IQ and glycidamide were obtained from Toronto Research Chemicals (Canada). MMS was purchased from Acros Organics (Geel, Belgium). Williams E medium was supplied by Eurobio (Les Ulis, France), foetal calf serum (FCS) from Perto (Brebières, France) and penicillin and streptomycin from Invitrogen Corporation (Illkirch, France). Hydrocortisone hemisuccinate was purchased from Upjohn Pharmacia (Gyancourt, France). All other chemicals were of the highest quality available.

Cell cultures

HepaRG cells were cultured as previously described (14). Briefly, for the present study, they were seeded at a density of 2.6 × 10^5 cells/cm^2 in the growth medium composed of Williams E medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine and 50 μM hydrocortisone hemisuccinate. Within a few hours, HepaRG cells appeared as elongated cells characterised by a clear cytoplasm with active division until they reached confluence. After 2 weeks, they were shifted to the same culture medium supplemented with 2% DMSO for two more weeks to obtain differentiated cultures (15,16). In the present study, HepaRG cells were used between passages 10 and 16.

Preparation of compound solutions

Acrylamide, NDMA, glycidamide, MMS and CPA were dissolved directly in FCS-free medium. AFB1, pyrene, PhIP, IQ and B[a]P were dissolved in sterile DMSO. For all assays, the final DMSO concentration never exceeded 1%.

Lactate dehydrogenase assay

Differrentiated HepaRG cells were exposed to chemicals for 3 or 24 h. Two 24-well plates were treated concomitantly: one for the lactate dehydrogenase (LDH) assay and the other for the comet assay (see below). The rate of LDH release was measured as an index of cytotoxicity. After treatment, LDH activity was determined in both extracellular (supernatant of cell culture) and release was measured as an index of cytotoxicity. After treatment, LDH activity was determined in both extracellular (supernatant of cell culture) and intracellular (after cell sonication by ultrasounds) media by the CytoTox96® non-radioactive cytotoxicity assay from Promega (Montluçon, France). LDH release was expressed as a percentage of total cellular LDH activity: [extracellular LDH/(intracellular LDH + extracellular LDH)] × 100. Experiments were performed in triplicate per experimental point.

Comet assay

The results of the comet assay were taken into account only when cell viability determined by the LDH assay was >70%, according to international recommendations (19). The comet assay was performed as described previously by Josse et al. (18). DNA was stained with propidium iodide (2.5 μg/ml in phosphate-buffered saline (PBS)) just before blind slide scoring with a fluorescence microscope (Leica DMR) equipped with a CCD-200E video camera. At least 100 cells per dose and treatment were scored using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK). The extent of DNA damage in individual cells was evaluated by the percentage of tail DNA. Three independent experiments were conducted.

Cytokinesis-block micronucleus assay

HepaRG cells were seeded at a density of 2.5 × 10^4 cells/cm^2 in 12-well plates. Once differentiation was reached, cells were exposed to chemicals in an FCS-free medium for 24 h. At the end of treatment, cells were washed twice with PBS, trypsinised and seeded at a density of 2.6 × 10^5 cells/cm^2 in a Lab-Tek II chamber slide system®. Twenty-four hours after seeding, the medium was replaced with a fresh one containing 10% FCS and 4.5 μg/ml of Cyt-B. Due to the doubling time of HepaRG cells (~30 h), the cells were incubated for 45 h with Cyt-B. At the end of Cyt-B treatment, the cells were washed twice with PBS and allowed to recover for 1.5 h in a 10% FCS-supplemented-fresh medium prior to fixation in 3% paraformaldehyde for 10 min. Slides were incubated with 0.5% Triton X-100 for 5 min and rinsed again twice with PBS. Staining of cells with DAPI (0.5 μg/ml) for 10 min and with phalloidin-FITC (1.2 μg/ml) for 30 min was followed by two washes with PBS. Slides were observed under a Leica fluorescence microscope.

All slides were scored blindly. The criteria used for identifying micronuclei were those recommended by the HUMN project (20). Micronuclei were scored in 200 binucleated cells per assay. Apoptotic, mitotic, mononucleated and polynucleated cells were also counted. Three independent experiments were conducted. For cytotoxicity assessment, the cytokinesis-block proliferation index (CBPI) was calculated: [CBPI = (number of mononucleated cells × 2 binucleated cells + 3 × polynucleated cells)/total number of cells)] (21).

Results

Cytotoxicity

Cell viability determined by the LDH assay remained >70% after 24 h of treatment for all compounds except IQ. IQ decreased cell viability up to 25% at 1000 μM.

Comet assay

DNA damage in the controls was low in all experiments. The positive control MMS (5 μg/ml) and the reactive acrylamide metabolite glycidamide (25 μM) induced important DNA fragmentation (Figure 1).

After 3 h exposure, the promutagens B[a]P, CPA, PhIP and NDMA induced only a slight increase of DNA damage in HepaRG cells (data not shown) while, after 24 h, a statistically significant response was observed with most of these compounds (Figure 1). B[a]P, CPA and PhIP were the most genotoxic compounds to HepaRG cells in the comet assay; they induced statistically significant increases of DNA fragmentation at 50, 200 and 40 μM, respectively. AFB1 increased DNA migration at 2.5 and 5 μM at both treatment times but a statistically significant difference was obtained only at the shorter time.

NDMA slightly increased the percentage of tail DNA at concentrations up to 500 μM, without a dose-dependent effect. IQ failed to induce any DNA damage, even at a cytotoxic concentration (1000 μM). Acrylamide increased DNA migration only at the highest doses, i.e. 5 and 10 mM. As expected, the negative control, pyrene, did not affect DNA migration.

Cytokinesis-block micronucleus assay

Except for NDMA and the negative control pyrene, all the chemicals were also evaluated in the micronucleus (MN) assay. In solvent controls, the percentage of binucleated cells was always >65% and the level of binucleated micronucleated (BNMN) cells varied between 2 and 4% (Figure 2). The positive control MMS (30 μg/ml) induced between 10 and 19% of BNMN cells.

After 24 h of treatment, AFB1, CPA, B[a]P and glycidamide statistically increased the number of BNMN cells at non-cytotoxic concentrations, considering the values of CBPI. CPA was the most active clastogenic compound, followed by AFB1, B[a]P and glycidamide. No induction of MN was observed with PhIP and IQ. Cytotoxicity but no genotoxicity was observed with acrylamide treatment.

Discussion

The aim of this study was to explore the capacity of the differentiated HepaRG cells to detect carcinogens requiring...
metabolic activation to form genotoxic compounds in humans, by using the comet and the MN assays. Our data provide evidence that these metabolically competent cells can activate different classes of promutagens to reactive metabolites. Positive results were obtained with CPA, B[a]P, PhIP and AFB1, as well as with the direct mutagens glycidamide and MMS, in at least one test.

Since the MN assay requires a cell division step for the occurrence of micronuclei, HepaRG cells are suitable to carry out this assay. However, some limitations should be considered with this cell line. Firstly, due to the methodology, it cannot be excluded that some hepatocytes, altered following chemical exposure, were unable to attach or divide after trypsinisation. Secondly, due to the presence of both hepatocyte-like and biliary-like cells in cell cultures, the proportion of micro-nucleated cells might be underestimated, particularly if the reactive metabolite is only produced in one of the two cell types.

In this study, various promutagens were tested and shown to cause DNA damage in HepaRG cells. In agreement with our preliminary observation (18), we demonstrated the induction of DNA damage by AFB1 with both the comet and the MN tests at 2.5 and 0.25 µM, respectively. This mycotoxin is converted to a very reactive 8,9-exo-epoxide metabolite by CYP3A4 and to a lesser extent by CYP1A2. These results are in accordance with the CYP3A4 and 1A2 expressions and activities found in HepaRG cells (16,22). Positive responses were also obtained with AFB1 in the HepG2 cell line (23–25).

B[a]P requires metabolic activation by CYP1A1 and CYP1B1 and the formation of the ultimate reactive species, a benzo[a]pyrene-7,8-dihydriodiol-9,10-epoxide, to react with DNA (26,27). We showed that B[a]P induced comet and MN formation in HepaRG cells in a dose-dependent manner, in agreement with the expression of CYP1A1 and 1B1 in these cells (data not shown).

After activation through 4-hydroxylation by several CYPs, including CYP2B1, 2B6, 2C9 and 3A4 (28,29), the cytotoxic bifunctional alkylating agent CPA can potentially produce DNA–DNA or DNA–protein cross links as well as single strand breaks by alkylating DNA at the N7 position of the guanine (30,31). As expected, CPA was effective in HepaRG cells, especially with the MN assay. It was the strongest clastogenic compound tested. Significant DNA damage was also detected by the comet assay after a 24 h exposure to CPA at a concentration as low as 100 µM.

The two heterocyclic aromatic amines PhIP and IQ are potent mutagenic and carcinogenic compounds (32,33); they are mainly oxidised via cytochrome P4501A2 to a hydroxylated intermediate that can be converted to a more reactive form via phase II metabolising enzymes, primarily N-acetyltransferases (34,35). Species differences have been reported. PhIP is preferentially converted to a reactive compound in humans, whereas detoxified products are mainly formed in rodents (35,36). In the present study, PhIP was the most potent genotoxic compound in the comet assay (Lowest Observed Effect Concentration, LOEC = 40 µM), whereas it failed to increase the number of micronucleated cells after 24 h of treatment. Conversely, after 24 h of exposure, IQ did not cause any DNA damage in both tests. By contrast, in the engineered human MCL-5 cell line which expresses CYP1A1 and
CYP1A2, DNA strand breaks were observed in the comet assay after both PhIP (LOEC = 200 μM) and IQ (LOEC = 480 μM) treatment (36). However, such effects were observed only in the presence of DNA repair inhibitors. Micronucleus formation was also demonstrated in binucleated MCL-5 cells and in a HepG2 subclone following treatment with 50 nM and 100 μM PhIP or IQ (37). The discrepancies of these results could be explained by different levels of the various enzymes involved in both activation and detoxication of these two amines.

Acrylamide is an important monomer formed in human food prepared by cooking at high temperatures (38). It is biotransformed by CYP2E1 into a reactive genotoxic compound, glycidamide (39). DNA fragmentation was observed in HepaRG cells, only with high acrylamide concentrations, in the comet assay. Similar observations have been made with HepG2 cells and the effects were interpreted as a consequence of oxidative DNA damage induced by intracellular reactive oxygen species and glutathione depletion (40,41). Since the activity of CYP2E1 is low and inconsistent in HepaRG cells, probably depending on

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**Fig. 2.** CBPI and frequency of BNMN cells in treated HepaRG cells. Graphs show the values of the frequency of BNMN cells and the cytotoxicity index CBPI of HepaRG cells exposed to AFB1, CPA, B[a]P, glycidamide, PhIP, IQ and acrylamide for 24 h. Data represent means ± SDs of three independent experiments. *P < 0.05 versus corresponding control (Pearson’s chi-square test).
cell culture conditions (16,42,43), it cannot be excluded that exposure to high acrylamide concentrations resulted in the formation of a small amount of glycidamide. As expected, the alkylating agent glycidamide induced DNA damages in both assays in HepaRG cells. This is in accordance with the genotoxicity of glycidamide reported in various cell lines (39).

The nitrosamine NDMA also requires metabolic activation, especially by CYP2E1, to exert its mutagenic and carcinogenic effects (44,45). In agreement with the results obtained with acrylamide and the inconsistency of CYP2E1 activity, no DNA damage was observed in HepaRG cells exposed to NDMA up to 5000 μM in the comet assay. Surprisingly, significant DNA fragmentation has been reported in HepG2 cells treated with 10 mM NDMA. However, it is unlikely that this genotoxic effect resulted from conversion of the nitrosamine by CYP2E1 into reactive metabolites (10,24).

These preliminary data on the use of differentiated HepaRG cells for genotoxic assessment of chemicals are encouraging. However, further experiments are obviously required with a larger number of genotoxic and non-genotoxic compounds, as recommended by Kirkland et al. (46), to determine the sensitivity and the specificity of this cell line for *vitro* genotoxicity testing.

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**References**


