A Mechanistic Basis for the Role of Cycle Arrest in the Genetic Toxicology of the Dietary Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

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Received July 29, 2004; accepted November 18, 2004

The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), formed during the cooking of meat, induces tumors of the prostate, colon, and mammary gland when fed to rats. PhIP is readily absorbed and efficiently metabolized to a genotoxic derivative by CYP1 enzymes. Although metabolism and mutational potential of PhIP have previously been well characterized, the intervening cellular and genomic responses to the chemical are not fully understood. We have examined the cellular response to PhIP exposure in human mammary epithelial MCF10A cells, which retain characteristics of normal breast epithelial cells. Because these cells fail to activate PhIP, they were cocultured with a human lymphoblastoid cell line MCL-5, which constitutively express CYP1A1, and have been transfected to express human CYPs1A2, 2A6, 3A4, and 2E1. The MCL-5 cells were irradiated (2,000 rads) prior to coculture, rendering them unable to replicate yet still retaining metabolic competency. MCF10A cells were treated (in the presence of MCL-5 cells) with PhIP (1–100 μM) and harvested at various time-points. Compared to DMSO control, treatment (24 or 48 h) with PhIP resulted in a significant dose-dependent fall in cell number. Cells treated for 48 h then cultured in the absence of PhIP (and MCL-5 cells) for a further 6 days showed a much greater dose-dependent reduction in cell number. Flow cytometric analysis indicated that PhIP treatment (48 h) resulted in a dose-dependent accumulation of cells in the G1 population. Western blotting revealed elevated expression of p53 and the cyclin dependent kinase inhibitor p21WAF1/CIP1 after PhIP treatment. Levels of MDM2, a negative regulator of p53, and the hypophosphorylated form of RB were observed after PhIP induction. All of these cell cycle effects are critical, as they enable cells to effect genome repair, accept mutation, or eliminate excessively damaged cells.

Key Words: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PhIP; cell cycle; p53; p21; MDM2; RB; cytotoxicity; breast epithelial cells.

Diet has been estimated to be associated with a large number of human cancers (Doll, 1992), a major factor being the consumption of red meat (Willett, 1990). A number of genotoxic heterocyclic amines (HCAs), formed during the cooking of muscle meats, have been identified. These compounds are highly mutagenic in bacterial tests (Felton and Knize, 1991; Sugimura et al., 1977) and carcinogenic in laboratory animals (Ohgaki et al., 1991; Sugimura et al., 1996). Epidemiological studies have suggested the possibility that HCAs play a part in the etiology of human breast cancer (Zheng et al., 1998). The most abundant HCA formed is PhIP, and it has been shown to induce lymphomas in mice (Esumi et al., 1989), colon and prostate tumors in male rats, and colon and mammary carcinomas in female rats (Ito et al., 1989; Shirai et al., 1997).

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is readily bioavailable following a cooked meat meal (Lynch et al., 1992) and requires metabolic activation via cytochrome P450-mediated N-hydroxylation to N-hydroxy-PhIP (Crofts et al., 1998; Zhao et al., 1994). This is followed by esterification of the N-hydroxy-PhIP, resulting in derivatives that are able to form DNA adducts, primarily at the C-8 position of guanine (Schut and Snyderwine, 1999). Formation of these adducts is believed to be premutagenic and, furthermore, thought to be critical in PhIP-induced carcinogenesis of the mammary gland (Ghoshal et al., 1995). Much work has been carried out on the genetic toxicology of PhIP, and it has been demonstrated that, once metabolized to its N-hydroxy active derivative, it is able to induce mutations in a number of gene loci both in vitro and in vivo. Dose-dependent increases in mutations at the hypoxanthine-guanine phosphoribosyl transferase (hprt) locus, and a mutagenic “fingerprint” were identified in both hamster and human cells (Morganthaler and Holzhauser, 1995; Yadollahi-Farsani et al., 1996) and in transgenic mice (Lynch et al., 1998; Okonogi et al., 1997).

The most frequent mutations induced by PhIP involved single base substitutions, mainly GC→TA transversions, although a high percentage of −1G frameshifts were also observed.

The ability to maintain genomic integrity is critical for normal cell growth and survival. A number of cell cycle
checkpoints exist that operate to prevent cells with DNA damage from proliferating. These act as a protective mechanism, allowing time for repair of damaged DNA or elimination of the cell via apoptosis if the damage is too severe. Arrest in the G1 phase of the cell cycle is mediated by the tumor suppressor gene p53 (Kastan et al., 1991), which is stabilized in response to DNA damage, and leads to increased levels of p21WAF1/CIP1 (el-Deiry et al., 1993; Xiong et al., 1993), an inhibitor of certain cyclin dependent kinases (Harper et al., 1993), which are required for cells to enter the next, DNA synthesis phase of the cell cycle (S-phase) (Levine, 1997). Defects in these checkpoints can result in mutations, chromosomal damage, or aneuploidy, thus contributing to tumorigenesis (Paulovich et al., 1997).

While the metabolic activation of PhIP and its ability to cause mutation are well characterized, little is known about its acute effects on genomic and cellular events following exposure. In order to further understand the early cellular responses to PhIP-induced DNA damage in an appropriate target cell type, we have examined the effects of PhIP upon cell cycle control and expression of key proteins in a human mammary epithelial cell line.

**MATERIALS AND METHODS**

**Chemicals.** All chemicals, unless otherwise stated, were from Sigma-Aldrich Chemical Co. (Poole, UK). Hams F12/DMEM (1:1) growth medium, horse serum, L-glutamine, penicillin/streptomycin, epidermal growth factor (EGF), and insulin were purchased from Invitrogen Corporation (Paisley, UK). Cholera Toxin was purchased from Merck Biosciences Ltd (Nottingham, UK). RPMI 1640 medium and Horse Serum for MCL-5 cell culture were from Gentest Corporation (Woburn, MA). PhIP was purchased from Toronto Research Chemicals Inc (Toronto, Canada). A Becton Dickinson FACScan flow cytometer and CellQuest software were used for analysis of cell cycle status. SDS–PAGE molecular weight standards were obtained from Bio-Rad (UK). Hybond nitrocellulose membrane and Herfilm autoradiography film were from Amersham Biosciences (Bucks, UK). BCA protein assay kit and SuperSignal West Pico chemiluminescent substrates were purchased from Perbio Science UK Ltd (Cheshire, UK). Mouse monoclonal anti-β-actin antibody, rabbit polyclonal anti-p53 (FL-393), mouse monoclonal anti-MDM2 (SMP14), and goat polyclonal anti-RB (C-15) antibodies were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Mouse monoclonal anti-p21WAF1/CIP1 (CP74) antibody was purchased from LabVision (Fremont, CA). Polyclonal goat anti-rabbit-horseradish peroxidase (HRP), goat anti-mouse-HRP and donkey anti-goat-HRP conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Western blot stripping solution was from Chemicon International (Temecula, CA). Nuclear extraction and GTBP DNA repair enzyme-linked immunosorbent assay (ELISA) kits were obtained from Active Motif (Rixensart, Belgium). Antibodies for CYP1A1 and CYP1A2 were a generous gift from Dr. Rob Edwards, Division of Medicine, Imperial College London.

**Cell lines.** MCF10A cells were purchased from the American Type Culture Collection (Manassas, VA). MCF10A cells are a spontaneously immortalized, adherent human mammary epithelial cell line with the phenotypic characteristics of normal breast cells. Cells were maintained in HamsF12/DMEM (1:1) medium supplemented with 5% horse serum, 2 mM L-glutamine, penicillin/streptomycin (100 IU and 100 μg per ml), 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone.

The human lymphoblastoid suspension cell line MCL-5 was obtained under license from Gentest Corporation (Woburn, MA). These cells constitutively express high levels of CYP1A1 and express transfected human CYPs 1A2, 2A6, 3A4, and 2E1, plus microsomal epoxide hydrolase. MCL-5 cells were cultured in RPMI 1640 medium without histidine but containing 2 mM histidine, supplemented with 9% horse serum, 2 mM L-glutamine and penicillin/streptomycin (100 IU and 100 μg per ml). At passage, hygromycin B (200 μg/ml in 0.035 M acetic acid) was added to ensure retention of the plasmids containing the P450 enzymes.

**Treatment.** MCF10A cells (3 x 10⁶) were seeded into T75 flasks and allowed to adhere overnight. The growth medium was then renewed and replaced with fresh MCF10A medium containing 6 x 10⁶ MCL-5 cells that had been irradiated (2000 rads), plus appropriate concentrations of PhIP (1–100 μM) or DMSO solvent control. Preliminary experiments showed that irradiation prevented the MCL-5 cells from replicating, yet did not kill the cells over a 48-h period. Cells were treated with PhIP for either 24 or 48 h.

**Cell viability and survival.** MCF10A cells were exposed to varying concentrations of PhIP under the coculture conditions described above for either 24 h or 48 h. Following treatment, medium, chemicals, and MCL-5 cells were removed by aspiration. The adherent MCF10A cells were then detached from the flask by trypsinization and collected by centrifugation at 1000 rpm for 5 min. For assessment of cell numbers, cells were counted on a hemocytometer with trypan blue exclusion.

**Flow cytometric analyses.** MCF10A cells (10⁶) were seeded into T25 flasks, allowed to adhere overnight, and treated under the coculture conditions described above. Following treatment (24 h or 48 h), chemicals and MCL-5 cells were removed by aspiration, and the adherent MCF10A cells were cultured for a further 18 h in fresh medium containing nocodazole (0.4 μg/ml). Nocodazole is an antimitotic agent that acts by depolymerization of microtubules to inhibit spindle formation. This results in an increase in the G2/M cell population as cells progress through the cycle yet cannot pass through mitosis. If treatment induces an arrest prior to G2/M, then the cells distribute to two cell populations, one at the point of cycle arrest and one at the G2/M phase, emphasizing the point of chemical-mediated arrest (Khan et al., 1997). The MCF10A cells were harvested, fixed in ice-cold 7% ethanol at −20°C for at least 24 h, washed with phosphate-buffered saline (PBS), treated with RNase A (1 mg/ml) at 37°C for 15 min, then stained with propidium iodide (10 μg/ml) for a further 20 min. Cell cycle distribution was determined by DNA content (propidium iodide staining), using flow cytometry, measuring 10,000 cells per analysis (Zhu et al., 2000).

**Western blotting.** Cells were treated in T75 flasks as described above and then harvested for immunoblot analysis of p53, p21WAF1/CIP1, MDM2, RB, CYP1A1, CYP1A2, caspase 3, and β-actin protein expression. Briefly, cells were washed twice with ice-cold PBS and then lysed on ice in buffer consisting of 50 mM Tris–HCl pH8.5, 150 mM NaCl, 1% Nonidet-P-40, 5 mM EDTA, supplemented with 10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 50 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were centrifuged at 14,000 x g at 4°C for 10 min, supernatants were collected, and protein content was determined by the Bradford method (Bradford, 1976). Proteins were separated by SDS–polyacrylamide gel electrophoresis (7 or 12.5% gels) and electroblotted onto a nitrocellulose membrane. Membranes were blocked for 30 min at room temperature in blocking buffer (PBS containing 0.01% Tween and 5% nonfat dried milk) and then incubated with primary antibody in fresh blocking buffer overnight at 4°C. Membranes were then washed three times in PBS-T (PBS containing 0.01% Tween) and then incubated in blocking buffer containing secondary antibody for 1 h at room temperature. Membranes were washed as above, and proteins were visualized using the enhanced chemiluminescence detection system (Perbio Science, UK). Equal protein loading was confirmed using an antibody to β-actin.

**GT binding protein (GTBP) ELISA.** GTBP (G/T binding protein), also known as hMSH6, content of nuclear extracts was analyzed using an enzyme
linked immunosorbent assay (ELISA), following the manufacturers protocol (Active Motif). GTBP is part of the mismatch repair (MMR) system that is involved in the recognition and repair of mismatched bases and insertion or deletion loops in double-stranded DNA (Marti et al., 2002). Along with hMSH2, GTBP forms the hMutSα heterodimer, which binds to G/T-mismatched base pairs (Palombo et al., 1995) and has also been shown to bind to DNA containing C8-guanine adducts of aminofluorene (AF) and N-acetyl-2-aminofluorene (AAF) (Li et al., 1996).

Cells were treated as described before. The direct-acting mutagen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 10⁻³ M), without coculture, was used as a positive control.

Following treatment, nuclear extracts were prepared using a nuclear extraction kit (Active Motif), according to the manufacturer’s instructions. Briefly, cells were washed with ice-cold PBS containing phosphatase inhibitors, then scraped and collected by centrifugation at 500 rpm for 5 min. Cells were resuspended in hypotonic buffer for 15 min, vortexed for 10 s, and then centrifuged at 14,000 g (10 min). The nuclear pellets were lysed in lysis buffer 30 min. The suspension was centrifuged for 10 min at 14,000 × g; the supernatant (nuclear fraction) was then collected and stored at −80°C until required.

Each well of the ELISA plate contains an immobilized linear oligonucleotide with a G/T mismatch. Binding buffer followed by 10 μg of nuclear extracts (containing GTBP) was loaded. Lysis buffer alone was added to the negative control wells. Plates were incubated for 1 h at room temperature. After wash, 100 μl of GTBP antibody (1:1000 dilution) was added, and the plate was incubated for 1 h at room temperature. Wells were again washed, and 100 μl of secondary antibody (1:1000 dilution) was added to each well, and incubated for 1 h. Washing was repeated, and 100 μl of TMB substrate (3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide solution in 20% v/v dimethylformamide) was added. The plate was incubated for 30 min at room temperature protected from direct light, after which 100 μl of stop solution (1 M sulfuric acid) was added to the wells. Absorbance of the wells was measured at 450 nm on a microplate reader (TiterTek) in order to assess the GTBP concentration of each sample.

RESULTS

Coculture of MCL-5 and MCF10A Cells

PhIP requires metabolic activation, via CYP1A2, to its N-hydroxy derivative in order to exert its genotoxic effects. Because MCF10A cells have minimal expression of CYP1A2 (see Fig. 1), we set up a coculture system, in which the adherent breast epithelial cells were cocultured with the suspension lymphoblastoid cell line, MCL-5. MCL-5 cells constitutively express high levels of CYP1A1 and have also been transfected with a number of other human CYP450 enzymes, including CYP1A2 (Fig. 1), and as such, are well able to metabolize PhIP (Pfau et al., 1999; Zhu et al., 2000). PhIP has been shown to be metabolically activated to genotoxic species and to induce micronuclei, cytotoxicity, and comet tail formation in these cells (Pfau et al., 1999). Irradiation of the MCL-5 cells leaves them effectively sterile and unable to replicate, but they retain their metabolic competency. The MCL-5 suspension cells are easily separated by aspiration from the adherent target MCF10A cells following treatment.

PhIP Inhibition of MCF10A Cell Proliferation and Colony Forming Ability

Trypan blue exclusion was used to count viable MCF10A cells following PhIP treatment. As demonstrated in Figure 2, the addition of PhIP under coculture conditions resulted in a reduction of cell number, compared to the DMSO control, that was dose dependent. Even at the lowest dose (5 μM), a 15% inhibition was observed following 48-h treatment. At the highest dose (100 μM), cell number was reduced (in comparison to the DMSO control) by almost 50%. No significant increase in nonviable cells (stained with trypan blue) was observed, implying that the inhibition of cell number was not a consequence of cell death.

In order to assess the longer-term survival of the cells following treatment, cell numbers were again determined 6 days after the 48-h treatment period. As seen in Figure 3, PhIP treatment resulted in impaired long-term survival, which was more pronounced than the short term reduction in cell number induced by PhIP at the three highest doses, implying that the compound caused long-term damage to the cells. At the highest dose, survival was reduced to 23.8 ± 5.3% of the control population (DMSO treated). No significant numbers of

![FIG. 1. Immunoblot showing the expression of CYPs 1A1 and 1A2 in MCF10A and MCL-5 cells. Levels of P450 enzymes were evaluated by Western blotting. Lanes 1, 2, and 3 are lysates for three separate MCF10A cell cultures. Lane 4: MCL-5 cell lysate. β-Actin operates as a loading control.](https://academic.oup.com/toxsci/article-abstract/84/2/335/1692266/243235/1692266)
detached dead cells were observed in any of the flasks, and cell numbers were well above the initial seeding density for all treatments, suggesting that apoptosis or necrosis was not primarily responsible for the decreased colony forming ability in the PhIP-treated cells. In the absence of cocultured MCL5 cells, treatment of MCF10A cells with PhIP (48 h) failed to affect cell number.

Induction of Cell Cycle Arrest by PhIP

In order to determine whether PhIP had any effect on progression through the cell cycle, MCF10A cells were treated under coculture conditions for 24 h or 48 h; then isolated MCF10A cells were cultured for a further 18 h in the presence of nocodazole (to prevent mitosis). As shown in Figure 4, exposure of PhIP for 24 h resulted in an increase in G1 population of cells that was dose dependent. There was little change in the cell cycle distribution of the cells given the lowest dose. Following 48 h treatment, there was again a dose-dependent increase in G1 population, alongside a concomitant fall in cells reaching G2/M phase. Again, the lowest dose had little effect on distribution, but the G1 arrest observed in the three highest dose treatment groups (25, 50, and 100 μM), was more marked than at the 24-h time point (2.9, 8.1, and 13.4% increases at 24 h, compared with 5.9, 12.7, and 17% increases at 48 h). The treatment had little effect on the sub-G1 cell population, implying that cell death was not a prominent feature at these time points. In the presence of PhIP and the absence of cocultured MCL5 cells, no changes in cycle were apparent. This implies that the G1 cell cycle arrest induced by PhIP was dependent on dose, length of exposure, and metabolic activation by the MCL5 cells.

PhIP Induces the Expression of DNA Damage Response Proteins

Enhanced expression of the transcription factor p53 and the downstream cyclin dependent kinase (CDK) inhibitor p21\(^{WAF1/CIP1}\) are often observed following DNA damage and are known to induce G1 cell cycle arrest (Di Leonardo et al., 1994; el-Deiry et al., 1994). We found that treatment of MCF10A cells with PhIP under coculture conditions for both 24 h and 48 h (Fig. 5) produced a strong induction of both p53 and p21\(^{WAF1/CIP1}\). The induction of both proteins was dose and time dependent. In the absence of MCL5 cells, neither p53 nor p21\(^{WAF1/CIP1}\) levels were affected by PhIP dose. MDM2, a protein that is trans-activated by p53 and promotes degradation of p53 as part of a regulatory negative feedback loop (Barak et al., 1993), was also upregulated in a dose- and time-dependent manner (Fig. 6).
The phosphorylation status of pRB is important in the control of the cell cycle. When pRB is hypophosphorylated it is able to bind to and inhibit the transcription factor E2F, thus inhibiting progression through the G1 phase of the cell cycle. This hypophosphorylation is mediated by p21WAF1/CIP1 (Lloyd et al., 1997). Cells treated with PhIP under coculture conditions showed an accumulation of the hypophosphorylated form of pRB, which was only slight following 24-h exposure, but more pronounced in cells treated for 48 h (Fig. 6). Again, this increase appeared to be dose dependent.

Caspase 3 Expression

Levels of caspase 3, an important effector of apoptosis (Porter and Janicke, 1999) were examined following PhIP treatment in the presence of MCL5 cells. As shown in Figure 7, no changes were observed in the levels of the inactive pro-form of caspase 3, nor was the active degradation product detected.

GTBP ELISA

The effect of PhIP on the activation of GTBP, a protein involved in the recognition and repair of damaged DNA, was examined by ELISA. Both PhIP (10⁻⁴ M) under coculture conditions and the positive control MNNG (10⁻⁵ M) marginally increased the activation of GTBP (130 ± 34 % and 130 ± 8 %, respectively, of DMSO control, n = 3), but neither increase was significant. No alteration of GTBP was detected when lower doses of PhIP (10⁻⁶ and 10⁻⁸ M) were used, nor when 10⁻⁴ M PhIP was administered in the absence of MCL-5 cells.

DISCUSSION

The data presented here examines the early molecular and cellular responses of a human epithelial breast cell line,
MCF10A, to PhIP. These cells were chosen because they are derived from a target tissue of PhIP; they are not transformed and have the phenotypic characteristics of normal breast cells (Soule et al., 1990). As MCF10A cells express very low levels of the enzymes required to metabolically activate PhIP, a coculture system was developed, whereby irradiated metabolically competent MCL-5 suspension cells, previously shown to activate PhIP to genotoxic derivatives that induce micronuclei and DNA strand breaks (Pfan et al., 1999), were cultured alongside the target adherent MCF10A cells.

Following exposure to PhIP for 24 h or 48 h under coculture conditions, cell growth and viability were significantly impaired in a dose-dependent manner, consistent with previous work, which has shown that N-hydroxy PhIP, but not the parent compound, inhibited proliferation in human mammary and prostate epithelial cells (Fan et al., 1995; Nelson et al., 2001). The impairment observed by Fan et al. (1995) and Nelson et al. (2001) was accompanied by an increase in DNA adduct levels, implying that the cytotoxicity observed was a consequence of the genotoxic effects of PhIP.

While MCF10A cell growth was inhibited by PhIP, there was little evidence of a sizeable increase in dead cells, as measured by trypan blue exclusion. In order to investigate whether the growth impairment observed was due to an arrest in cell growth rather than induction of cell death, the effects of PhIP on cell cycle distribution and proteins involved in the control of the cell cycle were examined.

The most widely studied checkpoint is at G1, although cell cycle arrest can also occur at S phase and G2/M transition. Following exposure to genotoxic agents, levels of the tumor suppressor protein p53 are frequently elevated, and this upregulates transcription of the CDK inhibitor p21WAF1/CIP1 (el-Deiry et al., 1994). p21WAF1/CIP1 binds to and inhibits D-type cyclins, thus preventing the activation of CDK4 and CDK6, resulting in pRB hypophosphorylation. This favors pRB to remain bound to E2F, which prevents E2F from inducing cells to transition to S phase thus precipitating cycle arrest in early G1 phase. The present study showed by flow cytometry that PhIP induced a dose-dependent G1 arrest in MCF10A cells following treatment under coculture conditions, and absence of a sizeable sub-G1 cell population indicated that cell death was not prominent. Consistent with this functional outcome, levels of p53, p21WAF1/CIP1, and the hypophosphorylated form of pRB were also increased in cells treated with PhIP under coculture conditions, thus providing a potential mechanism for the observed accumulation of cells in G1.

Six days after withdrawal of PhIP, the colony-forming ability of MCF10A cells under coculture conditions treated with PhIP was significantly reduced compared to cells treated with vehicle alone, implying that the cells had experienced a prolonged arrest at G1 phase, and/or the genetic damage inflicted by PhIP had not been efficiently repaired. While cell checkpoints are an essential protective mechanism for the prevention of damaged DNA becoming incorporated into the genome, they are not infallible. Prolonged G1 arrest pending repair but without apoptosis can lead to some “leakage” of cells into S phase, giving rise to mutation (reviewed by Roberts, 1998). There is also a danger of adaptation, in which the genetic damage remains unrepaired, and after a period of arrest the cell may resume progress through the cell cycle leading to accumulated mutational events (Sandell and Zakian, 1993).

We propose that the G1 arrest observed here was triggered by the genotoxic effects of PhIP. We showed that induction of p53 and p21WAF1/CIP1 by PhIP was dose dependent and draws parallels with a study by Binkova et al. (2000), which showed a significant correlation between dose-dependent increases in DNA adduct levels and p53 and p21WAF1/CIP1 levels in human embryonic lung diploid fibroblasts (HEL) treated with the carcinogenic polycyclic aromatic hydrocarbons benzo[a]pyrene (B[a]P) and dibenzo[a,1]pyrene (DB[a]P). When MCF10A cells were treated with PhIP in the absence of coculture (no metabolism of PhIP), no induction of p53 or p21WAF1/CIP1 was observed, consistent with the work of Fan et al. (1995), who reported induction of DNA damage by N-hydroxy PhIP but not PhIP itself.

PhIP induction of p53 and p21WAF1/CIP1 was also time dependent. Induction of p53 was greatest at 24 h, whereas p21WAF1/CIP1 was maximally elevated following 48-h exposure. The temporal effects on p53 are consistent with the findings of Binkova et al. (2000) and also those of Yang and Duerksen-Hughes (1998), who assessed the levels of p53 following treatment of mouse fibroblasts with a range of carcinogens and demonstrated an elevation of p53 levels peaking between 16 to 24 h after treatment.

The finding that MDM2 levels were also dose-dependently upregulated further demonstrates that the p53 induced by PhIP is transcriptionally active. In cells that have not undergone genotoxic stress, the p53 protein has a half-life of ~20 min (Maltzman and Czyzyk, 1984) and is maintained at a low level by ubiquitin-dependent degradation rapidly after synthesis (Chaudhary et al., 2000). MDM2 is trans-activated by p53 as part of a negative regulatory feedback loop and targets p53 for ubiquitin-dependent degradation by binding to its transactivation domain and acting as an E3 ubiquitin ligase (Haupt et al., 1997). MDM2 is also thought to regulate the nuclear export of p53 (Roth et al., 1998). Following DNA damage, binding of MDM2 to p53 can be disrupted by a number of mechanisms, such as phosphorylation of the amino terminus of p53 (Hecker et al., 1996; Hupp et al., 1992), and protein–protein interactions, such as binding of p16ARF to MDM2 (Pomerantz et al., 1998). This disruption of p53–MDM2 binding can lead to the stabilization of p53, and may explain the elevated levels of p53 after PhIP treatment, despite increased levels of MDM2.

In contrast to previous work with TK6 cells (Zhu et al., 2000), MCF10A cells arrested in G1 phase rather than S phase following exposure to PhIP, and no significant apoptosis was observed, as evidenced by little change in the sub G1 population of treated cells (Zhu et al., 2000) and the absence...
of degradation of pro-caspase 3 to its active form. The findings presented here therefore raise the possibility that the early response to PhIP may be dependent on the cell or tissue type. Previous studies have shown that benzo[a]pyrene induces S-phase arrest in human diploid lung fibroblasts (Binkova et al., 2000), and a G1 checkpoint is activated in 3T3 fibroblasts treated with the same chemical (Vaziri and Faller, 1997), demonstrating the importance of the cellular background on the genotoxic stress response. Several studies examining TK6 cells have noted an absence of G1 arrest following irradiation, despite elevation of p53 and p21WAF1/CIP1 levels, and induction of apoptosis, and one study observed that the cells instead arrested in G2/M phase (Hendriks et al., 2000; Little et al., 1995), suggesting that the G1 checkpoint may not be operational in this cell line. Irradiation has been shown to induce G1 and G2/M checkpoints in an immortal human mammary cell line (184A1N4) but only G1 arrest in MCF10AAs (Sheen and Dickson, 2002). It is therefore possible that particular cell lines are predisposed to arrest in particular phases of the cell cycle. Further work would be merited analyzing the effect of PhIP on the cell cycle of other target and nontarget cells, in order to gain a greater understanding of whether PhIP-mediated genetic damage produces a tissue-specific response.

The finding that PhIP caused an accumulation of the hypophosphorylated form of pRB provides another potential explanation for the lack of apoptosis in MCF10A cells. It has been proposed that p53-dependent growth arrest requires the activation of pRB, while inactivation of pRB is necessary for p53-dependent apoptosis to take place (Wang and Ki, 2001). Activation (dephosphorylation) of pRB following genotoxic stress enables it to interact with the transcription factor E2F, thus playing an essential role in p53-dependent growth arrest, demonstrated by the fact that pRB-deficient cells have a defective G1 checkpoint in response to DNA damage (Brugarolas et al., 1999; Knudsen et al., 2000). However, while E2F is important for the stimulation of proliferation, it also plays a role in apoptosis following DNA damage by inducing the expression of the pro-apoptotic factors Apaf-1, caspase 3, and p73 mRNA (Irwin et al., 2000; Matsumura et al., 2003; Moroni et al., 2000). The pro-apoptotic function of E2F-1 is dependent on p53 and p73, and following E2F-stimulated transcription of p73 mRNA, the protein stability and apoptotic function of p73 is stimulated by the c-Abi protein, which is also negatively regulated by pRB. By inhibiting E2F-mediated transcription of p73 mRNA and also preventing the c-Abi tyrosine kinase from stabilizing p73 protein, pRB is thus able to inhibit apoptosis at the same time as activating G1 cell cycle arrest. It therefore is interesting to speculate that the activation of pRB downstream of PhIP-induced damage in MCF10A cells is responsible for the checkpoint response observed, and also for the absence of apoptosis.

As the effects reported here are presumed to be in response to the genotoxicity of PhIP, a GTBP DNA repair ELISA was used to look for evidence of induction of the mismatch repair (MMR) protein GTBP (G/T binding protein), also called hMSH6. A human colorectal cancer cell line DLD-1, which has a mutated hMSH6 gene, was significantly resistant to the cytotoxic effects of PhIP under a mouse liver S9 metabolizing system compared to cells with functional hMSH6, and PhIP-induced mutation frequencies at the hprt locus were approximately three-fold higher in the hMSH6-defective cell line (Glaab and Skopek, 1999). Furthermore, MT1, an MMR-deficient derivative of TK6 which contains mutations in both alleles of its hMSH6 gene, is less responsive to PhIP-induced apoptosis and displays a significantly higher hprt mutation frequency than TK6 cells (Duc and Leong-Morganthaler, 2001; Leong-Morganthaler et al., 2001). Further support for the involvement of GTBP/hMSH6 in PhIP-induced mutagenesis is provided by comparison to the carcinogenic aromatic amines, aminofluorene (AF) and N-acetyl-2-aminofluorene (AAF). Like PhIP, AF and AAF produce bulky adducts at the C8 position of guanine, dG-C8-AF and dG-C8-AAF, with a mutational specificity of G:C to T:A transversions (Lambert et al., 1992; Schaapen et al., 1990). hMSH6 forms the hMutSα complex with hMSH2, and this heterodimer binds to both the dG-C8-AF and dG-C8-AAF adducts (Li et al., 1996) and is believed to be involved in the processing and repair of this type of DNA damage. Given the similarities in adduct structure and mutational specificity of the PhIP adduct dG-C8-PhIP and dG-C8-AAF, it is hypothesized that MMR proteins are also involved in recognizing dG-C8-PhIP adducts (Duc and Leong-Morganthaler, 2001; Glaab et al., 2000). We attempted to examine this proposal using a GTBP ELIZA assay. However, treatment of MCF10A cells under metabolizing conditions with a known mutagenic dose of PhIP (100 μM) only resulted in a marginal induction (30%) of GTBP/hMSH6, although this response was quantitatively similar to that found with the positive control N-methyl-N′-nitro-N-nitrosoguanidine (NMMG), an alkylating agent known to trigger the accumulation of GTBP.

Our experiments demonstrate that the early cellular and molecular response to PhIP-induced genotoxic stress in human breast epithelial cells involves a prolonged growth inhibition via a p53- and p21WAF1/CIP1-mediated G1 cell cycle checkpoint. GTBP/hMSH6, a protein involved in the mismatch repair system, is also elevated following PhIP exposure, consistent with the presence of guanine-damaged DNA. Administration of PhIP in the absence of activating metabolism did not induce signs of genotoxicity in MCF10A cells as measured by p53, p21, or GTBP/hMSH6 induction. In summary, we suggest that cell cycle events are critical to enable cells to affect genome repair or for acceptance of mutation or elimination of excessively damaged cells by necrotic or apoptotic cell death. Clearly, impairment of the functioning of any of these key protein effectors will influence the susceptibility of the cell to accumulate genetic damage.

ACKNOWLEDGMENT

This work was supported in part by the UK Food Standards Agency. S.C. thanks Imperial College London for the award of a scholarship.
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