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Role of p53 and mismatch repair in PhIP-induced perturbations of the cell cycle

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Abstract

Heterocyclic amines, found ubiquitously in our diet, are carcinogenic and mutagenic. Among this class of compounds, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) is the most abundant. To further understand the carcinogenesis of this compound, we studied the effects of PhIP on the progression of human lymphoblastoid cells through the cell-cycle. Cells differing in p53 or mismatch repair status were used to evaluate the role of those proteins. Following PhIP-treatment, a dose and time-dependent accumulation of p53 was found in cells containing functional p53. The augmentation of the p53 protein, accompanied by increases in p21-WAF1, confirms that the p53 is activated. The increase in p53 was independent of the mismatch repair status of the cells. Perturbations in the cell-cycle were also observed. Twenty-four hours after PhIP treatment, the activation of the G2-M checkpoint was evident. Functional p53 and mismatch repair were not required for the PhIP-induced G2-M arrest. The G2-M arrests were reversible and are interpreted as necessary for the repair of the PhIP-DNA lesions. Under treatment conditions where less than 5% of the cells survived, the G2-M arrests were absent.

Keywords: p53; 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine

1. Introduction

Human exposure to heterocyclic amines (HCAs) is substantiated by the presence of HCAs and their metabolites in urine, and the detection of HCA–DNA-adducts in different tissue samples of healthy people consuming a normal diet (reviewed in [1]). HCAs are carcinogenic in mice, rats and non-human primates and are potent mutagens in the Ames test ([2] and references therein). The most abundant heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP), is found at levels ranging from less than 1 ppb to over 200 ppb in various cooked meats and fish [3].

PhIP requires metabolic activation by the cytochrome P-450 enzymes and the *N*-*O*-Acetyl transferase to exert its biological effects (reviewed in [4]). The presence of PhIP–DNA and PhIP-Protein adducts have been found in the colon of human volunteers showing that PhIP is bioavailable to the colon at dietary-relevant doses [5]. Metabolically activated PhIP is cytotoxic and mutagenic, inducing predominantly G → T transversions and −1 G frameshift mutations, in human cells [6]. Cells deficient in mismatch repair are hypermutable by PhIP [7] indicating a role of mismatch repair proteins in this pathway.

Following DNA damage, a cascade of biological effects is elicited in human cells. One of the most common responses following genotoxic stress is the activation of p53 (reviewed in [8]). Different chemical and physical agents, including ionizing and UV radiation, methyl methanesuiphonate, mitomycin C, cisplatin and BPDE are potent inducers of p53 [9–11]. The signal from DNA damaging agents that triggers the induction of p53 is still not clear. DNA strand breaks and inhibition of RNA polymerases have been postulated to play a role [12,13].

To provide cells time to repair the damage, controls are present to delay entry into the S and M-phases in the presence of DNA-damage. G1-S arrest is observed in cells following DNA-damage primarily through the activation of p53 and p21-WAF1 genes [14]. The increase in p21-WAF1 results in the inhibition of the cyclin–cdk complexes and eventually leading to arrest at the G1-S checkpoint. The arrest at the G1-S checkpoint appears to be p53 dependent [15].

The mechanisms of DNA-damage related delays within the S-phase or G2-M are less well understood. Both

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p53-dependent and p53-independent pathways exist to regulate the G2-M transition in response to DNA-damage. The G2-M arrest following DNA damage when independent of p53, could be sustained by p53 in some cells [16]. Mismatch repair proteins have been implicated in G2-M arrests resulting from treatment with alkylating agents and ionizing radiation [17–19]. Mismatch repair proteins have also recently been reported to play an important role in the activation of the S-phase checkpoint [20].

Treatment of cells with CYP1A2 activated PhIP results in a S-phase delay, but does not activate either the G1 or G2-M checkpoints in the human lymphoblastoid cell line TK6 [21]. In this article, we report that PhIP activated by the metabolizing enzymes in rat liver S9 induces a G2-M cell-cycle arrest through a p53-independent mechanism. We also show that following PhIP treatment, activation of p53 and induction of p21-WAF1 protein together with a G1-S arrest occurs only in cells with wild-type p53 (TK6). Mismatch repair proteins do not appear to play a role in the induction of those cell-cycle checkpoints by PhIP.

2. Materials and methods

2.1. Chemicals

All chemicals, unless otherwise stated were from Fluka (Buchs, SG, Switzerland). RPMI 1640, glutamine, penicillin/streptomycin and fetal calf serum were obtained from Gibco, Life Technologies, Basel, Switzerland. PhIP was purchased from Toronto Research Chemicals, Ontario, and Moltox arochlor1254 induced rat liver S9 was from Molecular Toxicology, Maryland, USA. DTT, PMSF, RNAse and protease inhibitors were from Roche Molecular Biochemicals. Nitrocellulose membranes were obtained from Schleicher&Schuell (Switzerland). Antibodies against p53 (Ab-6) and p21-WAF1 were from Oncogene Research Products, (Massachusetts, USA) and secondary antibodies were from Amersham Life Science Products (Switzerland) and from Biorad Laboratories, (Switzerland). The ECL kit was from Amersham Pharmacia Biotech (Switzerland).

2.2. Cell culture and PhIP treatment

TK6, MT1 and WTK1 human lymphoblastoid cells were cultured in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal calf serum. WTK1 (p53 mutant), MT1 (hMSH6 mutant) and TK6 (p53 and hMSH6 wild-type) were derived from the same donor [22,23]. Cells were treated with different concentrations of PhIP in the presence of an exogenous metabolising system (2% rat liver S9) for 4 h. Treated cells were maintained as stationary cultures in T-flasks with daily dilution to 4.5×10^5 cells/ml and cells were sampled at the times indicated for cell-cycle analysis and protein preparation.

2.3. Cell-cycle analysis

The DNA content of cells was assayed following fixation and staining with propidium iodide as described [24]. Briefly, 1×10^6 cells were suspended in 500 μl of 70% ethanol and placed at −20 °C for at least 1 h. The ethanol was then removed and the cells suspended in 500 μl PBS containing 150 μg/ml RNA and incubated at 37 °C for 45 min. The cells were centrifuged, suspended in 300 μl containing 50 μg/ml propidium iodide, and analysed by flow cytometry.

2.4. Western blot

Cells were sampled (3×10^6 cells each) at the indicated times following treatment. Protein extracts were prepared as described in [25]. Fifty micrograms of protein were loaded on SDS-polyacrylamide gels. After separation, the proteins were transferred onto nitrocellulose membranes. The membrane was blocked with 5% nonfat milk in TBS-Tween, incubated with the primary antibody, p53 (Ab-6) or WAF1 (Ab-1) from Oncogene Research Products, (Massachusetts, USA) and washed with TBS-Tween. After incubation with peroxidase-conjugated rabbit anti-mouse secondary antibody, signals were detected with the Amersham-Pharmacia ECL kit. The signals on the blots were quantified using the Bio-1D programme, version 6.01 from Vilber–Lourmat.

3. Results

3.1. Cell cycle distribution of wt p53 TK6 cells following PhIP treatment

The activation of either a G1-S or G2-M cell-cycle arrest by PhIP in human lymphoblastoid cells was evaluated 8, 24 and 48 h after PhIP treatment by staining cells with propidium iodide and flow cytometry analysis. Differences between the treated cultures and the control were most evident 24 h post treatment. Fig. 1 shows the distribution of TK6 cells 24 h after PhIP treatment from a representative experiment. At the lowest concentration tested (1.25 μg/ml) where about 80% survival was observed, a slight increase (4%) in the G1 population and a decrease (6%) in the S population relative to the control cultures were seen in this experiment. These differences were not found to be significant at $\alpha < 0.05$ when three independent experiments were analysed (Table 1). At the intermediate concentration (40% survival), significant decreases of cells in G1 with increases of cells in G2-M were found. Total 34 and 37% of the treated cells were in the G1 and G2-M phases, respectively. The non-treated culture contained 48 and 26% of its cells in those two phases. These results indicated an arrest at the G2-M checkpoint, but did not allow us to conclude if the Gl-S checkpoint had been activated or not. As a result of accumulation of cells at G2-M, the presence of G1-S arrest will not be manifested as an increase in G1 cells. At the highest

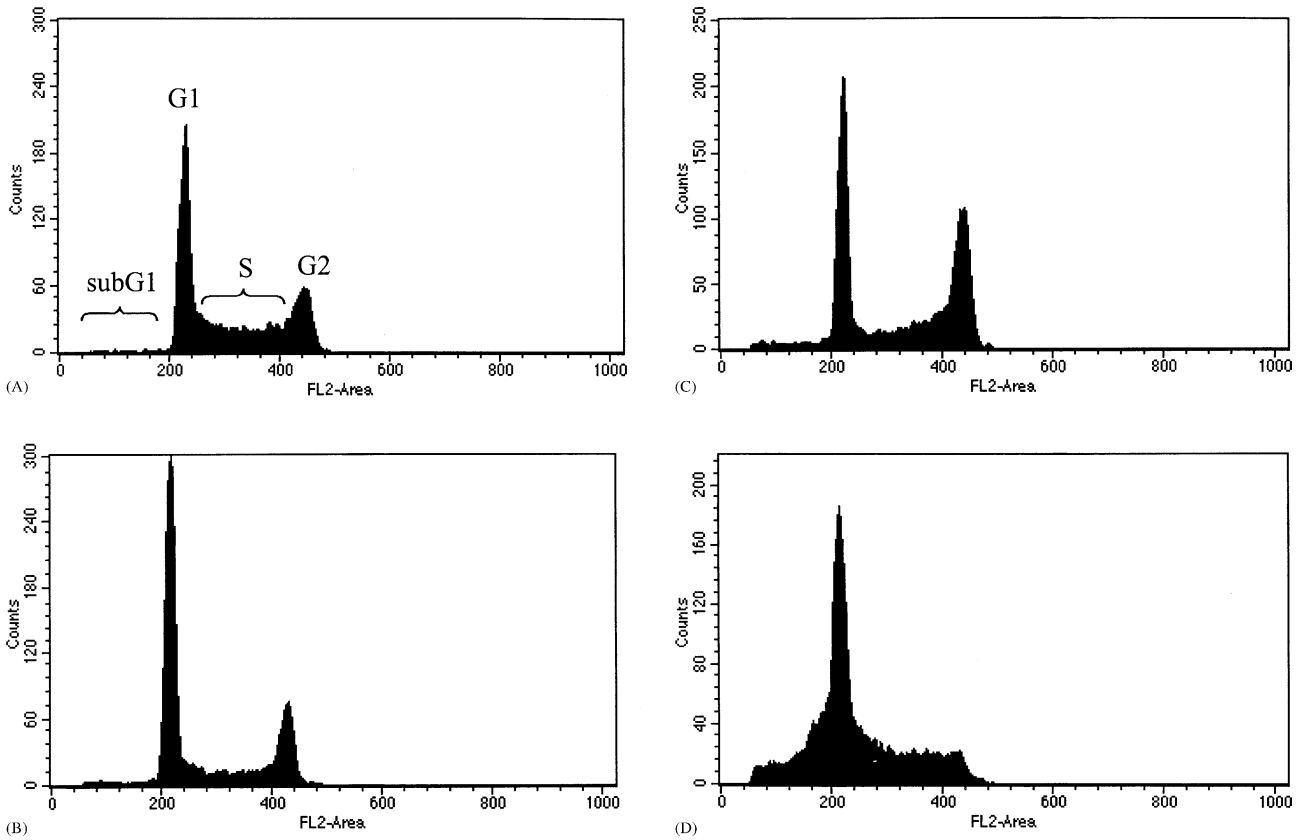

Fig. 1. Analysis of the cell-cycle distribution of TK6 24 h following PhIP treatment. Cells were stained with propidium iodide and subjected to flow cytometric analysis of DNA content as described in the text. The positions of subG1, G1, S and G2-M DNA are noted in the control. (A) Control (B) 1.25 μg/ml PhIP (C) 2.5 μg/ml PhIP (D) 5.0 μg/ml PhIP.

concentration (less than 5% survival) the contrary was observed. An increase of cells in G1 and a decrease of cells in G2-M were found. After 48 h, the perturbations in the cell cycle were not observed in the lower two PhIP concentrations used (data not shown). At the highest concentration, the decrease in the G2-M fraction was still evident.

Table 1
Distribution[a] of human lymphoblastoid cells 24 h after treatment with PhIP

Treatment	Cell-cycle phase	TK6	WTK1	MT1
Control	G1	48.0 (4.0)	44.0 (1.9)	46.5 (1.2)
	S	25.7 (3.0)	25.0 (2.1)	25.5 (1.0)
	G2-M	25.8 (1.6)	31.0 (2.2)	28.0 (1.4)
1.5 μg/ml PhIP	G1	45.0 (2.0)	30.6 (6.6)[b]	45.3 (0.7)
	S	19.8 (2.3)	21.3 (3.5)	23.2 (1.3)
	G2-M	35.6 (2.5)[b]	48.1 (9.5)[b]	31.6 (1.0)
2.5 μg/ml PhIP	G1	34.0 (1.0)[b]	25.6 (4.3)[b]	39.1 (2.5)
	S	29.2 (2.5)	37.0 (5.7)[b]	24.3 (2.1)
	G2-M	36.8 (3.0)[b]	37.3 (1.3)[b]	36.6 (2.5)[b]
5.0 μg/ml PhIP	G1	57.0 (3.6)	43.0 (1.7)	54.3 (3.8)
	S	28.0 (2.0)	36.9 (3.1)[b]	25.8 (1.0)
	G2-M	14.0 (1.0)[b]	20.0 (3.7)[b]	19.9 (4.0)

The values in parentheses show the S.E.M., $n = 3$.

[a] The percentages of cells in the different phases of the cell-cycle are shown. Cells containing DNA in sub G1 were not counted.

[b] The values are different ($\alpha < 0.05$) from the control.

3.2. Induction of p53 by PhIP

To determine if p53 played a role in the cellular responses to PhIP, the accumulation and transcriptional activity of p53 was evaluated in protein extracts of cells sampled at various times after treatment with 2.5 and 5 μg/ml PhIP. The time-course of the induction of p53 was determined by Western blot analysis using a p53 specific antibody (Fig. 2). The accumulation of p53 was already evident at the earliest time analysed, 4 h after PhIP treatment. After this augmentation, the level of p53 continued to increase to reach its maximum at about l4–16 h, after which the p53 levels remained stable until 20 h. The p53 levels then decreased but at 42 h, the p53 levels had not yet returned to basal levels.

3.3. Accumulation of p53 is accompanied by induction of p21-WAF1

The transcriptional activation potential of p53 was verified by the induction of the p21-WAF1 protein. The presence of p21-WAF1 was determined by Western blots using PhIP treated cell extracts and an antibody specific for

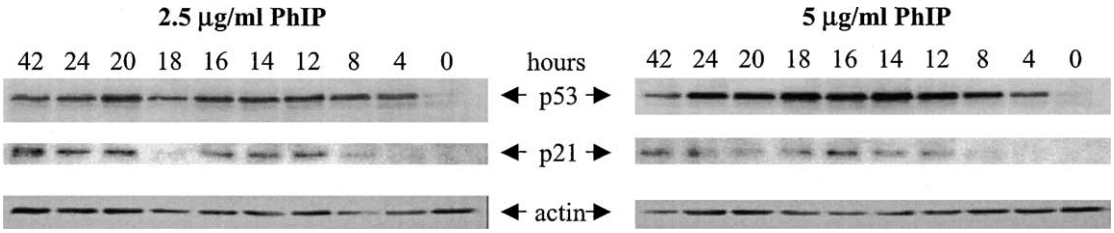

Fig. 2. Time course of induction of p53 following PhIP treatment. Western blot analysis of protein extracts from cells sampled at the times noted from a representative experiment. Cells were treated with 2.5 or 5.0 μg/ml PhIP. An amount of 5.0 μg of proteins were loaded per lane. The blots were hybridized to an antibody against p53 (Ab-6) or p21-WAF1 (Ab-1) and the signal detected by ECL system. Uniformity of loading was checked by hybridization to an antibody against actin.

p21-WAF1 (Fig. 2). Increases in p21-WAF1 protein were observed about 8 h following treatment. The accumulation of p53 accompanied by the augmentation in the p21-WAF1 protein confirmed that the p53 is active.

3.4. Cell cycle distribution of a p53 mutant line (WTK1) following treatment with PhIP

In order to dissect the role of p53 in the observed cell-cycle arrests, we next analyzed WTK1, which contains a C → T missense mutation in codon 237 of the p53 gene [23]. The distribution of PhIP-treated WTK1 cells 8, 24 and 48 h after treatment showed that differences between the control and treated cultures were most evident 24 h after treatment (data not shown). Table 1 shows the distribution of WTK1 24 h after PhIP treatment. Decreases of cells in G1 with concomitant increases of cells in G2-M were observed in the cultures treated with 1.25 and 2.5 μg/ml PhIP. These results clearly indicated activation of the G2-M checkpoint. The G2-M arrest was more pronounced in the lowest dose where a 1.5-fold increase of cells in G2-M was observed. Increases in cells in S-phase were also observed in the 2.5 μg/ml PhIP treated populations. At the highest concentration (less than 5% survival), increases in cells in S-phase and decreases in cells in G2-M were observed.

3.5. Lack of induction of p53 in WTK1 by PhIP

The accumulation of p53 in WTK1 was analysed by Western blots using protein extracts from control and treated cells. The basal level of the p53 protein was higher in WTK1 than TK6. Following treatment with PhIP, only slight increases in the accumulation of the p53 protein were observed. In spite of the high levels of p53, no induction of the p21-WAF1 was observed (Fig. 3).

3.6. Cell cycle distribution of a mismatch repair deficient line (MT1) following treatment with PhIP

The percentage of cells in the G1, S and G2-M of MT1 24 h after PhIP treatment is also shown in Table 1. The distribution of MT1 cells treated with the lowest concentration 1.25 μg/ml PhIP was not different from the control. At 2.5 μg/ml PhIP, increases in cells in G2-M were observed. At the highest concentration (5 μg/ml) of PhIP, no significant differences were found in the treated and the control populations.

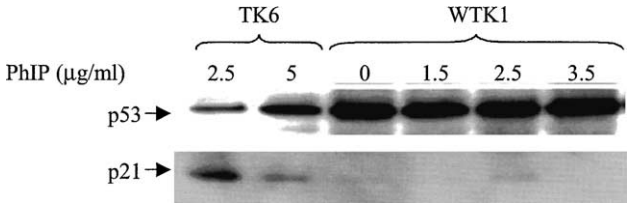

Fig. 3. Lack of accumulation and induction of transcriptional activation of p53 in WTK1 following treatment with PhIP. Western blot analysis of protein extracts from cells 24 h after treatment with different concentrations of PhIP. An amount of 50 μg of proteins were loaded per lane. The blots were hybridized to an antibody against p53 (Ab-6) or p21-WAF1 (Ab-1) and the signal detected by ECL system. Lanes 1, 2: TK6; lanes 3–6: WTK1; lane 3: no treatment; lane 4: 1.25 μg/ml PhIP; lanes 1 and 5: 2.5 μg/ml PhIP; lanes 2 and 6: 5 μg/ml PhIP.

4. Discussion

Treatment with PhIP (activated by rat liver S9) triggered the signal for p53 accumulation (Fig. 2). The accumulation of p53 was rapid (within 4 h) and was sustained for at least 42 h. A concomitant increase in p21-WAF1 protein was observed in TK6 and MT1 ([26], this study) confirming that the endogenous p53 is functional. The nature of the signal(s) initiated by PhIP to is not known. Similar levels of p53 accumulation were observed in mismatch repair deficient cells indicating that the trigger did not involve mismatch repair proteins [26].

Cell-cycle arrests were found at different levels of PhIP-induced cytotoxicity. PhIP activated the G2-M checkpoint in all three cell-lines. It is clear from the results that functional p53 and mismatch repair were not required for the PhIP-induced G2-M arrest. In the case of TK6 and WTK1, the G2-M arrests were already evident at the lowest concentration of PhIP used where survival was 85%. These results show that cells deficient in mismatch repair may be

less sensitive to PhIP-induced G2-M responses. PhIP can activate the G2-M checkpoint in mismatch repair deficient cells, albeit at higher concentrations. This is in contrast to the responses triggered by alkylating agents where mismatch repair deficient cells do not exhibit the G2-M arrest [17,18]. The arrests observed 24 h after treatment were not present at 48 h and were, therefore, reversible. The prolonged G2 would provide cells sufficient time to repair the DNA damage after which, the cells continued through the cell cycle.

The increase in G2-M cells at the lowest PhIP concentration was accompanied by a decrease in cells in G1 in WTK1 only. Comparison of the responses in the cells with functional p53 (TK6) to WTK1 (mutant p53) showed less Gl cells in WTK1. Because treatment with PhIP resulted in p53 accumulation and concomitant increase in p21-WAF1, and the induction of p21-WAF1 by DNA damaging agents has been shown to lead to G1 arrest [14], we postulate that the differences TK6 and WTK1 could reflect a G1-S arrest in TK6 following PhIP treatment.

Under conditions of high cytotoxicity (cell survival less than 5%), significant decreases ($\alpha < 0.05$) of cells at G2-M following PhIP treatment were observed only in TK6 and WTK1. Whether the different result observed in MT1 could be due to its lower PhIP-induced apoptotic response [26] warrants further studies. Unlike the effects observed in the lower concentrations, the effects on the cell cycle in the presence of high cytotoxicity were irreversible. After 48 h, the decreases in the G2-M fraction were still evident. In spite of the activation of p53 and induction of p21-WAF1, the accumulation of cells at the G1-S checkpoint was not observed in TK6. Because of the low survival, it is difficult to evaluate whether the G1-S and G2-M checkpoints were not activated or whether the cells were arrested transiently before cell death. These effects at high cytotoxicity do not reflect the response in humans who are normally exposed to lower concentrations of PhIP.

Our data shows that PhIP can induce a reversible G2-M arrest independent of p53 -transactivation competence and functional mismatch repair. The results also suggested the presence of a p53-dependent G1-S arrest. Increases in S-phase cells were observed in WTK1 but there was no evidence of a S-phase delay in TK6. These results are different from those of Zhu et al. [21] where PhIP transformed by cells expressing human cytochrome P-450-1A2 was reported to uniquely activate the S-phase checkpoint. In our study, PhIP was activated by a rat liver homogenate (S9). Rat liver S9 transforms PhIP to the N-hydroxy and 4′-hydroxy forms, while human cytochrome P-450-1A2 produces *N*-hydroxy PhIP only [27]. Further studies are needed to elucidate the mechanism that led to the different cellular responses when the two methods of activation of PhIP were used.

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