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Impairment of cell cycle progression by aflatoxin B1 in human cell lines

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Aflatoxin B1 is a mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticum*, which may be present as a food contaminant. It is known to cause acute toxic effects and act as a carcinogenic agent. The carcinogenic action has been related to its ability to form unstable adducts with DNA, which represent possible mutagenic sites. On the other hand, the primary cellular target responsible for its toxic action has not yet been clearly identified. Previous data suggested a possible correlation between cell proliferation and responsiveness to aflatoxin toxicity. These observations led us to investigate the effect of the toxin on cell cycle progression of three human cell lines (HepG2, SK-N-MC and SK-N-SH derived from liver and nervous tissue tumours); they were shown to display different responses to toxin exposure and have different growth kinetics. We performed analysis of the cell cycle, DNA synthesis and expression of p21 and p53 in the presence and absence of the toxin in all cell lines exposed. The results of cell cycle cytofluorometric analysis show significant alterations of cell cycle progression as a result of toxin treatment. In all cell lines exposure to a 24 h toxin treatment causes a dose-dependent accumulation in S phase, however, the ability to recover from impairment to traverse S phase varies in the cell lines under study. SK-N-MC cells appear more prone to resume DNA synthesis when the toxin is removed, while the other two cell lines maintain a significant inhibition of DNA synthesis, as indicated by cytofluorimetry and [^{3}H]dTR incorporation. The level of p53 and p21 expression in the three cell lines was examined by western blot analysis and significant differences were detected. The ready resumption of DNA synthesis displayed by SK-N-MC cells could possibly be related to the absence of p53 control of cell cycle progression.

Introduction

Aflatoxin B1 (AFB1) is a well-known mycotoxin produced by different strains of *Aspergillus flavus* and *Aspergillus parasiticum*. In humans and various animal species it has been reported as a potent hepatotoxic and hepatocarcinogenic agent (Massey *et al*., 1995; McLean and Dutton, 1995). AFB1 is readily transported across the plasma membrane and interacts with nucleic acids and proteins, altering various cellular activities. Its interaction with protein(s) carrying a nuclear localization signal may be responsible for its nuclear translocation and subsequent binding to DNA. A well-characterized effect of AFB1 is the formation of covalent adducts with DNA, which impair DNA, RNA and ultimately protein synthesis (Meneghini and Schumacher, 1977; Yu, 1981; Amstad and Cerruti, 1983; Hsieh, 1986). AFB1, or more precisely its epoxide derivative, forms DNA adducts primarily with guanine and cytosine. Although in an *in vitro* system the formation of cytosine adducts has been reported (Yu *et al*., 1994), in humans and experimental animals guanine adducts only have been observed (Wogan, 1992). K-*ras* activation (Soman and Wogan, 1993) and *p53* mutation (Aguilar *et al*., 1994) are also induced by AFB1 and may contribute to carcinogenesis in AFB1-exposed animals.

Aflatoxin is also capable of causing acute toxic effects *in vivo* (McLean and Dutton, 1995). While molecular targets for the mutagenic and carcinogenic actions of AFB1 have been identified (Meneghini and Schumacher, 1977; Wogan, 1992), cellular components responsible for AFB1 cytotoxicity have not been clearly determined. Decreased cell survival has been reported in cultured cells in the presence of AFB1 (Iwaki *et al*., 1990; Bonsi *et al*., 1996). The decrease in cell survival reported both in primary cultures and stable lines could be related to either DNA damage caused by the toxin or to a direct impairment of specific protein functions. AFB1 has been reported to alter protein phosphorylation in liver (Viviers and Schabort, 1985) and the activity of enzymes such as protein kinase (Van den Heever and Dirr, 1991; Mistry *et al*., 1996), Ca^{2+}-ATPase (Adebayo *et al*., 1995) and cyclic nucleotide phosphodiesterase (Bonsi *et al*., 1999) can be altered by AFB1.

Cell lines have been widely used as a suitable system for the characterization of xenobiotic toxic effects and the identification of their mechanism of action. We have previously reported that AFB1 exerts a cytotoxic effect on three human cell lines, SK-N-MC, SK-N-SH and HepG2; the response of the different lines to the toxin, as measured by cell survival, varies and may be related to differences in their cell growth (Bonsi *et al*., 1996). These observations led us to consider whether AFB1 causes similar alterations of cell cycle progression in the three cell lines, which may help to establish the primary event of its cytotoxic action. To this end a comparative cytofluorimetric analysis of the cell cycle in the presence of AFB1 was performed on the previously studied cell lines.

Materials and methods

Materials

Aflatoxin B1 was purchased from Sigma (St Louis, MO). All other reagents were of analytical grade and were purchased from various sources. Culture media and sera were obtained from HyClone (Logan, UT). The SK-N-MC, SK-N-SH and HepG2 cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Cell cultures

Human cell lines SK-N-MC and SK-N-SH were originally isolated from neuroblastoma metastases (Biedler *et al*., 1973); they were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, non-essential amino acids (100× solution) and sodium pyruvate (100 mg/l).

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Under these experimental conditions, doubling time was 28 h for the SK-N-MC and 48 h for the SK-N-SH cell line. Both cell lines display a rather immature morphology, although in response to various molecules (e.g. retinoic acid, cAMP and 12-*O*-tetradecanoyl phorbol-13-acetate) they can grow neuritic processes with terminal arborization. SK-N-SH cultures are characterized by the presence of neuroblastic and epithelial-like morphological variants, which have been subcloned; the former variants express tyrosine hydroxylase and dopamine β-hydroxylase, showing a cathecolaminergic phenotype. Conversely, SK-N-MC cells express choline acetyltransferase, the biosynthetic enzyme of cholinergic neurons (Israel and Thiele, 1994).

The hepatoma cell line HepG2, derived from a human liver carcinoma, was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum; the doubling time was 36 h. HepG2 cells morphologically resemble liver parenchymal cells and secrete major plasma proteins (Knowles *et al*., 1980).

In order to evaluate AFB1 effects, 24 h after plating toxin was added to the culture medium at the appropriate concentration, as indicated in the figure legends; 24 h later the medium was renewed and culture continued in the absence of toxin for an additional 24, 48 and 72 h (0, 24 and 48 h post-treatment, respectively).

[^{3}H]thymidine incorporation

SK-N-MC, SK-N-SH and HepG2 cells were plated at a density of 1×10^4 in 24-well Falcon trays. Twenty-four hours after plating the cells were exposed to AFB1 for 24 h at the following concentrations: 9.6, 19.2 and 40 μM for HepG2, SK-N-MC and SK-N-SH cells, respectively. On the basis of previously determined sensitivity to AFB1 of each cell line (Bonsi *et al*., 1996), toxin concentrations were chosen in such a way that DNA synthesis could be significantly evaluated.

At the end of the treatment 1 μCi/ml [^{3}H]thymidine was added to each well for 2 h. Cultures were then washed twice with 0.5 ml of phosphate-buffered saline at 4°C, followed by solubilization with 0.2 ml of 1% Triton X-100 at 4°C for 5 min. Lysates were collected, supplemented with 10% trichloroacetic acid (TCA) and kept at 4°C for 15 min. Precipitates were then collected on GF/C Whatman filters, washed once with 10% TCA and twice with 1% TCA. Filters were then transferred to scintillation vials, 10 ml of scintillation mixture (Filter-Count; Packard) was added and the radioactivity determined using a scintillation counter (TriCarb 4000; Packard).

[^{3}H]thymidine incorporation was also evaluated 48 h after AFB1 treatment was ended.

Western blotting

After determination of the protein concentration by the Bradford method (Bradford, 1976) crude cell extracts were denatured by boiling for 5 min in Laemmli sample buffer to evaluate p21 and p53 levels. Aliquots corresponding to equal protein content were subjected to SDS–PAGE on 7.5% polyacrylamide gels under reducing conditions. Proteins were then electrotransferred to PVDF filters (Amersham) at 30 V overnight at 4°C. Non-specific sites were blocked by incubation of the membranes with 20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.1% Tween-20 containing 5% bovine serum albumin (BSA) for 1 h at room temperature. Filters were then incubated with a mouse polyclonal anti-p53 IgG1 antibody (FL393; SantaCruz) and a rabbit polyclonal anti-p21 IgG antibody (C19; SantaCruz).

Following 1 h incubation with the primary antibody, filters were washed three times with TBS (10 mM Tris–HCl, pH 8, 150 mM NaCl) and immunoblots were developed using anti-rabbit and anti-mouse alkaline phosphatase conjugated secondary antibodies. The ECF (Amersham) substrate was used to develop the reaction (chemifluorescence).

Fluorescence was acquired by a Storm 840 phosphorimager/fluorimager (Molecular Dynamics, Sunnyvale, CA), which gives a volume report by integrating the area of the band and its density.

Flow cytometry analysis of DNA content and bromodeoxyuridine incorporation

Exponentially growing neuroblastoma and hepatoma cells were exposed to AFB1 for 24 h. The following toxin concentrations were used: 9.6, 19.2 and 32 μM for SK-N-MC and HepG2 and 19.2, 80 and 160 μM for SK-N-SH; the higher toxin concentrations for the latter were due to its lower sensitivity, as previously reported (Bonsi *et al*., 1996).

In order to identify cells in S phase, DNA content and bromodeoxyuridine (BrdUrd) incorporation were determined, by simultaneous analysis of propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd fluorescence. Cells were exposed to 45 μM BrdUrd for 15 min before harvesting, collected by trypsinization and fixed in a 1:1 absolute methanol/phosphate-buffered saline (PBS) mixture. Partial DNA denaturation was

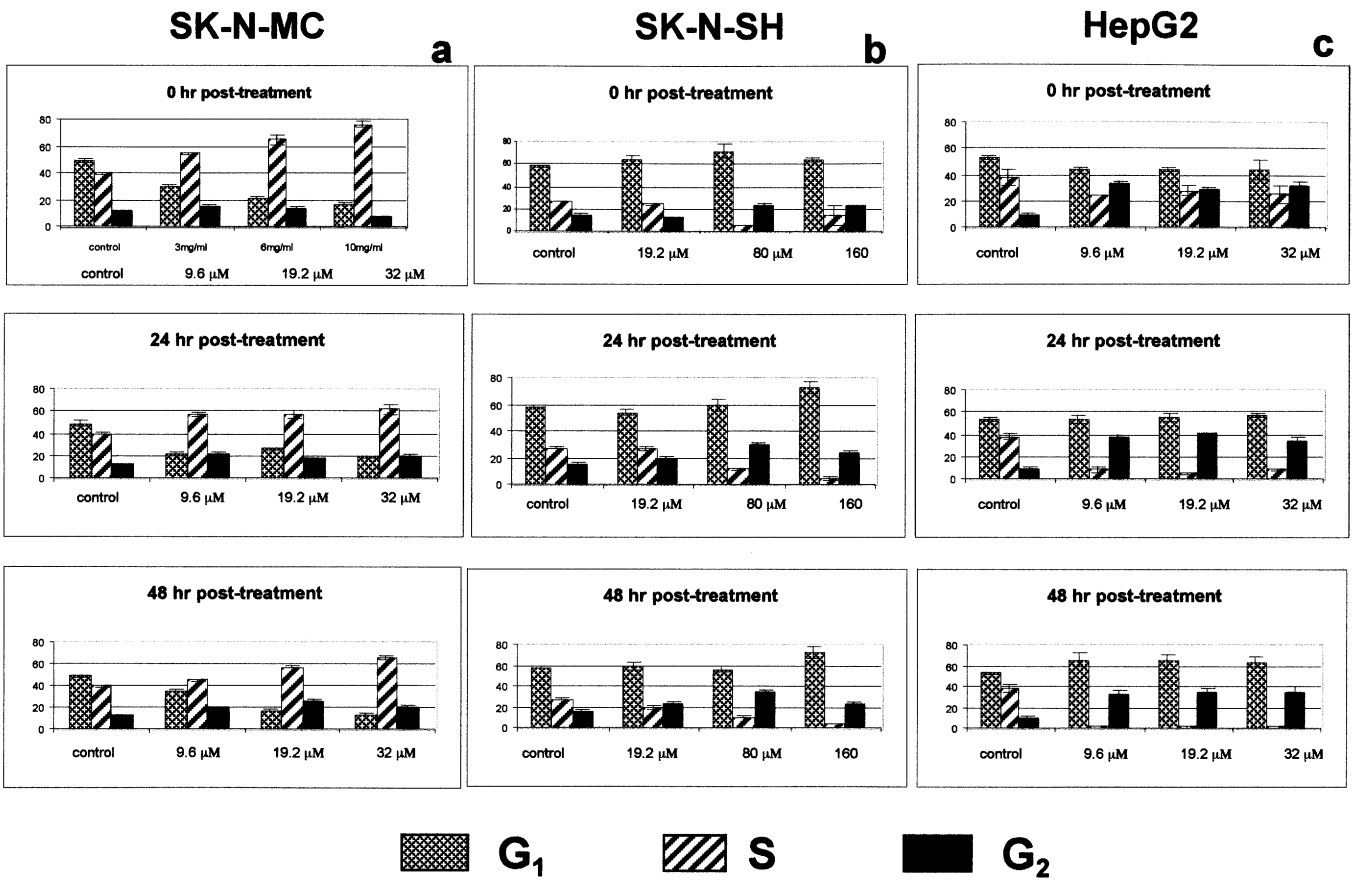

Fig. 1. Histograms showing percentage of SK-N-MC, SK-N-SH and HepG2 cells in the G_1, S and G_2 phases after treatment with increasing doses of AFB1 at 0, 24 and 48 h post-treatment. Data are reported as the averages of three independent experiments. Bars indicate SD.

performed by incubating cells in 3 N HCl for 30 min, followed by neutralization with 1 ml of 0.1 M sodium tetraborate. Samples were then incubated with a mouse monoclonal anti-BrdUrd antibody (DAKO, CA) for a further 30 min at room temperature in the dark, washed twice with 0.5% Tween-20 in PBS and incubated for 30 min with anti-mouse FITC-conjugated anti-IgG antibody (Vector Laboratories, CA). Samples were washed twice with PBS and finally stained with 20 μg/ml PI for 15 min at room temperature.

Flow cytometry analysis was performed with a FACStar Plus flow cytometer (Becton Dickinson) equipped with a 5 W Innova 90 coherent laser with 488 nm wavelength excitation. 10 000 events were collected for each sample. Monoparametric (DNA histograms) and biparametric (DNA content versus BrdUrd content) analyses were performed using WinMDI 2.7 software.

Results

Cell cycle distribution was determined by incubating the cells with 45 μM BrdUrd for 15 min to label cells active in DNA synthesis at 0, 24 and 48 h after a 24 h exposure to three doses of AFB1. Cells were fixed, incubated with an anti-BrdUrd monoclonal antibody and stained with PI. The cells were then analysed using a flow cytometer, as described in Materials and methods.

Toxin concentrations used for the three cell lines were chosen on the basis of their sensitivity to the toxin, as estimated by cell survival (Bonsi *et al*., 1996).

SK-N-MC cells

In SK-N-MC cells AFB1 treatment causes, immediately after treatment, a dose-dependent increase in cells in S phase, with a parallel decrease in cells in G_1 and G_2 (Figure 1a). The high percentage of cells in S phase, with a concomitant decrease in cells in G_1 and G_2, suggests that AFB1 induces a dose-dependent delay through S phase and in the transition from S to G_2/M phase in SK-N-MC cells. When the toxin is removed the block of cells in S phase is still observed after 24 and 48 h (post-treatment experiments). However, it is interesting to note that at the lowest dose cell distribution in the cycle phases is restored to normal 48 h after toxin removal, as shown by the percentage of cells in S phase (Figure 1a). However, 24 and 48 h after exposure to the higher doses of AFB1 is ended a significant slowing down of S phase traverse is persistent, as is also shown in Figure 1a.

In order to study the kinetics of S phase traverse after AFB1 treatment, we performed a biparametric analysis using BrdUrd incorporation and subsequent immunostaining with an anti-BrdUrd monoclonal antibody. The results of flow cytometric analysis are shown in Figure 2.

At all toxin doses the region corresponding to early S phase (left region of the BrdUrd-positive cells) remained nearly empty 24 h after toxin removal, while at 48 h a replenishment of this region can be observed only for the low dose treated cells, indicating a progressive recovery of cell cycle distribution. At the intermediate dose we can observe a progressive increase in the middle and late S regions. At the highest dose and at 48 h post-treatment a more significant delay in S phase traverse is demonstrated by an accumulation of cells in the middle S phase region without any significant increase, at this time, in the late S phase region, as shown in Figure 2.

SK-N-SH cells

In contrast to the results obtained for SK-N-MC cells, AFB1 treatment of SK-N-SH cells causes a dose-dependent decrease in cell ability to traverse S phase, as shown by the lower number of cells in S as compared with control cells (Figure 1b). This effect is more evident in the biparametric DNA/BrdUrd analysis (Figure 3). At the lowest dose (19.2 μM) AFB1 does not substantially modify the cell cycle distribution with respect to the control cells. However, at 80 μM we can observe, after the 24 h AFB1 treatment, a dramatic decrease in cells labelled by BrdUrd in the S phase region. At the same time, a consistently high number of unlabelled cells are present in a region corresponding to an intermediate content of DNA ('negative' S cells); this behaviour becomes more evident at the highest dose (160 μM) (Figure 1b) and indicates that cell progression through S phase is highly impaired. Thus cells remain in S phase, but their ability to synthesize DNA and to incorporate BrdUrd is blocked. In contrast to the toxin effect on SK-N-MC cells, in this case AFB1 treatment causes an arrest of cells in the G_1 and G_2 phases, as indicated by the substantially unmodified numbers of cells in G_1 and by the increase in G_2 cells.

These results suggest that in SK-N-SH cells AFB1 causes an activation of G_1 and G_2 checkpoints leading to a block of cell cycle progression. The presence of a fraction of cells with S phase DNA content, which, however, do not incorporate BrdUrd, indicates that in the presence of high doses of the toxin cells become unable to complete DNA synthesis and exit from S phase. The position of these cells on the right side of the S phase region suggests that they are arrested in late S at the S/G_2 transition.

HepG2 cells

Figure 1c shows the cell cycle distribution of HepG2 cells. In this instance AFB1 treatment induces a similar cell cycle perturbation to that observed for SK-N-SH cells, with a more evident dose-dependent effect (Figure 1c). In the histogram analysis we can observe a consistent number of cells with a DNA content intermediate between the peaks corresponding to the G_1 and G_2 phases. The analysis of S phase performed with BrdUrd (Figure 4) reveals that these cells have an S phase-like DNA content, but incorporation of BrdUrd is nearly absent, suggesting that, as observed in SK-N-SH cells, these cells are unable to complete S phase after toxin exposure. The increase in the G_2 phase cell population, with a corresponding decrease in the G_1 cell fraction, indicates a complete block of cell cycle progression, persisting after 48 h recovery.

[^{3}H]thymidine incorporation

The data reported in Figures 1–4 suggest that AFB1 brings about a persistent impairment to the resumption of DNA synthesis in SK-N-SH and HepG2 cells, while SK-N-MC cells appear to be capable of recovering from the block over a 48 h period after toxin exposure is stopped. In order to confirm these observations the ability of the cell lines to incorporate tritiated thymidine was estimated under the same experimental conditions as adopted for cell cycle analysis. As shown in Figure 5, a marked inhibition of [^{3}H]thymidine incorporation was observed for the three cell lines after 24 h exposure to the toxin. However 48 h after the toxin had been removed [^{3}H]thymidine incorporation in SK-N-MC cultures resumed, while in HepG2 and SK-N-SH cells no increase in thymidine incorporation was evident, with respect to the values observed at the end of toxin exposure. These data show the ability of SK-N-MC cells to resume DNA synthesis, otherwise absent in the other two cell lines.

Western blots of p53 and p21

In order to investigate whether the effects of AFB1 on progression through the cell cycle could be related to the expression of p21 and p53, we performed western blot analyses

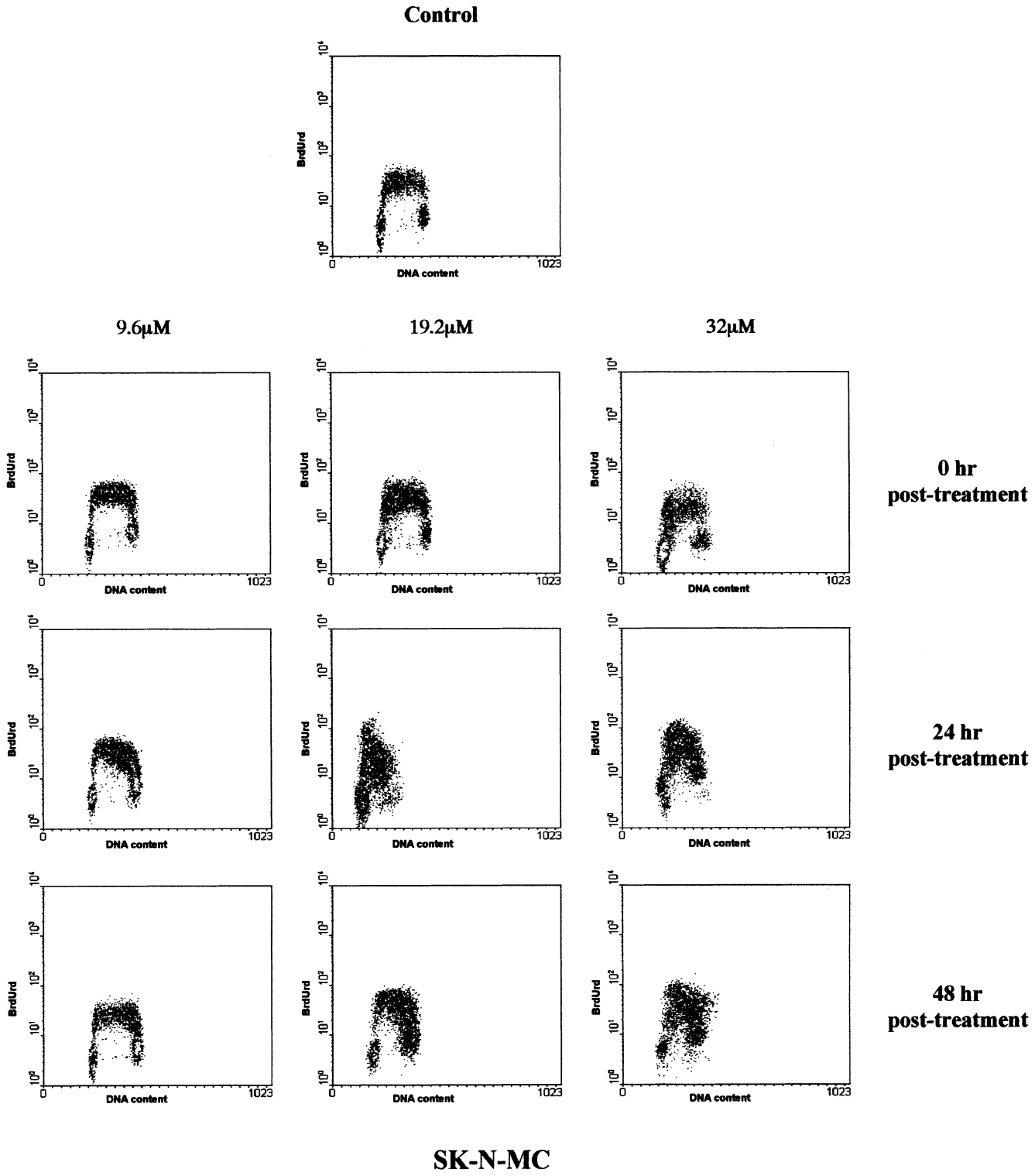

Fig. 2. Bivariate analysis of BrdUrd incorporation (ordinate) and DNA content (abscissa) in SK-N-MC cells at 0, 24 and 48 h after AFB1 treatment.

on total cell extracts. As shown in Figure 6, p21 could not be detected in either control or toxin-treated SK-N-MC cells. Similar results were obtained for the expression of p53, indicating the absence of this protein in SK-N-MC cell lysates. The absence of both proteins suggests a p21/p53 null phenotype for this cell line.

A similar analysis was performed on SK-N-SH cells. In this line AFB1 treatment leads to a time-dependent increase in the level of p21. We also observed a significant induction of p53 showing an increase from 0 to 48 h after AFB1 treatment.

The fluctuations of both proteins were also analysed in the HepG2 cell line, which expresses levels of p53 and p21 similar to those found in SK-N-SH cells.

These findings suggest that the lack of expression of proteins involved in control of the cell cycle, and more specifically in the control of DNA integrity, such as p53 and p21, may be

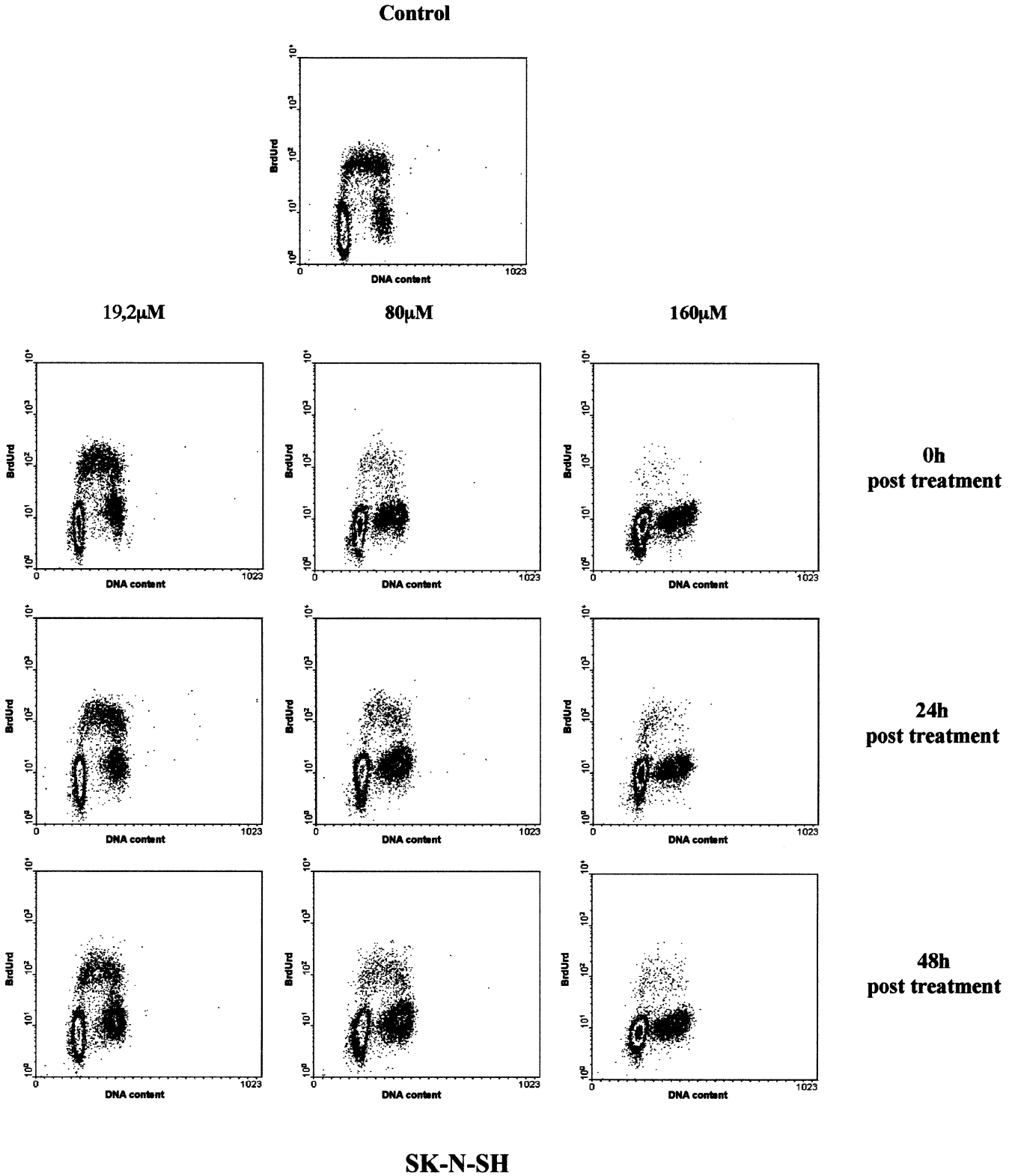

Fig. 3. Bivariate analysis of BrdUrd incorporation (ordinate) and DNA content (abscissa) in SK-N-SH cells at 0, 24 and 48 h after AFB1 treatment.

responsible for the higher ability of SK-N-MC cells to resume progression through the cell cycle when the toxin is removed, regardless of possible DNA damage caused by the toxin.

Discussion

DNA damage in mammalian cells leads to inhibition of cell cycle progression, in order to allow repair of damaged DNA before its replication. Thus arrest at the cell cycle checkpoints prevents accumulation of alterations in the DNA.

p53 is a key protein in the cell cycle regulation mechanism, depending on the physiological state of the cells, the cell type and possible DNA damage (Sanchez and Elledge, 1995). It is well known that DNA damage induces p53 expression and checkpoint activation, blocking cells in the G_1 phase so as

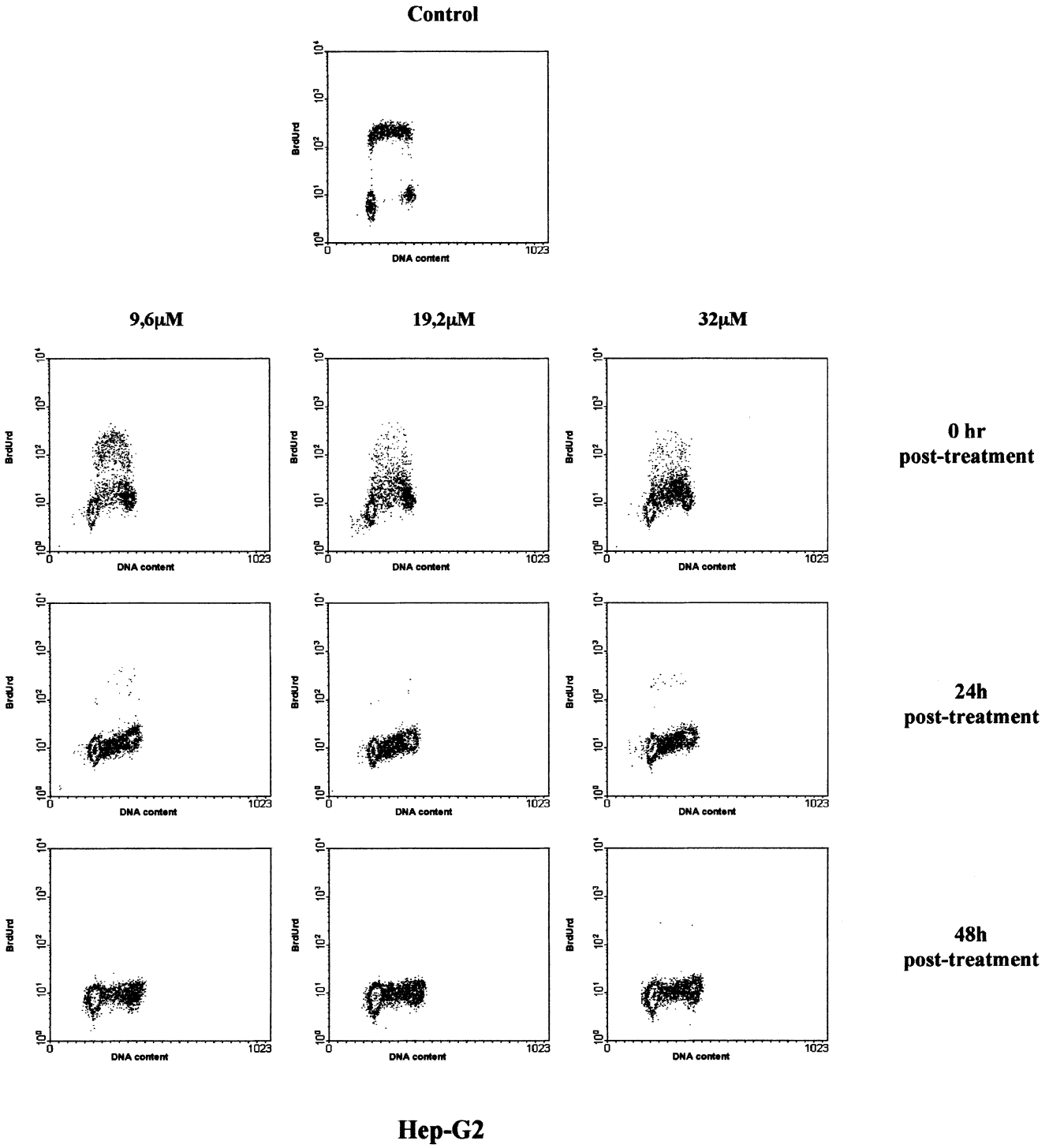

Fig. 4. Bivariate analysis of BrdUrd incorporation (ordinate) and DNA content (abscissa) in HepG2 cells at 0, 24 and 48 h after AFB1 treatment.

to extend the time available for DNA repair; alternatively, replication of damaged cells is avoided through activation of apoptosis (Guo and Hay, 1999; Lundberg and Weinberg, 1999).

In this paper we have provided evidence that the DNA-damaging agent AFB1 induces a cell cycle perturbation in two human neuroblastoma and a human hepatoma line, although their responses are somewhat different. The different response to AFB1 treatment can be related to differences in p53 and p21 expression in the cells.

SK-N-MC cells show a marked dose-dependent S/G_2 delay that, at the lower dose, is reversed after 48 h recovery. The biparametric cytofluorimetric analysis of S phase progression in these cells shows that after AFB1 treatment the cells are not completely blocked in their progression through S phase, although displaying a marked slowing down from medium to late S. It also shows that DNA synthesis can be resumed after the end of treatment. The presence of a consistent number of cells that incorporate BrdUrd indicates that the cells are able

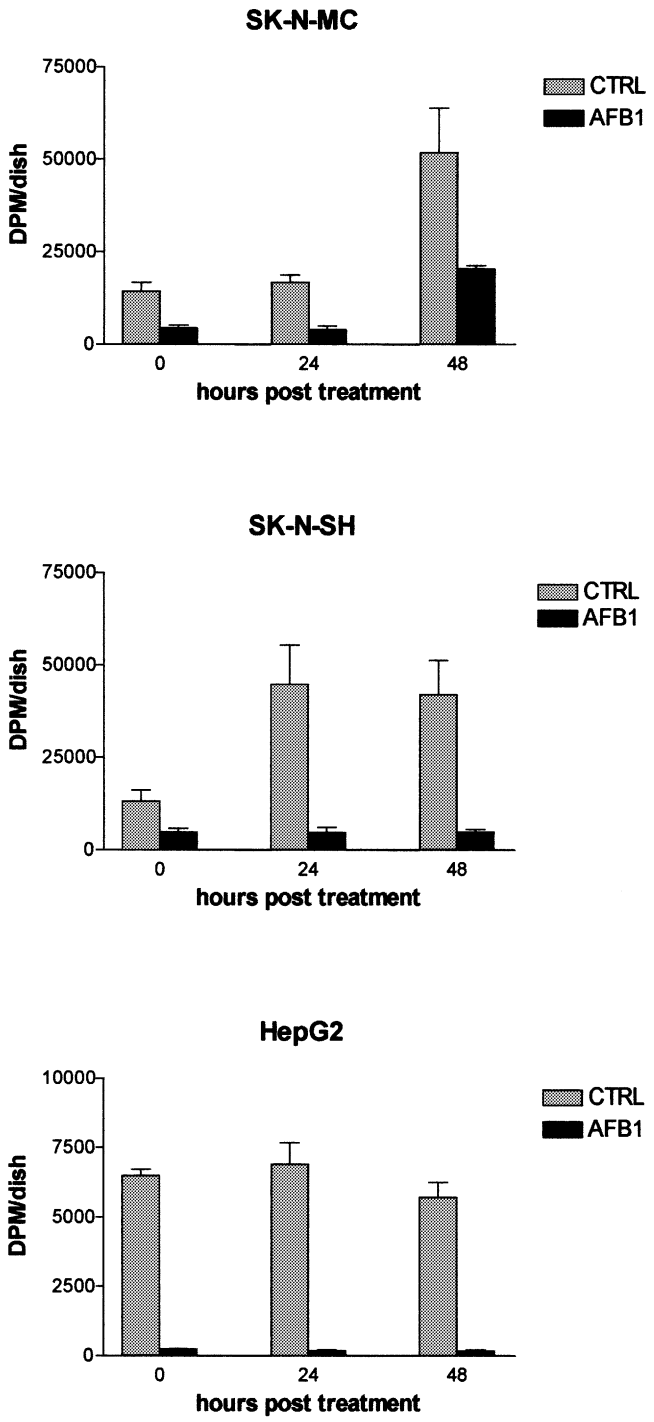

Fig. 5. [^{3}H]thymidine incorporation. [^{3}H]thymidine incorporated by SK-N-MC, SK-N-SH and HepG2 cells was determined as described in Materials and methods. Cultures treated without or with AFB1 (9.6, 19.2 and 40 μM, respectively, for HepG2, SK-N-MC and SK-N-SH) for 24 h were incubated with [^{3}H]thymidine for 2 h at the end of treatment (0 h) and 24 and 48 h after the end of treatment. The data are means ± SEM of three independent experiments. The statistical significance of differences between control and treated cells was determined by Student's *t*-test.

to continue DNA synthesis; in this case it is also evident that the cells accumulate in late S/G_2 phase and that the decrease in cells in G_1 confirms the ability of the cells to get through the G_1/S checkpoint.

The behaviour of SK-N-SH and HepG2 cells is significantly different. They show a substantially dose-dependent inhibition of cell cycle progression that is persistent 48 h after AFB1 was removed. Cell distribution in the cell cycle phases remains substantially unaltered over 48 h recovery and the number of cells exhibiting active BrdUrd incorporation decreases dramatically in a dose-dependent manner, BrdUrd-positive cells being nearly absent in HepG2 cultures exposed to the toxin. The presence of a cell population with a DNA content intermediate between G_1 and G_2, but not exhibiting active DNA synthesis, indicates the inability of these cells to progress through S phase. The persistent inability of HepG2

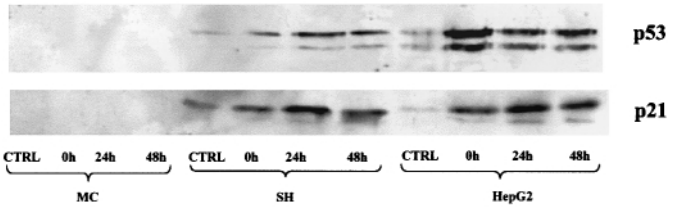

Fig. 6. Effects of AFB1 on p53 and p21 expression in SK-N-MC, SK-N-SH and HepG2 cells. Cell were grown for 24 h in the absence or presence of AFB1 at the following concentrations: 32 μM for SK-N-MC and HepG2 and 50 μM for SK-N-SH cells. Cells were collected 0, 24 and 48 h after toxin treatment. Equal amounts (5 μg/sample) of proteins were loaded in all lanes. Lanes 1, 5 and 9, control cultures; lanes 2, 6 and 10, end of treatment; lanes 3, 7 and 11, 24 h post-treatment; lanes 4, 8 and 12, 48 h post-treatment.

and SK-N-MC cells to resume DNA synthesis following a 24 h exposure to the toxin is confirmed by the [^{3}H]thymidine incorporation experiments (Figure 5).

S phase delay following DNA damage is related to a block of initiation of DNA replication and an inhibition of DNA elongation (Paulovich and Hartwell, 1995). The G_1/S phase transition is regulated by the activity of cyclin and cyclin-associated kinases; overexpression of p53 protein induced by DNA damage and the consequent increase in p21 transcription, which inhibits cyclin-associated kinase activity, thus delays the G_1/S transition and allows DNA repair prior to its replication. No or low expression of p53 may result in replication of damaged DNA, which in turn may lead to accumulation of genetic alterations in the cell and eventually to cell death.

The DNA-damaging action of AFB1 becomes manifest during DNA synthesis; thus disruption of the G_1/S checkpoint due to deficiency of the *p53* gene product may be responsible for the high cytotoxicity of AFB1 in SK-N-MC cells. Moreover, p53 might also play a role in the S/G_2 checkpoint, binding to the damaged DNA and facilitating DNA repair (Jayaraman and Prives, 1995; Smith *et al*., 1995); disruption of p53 function has also been reported in cells showing a G_2 checkpoint deficiency (Fan *et al*., 1995; Russel *et al*., 1995).

More recently, a further mechanism has been suggested by which p53 may play a role in the G_2 checkpoint (Passalaris *et al*., 1999). p53 induction in response to induced DNA damage leads to expression of GADD45 (Zhan *et al*., 1994, 1996), which *in vitro* destabilizes Cdc2/cyclin B complexes, suggesting possible inactivation of this G_2 checkpoint (Zhan *et al*., 1999). On the other hand, GADD45 and p21 appear to cooperate in inducing growth arrest (Dotto, 2000) and p21 interaction with PCNA by binding to its C-terminal domain causes an inhibition of S phase progression (Cayrol *et al*., 1998).

AFB1 has been reported to induce p53 expression in liver as a consequence of DNA damage (Van Gijssel *et al*., 1997). p53 induction blocks hepatocyte proliferation; at the same time preneoplastic cells, which may arise as a consequence of the toxin interaction with DNA, escape this block and may give rise to a cell population with altered growth control. On the other hand, AFB1 has been associated with *p53* mutations at codon 249 in hepatocellular carcinomas (Shimizu *et al*., 1999). It thus appears reasonable that the absence of p53 in SK-N-MC cells slows down DNA synthesis and cell progression through S phase, but the block of G_1/S progression is not activated and the cells can readily recover normal cycle progression, once the toxin has been removed. On the other hand, in the cell lines, where p53 is expressed, DNA damage caused by the toxin activates a G_1/S as well as G_2/M block and causes an irreversible accumulation of cells in S phase, although they are not capable of active DNA synthesis.

In conclusion, our study suggests that the S phase checkpoint dependent on p53 (which induces a DNA replication delay in the presence of DNA damage) may be responsible for the different abilities of the studied cell lines to recover from AFB1 treatment. It also suggests that the cytotoxic action of AFB1 may be due to its ability to interfere with the molecular mechanism of cell cycle regulation.

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