Apoptosis can be a confusing factor in *in vitro* clastogenic assays

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Among the tests used to determine the mutagenic potential of chemicals, the chromosomal aberrations and micronucleus assays play an important role. These tests score either chromosomal structural aberrations at metaphase or micronuclei at interphase. One of the hallmarks of apoptosis is DNA fragmentation into 50–300 kbp leading to oligonucleosomal fragmentation that can interfere with the results of clastogenic assays. In this case, apoptosis may be a confusing factor in the evaluation of the mutagenic potential of molecules and lead to false positive results. For these reasons we have developed a cell line able to demonstrate the interference of apoptosis in two mutagenicity tests: the *in vitro* micronucleus test and metaphase analysis *in vitro*. We used a murine cytotoxic T cell line, CTLL-2 Bcl2, in which a stably transfected *bcl2* gene is known to protect these cells from apoptosis induced by various stimuli. A comparison between results obtained in parental CTLL-2 cells and in CTLL-2 Bcl2 cells treated with non-genotoxic apoptosis inducers, such as dexamethasone or glutathione, leads us to conclude that apoptosis could give false positive results due to DNA fragmentation. Moreover, with etoposide, a clastogen that also induces apoptosis, we observed that the percentages of aberrant cells and numbers of micronuclei were significantly increased in CTLL-2 cells compared with CTLL-2 Bcl2 cells. This observation suggests that apoptosis leads to an overestimation of the genotoxic potential of chemicals. Finally, with nocodazole, an aneugen, we confirm that this model can also detect agents that have only genotoxic potential and thus allows a better estimation of the genotoxic threshold in studies with aneugens, thus avoiding overestimation of the mutagenic risk of such a compound.

Introduction

The development of new chemicals requires investigation of their mutagenic potential. Several tests are now routinely performed and are required for mutagenic risk assessment before approval by various regulatory agencies. These include the Ames assay, the *in vitro* gene mutation assay in eukaryotic cells, the *in vitro* chromosome aberration assay and the *in vivo* micronucleus test (ICH, 1997).

The process of repairing damaged DNA is of primary importance since DNA damage can be converted into mutations if the cell replicates before completing DNA repair (Moustacchi, 2000). Indeed, oxidants and reactive nitrogen species provoke the accumulation of mutations leading to metaplasia and dysplasia, particularly if these mutations occur in genes regulating cell division and the cell cycle (Correa and Miller, 1998). Cells respond to the burden of DNA damage by two mechanisms: DNA repair or apoptosis. The latter process removes cells with damaged DNA from the pool of replicating cells, avoiding the introduction of mutations into the genome and the associated risk of cancer (Roberts et al., 1997; Correa and Miller, 1998). Cell death may be genetically controlled and the ability of a cell to survive or die is determined by the repertoire of gene products, i.e. *p53*, *bax*, *bcl2* and *bcl-xL* (Denis et al., 1998). Their differential expression is a critical determinant of cell and tissue sensitivity to a toxicant (Roberts et al., 1997).

Apoptosis is a form of cell death occurring under physiological conditions or in response to external stimuli, such as DNA-damaging agents, growth factor deprivation or death receptor triggering (Nagata, 1997; Ashkenazi and Dixit, 1998; Evan and Littlewood, 1998; Green and Reed, 1998). Apoptosis is characterized by biochemical features including the activation of cysteine proteases named caspases, mitochondrial permeability transition, cell membrane exposure of phosphatidylserine and DNA cleavage leading to the typical morphology of apoptotic cells, in which the nucleus appears condensed and fragmented. In most cell types DNA cleavage occurs after irreversible activation of endonucleases. An initial cleavage of DNA into 50–300 kbp induces chromatin condensation and in most cell types oligonucleosomal fragmentation follows due to double-stranded cleavage of DNA in the linker region of nucleosomes (Saraste and Pulkki, 2000).

During the process of apoptosis and at the stage of chromatin condensation the original nucleus splits into a number of dense micronuclei, scattered throughout the cytoplasm (Di Baldassarre et al., 2000). These micronuclei generally appear surrounded by a double membrane system, externally outlined by ribosomes. The functional role of these micronuclei is still unknown, but it is generally accepted that they contain sequestered inactive genetic material (Columbaro et al., 1998). Consequently, in the *in vitro* micronucleus test a possible problem is that the very early steps of chromatin condensation due to apoptosis are not easily distinguishable from micronuclei induced by chemicals using Giemsa staining. Moreover, Simkó et al. (1998) showed in human cell lines that the increase in apoptotic cells is positively correlated with the appearance of micronuclei. Pom et al. (1999) have observed that proliferating lymphocytes undergoing apoptosis after saporin treatment showed DNA damage during the first metaphase, after which the cells die and no metaphases are observable.

A number of genes have been discovered that are involved in apoptosis, such as those belonging to the *bcl2* family. The
family of Bcl2-related proteins constitutes one of the most biologically relevant classes of apoptosis regulatory gene products acting at the effector stage of apoptosis, with some members functioning as suppressors of apoptosis and others as promoters of cell death (Basu and Haldar, 1996; White, 1996; Kroemer, 1997; Reed, 1997). The relative ratio of these pro- and anti-apoptotic members of the Bcl2 family (Oltvai et al., 1993; Yang and Korsmeyer, 1996) has been shown to determine the ultimate sensitivity or resistance of cells to diverse apoptotic stimuli, including chemotherapeutic drugs, radiation, growth factor deprivation and hypoxia (Selvakumaran, 1994; Kitada et al., 1996).

The properties of Bcl2 were utilized in two murine lymphocyte cell lines: CTLL-2 and CTLL-2 Bcl2. In the latter the apoptosis inhibitor protein Bcl2 is overexpressed after stable transfection. Its capacity to promote cell survival is associated with the p53-sensitive apoptosis inhibitor protein Bcl2 is overexpressed after stable transfection. Its capacity to promote cell survival is associated with CTLL-2 or CTLL-2 Bcl2 cells (75 000 cells/ml) cultured in complete RPMI medium containing 25 pg/ml IL-2 (a concentration that avoids apoptosis due to IL-2 deprivation for the duration of the assay; Singh et al., 1994). The cells were centrifuged for 15 h with the compounds at different concentrations. Cells were harvested after centrifugation for 5 min at 200 °C and resuspended in a hypotonic solution (1 vol. RPMI 1640, 0.6 vol. water, 2% FCS) for 5 min. The cells were centrifuged for 5 min at 200 g and fixed with 10 ml of Carnoy II fixative mixture (3 vol. ethanol, 1 vol. acetic acid) for 10 min. After another centrifugation the cells were spread on slides and stained with Giemsa dye (Sigma) diluted at 5% in water. Micronucleated cells were scored in at least 1000 cells/culture of three parallel cultures (3000 cells/dose) at 500× magnification. The criteria for micronucleus determination are that the intensity of stained micronuclei is inferior to the principal nucleus, its diameter is inferior to the principal nucleus, it is round with a nuclear membrane, it is not connected to the principal nucleus, there is no overlap with the principal nucleus and it is within the cytoplasm.

In vitro micronuclear test

CTLL-2 or CTLL-2 Bcl2 cells (75 000 cells/ml), cultured in complete RPMI medium containing 25 pg/ml IL-2, were treated for 15 h with the compounds at different concentrations. Cells were harvested after centrifugation for 5 min at 200 g and resuspended in a hypotonic solution (75 mM KCl at 37°C) for 5 min. Cells were centrifuged for 5 min at 200 g and fixed with 10 ml of Carnoy I fixative mixture (3 vol. methanol, 1 vol. acetic acid) for 12 min. After a further centrifugation the cells were spread on slides and stained with Giemsa dye (Sigma) diluted at 5% in water. Micronucleated cells were scored in at least 1000 cells/culture of three parallel cultures (3000 cells/dose) at 500× magnification. The criteria for micronucleus determination are that the intensity of stained micronuclei is inferior to the principal nucleus, its diameter is inferior to the principal nucleus, it is round with a nuclear membrane, it is not connected to the principal nucleus, there is no overlap with the principal nucleus and it is within the cytoplasm.

Materials and methods

Cell lines and culture conditions

CTLL-2 is a subclone of cytoxic T lymphocytes from mouse strain C57Bl/6. The cells were routinely cultured in complete RPMI 1640 medium (Gibco BRL, Paisley, UK) containing 10% foetal calf serum (FCS) (Gibco BRL) heat-inactivated for 30 min at 56°C, 20 mg/ml sodium pyruvate (Sigma-Aldrich Chemical Co., l’Ile d’Abeau Chesnes, France), 2 mM l-glutamine (Gibco BRL), 2 mM HEPES (Sigma), 100 U/ml penicillin (Gibco BRL), 0.1 mg/ml streptomycin (Gibco BRL) and 5×10⁻³ M β-mercaptoethanol (Merck, Nogent-sur-Marne, France) supplemented with 1 mg/ml interleukin-2 (IL-2) (Chiron, France).

The CTLL-2 Bcl2 cell line was produced by stable transfection of CTLL-2 with the pSFFV neo Bcl2 plasmid (a kind gift of Dr S.Korsmeyer) containing a 1.9 kb EcoRI insert encoding the human Bcl2 protein downstream of the SFFV promoter and resistance genes to ampicillin and gentamicin (Di Baldassarre et al., 2000). Briefly, CTLL-2 cells were electroporated with 10 µg plasmid using a Bio-Rad gene pulser set at 250 V and 960 µF. CTLL-2 Bcl2 cells were selected in complete medium containing 800 µg/ml G418 (Gibco) for 2 weeks and cloned by limiting dilution. Expression of Blc2 (reaching 90.2%) was measured by intracellular staining using anti-human Bcl2 (Dako, France) labelled with FITC and flow cytometry. Both cell lines were cultured in a humidified incubator containing 5% CO2 at 37°C.

Compounds

Dexamethasone, glutoxin, etoposide and nocardazole were purchased from Sigma (Sigma-Aldrich Chemical Co.), methane methylsulphonate (MMS) was purchased from Aldrich. The test compounds were dissolved in DMSO (final concentration not exceeding 0.2%) and stored at −20°C. The first two compounds are not carcinogens in humans whereas the others are possible carcinogens (class 2B in the IARC classification).

These compounds were studied up to concentrations inducing apoptosis and/or cytotoxicity (measured by Trypan Blue exclusion) in >50% of the cells according to OECD guidelines (OECD, 1997).
Apoptosis in _in vitro_ clastogenic assays

**Fig. 1.** Electrophoresis of extracted DNA from CTLL-2 cells treated for 15 h with 50 and 100 nM dexamethasone and CTLL-2 Bcl2 cells treated with 50, 100 and 200 nM dexamethasone. DX, dexamethasone; MW, molecular weight.

overnight at 65°C with proteinase K (1.25 mg/ml), 10× proteinase K buffer (100 mM Tris, 100 mM EDTA, 250 mM NaCl) and extraction buffer. The proteins were extracted with water-saturated phenol. The aqueous phase was harvested and phenol was eliminated with chloroform:isoamyl alcohol (24:1).

**Fig. 2.** Effect of dexamethasone on CTLL-2 and CTLL-2 Bcl2 cells. The DNA from the aqueous phase was precipitated with 1/10 vol. 3 M sodium acetate and 2 vol. cold absolute ethanol for 1 h at −80°C. The DNA was washed in cold absolute ethanol and dissolved in extraction buffer. DNA (10 µg) was separated electrophoretically at 30 mA for 2–3 h on a 1.2% agarose gel containing 0.5 µg/ml ethidium bromide and visualized with UV light.

**Statistical analysis**

Statistical analysis of the micronucleus test results was performed by analysis of variance (ANOVA) and multiple comparisons were made by Dunnett analysis (Statview v.4.0) to assess where the difference in the dependent variable arose in each cell line. The comparisons between pairs of groups for each dose in the two cell lines were made by Student’s _t_-test. The two types of aberrations (gaps and breaks) were analysed using Student’s _t_-test, because although the standard deviations found did not demonstrate that the distribution was normal, this test can be used because of the large number of samples (100 cells/culture/dose). In the figures the values for number of micronucleated cells differ significantly from the corresponding control value (_P_ < 0.05, _bP_< 0.01) and the number of micronucleated CTLL-2 cells differs significantly from the corresponding CTLL-2 Bcl2 value at the same dose (_P_ < 0.01).

**Results**

**Expression of p53 in CTLL-2 and CTLL-2 Bcl2 cells**

Apoptosis can occur by a _p53_-dependent pathway as well as by a _p53_-independent pathway (Vogt Sionov and Haupt, 1999). Therefore, it is important to verify that CTLL-2 cells express this protein.

Genotoxins are known to induce _p53_ expression in order to control the cell cycle (Yang and Duersken-Hugues, 1998). CTLL-2 and CTLL-2 Bcl2 cells were treated with 10 and 100 µM MMS, an alkylating agent known to induce _p53_ expression (Zhan _et al._, 1993), and were examined by western blotting for expression of _p53_ and its induction by genotoxic stress. The results showed that wild-type _p53_ is normally present in the two cell lines without genotoxic treatment and its expression is increased by treatment with MMS in both cell lines.

**DNA fragmentation assay**

We first verified that in CTLL-2 cells DNA fragmentation could be seen when apoptosis occurs in this model. DNA electrophoresis from CTLL-2 cells treated with dexamethasone showed a typical DNA ladder, indicating that oligonucleosomal fragmentation was present (Figure 1). However, no DNA fragmentation was present when CTLL-2 Bcl2 cells were treated with dexamethasone, demonstrating that overexpression of Bcl2 in CTLL-2 cells protects them from apoptosis (Figure 2).

**Evaluation with compounds inducing apoptosis in CTLL-2 cells**

To assess whether apoptosis can lead to chromosome and chromatid breakage we compared the results obtained in parental CTLL-2 cells with those obtained in CTLL-2 cells stably transfected with the apoptosis inhibitor gene _bcl2_ and treated with either dexamethasone or gliotoxin, which are apoptotic but not genotoxic compounds. Two tests were used: the _in vitro_ micronucleus test and metaphase analysis. Apoptosis was evaluated with Annexin V and YOPRO-1 staining, which quantify two different outcomes of apoptosis.

In the _in vitro_ micronucleus test we observed a dose-
Table I. Effects of dexamethasone, gliotoxin and etoposide on the frequencies of chromosome and chromatid-type aberrations in CTLL-2 cells

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aP < 0.05 versus control by Student’s t-test.
bP < 0.01 versus control by Student’s t-test.
cNot interpretable.

Table II. Effects of dexamethasone, gliotoxin and etoposide on the frequencies of chromosome and chromatid-type aberrations in CTLL-2 Bcl2 cells

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aP < 0.05 versus control by Student’s t-test.
bP < 0.01 versus control by Student’s t-test.
cNot interpretable.

dependent increase in the number of micronucleated cells in CTLL-2 cells treated with dexamethasone, with maxima of 38.3 and 39.6% at 75 and 150 nM, respectively. This increase was statistically significant at doses of 18.75 nM and higher (Figure 2A). Similar results were obtained in the in vitro metaphase analysis, where dexamethasone significantly increased the percentage of aberrant cells at doses of 37.5 nM and higher (Figure 2A). The percentage of aberrant cells was reduced at the highest dose of dexamethasone used (150 nM). This effect could be the consequence of the anti-proliferative action of glucocorticoids. Chromosome and chromatid breaks, but never chromatid exchanges, were observed following dexamethasone treatment (Tables I and II). However, when CTLL-2 Bcl2 cells were treated with dexamethasone we did not observe an increase in micronucleated cells or aberrant cells at any of the doses tested (Figure 2A and B). Gliotoxin, another apoptosis inducer, was studied at a range of doses from 25 to 200 nM (Figure 3). In the in vitro micronucleus test gliotoxin was positive at 100 and 200 nM in CTLL-2 cells (P < 0.01, Figure 3A) whereas in the
metaphase analysis test a statistically significant difference was present at >50 nM (Figure 3B). Concentrations in the metaphase analysis assay were not tested over 50 nM in CTLL-2 cells due to the absence of mitosis. Apoptosis was present at 100 and 200 nM using either Annexin V–FITC staining or the YOPRO-1 assay (Figure 3C and D). Again, when CTLL-2 Bcl2 cells were treated with gliotoxin neither micronuclei, aberrant cells nor apoptosis were present at a significantly higher level compared with control cells at concentrations up to 200 nM (Figure 3A–D).

These studies using compounds known to induce apoptosis but devoid of genotoxicity demonstrated: (i) micronucleated cells or aberrant cell number did not increase following treatment with either dexamethasone or gliotoxin in CTLL-2 Bcl2 cells whereas they did increase in parental CTLL-2 cells; (ii) the presence of micronucleated cells or aberrant cells was correlated with the occurrence of apoptosis.

**Evaluation with compounds inducing both genotoxicity and apoptosis in CTLL-2 cells**

We compared the results obtained in the two murine cell lines with compounds that are both genotoxic and apoptotic to test whether apoptosis could lead to an overestimation of the genotoxic potential of chemicals using mutagenicity tests.

Etoposide, studied in a range of concentrations from 62.5 to 500 nM, induced a dose-dependent increase of the number of micronucleated cells in both CTLL-2 and CTLL-2 Bcl2 cells (Figure 4A). This effect was also observed in the metaphase analysis test, with a dose-dependent increase in the percentage of aberrant cells in both cell lines. This increase was statistically significant from 62.5 nM in CTLL-2 cells and from 125 nM in CTLL-2 Bcl2 cells (Figure 4B and Tables I and II). Apoptosis was present in CTLL-2 cells treated with etoposide, with 29% apoptotic cells at the higher dose, but not in CTLL-2 Bcl2 cells, suggesting that etoposide provoked genotoxicity independently of apoptosis. However, the numbers of micronucleated cells and the percentages of aberrant cells were always significantly higher in parental CTLL-2 cells compared with CTLL-2 Bcl2 cells. This difference is probably the consequence of DNA fragmentation during apoptosis that occurs in parental CTLL-2 cells leading to higher numbers in both tests compared with CTLL-2 Bcl2 cells.

It is interesting to note that with the non-genotoxic apoptosis inducers (dexamethasone and gliotoxin) chromosome breaks but no exchanges were observed (Tables I and II). In contrast, with etoposide chromosome breaks and exchanges were observable. On the one hand, this difference arises from the difference in primary DNA damage. On the other hand, this difference is due to the fact that in apoptotic cells no DNA repair occurred while in cells with primary DNA damage repair occurred.
leading to chromosome exchanges. In practice, a high level of breaks without exchanges induced by a test compound could alert one to suspect a role of apoptosis in breakage induction.

The aneugenic molecule nocodazole, studied over a range of doses from 40 to 100 nM, was also able to induce both an increase in the number of micronucleated cells (354/1000 at the highest concentration) and in the percentage of apoptotic cells (22% at the highest concentration) in parental CTLL-2 cells (Figure 5). In CTLL-2 Bcl2 cells nocodazole did not induce apoptosis but significantly increased in a dose-dependent manner the number of micronucleated cells (Figure 5A), leading to a statistically significant difference in the number of aberrant cells between the two cell lines. No metaphase analyses were conducted since the OECD guidelines (OECD, 1997) note that this assay is not pertinent to the study of aneugenic compounds. Again, like etoposide, the number of micronucleated cells was higher in parental cells compared with CTLL-2 Bcl2 cells, suggesting that apoptosis participates in the production of micronucleated cells in the parental CTLL-2 line (Figure 5A).

**Discussion**

The overestimation of chromosomal aberrations due to interference of apoptotic DNA fragmentation is a problem previ-}

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to conclude a genotoxic effect for the compounds, which constituted false positive responses.

We then addressed the question whether apoptosis can lead to an overestimation of the genotoxic potential of a molecule assessed by mutagenicity assays. Cells were treated with etoposide, a clastogenic agent that is also an apoptosis inducer. Etoposide leads to apoptosis by a p53-dependent pathway and recent studies suggest that activation of tyrosine kinases plays an important role in the apoptotic process induced by etoposide (Usami et al., 1998) as well as by topoisomerase II inhibition (Ferguson, 1998). Moreover, Kamesaki et al. (1993) showed that Bcl2 inhibits apoptosis induced by etoposide. Etoposide induced apoptosis, micronucleus formation and structural aberrations in CTLL-2 cells, whereas in the transfected cell line these anomalies were fewer, but still present. Moreover, metaphase analyses and micronucleus tests demonstrated that apoptosis could also give excessive positive results with genotoxic agents and apoptosis inducers. In the case of indirect mechanisms of mutagenesis it was proposed that we can determine a threshold of activity (Henderson et al., 2000; Müller and Kasper, 2000). As etoposide acts by topoisomerase inhibition it should therefore have a threshold for mutagenic activity. In the results of the clastogenicity assay apoptosis leads to an overestimation of the genotoxic effect and lowers the threshold. Under these conditions the results can lead to a false overestimation of genotoxic risk.

The induction of aneuploidy by spindle inhibitors produces a characteristic dose–response curve including a threshold (Singh et al., 1994; Kirsh-Volders et al., 2000). We noted this phenomenon in the studies of nocodazole: a statistically significant difference from the control was observed in CTLL-2 cells with 40 nM compound, whereas the threshold was shifted to 55 nM compound in the transfected cell line. Therefore, the lowest effective dose was different in the apoptotic versus non-apoptotic lines because apoptosis lowers this value. This indicates that suppression of the apoptotic effects permitted a better evaluation of the threshold value to take account of biologically relevant events and thus not overestimate the risk for the compound tested.

Since transfection with bcl2 diminished and even abolished the apoptotic component, the model developed can be envisaged as a preliminary short-term screening assay to detect true genotoxic compounds at an early stage of their development. The micronucleus test in these cells has good specificity: indeed, the two apoptosis inducers (dexamethasone and glictoxin) gave negative results in terms of genotoxicity in CTLL-2 Bcl2 cells. It also has good sensitivity, since the two genotoxic compounds (etoposide and nocodazole) gave positive results in the two cell lines.

In order to validate the model, other aneugenic or clastogenic compounds will need to be studied. This will allow us to confirm the ability of the transfected cells to minimize the apoptotic factor observed in determination of the genotoxic potential, even if not all the apoptotic compounds induce a pathway controlled by Bcl2.

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References

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