

Analysis of Cell Cycle Disruptions in Cultures of Rat Pleural Mesothelial Cells Exposed to Asbestos Fibers

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The control of DNA integrity in mammalian cells is important to maintain the cell homeostasis and prevent neoplastic transformation. Control of cell division and cell death permits repair or elimination of damaged cells. Since asbestos fibers can produce DNA damage, chromosome alterations and apoptosis in several sorts of cells, including mesothelial cells, it was interesting to investigate cell cycle disturbances in rat pleural mesothelial cells (RPMC) treated with asbestos fibers. Cell cycle analyses were performed in RPMC exposed to crocidolite (10 and 20 $\mu\text{g}/\text{cm}^2$) and chrysotile (5 and 10 $\mu\text{g}/\text{cm}^2$) for different times (4 to 48 h). Both fiber types entailed a G2/M accumulation in agreement with a delay in the mitosis course. Chrysotile fibers produced a G0/G1 accumulation associated with a time-dependent p53 and p21 expression. Crocidolite exposure resulted in a delay in the G1/S transition paralleling a low rate of p53 expression. These results are in agreement with a DNA damaging potential of asbestos fibers since similar results were found following RPMC exposure to γ rays. In asbestos-treated RPMC, a low rate of apoptosis was found suggesting that RPMC may follow a DNA repair pathway that could contribute to the formation of DNA lesions. In addition, the cell cycle disturbances at the G2/M checkpoint suggest that genetically altered cells have progressed through the cycle and support the already published findings on the ability of asbestos fibers to impair cell division. Levresse, V., A. Renier, J. Fleury-Feith, F. Levy, S. Moritz, C. Vivo, Y. Pilatte, and M.-C. Jaurand. 1997. Analysis of cell cycle disruptions in cultures of rat pleural mesothelial cells exposed to asbestos fibers. *Am. J. Respir. Cell Mol. Biol.* 17:660-671.

Human exposure to asbestos fibers produces malignant mesothelioma, a primary tumor due to the neoplastic transformation of mesothelial cells. The interactions between mesothelial cells and asbestos fibers are complex and the mechanisms by which asbestos produce cell damage are still a matter of controversy. Mesothelial cells exposed to asbestos *in vitro* exhibit a wide range of molecular and cellular changes. These include the release of inflammatory factors (1, 2), the production of reactive oxygen derivatives, the induction of several genes (c-Fos, c-Jun, NF κ B), DNA and chromosome damage (3-6) and neoplastic transformation (7). In previous studies, we found that asbestos induced a dose-dependent reduction in cell pro-

liferation, as shown by an increase in the mean population doubling time (8). In terms of regulation of cell proliferation, this means that the cell cycle progression is blocked and/or that some cells are engaged in the cell death pathway. Since DNA damage, cell proliferation and cell death are important processes in carcinogenesis, it is of great importance to understand the molecular events associated with the cellular changes induced by asbestos fibers in mesothelial cells.

Several studies have reported that, in many cell types, alterations in cell cycle progression occur after exposure to DNA damaging agents. This is likely to permit DNA repair before the continuing of DNA synthesis or mitosis. Delays take place at both G1 and G2 phases of the cell cycle (9). Failure in repair processes could result in fixation and propagation of genomic changes necessary for neoplastic transformation. Under certain circumstances, DNA damage may trigger a rapid programmed death of the cells, apoptosis, a process that has already been described in asbestos-exposed mesothelial cells (10, 11). This cell death is a useful process for eliminating highly damaged cells and avoiding propagation of abnormalities. Conversely, dysregulation of the cell response to apoptotic signals may be an important event in neoplastic transformation.

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Abbreviations: 5-bromo-2'-deoxyuridine, BrdUrd; May Grünwald/Giemsa, MGG; Rat pleural mesothelial cells, RPMC; SV 40 large T antigen, T SV40.

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A critical regulator of the cellular response to genotoxic stress is the transcription factor encoded by the p53 tumor suppressor gene (12, 13). Several studies suggest that the nuclear phosphoprotein p53 participates in the control of cell cycle progression following certain types of DNA damage; it could also be involved in the performance of some forms of apoptosis. Ionizing radiations, UV radiations, or chemotherapy can increase the nuclear p53 level (14). In many cell types, this increase correlates temporarily with an arrest of the irradiated cells in the G1 phase of the cell cycle (15, 16) or in the G2 phase (17, 18). Several experiments show that the wild type p53 protein can stimulate the transcription of some crucial genes such as the gene encoding for the p21/WAF1/CIP1, an inhibitor for cyclin-dependent kinase (19, 20). By a direct interaction with several cyclins and cyclin-dependent kinases, this protein inhibits the kinase activity and leads to a disruption of both initiation and progression through the S phase and mitosis course (21, 22). The p21/WAF1/CIP1 protein can also stop DNA synthesis by directly inhibiting some proteins involved in the replication machinery, such as the Proliferating Cell Nuclear Antigen (PCNA), a DNA polymerase δ auxiliary (23). P53 could be a determinant factor between DNA repair and elimination of damaged cells. Besides, the balance between DNA repair and apoptosis seems to be a key point in the understanding of cancer induction. In the present study, we observed cell cycle progression of RPMC treated with asbestos fibers and also determined the associated rate of apoptosis. We observed cell cycle disruptions at G1 and G2 phases in response to asbestos exposure and a low apoptosis yield. These responses correlated with the expression of p53 and p21 proteins.

Materials and Methods

Cells

Normal rat pleural mesothelial cells (RPMC) were obtained from rat parietal pleura according to a method described previously (24). They were cultured at 37°C in Ham's F10 medium (ATGC, Noisy le Grand, France) supplemented with 10% fetal bovine serum (FBS) (ATGC), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes) (Eurobio, Les Ulis, France), 100 IU/ml penicillin, 50 μ g/ml streptomycin (Eurobio). Confluent monolayers were routinely subcultured by detachment with a mixture of 0.25% trypsin (Eurobio) + 0.02% EDTA (Merck, Nogent sur Marne, France). Only diploid cells and cultures not older than 25 passages were used in this study. In some experiments, rat pleural mesothelial cells infected with a retroviral recombinant large T-antigen of SV40 were used as a reference (TSV40-RPMC) for p53 expression in Western blots. These cells were cultured as described above in RPMI 1640 medium (ATGC) supplemented with the same constituents as Ham's F10 medium.

Reagents, Drugs, Particles and Antibodies

Camptothecin was purchased from Sigma (La Verpillière, France). A stock solution (1 mg/ml) was made and kept frozen in dimethylsulfoxide (DMSO) (Sigma). Two types of asbestos fibers obtained from the Union Internationale Contre le Cancer (UICC) were used: Rhodesian chrysotile

and crocidolite. Mean length was $1.7 \pm 2.2 \mu\text{m}$ and $2.1 \pm 3.6 \mu\text{m}$, respectively (25). Fibers (1 mg/ml) were dispersed by sonication in complete Ham's F10 medium for 5 min (50 watts; Vibra cell, BioBlock, Illkirch, France) to permit a homogenous suspension of the fibers.

A monoclonal antibody against human p53 protein (HR 231) was purchased from Eurobio. It cross-reacted with rat p53 and was specific of the wild type p53. It was used at a final concentration of 2 $\mu\text{g/ml}$. Polyclonal antibodies against bcl2 protein (N-19) and p21/WAF1/CIP1 protein (M-19) were purchased from TEBU (Le Perray en Yvelines, France) and were used at a final concentration of 0.1 $\mu\text{g/ml}$.

Treatments

All treatments were performed with log phase RPMC. Camptothecin was used as a reference for a p53-dependent cell cycle arrest and apoptosis induction (26). RPMC were treated for 1, 4, 24, 48 and 72 h with 10 $\mu\text{g/ml}$ of camptothecin dissolved in DMSO. DMSO was added to control cell culture at the concentration used in the treated cells. This concentration never exceeded 1% and did not affect cell survival. Fibers were used at concentrations of 0 (untreated), 0.5, 2, 10 and 20 $\mu\text{g/cm}^2$; 48 h after seeding, the medium was discarded and replaced with the same medium containing fibers for 1, 4, 24, 48 and 72 h.

RPMC were γ -irradiated at a dose of 24 Gy (1.46 Gy/min) by exposure to a ^{60}Co source (Alcyon from General Electric Medical System, France).

Cell Cycle Analysis

Cells were seeded in 75 cm^2 flasks (Costar, Brumath, France) in 12 ml of complete Ham's F10 medium. Forty-eight hours after seeding, cells were treated as described above in 10 ml of medium. The study of cell cycle progression was performed according to the method of Dolbeare and associates (27). Asbestos- or camptothecin-treated cells were incubated in a medium containing 18 $\mu\text{g/ml}$ of bromodeoxyuridine (BrdUrd) (Sigma) for different lengths of time. After fixation at 4°C in 70% ethanol, cells were washed and resuspended in 2 M HCl for 30 min at room temperature. After washing, cells were incubated with BrdUrd antibodies (Dako, Trappes, France) for 1 h at room temperature and then stained with propidium iodide for 1 h. Flow cytometry analysis was made with a Coulter (Margency, France) Epics XL W05039 cytometer. This method allows the determination of parameters representing the proportion of both cycling and noncycling cells, that is BrdUrd incorporated into cells in the S phase and, depending on the duration of BrdUrd incorporation and rate of cell progression, distributed into G2/M and G0/G1. Propidium iodide incorporation allows us to assign the cells to a 2N, 2N to 4N, or 4N compartment. Data were analyzed according to the Multicycle analysis software distributed by Coulter. Statistical analysis was performed using a variance analysis followed by a two-tailed Student's test. The percentages of treated and untreated cells in each population (replicative phase or G2/M phase) were compared.

Apoptosis Analysis

Analysis of DNA cleavage. Forty-eight hours after seeding in 75- cm^2 flasks, cells were treated as described above

in 10 ml of complete Ham's F10 medium. After different lengths of treatment, the cells were washed in PBS (Gibco, Cergy Pontoise, France), trypsinized, resuspended in STE buffer (NaCl: 0.1 M, Tris HCl: 10 mM, EDTA: 1 mM) with 0.1% SDS 10% and 200 µg/ml of proteinase K (Boehringer Mannheim, Meylan, France) and incubated for 3 h at 56°C. DNA was extracted with a phenol/isoamyl alcohol mixture (Fluka, Saint Quentin Fallavier, France) and precipitated at -20°C with ethanol and 3 M sodium acetate. The DNA pellet was resuspended in TE buffer, pH = 7.4 (Tris HCl: 10 mM, EDTA: 1 mM). The characterization of DNA cleavage was then achieved by submitting the samples to an electrophoresis on 1.5% agarose gel in TBE buffer (Tris base: 2 M, Boric acid: 2 M, EDTA: 0.01 M).

Since DNA damage could occur following the incubation of asbestos fibers with DNA (28), we investigated DNA stability in the presence of asbestos fibers by incubating 5 µg of salmon sperm DNA with 30, 125 or 1,000 µg/ml of fibers in TE buffer, at 37°C, i.e., under the same conditions as for cell DNA extraction. After extraction, an electrophoresis on 1.5% agarose gel was performed to determine whether strand breakage of DNA had occurred. Furthermore, the low molecular weight DNA fragments were loaded onto 1.5% agarose gel in presence of crocidolite fibers to determine whether the presence of fibers would impair DNA migration. For this purpose, low molecular weight markers were incubated in TE buffer with fibers (30, 125 or 1,000 µg/ml) at 37°C for 48 h to mimic the DNA migration conditions in asbestos-treated cells.

May Grünwald/Giemsa (MGG) staining. RPMC were seeded on 9 cm² slide flasks (Costar, Brumath, France) in 3 ml of complete Ham's F10 medium. Forty-eight hours after seeding, cells were treated as described previously. After different lengths of time of treatment, cells were fixed in methanol/acetic acid (3:1 vol:vol), stained in May Grünwald and in 3% Giemsa (CML, Nemours, France). Observation was made by light microscopy on 2,000 cells per slide. Four slide flasks were analyzed per treatment. Statistical analysis was performed using a variance analysis followed by a two-tailed Student's test.

Viability Test: MTT

This test was performed according to Mosmann's method (29). Cells were seeded in 96-well cell culture plates (Costar) at a concentration of 7,000 cells/ml. When cells reached confluence, treatments with asbestos fibers were performed under the same conditions as for the other analyses. At the end of treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Sigma) was added at a final concentration of 0.4 mg/ml and culture plates were incubated at 37°C for 3 h. The supernatant was then carefully removed and 200 µl of DMSO were added to dissolve the reduced MTT. The amount of reduced MTT was determined by the measurement of absorbance (A) at 540 nm. For each treatment, 8 wells were analyzed and 4 independent experiments were carried out. The results are expressed as loss of cell viability in comparison with the untreated control:

$$\frac{(A \text{ untreated} - A \text{ treated})}{A \text{ untreated}} \times 100$$

Statistical analysis was performed using a variance analysis followed by Student's *t* test.

Protein Extraction and Western Blotting

Western blot analysis was carried out on cytoplasmic and nuclear fractions as well as on total cell extracts.

Total protein extraction. After treatments, cells were washed with PBS and lysed by scraping in the lysis buffer: 1% NP40 (Nitrophenoxy 40, used as a detergent), 10 mM EDTA, 25 µM NPGB (pNitro-guanidinobenzoate), used as protease inhibitors, in PBS 1X. After an incubation on ice, cells were homogenized by using a loose fitting glass-
teflon homogenizer and centrifuged at 16,000 × *g* for 15 min. Protein content was determined in the supernatant by Lowry's method (30) (Kit Biorad, Ivry/Seine, France).

Nuclear and cytoplasmic protein extraction. After washing in PBS, cells were scraped in a sucrose buffer (0.25 M sucrose, 3 mM imidazole, 1 mM EDTA) and then homogenized as described above. After centrifugation at 1,500 rpm for 15 min, the cytoplasmic protein content was determined in the supernatant. The pellet which contained nuclei and unbroken cells was resuspended in a lysis buffer and centrifuged at 12,000 × *g*, for 15 min prior to protein measurement.

Electrophoretic procedures. Proteins (25–35 µg) were electrophoretically analyzed on a 10% (for p53 analysis) or 14% (for bcl2 and p21 studies) polyacrylamide discontinuous mini slab gels in a mini Protean II Cell (Bio-Rad). The electrophoretic transfer of proteins from SDS-PAGE slab gels onto nitrocellulose membrane (Schleicher et Schuell-Cera Labo, Ecqueville, France) was performed in a mini transblot cell (Bio-Rad) for 1 h on ice at 250 mA, according to the method described by Towbin and associates (31). Prestained molecular weight markers (Bio-Rad) were used to assess transfer efficiency and determine the size of blotted proteins.

After transfer, the membrane was blocked 1 h at room temperature with 5% (wt/vol) skimmed milk in 0.05 M Tris buffer pH 7.4 containing 1 M PBS and 0.05% Tween-20 and then incubated with primary antibodies overnight at room temperature. Blots were developed using enhanced chemical luminescence (Amersham-Life Science, Les Ulis, France).

Results

Cell Cycle Analysis

Cell cycle analysis was performed using a double labeling method with BrdUrd and propidium iodide. Kinetics of BrdUrd incorporation, from 30 min until 9 h, gave a dynamic view of the cycle progression. The dot plots allow the quantification of the replicating cells, i.e., cells incorporating BrdUrd. The quantification of the G2/M cells is based on the analysis of the propidium incorporation results. The cell cycle distribution of untreated RPMC was studied under the same experimental conditions as asbestos-treated cells, i.e., at times corresponding to 4, 24, and 48 h of treatment. The percentage of cells in the replicative phase in untreated cultures decreased with time, indicating that the cells progressed toward confluence (Table 1). At 48 h, 10% of the cells were in the replicative phase. The

TABLE 1

Time-dependent percentage of cells in replicative and G2/M phases as determined on the basis of BrdUrd incorporation and propidium iodide content, respectively (mean $\pm$ standard deviation on 3 experiments)

Cell cycle phases	Agent	Concentrations	Duration of treatments (h)		
			4	24	48
Replicative	Chrysotile, $\mu\text{g}/\text{cm}^2$	0	35.4 $\pm$ 3.9	22.5 $\pm$ 2.0	10.5 $\pm$ 0.4
		5	32.6 $\pm$ 1.9*	11.5 $\pm$ 0.9 [†]	6.6 $\pm$ 0.7 [†]
		10	33.0 $\pm$ 2.1*	7.8 $\pm$ 1.8 [†]	1.5 $\pm$ 0.9 [†]
	Crocidolite, $\mu\text{g}/\text{cm}^2$	0	34.0 $\pm$ 4.6	19.4 $\pm$ 2.1	10.0 $\pm$ 0.5
		10	22.1 $\pm$ 7.1*	14.8 $\pm$ 4.0*	9.7 $\pm$ 0.6*
		20	29.0 $\pm$ 1.3*	14.9 $\pm$ 0.8*	8.8 $\pm$ 0.9*
	Camptothecin, $\mu\text{g}/\text{ml}$	0	33.2 $\pm$ 2.1	20.4 $\pm$ 2.1	nd [‡]
		10	38.7 $\pm$ 6.3*	0.6 $\pm$ 0.3 [†]	nd
G2/M	Chrysotile, $\mu\text{g}/\text{cm}^2$	0	15.7 $\pm$ 7.4	10.3 $\pm$ 1.1	7.0 $\pm$ 1.5
		5	15.6 $\pm$ 5.8*	27.8 $\pm$ 1.4 [‡]	33.2 $\pm$ 4 [‡]
		10	18.0 $\pm$ 4.5*	26.4 $\pm$ 3.2 [‡]	18.1 $\pm$ 7.6 [‡]
	Crocidolite, $\mu\text{g}/\text{cm}^2$	0	16.7 $\pm$ 2.1	9.9 $\pm$ 1.6	6.5 $\pm$ 0.9
		10	14.4 $\pm$ 6.1*	12.9 $\pm$ 5.6*	21.0 $\pm$ 2.4 [†]
		20	16.1 $\pm$ 7.1*	20.2 $\pm$ 3.0*	25.1 $\pm$ 6.5 [†]
	Camptothecin, $\mu\text{g}/\text{ml}$	0	20.6 $\pm$ 1.2	12.6 $\pm$ 3.7	nd [‡]
		10	3.0 $\pm$ 2.0 [‡]	0.5 $\pm$ 0.4 [†]	nd

* Not significant, [†] $P < 0.05$, [‡] not done.

percentage of G2/M cells accordingly showed a time-dependent decrease (Table 1).

Treatment with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile resulted in several disruptions of cell growth. Cell cycle analyses performed after 30 min of BrdUrd incorporation showed modifications suggesting disruptions in the cell cycle at two locations, G0/G1 and G2/M (Table 1). An alteration of the G1/S progression is suggested by the significant reduction in the amount of cells entering the replicative phase in comparison with untreated cells. A time-dependent reduction in the percentage of replicating cells was observed in both untreated and asbestos-treated RPMC, but it was greater in chrysotile-treated cells (76% between 4 and 24 h) than in untreated cells (36%). After 48 h of contact with 10 $\mu\text{g}/\text{cm}^2$ chrysotile, only 1.5% of the cells were engaged in the replicative phase. The microscopic observations illustrate that the decrease in the replicating cell population did not result from a confluent state in asbestos-treated cells. In contrast, the number of cells was much lower in asbestos-treated cells than in untreated cells as already described (8). To distinguish a cell cycle delay in G1/S transition and an arrest in the G1 phase, we increased the time of BrdUrd incorporation to 9 h. On that condition, if cells are only delayed in G1, they would further progress through G1/S. Thus, after 9 h, BrdUrd labeled cells would have been found. The dot plots presented in Figure 1 show that no BrdUrd labeled cells were detected after 48 h of treatment with chrysotile, confirming the induction of a blockade in the G1 phase.

Table 1 shows that the percentage of cells in G2/M phase was enhanced following chrysotile exposure in comparison with that of untreated cells, suggesting that chrysotile fibers were able to induce a G2/M delay. G2/M accumulation was also observed on the dot plots (Figure 1A);

it was associated with the appearance of cells with a DNA content greater than 4N. The microscopic observations indicated that this population corresponded to bi- or multinucleated cells (Figure 2).

In crocidolite-treated cultures, the proportion of replicating cells was slightly modified after 4 h of treatment in comparison with untreated cultures. A reduction in the percentage of cells in the replicative phase was observed, but it always remained lower than after chrysotile treatment. Moreover, it was attenuated with the length of treatment. When compared to untreated cells, the percentage of cells incorporating BrdUrd was reduced to 65, 76, and 97% after treatment with 10 $\mu\text{g}/\text{cm}^2$ for 4, 24, and 48 h, respectively (Table 1). The analysis of dot plots obtained after 9 h of BrdUrd incorporation (Figure 1B) showed that the cell cycle progression was not totally arrested. This suggests that crocidolite fibers induce a delay rather than an arrest in the transition between the G1 and S the phase. The propidium iodide incorporation data showed a time-dependent G2/M accumulation in crocidolite-treated cells. It was 1.3 and 3.2 times more than that of untreated cells after exposure to 10 $\mu\text{g}/\text{cm}^2$ of crocidolite for 24 and 48 h respectively (Table 1). The difference was significant after 48 h of treatment.

From data we previously obtained, the number of fibers in the chrysotile sample is 4 times higher than that of crocidolite: 22×10^6 versus 5.3×10^6 per μg , respectively. By comparing results obtained with 5 $\mu\text{g}/\text{cm}^2$ (110×10^6 fibers/ cm^2) of chrysotile to those obtained with 20 $\mu\text{g}/\text{cm}^2$ of crocidolite (106×10^6 fibers/ cm^2), we observed that even on a per number of fiber basis, the chrysotile sample presented a greater ability, in comparison with crocidolite, to induce cell cycle disturbances, especially at the G1/S transition.

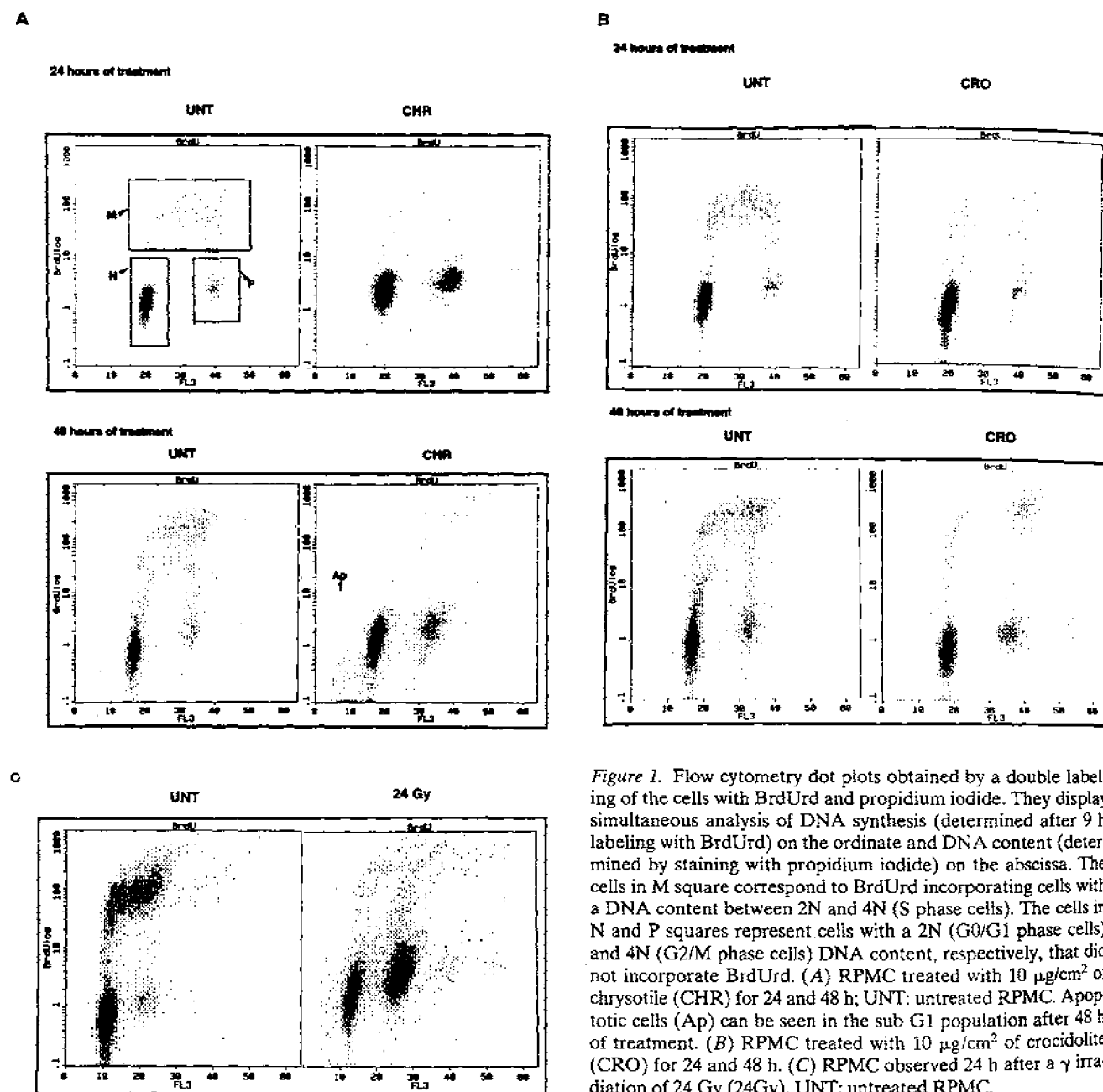


Figure 1. Flow cytometry dot plots obtained by a double labeling of the cells with BrdUrd and propidium iodide. They display simultaneous analysis of DNA synthesis (determined after 9 h labeling with BrdUrd) on the ordinate and DNA content (determined by staining with propidium iodide) on the abscissa. The cells in M square correspond to BrdUrd incorporating cells with a DNA content between 2N and 4N (S phase cells). The cells in N and P squares represent cells with a 2N (G0/G1 phase cells) and 4N (G2/M phase cells) DNA content, respectively, that did not incorporate BrdUrd. (A) RPMC treated with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile (CHR) for 24 and 48 h; UNT: untreated RPMC. Apoptotic cells (Ap) can be seen in the sub G1 population after 48 h of treatment. (B) RPMC treated with 10 $\mu\text{g}/\text{cm}^2$ of crocidolite (CRO) for 24 and 48 h. (C) RPMC observed 24 h after a γ irradiation of 24 Gy (24Gy). UNT: untreated RPMC.

Treatment with camptothecin for 24 h resulted in different modifications in the cell cycle since no G2/M accumulation was observed. The blockade of the cell cycle progression occurred at the G1/S transition, in agreement with results in the literature. A reduction in the percentage of cells incorporating BrdUrd was also found after camptothecin treatment in comparison with untreated cells.

In order to determine whether the cell cycle alterations observed after asbestos exposure were of the same type as those produced by reactive oxygen species, RPMC were exposed to γ irradiation. The cell cycle alterations occurring 24 h after γ irradiation (Figure 1C) were similar to those observed after chrysotile exposure, i.e., an arrest at

the G1/S transition and a cell accumulation in the G2/M phases.

p53 Protein Induction

Since several studies have shown an association between a p53 up-regulation and cell cycle arrest (17, 32), it was interesting to determine whether the cell cycle alterations observed after asbestos exposure were accompanied by an increase in p53 expression. As shown in the typical experiment presented in Figure 3, Western blotting analyses of total protein extracts indicated that p53 protein was present in untreated cells but at a low level. Treatment of RPMC with asbestos fibers resulted in a time-dependent

Figure 1
SDS-PAGE
TSV40:
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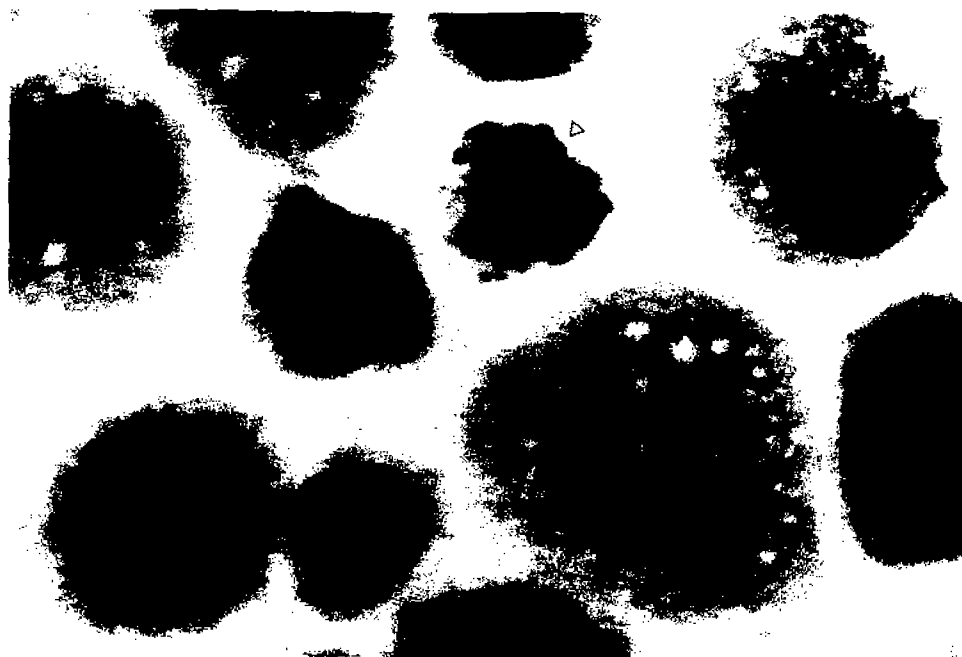


Figure 2. Binucleated cells in RPMC cultures treated with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile for 48 h. An apoptotic cell can be seen (arrow). After treatment, cells were fixed with methanol/acetic acid and stained with MGG.

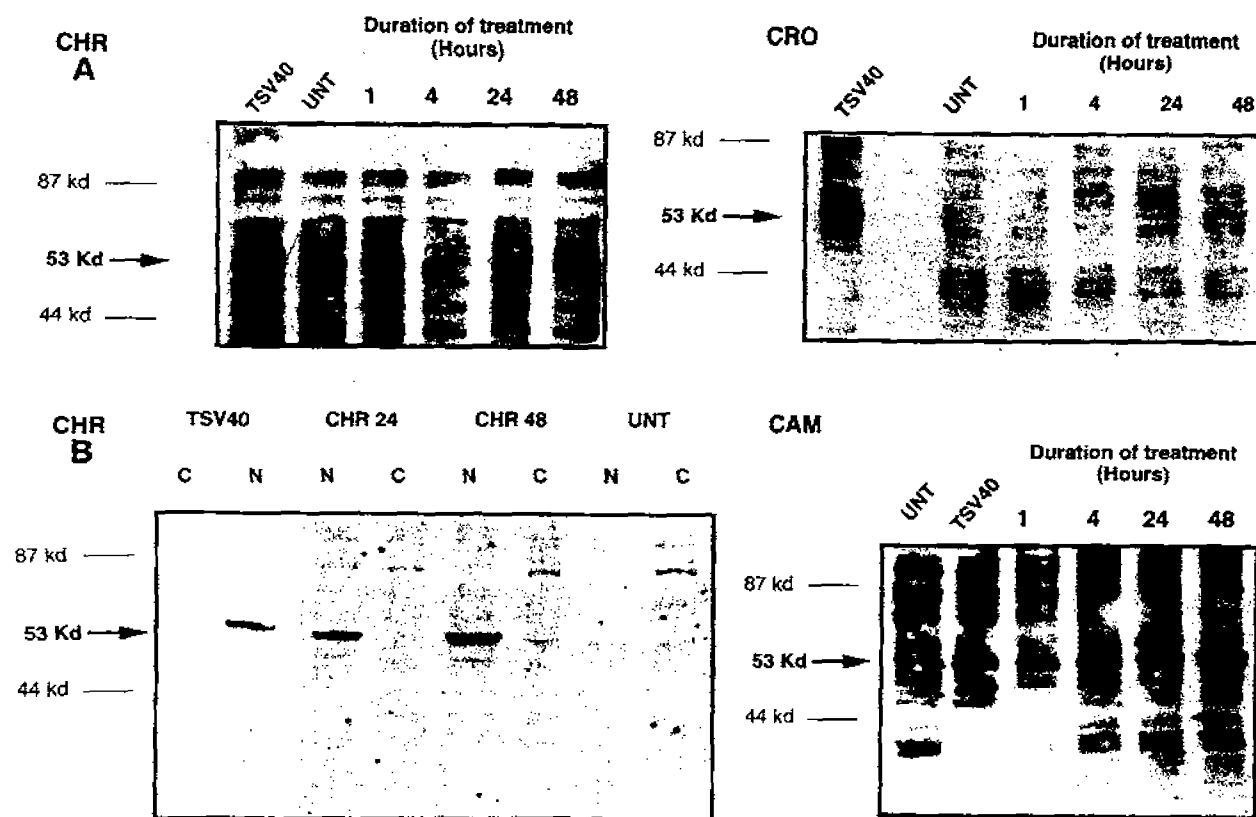


Figure 3. p53 protein expression in RPMC. Total proteins were extracted after each treatment. Separation was performed in reduced SDS-PAGE on 10% acrylamide gel (35 $\mu\text{g}/\text{lane}$). After blotting, p53 was probed by using a monoclonal antibody (HR 231; Eurobio). TSV40: RPMC infected with retroviral recombinant TSV40, UNT: untreated RPMC. (CHR A): Total cell extracts, RPMC treated with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile for 1, 4, 24, and 48 h. (CHR B): Cytosolic (C) and nuclear fractions (N). CHR24: RPMC treated with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile for 24 h, CHR48: RPMC treated with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile for 48 h. CRO: Total cell extracts, RPMC treated with 10 $\mu\text{g}/\text{cm}^2$ of crocidolite for 1, 4, 24, and 48 h. CAM: Total cell extracts, RPMC treated with 10 $\mu\text{g}/\text{ml}$ of camptothecin for 1, 4, 24, and 48 h.

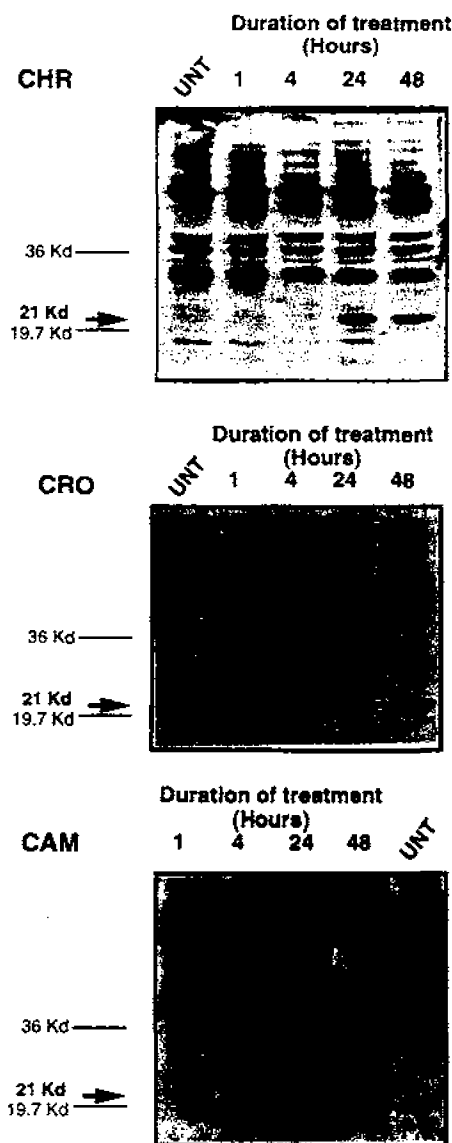


Figure 4. p21/WAF1/CIP1 protein expression studied by western blotting on a 14% SDS-PAGE gel, using 30 $\mu\text{g}/\text{lane}$ of protein. CHR: 10 $\mu\text{g}/\text{cm}^2$ of chrysotile for 1, 4, 24, and 48 h. CRO: 10 $\mu\text{g}/\text{cm}^2$ of crocidolite for 1, 4, 24, and 48 h. CAM: 10 $\mu\text{g}/\text{ml}$ of camptothecin for 1, 4, 24, and 48 h. UNT: untreated cells.

induction of p53: After 1 h of treatment with chrysotile (10 $\mu\text{g}/\text{cm}^2$), the p53 level was the same as in untreated cells but an enhancement was detected after 24 and 48 h (Figure 3). Analysis of cytoplasmic and nuclear protein fractions allowed the localization of p53 in the nuclear fractions. The p53 level in cytoplasmic extracts was very low and did not change with the duration of treatment. After crocidolite treatment (Figure 3), p53 induction was lower than in chrysotile-treated cells. By immunocytochemistry, no nuclear p53 was detectable in asbestos-treated RPMC whereas UV irradiation induced a p53 nuclear localization (data not shown).

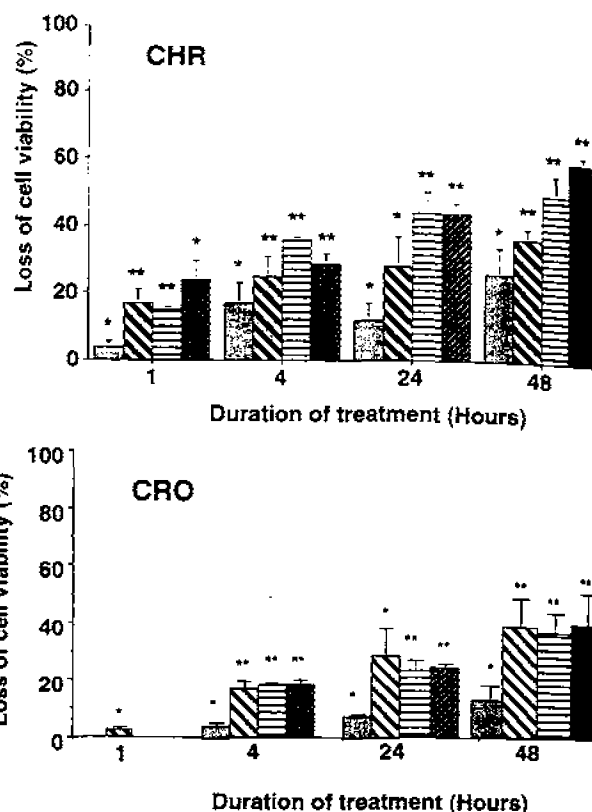


Figure 5. RPMC viability determined by the MTT assay. The figure represents the time-dependent percentage of loss of cell viability (untreated control = 0%). Confluent cells have been treated with: chrysotile (CHR) at 0.5 ($\square$), 2 ($\square$), 5 ($\square$), 10 ($\blacksquare$) $\mu\text{g}/\text{cm}^2$, crocidolite (CRO) at 0.5 ($\square$), 2 ($\square$), 5 ($\square$), 10 ($\blacksquare$) $\mu\text{g}/\text{cm}^2$. Data represent the mean $\pm$ standard deviation of 4 experiments. *t* test: * $P < 0.05$; ** $P < 0.005$ in comparison with untreated cultures.

Treatment with camptothecin (Figure 3) also resulted in an increase in p53 expression. This p53 induction was observed from 1 h after treatment and increased in a time-dependent manner until 48 h of treatment.

In all experiments performed on total protein extracts, several extrabands appeared despite the use of a monoclonal antibody. Control experiments, performed with no primary antibody, have ensured the specificity of the p53 signal, since, under those conditions, no band appears in the molecular weight range of the p53 protein (data not shown). However, to be sure to detect the presence of the p53 protein even at a very low level, we have used a high antibody concentration that could partly explain the high background.

p21/WAF1/CIP1 Protein Expression

Several reports in the literature have associated the p53 protein induction with an increased level of the p21/WAF1/CIP1 protein. Regarding the p53 upregulation after asbestos exposure, it was interesting to study the p21/WAF1/CIP1 expression in asbestos-treated RPMC.

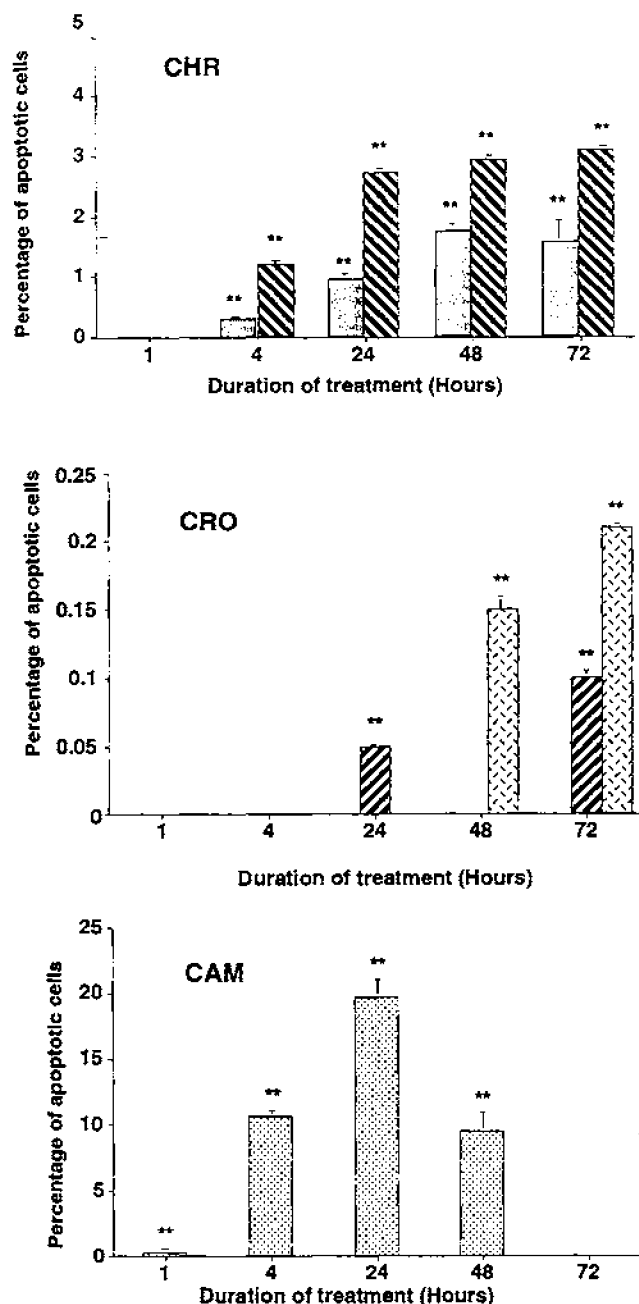


Figure 6. Percentage of apoptotic cells in RPMC cultures treated with chrysotile (CHR) at 2 (□) and 10 (▨) $\mu\text{g}/\text{cm}^2$; crocidolite (CRO) at 10 (▨) and 20 (□) $\mu\text{g}/\text{cm}^2$ and camptothecin (CAM) at 10 $\mu\text{g}/\text{ml}$. After fixation and MGG staining, 2,000 cells per slide were counted. For each treatment, 5 slides were analyzed. Data are mean $\pm$ standard deviation of 5 experiments. ** $P < 0.005$. Untreated control at 1 and 4 h: 0.2%; Untreated control at 24 and 48 h: 0.5 and 1%, respectively.

As shown in the typical experiment reported in Figure 4, it appeared that the p21 protein was expressed after 24 and 48 h of chrysotile treatment (10 $\mu\text{g}/\text{cm}^2$), but not earlier. The time-course of the p21 protein expression following crocidolite treatment was slightly different: p21 protein was only detected after 48 h. For the shorter lengths of

treatment, the p21 protein was not detected. In untreated cells, the p21 protein level was very low.

A time-dependent induction of p21 occurred in camptothecin-treated RPMC (Figure 4). After 1 h, the level of p21 expression was the same as in control cells but after 4 h of treatment, the protein expression increased until 24 h and remained stable.

Analysis of RPMC Viability and Apoptosis

Analysis of RPMC viability performed with the MTT assay shows a decrease in cell viability in comparison with untreated controls (Figure 5). On a per weight basis, chrysotile was more cytotoxic than crocidolite. The maximal loss in cell viability in RPMC treated with chrysotile reached 60% whereas it did not exceed 40% with crocidolite. Moreover, cytotoxicity appeared later after the treatment with crocidolite than with chrysotile (after 1 h and 4 h, respectively). In RPMC treated with chrysotile fibers, cytotoxicity was time- and dose-dependent whereas increasing concentrations of crocidolite above 2 $\mu\text{g}/\text{cm}^2$ did not significantly enhance its effect. RPMC viability was greatly impaired with 10 $\mu\text{g}/\text{ml}$ of camptothecin (74% loss of cell viability after 72 h of treatment).

To determine to what extent loss of cell viability was due to apoptosis, we quantified the number of apoptotic cells induced by the different treatments, by morphological observation following MGG staining. The spontaneous rate of apoptosis observed in untreated cultures was 0.05% and 0.1% after 24 and 48 h of culture, respectively. Chrysotile fibers induced a dose-dependent apoptosis in RPMC. The highest percentage of apoptotic cells (4%) was observed after 72 h of treatment with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile. Apoptosis, in cells treated with crocidolite, appeared at the highest concentrations tested and at a low rate (lower than 1%) (Figure 6). As expected, camptothecin, used as a positive control, triggered a rapid apoptosis in RPMC. Apoptosis was also detected by DNA fragmentation analysis, with the appearance of a DNA ladder in cultures treated with fibers and with camptothecin (Figure 7) not observed in experimental controls (Figure 8).

Bcl2 Protein Expression

The mitochondrial bcl2 protein is thought to be involved in the control of programmed cell death; it was therefore interesting to study the bcl2 protein expression during asbestos treatment. Figure 9 presents the results obtained after chrysotile asbestos and camptothecin treatment. No modification of the bcl2 protein was detected after the different lengths of treatment with 10 $\mu\text{g}/\text{cm}^2$ of chrysotile, when compared with untreated cells. The same results were observed after camptothecin treatment.

Discussion

In the present study, we investigated cell cycle progression in asbestos-treated cells. We found that asbestos treatment provoked complex changes in cell cycle progression occurring in both G1/S and G2/M locations. Chrysotile exposure led to a time-dependent decrease in the percentage of cells in the replicative phase appearing after 4 h of treatment and persisting later. Results obtained after 9 h of BrdUrd

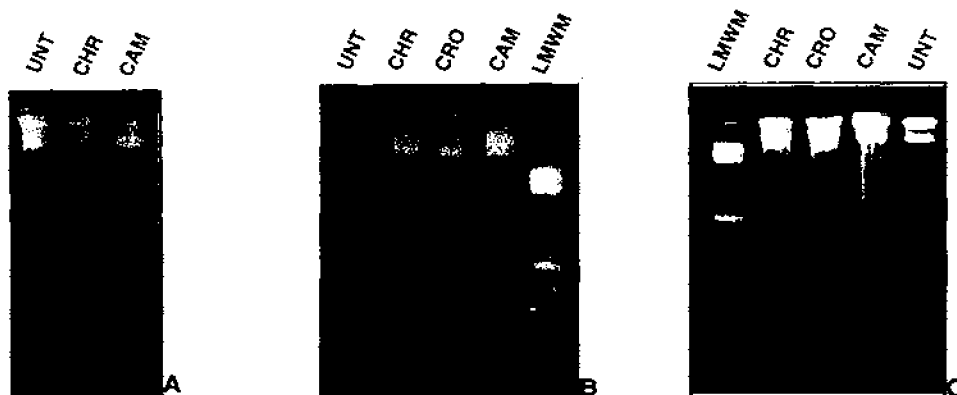


Figure 7. Kinetics of DNA fragmentation in RPMC treated with: 10 $\mu\text{g}/\text{cm}^2$ of chrysotile, 10 $\mu\text{g}/\text{cm}^2$ of crocidolite or 10 $\mu\text{g}/\text{ml}$ of camptothecin. (A) 4 h, (B) 24 h, (C) 48 h of treatment. CAM: camptothecin, CHR: chrysotile, CRO: crocidolite, LMWM: low Molecular Weight Marker, UNT: untreated cells. After DNA extraction in phenol/isoamyl alcohol mixture, samples were submitted to an electrophoresis on 1.5% agarose gel, in TBE buffer, as described in MATERIALS AND METHODS.

incorporation showed that RPMC accumulated in G0/G1 after 24 h of treatment and were further unable to enter the replicative phase. These data suggest that chrysotile can induce a blockade in G0/G1. Crocidolite also limited the cell cycle progression after 4 h and 24 h of treatment, in agreement with a G1 arrest. However, the decrease in the percentage of cells in the replicative phase was moderate after 48 h. These results suggest that crocidolite delayed the cell cycle progression rather than provoking a blockade through G1/S progression.

The second evident disruption observed after asbestos exposure was localized at the G2/M checkpoint: an evident cell accumulation in G2/M phases occurred after 4 and 24 h of treatment with chrysotile and crocidolite, respectively. The formation of bi- or multinucleated cells after both treatments showed that the G2/M accumulation may not entirely reflect a G2 arrest. It may be due, at least partly, to the alteration of mitosis, especially to chromosome seg-

regation, as demonstrated in several studies (33, 34). Bi- and multinucleated cells have also been found following treatment of human mesothelial cells with asbestos (35). Several studies have suggested that, in many cell types, a spindle checkpoint exists to ensure the maintenance of diploidy (36). Therefore, our results suggest that asbestos fibers might impair the spindle checkpoint.

The commonly held view on growth arrest at the G1/S and G2/M transition points proposes that DNA damaged cells stop their cell cycle progression to allow DNA repair before entering either the replicative phase or mitosis (37). Clopton and colleagues (38) demonstrated that CHO cells respond to a low level of oxidative stress by a G1/S and a G2/M arrest. For Hawn and associates (39), a G2/M arrest is a hallmark of treatment with DNA damaging agents such as γ irradiation (via the production of oxygen species) or agents that induce base mispairings. The results we obtained after RPMC exposure to γ irradiation for 24 h were similar to those obtained with chrysotile fibers and support this hypothesis. Therefore, the results obtained with asbestos could be related to the fibers' ability to induce such DNA damage, via the generation of reactive oxygen species. Furthermore, for Seychab and colleagues (40), the severity of the cell cycle disturbance (delay or arrest) may be dependent on the degree of DNA repair. Accordingly, the differences in cell cycle disruptions between chrysotile and crocidolite might reflect differences in DNA damage induction.

In order to study the mechanisms involved in cell cycle alterations, we investigated the expression of the tumor suppressor gene product p53 which would be a determinant factor in cell cycle control. While p53 is recognized as playing a role in the control of G1/S transition, its involvement at the G2/M checkpoint is at present debatable and the role of the protein may be dependent on cell type or on experimental conditions. Cross and associates (36) proposed that p53 is involved in the G2/M checkpoint control and observed that p53 defective cells develop a tetraploid population after treatment with spindle interacting agents. Under our conditions, an association between p53 induction and G2 delay is not evident. The accumulation of cells in the G2/M phase appeared clearly after 4 h of chrysotile exposure whereas no p53 accumulation was detectable. In contrast, a correlation between G1/S arrest and p53 ex-

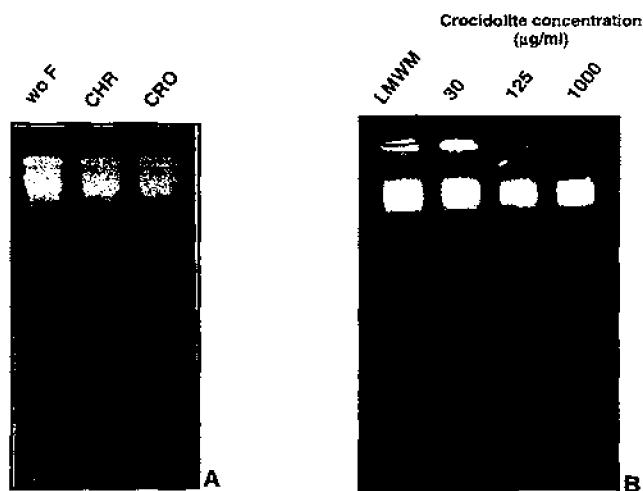


Figure 8. Analysis of DNA stability in the presence of asbestos fibers. (A) Five μg of salmon sperm DNA were incubated in TE buffer, at 37°C for 48 h without fiber (wo F) or with 1 mg/ml of chrysotile (CHR) or crocidolite (CRO). (B) Migration of low molecular weight DNA fragments after incubation without (LMWM) or with 30, 125 or 1,000 $\mu\text{g}/\text{ml}$ of crocidolite during 48 h.

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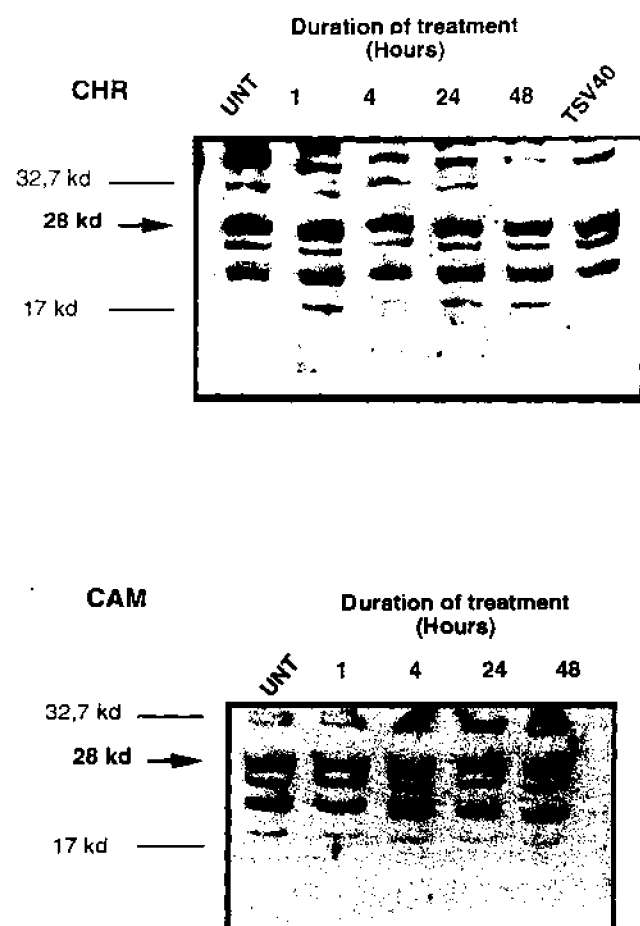


Figure 9. Bcl2 expression in RPMC. Extracted proteins (30 μ g/lane) were separated by SDS-PAGE on 12% acrylamide gel. After transfer, visualization of bcl2 was performed by using a polyclonal antibody (N-19, Tebu). CHR: 10 μ g/cm² of chrysotile for 1, 4, 24, and 48 h. CAM: 10 μ g/ml of camptothecin for 1, 4, 24, and 48 h. UNT: untreated RPMC. TSV40: RPMC transduced with TSV40.

pression exists: after chrysotile exposure, a clear limitation in G1/S transition after 48 h was associated with an increased p53 level that persisted after 48 h. In agreement with several reports (19, 41), this G0/G1 arrest seems to be mediated by the p53-dependent transcription of the p21/WAF1/CIP1 since a coexpression of p21 protein and p53 appeared after treatment with chrysotile. The delay in G1/S transition observed after crocidolite exposure correlates with the low level of p53 expression and the absence of p21 expression. The results obtained with camptothecin clearly show that the irreversible G0/G1 arrest, observed after 24 h of treatment, was associated with both p53 and p21 accumulation, in agreement with other studies (26).

Cell cycle disruptions are associated with apoptosis induction as shown in several studies (42, 43). We therefore investigated apoptosis induced by asbestos fibers in the present study. A global analysis of DNA has demonstrated a DNA fragmentation characteristic of apoptosis. The apop-

tosis yield was determined after MGG staining; it did not exceed 4%. A very low rate of spontaneous apoptosis occurred in untreated RPMC (0.05% to 0.1% after 24 or 48 h of culture). After asbestos exposure, a time- and dose-dependent enhancement of apoptosis was found. In our experiment, the higher potential of chrysotile to induce apoptosis is in agreement with the MTT results demonstrating a higher loss of cell viability after chrysotile treatment than after crocidolite exposure. Apoptosis induction after asbestos exposure has already been reported by others (10, 11) but the level of apoptosis found in the present study was lower than that reported in those studies. Several parameters could account for the differences. First, the origin of asbestos samples was different (NIEHS versus UICC in the present study) that is, each mean sample length differs from another (11.4 μ m for NIEHS crocidolite versus 2.1 μ m for UICC crocidolite). Regarding the greater effect of long fibers in comparison with short fibers in asbestos cytotoxicity, the differences in fiber dimensions could account for the different results obtained. Second, the culture conditions were not identical in the different studies. In the study performed by Bérubé and colleagues (10), mesothelial cells were serum deprived for 24 h prior to fiber exposure, a condition known to enhance apoptosis in many cell systems (44, 45). In the Broadus study (11), the cells were cultured in a serum-free medium for one hour before fiber treatment. The differences between our study and others are unlikely to be due to loss of apoptotic cells because we collected all the cells in suspension in the medium and our methodology allowed us to detect a high percentage of apoptotic cells after camptothecin treatment. The finding of cell cycle disturbances not associated with a high amount of apoptosis suggests that the amount of DNA damage permits DNA repair. It would be interesting to investigate the balance between apoptosis and repair of damage, because the proliferation of unrepaired or misrepaired cells might be of worse prognosis than the formation of apoptotic cells. Stimulation of lung and mesothelial cell proliferation has been reported within days after *in vivo* exposure of rodents to fibers (46–49).

The increase in p53 protein expression, associated with a nuclear localization, found in asbestos-treated RPMC parallels the appearance of apoptotic cells. Following crocidolite treatment, p53 induction was lower than after chrysotile treatment, in agreement with the lower rate of apoptosis. Thus, the apoptotic process observed in RPMC after asbestos treatment appears to be associated with a p53 induction. The data obtained with camptothecin are in agreement with this association. These results suggest that both apoptosis and p53 induction are directly dependent on the agent's ability to induce DNA damage. Another candidate that may be responsible for the cell commitment to apoptosis is the mitochondrial bcl2 protein. Many reports have demonstrated that in some cell lines, an enforced bcl2 expression can block p53-induced apoptosis (50, 51). On the other hand, a downregulation of bcl2 expression, for instance, after growth factor withdrawal, can be responsible for the increased tendency of some cell lines to undergo a p53-mediated apoptosis (52). In our study, no change in the bcl2 protein was found after treatment with chrysotile.

In conclusion, our results demonstrate that asbestos fibers behave as known DNA damaging agents (camptothecin, γ irradiation) by inducing cell cycle disruptions associated with a low rate of apoptosis. This may suggest that the DNA repair pathway can be preferentially chosen and could therefore contribute to the fixation of DNA lesions. Furthermore, the cell cycle alterations indicate that asbestos fibers might impair some cell cycle checkpoints, critical to maintaining the genome integrity.

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