Influence of caspase activity on micronuclei detection: a possible role for caspase-3 in micronucleation

Ilse Decordier^{*}, Enrico Cundari¹ and Micheline Kirsch-Volders

Vrije Universiteit Brussel, Laboratorium voor Cellulaire Genetica, Pleinlaan 2, B-1050 Brussel, Belgium and ¹Istituto di biologia e patologia molecolari, C.N.R., Via degli Apuli 4, I-00185 Roma, Italy

Aneugenic and clastogenic agents are good inducers of both micronuclei and apoptosis. In its turn, apoptosis may modify the threshold values for the induction of micronuclei. This is of major concern for accurate assessment of hazard related to exposure to mutagens. In the present work we studied the influence of caspases, the key regulators of the apoptotic process, on the induction of micronuclei in the cytokinesis block micronucleus assay. For this, we applied a combined approach in which both human peripheral blood mononucleated cells (PBMC) and the paired human breast carcinoma cell lines MCF-7, which is caspase-3 deficient, and the caspase-3 transfected MCF-7 (MCF-7^{casp-3}) were used to study the influence of caspase activity on micronuclei. When nocodazole induced apoptosis was inhibited by the use of inhibitors of the two main apical caspases-8 and -9 in PBMC, the frequencies of micronucleated binucleates (MNCB) increased with inhibition of these caspases confirming that apoptosis can eliminate micronucleated cells. On the contrary when caspase-3 was inhibited, the frequencies of MNCB was lower, suggesting a role of caspase-3, also in micronuclei formation. To verify this hypothesis, we compared the induction of apoptosis and micronuclei by the aneugen nocodazole, the clastogen methyl methane sulfonate (MMS) and the non-mutagenic apoptogen staurosporin in MCF-7 and MCF-7^{casp-3} cells. The results showed that when caspase-3 activity was impaired, in the parental MCF-7 cell line or in the MCF-7^{casp-3} cells in the presence of the caspase-3 inhibitor, the frequencies of nocodazole or MMS induced micronuclei decreased. These results suggest that caspase-3, besides its function as an effector caspase in the apoptotic pathway, is also involved in the formation of micronuclei.

Introduction

Micronuclei (MN) can be induced following exposure to aneugenic and clastogenic agents and their quantification provides a sensitive tool for the assessment of genotoxicity (1,2). However, the nature and the destiny of MN have still to be elucidated. Agents interfering with microtubule dynamics or assembly are good inducers of MN and at the same time trigger a significant apoptotic response (3). Microtubules serve as an intracellular scaffold and their unique polymerization dynamics are critical for many cellular functions, such as intracellular transport, interactions of cells with their extracellular matrix, immune cell infiltration and the formation and function of the mitotic spindle to ensure a correct segregation of the chromatids (4). Restructuring of the microtubule network contributes to the commitment of a cell to grow, differentiate, move and divide or undergo apoptosis.

The key regulators of apoptosis are the proteinases belonging to the family of caspases. Activation of the caspases results in the cleavage of different substrates in the cytosol and the nucleus resulting in the systematic and orderly disassembly of the dying cell [for a review see (5)]. Different apoptotic stimuli converge to the apical caspases 8 and 9, which initiate the caspase cascade carried out by effector caspases, such as caspase-3, -6 and -7 (6).

Since apoptosis can eliminate micronucleated cells, it may modify the threshold values, defined previously in our laboratory (7,8) for the induction of MN by aneugens. Moreover, Meintières *et al.* (9), using mouse CTLL-2 (a stable subclone of cytotoxic T-lymphocytes from a C57BL/6 mouse) and CTLL-2 *bcl2* cells transfected with the *bcl2* gene, showed that the ectopic overexpression of the paradigmatic apoptosis inhibitor gene *bcl-2* strongly influenced the detection of MN induced by a panel of differentially acting chemical agents.

In the present work, we addressed the influence of caspase activity on the detection of MN. We applied a combined approach where both human peripheral blood mononucleated cells (PBMC) and the paired human breast carcinoma cell lines MCF-7, which is caspase-3 deficient, and the caspase-3 transfected MCF-7 (MCF-7^{casp3}) were exposed to the spindle inhibitor nocodazole, to the clastogen methyl methane sulfonate (MMS) and to the non-genotoxic apoptogen staurosporine. We determined the induction of apoptosis by annexin-V staining and the frequency of MN in cytochalasin B blocked cells. We confirmed our previous observations that apoptosis can eliminate micronucleated cells induced by nocodazole and demonstrated that apoptosis induced by nocodazole involves caspase-9, -8 and -3. Furthermore, our data suggest that caspase-3, in addition to its function in apoptosis, is also involved in the formation of MN.

Materials and methods

Cell cultures

Human peripheral blood samples were obtained from two healthy non-smoking female donors of <35 years of age. Lymphocytes were isolated using Ficoll-Pacque (Pharmacia Biotech, Uppsala, Sweden) and were cultured in Ham's F-10 medium and 25 mM L-glutamine (Gibco BRL, Paisley, UK), supplemented with 15% fetal calf serum (Gibco BRL) and 2% phytohaemagglutinin HA 16 (PHA; Murex Biotech Ltd, Dartford, UK) and incubated in 5% CO₂ in a humidified incubator at 37°C.

Untransfected MCF-7 cells were cultured in RPMI 1640 containing 10% FCS, non-essential amino acids, 1 mM sodium pyruvate, 10 µg/ml insulin, penicillin (50 U/ml) and streptomycin (50 µg/ml). The MCF-7^{casp-3} cell line, stably transfected with the pcDNA3 vector containing the full-length Yama

*To whom correspondence should be addressed. Tel: +32 2 629 34 28; Fax: +32 2 629 27 59; Email: idecordi@vub.ac.be

© The Author 2005. Published by Oxford University Press on behalf of the UK Environmental Mutagen Society.

All rights reserved. For permissions, please email: journals.permissions@oupjournals.org

I.Decordier, E.Cundari and M.Kirsch-Volders

(CASP-3) (10), was cultured in the same medium containing 750 μ g/ml G418. Both cell lines were maintained in a humified atmosphere of 5% CO₂ at 37°C.

Drug treatment

Nocodazole (Acros Organics, Belgium), the specific caspase inhibitors Ac-DEVD-CHO (caspase-3), Ac-LEHD-CMK (caspase-9) and Boc-AEVD-CHO (caspase-8) (Bachem, Bubendorf, Switzerland) and staurosporine (Sigma) were dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and MMS in phosphate-buffered saline (PBS). After 24 h PHA stimulation, the PBMC were incubated with nocodazole either alone or concurrent with Ac-DEVD-CHO, Ac-LEHD-CMK and Boc-AEVD-CHO; the final concentration of DMSO did not exceed 0.5%. The control cultures were treated with 0.5% DMSO. The PBMC cultures were harvested after the specified periods of culture time.

The MCF-7 cell lines were seeded into 25 cm² flasks and were allowed to attach for 24 h. After 24 h the medium was replaced with fresh medium and the test substances were added for 48 h.

Annexin staining

Cells were collected by centrifugation and resuspended in 100 μ l annexin labeling solution consisting of 2% annexin-V-FLUOS (Roche Diagnostics, Belgium) and 0.1 μ g/ml propidium iodide in HEPES buffer containing 10 mM HEPES, 140 mM NaCl, 2 mM CaCl₂, 5 mM KCl and 1 mM MgCl₂, pH 7.4. Cells were incubated in the dark in this solution for 15 min. Slides were prepared by dropping the cell suspension on them. The simultaneous application of propidium iodide allows the discrimination of necrotic cells from the annexin-V positive cells, since necrotic cells also expose phosphatidyl serine according to the loss of membrane integrity.

Cell preparations were analysed with a Zeiss Axioscop fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at a magnification of \times 400, equipped with a double band pass filter, no. 24 (Zeiss) to visualize the annexin-V-FLUOS labeled apoptotic cells and the necrotic cells stained by propidium iodide and viable cells were analysed with normal transmitted light. Early apoptotic cells (positive for annexin-V but negative for propidium iodide) were identified. One thousand cells per culture were scored.

In vitro cytokinesis blocked MN test

For the PBMC cultures. Cytochalasin B (Sigma Chemical Co., Belgium) was added at 6 μ g/ml culture at 44 h. After 72 h, cells were put directly onto slides using cytospin (Shandon) at 700 r.p.m. for 5 min and fixed immediately with methanol. Staining was achieved with 5% Giemsa (Merck, Darmstadt, Germany) in Sörensen buffer, pH = 6.8 (Prosan, Gent, Belgium) for 20 min.

For the cell lines. The cells were seeded into 25 cm^2 flasks and were allowed to attach for 24 h. After 24 h, the medium was replaced with fresh medium and the test substances were added; after 6 h, 2.5 µg/ml culture cytochalasin B was added. After 48 h, incubation cells were harvested after trypsinization and put directly onto slides using cytospin (Shandon) at 5000 g for 5 min and immediately fixed with methanol. Staining was achieved with 5% Giemsa (Merck, Darmstadt, Germany) in Sörensen buffer, pH = 6.8 (Prosan, Gent, Belgium) for 20 min.

MN analysis. Two cultures per concentration of the test substance were analysed; 1000 binucleated cells (CB) were examined per culture for the presence of one, two or more MN and expressed per thousand MNCB. The scoring criteria included round or oval shaped MN with no connection to the main nuclei, a size between 1/16 and 1/3 and similar staining characteristics of the main nuclei. In addition, the percentage of CB, polynucleated cells and mononucleated cells containing MN were recorded. The slides were coded and analysed on a Zeiss transmitted light microsope at a magnification of \times 1000. From the data of the MN analysis the cytokinesis-block proliferation index (CPBI) (11) was calculated as follows:

CBPI = (number mononucleate cells + 2 × number binucleate cells +3 × number multinucleate cells)/Total number of cells

Colorimetric caspase-activity assay

Caspase-3 activity was measured with the Caspase-3 Colorimetric Protease Assay Kit (MBL, Japan). Cell lysates were prepared by resuspending cells in 100 μ l lysis buffer and lysing by three rounds of freezing in liquid nitrogen and thawing on ice. The lysates were then centrifuged for 10 min at 13 400 g. Supernatants were measured in a 96-well plate for caspase-3 activity using the DEVD-pNA, labelled substrate (200 μ M final concentration), in the reaction buffer containing 10 mM dithiothreitol (DTT). After incubation at 37°C for 2 h samples were read at 405 nm in a microplate reader. In each experiment, the OD values measured for untreated samples were similar to those of the negative controls, which contained no cell lysate. The background level reported in

Statistical analysis

Statistical analysis was performed using Mann–Whitney *U*-test to determine significant differences in lymphocytes treated with different experimental conditions and Student's *t*-test to compare between pairs of groups for each dose in the two cell lines. Analysis of variance (one-way ANOVA) was also applied to the results of the various treatment groups, followed by *post-hoc* comparison using Dunnett analysis (SPSS 12.0).

Results

Caspase-8, -9 and -3 inhibitors affect the frequencies of nocodazole induced MN in human PBMC

To elucidate the upstream caspase pathway leading to nocodazole-induced apoptosis, we used preferential inhibitors of the two initiator caspases 8 (Boc-AEVD-CHO) and 9 (Ac-LEHD-CMK) (13), and the executioner caspase-3 (Ac-DEVD-CHO) (14). After 24 h PHA stimulation parallel cultures of isolated PBMC of two donors were exposed *in vitro* to 0.303 μ M nocodazole for 48 h in the presence or absence of the three caspase inhibitors, all at the concentration of 300 μ M. It was shown by previous studies in our laboratory that this concentration of nocodazole is situated above the threshold value for chromosome loss (7,8) and induces apoptosis in both primary human lymphocytes and in the human erythroleukemia cell lines K562 and KS (15).

The induction and inhibition of apoptosis was quantified by annexin-V staining (3). A significant reduction of nocodazoleinduced apoptosis was observed in the presence of all inhibitors used (P < 0.05) (Figure 1). Inhibition of the effector caspase-3 provoked the highest decrease of apoptosis. A lower inhibition was observed in presence of the caspase-8 and -9 inhibitors.

Micronucleated cells generated by treatments with aneugenic agents are preferentially eliminated by apoptosis (3). Inhibition of apoptosis should, hence, increase the proportion of micronucleated cells as determined in the cytochalasin B-block MN test (2). The frequencies of MNCB (corresponding to cells which divided once in culture) were, therefore, analysed for two donors after 48 h treatment with 0.303 μ M nocodazole in the presence or absence of the caspase inhibitors

PBMC

🖾 control

🖾 noc + inh

caspase-3

🖸 inh

noc

60

50

40

30

20

10

0

% apoptotic cells



caspase-8

caspase-9



Fig. 2. Frequencies of nocodazole-induced MNCB in presence of caspase-9 inhibitor Ac-LEHD-CMK, the caspase-8 inhibitor Boc-AEVD-CHO and the caspase-3 inhibitor Ac-DEVD-CHO. Each bar represents the mean of two donors, per donor two parallel cultures and per culture 1000 binucleates were scored for the incidence of MN. *P < 0.05, presence of the caspase inhibitor versus absence of the caspase inhibitor.

at 300 μ M. As far as caspase-8 and -9 inhibition are concerned (Figure 2) inhibition resulted, as expected, in a higher proportion of nocodazole-induced MNCB compared with MNCB frequency measured in the absence of inhibitors. Unexpectedly, however, in presence of the caspase-3 inhibitor the frequency of nocodazole-induced MNCB was lower, suggesting that caspase-3 is not involved in the elimination of micronucleated cells but rather in their formation.

Induction of MN in the MCF-7 cell line and the caspase-3-transfected derivative MCF-7 cell line by the aneugenic agent nocodazole

In order to further investigate the possible involvement of caspase-3 in the formation of MN, the human MCF-7 breast carcinoma cell line, which is deficient for caspase-3 by a mutation in the CASP-3 gene, and a paired MCF-7 derivative cell line stably transfected with a caspase-3 expression vector (MCF- 7^{casp-3}) (10) were used. These two cell lines are, therefore, isogenic and in particular both harbour a functional *p53* gene (16).

Cells were exposed to 0.1212, 0.2424 and 0.3030 μ M nocodazole, in order to compare the efficiency of MN induction in the two cell lines. The reliability of our experimental conditions was also verified by determing (i) caspase-3 activity, (ii) apoptosis induction by the aneugen, (iii) apoptosis inhibition by inhibitors of the caspases-8 and -9 and (iv) cell proliferation index in order to avoid artifacts owing to different cell cycle dynamics.

The activity of caspase-3 was measured in untreated and treated cells of both cell lines and a 3-fold increase of caspase-3 activity over the background was observed following treatment with nocodazole at all concentrations tested in the MCF-7^{casp3+} cells. On the contrary, MCF-7 cells were completely devoid of caspase-3 activity, as expected (see online Supplementary Material).

The analysis of apoptosis, as determined by annexin-V staining, showed that nocodazole induced apoptosis in both cell lines (Figure 3A). As expected, taking into account that apoptosis can also be induced through a caspase-3-independent pathway (16). Comparison between the two cell lines showed that the apoptotic response was very weak, although significant



Fig. 3. Nocodazole-induced apoptosis after 48 h treatment in the MCF-7 cell line: (**□**), the MCF-7 ^{casp-3+} cell line: (**□**), and in the MCF-7 ^{casp-3+} cell line in the presence 300 μ M of the caspase-3 inhibitor Ac-DEVD-CHO: (**□**) (**A**); in the MCF-7 cell line: (**□**), in the presence of the caspase-8 inhibitor Boc-AEVD-CHO: (**□**), and the caspase-9 inhibitor Ac-LEHD-CMK: (**□**) (**B**); and in the MCF-7 ^{casp-3+} cell line: (**□**), in the presence of the caspase-8 inhibitor Boc-AEVD-CHO: (**□**), and the caspase-9 inhibitor Ac-LEHD-CMK: (**□**) (**B**); and in the MCF-7 ^{casp-3+} cell line: (**□**), in the presence of the caspase-8 inhibitor Boc-AEVD-CHO: (**□**), and the caspase-9 inhibitor Ac-LEHD-CMK: (**□**) (**C**). Each bar represents the mean ±SD of 4–9 cultures; plus symbols indicate *P* < 0.05 compared with the control; asterisks indicate *P* < 0.05 MCF-7 ^{casp-3+} compared with MCF-7; crosses indicate *P* < 0.05 with inhibitor as compared without.

in the MCF-7 cells at all tested concentrations of nocodazole, while a significantly higher frequency of apoptotic cells was observed in MCF-7^{casp-3} cells. When caspase-3 was specifically inhibited in the MCF-7^{casp-3} cells, the frequency of apoptosis decreased to values comparable with those measured in the MCF-7 cell line. The same phenomenon was observed when the two apical caspases-8 and -9 were inhibited by the



Fig. 4. CBPI in the MCF-7 cell line and the MCF-7^{casp-3+} cell line after treatment with NOC. Each point represents the mean \pm SD of 4–9 cultures.

use of the specific inhibitors Boc-AEVD and Ac-DEVD-CHO, respectively (Figure 3B and C).

We then carried out the cytochalasin-B blocked MN test to verify whether, in the presence of caspase-3, the frequency of micronucleated cells increased similarly to that described previously on lymphocytes. We first determined the CBPI (11) to compare the cell cycle kinetics of the two cell lines and obtained similar CBPI patterns in both MCF-7 and MCF-7^{casp3+} cells (Figure 4).

As far as the induction of MN is concerned (Figure 5) no difference in the frequencies of MN was observed between the two cell lines in the control cultures. Interestingly, while the frequencies of MNCB induced by nocodazole at the concentration of 0.1212 µM was similar in both cell lines, above the dose of 0.1212 µM, a significant increase of micronucleated cells was recorded only in the caspase-3 proficient cell line (Figure 5A). Furthermore, when caspase-3 was inhibited in the caspase-3 proficient cell line, micronucleated cells significantly decreased at both concentrations tested. On the contrary and consistent with our previous observations in PBMC when caspase-8 or -9 were inhibited, the frequency of micronucleated cells increased (Figure 5B and C). Irrespective of the presence or absence of the inhibitor, the frequency of micronucleated cells was always higher in the caspase-3 proficient cell line.

Influence of caspase-3 on MN induced by the clastogen MMS

To assess the specificity of the results found with the aneugenic agent nocodazole, similar experiments were performed with a typical clastogenic agent MMS.

MMS induced a dose-dependent increase in caspase-3 activity ranging between two and three times the background value in the MCF-7^{casp3+} cell line (see Supplementary online data). A strong dose-dependent apoptotic induction was recorded in both MCF-7 and MCF-7^{casp3+} cells in this case as well. A possible explanation is that a caspase-3 independent mechanism is prominent in MMS-induced apoptosis. This is consistent with the observations that the specific caspase-3 inhibitor provoked a weaker inhibition after MMS treatment compared with nocodazole-treated cells (Figure 6).

In the MN assay, MMS affected cell proliferation as determined by CBPI in a quite similar way in the two cell lines



 A^{120}

Fig. 5. Nocodazole-induced MN after 48 h treatment in the MCF-7 cell line: (I), the MCF-7^{casp-3+} cell line: (I), and in the MCF-7^{casp-3+} cell line in the presence 300 µM of the caspase-3 inhibitor Ac-DEVD-CHO: (I) (A); in the MCF-7 cell line: (I), in the presence of the caspase-8 inhibitor Boc-AEVD-CHO: (I), and the caspase-9 inhibitor Ac-LEHD-CMK: (I) (B); and in the MCF-7^{casp-3+} cell line: (I), in presence of the caspase-8 inhibitor Boc-AEVD-CHO: (I), and the caspase-9 inhibitor Ac-LEHD-CMK: (I) (C). Each bar represents the mean ± SD of 4–9 cultures; plus symbols indicate P < 0.05 compared with the control; asterisks indicate P < 0.05 MCF-7^{casp3+} compared with MCF-7; crosses indicate P < 0.05 with inhibitor as compared without.

(see Supplementary online data). Interestingly, the frequency of MMS-induced MN was, at all concentrations tested, higher in the caspase-3 positive cell line than in the MCF-7 cells. Moreover, in this case when caspase-3 was inhibited in the MCF-7^{casp3+} cell line a decrease of micronucleated cells was observed (Figure 7). It is to be stressed that the caspase-3



Fig. 6. MMS-induced apoptosis after 48 h treatment in the MCF-7 cell line: (**(**)), the MCF-7 ^{casp-3+} cell line: (**(**)) and in the MCF-7 ^{casp-3+} cell line in presence 300 μ M of the caspase-3 inhibitor Ac-DEVD-CHO: (**(**)). Each bar represents the mean \pm SD of 4–9 cultures; plus symbols indicate P < 0.05 as compared with the control; asterisks indicate P < 0.05 MCF-7^{casp3+} compared with MCF-7; crosses indicate P < 0.05 with inhibitor as compared without.



Fig. 7. MMS-induced MN after 48 h treatment in the MCF-7 cell line: (**□**), the MCF-7^{casp-3+} cell line: (**□**) and in the MCF-7^{casp-3+} cell line in presence 300 μ M of the caspase-3 inhibitor Ac-DEVD-CHO: (**⊡**). Each bar represents the mean ±SD of 4–9 cultures; plus symbols indicate *P* < 0.05 compared with the control; asterisks indicate *P* < 0.05 MCF-7^{casp3+} compared with MCF-7; crosses indicate *P* < 0.05 with inhibitor as compared without.

inhibitor was much more efficient in inhibiting MMS-induced MN than MMS-induced apoptosis.

Pure apoptogenic agents do not induce MN

In order to exclude the possibility that the observed micronucleated cells correspond to pre-apoptotic or apoptotic cells, we carried out similar experiments (48 h treatment) using staurosporine, which is a non-genotoxic strong apoptogenic agent. As expected, staurosporine induced, at all concentrations tested, a strong increase in caspase-3 activity (see Supplementary online data) and a corresponding increase in apoptotic cell frequencies (Figure 8). However, staurosporine did not induce MN either in MCF-7 cells or MCF-7^{casp3+} cells over the background (Figure 9). MN induction was also assessed after short exposure time (4 h); again no increase of MN was observed in either MCF-7 cells or MCF-7^{casp3+} cells (see online Supplementary Material).



Fig. 8. Staurosporine-induced apoptosis after 48 h treatment in the MCF-7 cell line: (**(**), the MCF-7 ^{casp-3 +} cell line: (**(**). Each bar represents the mean \pm SD of 4–9 cultures; plus symbols indicate *P* < 0.05 compared with the control; asterisks indicate *P* < 0.05 MCF-7^{casp3+} compared with MCF-7.



Fig. 9. Staurosporine-induced MN after 48 h treatment in the MCF-7 cell line: (a), the MCF-7^{casp-3+} cell line: (a). Each bar represents the mean \pm SD of 4–9 cultures.

Discussion

Determination of thresholds for risk assessment of humans exposed to mutagens is at the present time based on results from *in vitro* studies with the cytochalasin B-blocked MN test (2) or the *in vivo* erythrocyte MN test (18). Our previous studies demonstrated *in vitro* in PBMC the existence of thresholds for the induction of chromosome non-disjunction and chromosome loss by microtubule inhibitors (7,8). It was also shown by our laboratory that the microtubule inhibitors nocodazole and carbendazim are capable of inducing apoptosis and that micronucleated cells can be eliminated by apoptosis (3). The fact that apoptosis can modify the frequencies of micronucleated cells is of concern for the accurate assessment of mutagenicity related to exposure to mutagens (clastogens and aneugens), which are also apoptogens, and for risk assessment of aneugens.

Nocodazole-induced apoptosis and MN in human PBMC

In a previous study of our laboratory (3) where we analysed MN frequencies in the apoptotic cell fraction, we showed that the presence of MN in a cell, induced by microtubule inhibitors such as nocodazole, is related to the induction of apoptosis and

I.Decordier, E.Cundari and M.Kirsch-Volders

that, therefore, micronucleated cells can be eliminated by apoptosis. We confirmed our previous results on the specific elimination of micronucleated peripheral blood lymphocytes showing that, when apoptosis is inhibited by the use of inhibitors of caspase-9, and to a lesser extend caspase-8, the frequency of MNCB increases. These observations showed that a significant number of cells undergoing micronucleation is eliminated by apoptosis. When caspase-3 was inhibited, however, nocodazole-induced apoptosis decreased as expected but the frequency of nocodazole-induced MNCB was lower than without the inhibitor. This substantially differentiates the role of caspase-3 from that of caspases-8 and -9, suggesting for caspase-3 a function other than the elimination of micronucleated cells by apoptosis, in particular the formation of MN.

Caspase-3 activity is involved in MN formation induced by aneugens and clastogens

To further assess the possible involvement of caspase-3 in MN formation, we compared the induction of MN by the aneugen nocodazole, the clastogen MMS and the non-mutagenic apoptogen staurosporin (9) in two human paired MCF-7 cell lines, one expressing caspase-3 and one deficient for caspase-3. It has been described in literature (19,20) that p53 accumulates into MN, although its role has not yet been elucidated. The two cell lines used in the present study are isogenic and, in particular, both express wild-type p53 (16), which rules out the possibility that the differences we describe are owing to a difference in p53 expression between the two cell lines.

To consolidate the results obtained in the caspase-3 deficient cell line, inhibition experiments by addition of the caspase-3 inhibitor Ac-DEVD-CHO in the caspase-3 proficient cell line were performed in parallel. In each case where caspase-3 was inactive, i.e. in the parental MCF-7 cell line or in the MCF-7^{casp-3} cells in the presence of the caspase-3 inhibitor, the frequencies of nocodazole or MMS-induced micronucleated cells decreased, suggesting that caspase-3 contributes to the formation of MN. This seems to be irrespective of the constitution of the induced MN, since the same effect of caspase-3 activity on MN formation was observed for both MN originating from chromosome loss (nocodazole), as well as for those originating from chromosome fragments (MMS).

The fact that at 0.1212 μ M nocodazole the frequency of micronucleated cells was similar in both cell lines, while above that dose of 0.1212 μ M, a significant increase of micronucleated cells was observed only in the MCF-7^{casp-3} proficient cell line suggests that the existence of an interplay between caspase-3 and microtubule integrity possibly modulates a non-apoptotic function such as micronucleus formation.

The caspase-3 specific inhibitor provoked a much stronger decrease of MMS-induced micronucleated cells than of MMS-induced apoptosis. This suggests that, at least for MMS, caspase-3 can be dispensable for apoptosis and other effector caspases, such as caspase-6 or -7, could compensate the lack of caspase-3 activity. However, the role of caspase-3 in MN formation seems to be more difficult to be compensated.

Experiments with STP were performed to exclude that the observed micronucleated cells corresponded to apoptotic bodies. In fact, the latter figures were discarded from countings and only CB without chromatin hypercondensation were taken into account for the determination of MNCB (21). Staurosporine, which in our hands actively induced apoptosis in MCF-7^{casp-3} cells, did not significantly induce MN at any concentration or sampling time tested and in any cell line challenged. These data

suggest that the scored MN are morphologically and functionally distinct from apoptosis.

With the MCF-7 and MCF-7^{casp-3} cell lines, we also aimed at confirming the results observed in lymphocytes for nocodazole-induced apoptosis and MN. The results recorded in the MCF-7^{casp-3} cell line, corresponding to the same condition as in lymphocytes, confirm that when the apoptotic pathway is obstructed by inhibiting the apical caspases-8 and -9, the frequency of micronucleated cells increases. Comparison between the two cell lines and lymphocytes indicates that in each condition where caspase-3 was deficient the frequencies of MN decreased. Even when frequencies of micronucleated cells increases of or -9, this increase was significantly stronger in all conditions where caspase-3 was active (Figure 3B and C).

It has been shown by Shimizu *et al.* (22) that following gene amplification double minutes-containing MN can be actively extruded from the cell. Although we cannot exclude the possibility that some genotoxic agent-induced MN are extruded, the phenomenon that we describe here seems to be much more complex. For example, depending on which caspase is inhibited, we observed either an increase or a decrease of micro-nucleated cells. We are therefore in favour of the hypothesis that caspase-3 is directly involved in the formation of MN, independently of apoptosis, rather than in their extrusion.

Recent literature has highlighted non-apoptotic functions of caspases. It was shown by Miossec et al. (23) and Wilhelm et al. (24) that caspase-3 like enzymes are activated in nonapoptotic human T-lymphocytes stimulated by IL-2, suggesting an involvement of caspase-3 in cell proliferation and/or nuclear division. Furthermore Zermati et al. (25) and Carlile et al. (26) demonstrated that normal erythroid differentiation requires the activation of caspases such as caspase-3. Regulation of apoptosis is associated with cell cycle (27) and cell division (28) and proteolysis of different substrates is critical for a wide range of processes in both apoptotic and nonapoptotic conditions. The exact mechanisms of MN formation has still to be elucidated. One can assume that potential targets for caspase-3 in this non-apoptotic process include proteins involved in integrity of the nucleus such as the nuclearmitotic apparatus protein, which is involved in mitotic assembly and nucleus formation after completion of mitosis (29) and lamin B, a component of the nuclear envelope (30), which has been shown to be involved in micronucleation following impairment of DNA replication (31). In conclusion, we confirmed that apoptosis can eliminate micronucleated cells induced by the aneugen nocodazole. We demonstrated that apoptosis induced by nocodazole involves caspases-9, -8 and -3. Moreover, we provided additional evidence for the role of caspase-3 in non-apoptotic cellular processes and more specifically in the formation of MN.

Supplementary Material

Supplementary material can be found at: http://www.mutage. oupjournals.org/

Acknowledgements

Dr Kaina (German Cancer Research Center, Heidelberg, Germany), Dr Jänicke (Institute of Molecular Medicine, Dusseldorf, Germany) and Dr Kaufmann (Mayo Clinic College of Medicine, Rochester) are gratefully acknowledged for providing the MCF-7 and the MCF-7^{casp-3} cell lines. This study was supported by the EU research program QLK4-CT-2000-00058.

References

- Kirsch-Volders, M., Elhajouji, A., Cundari, E. and Van Hummelen, P. (1997) The *in vitro* micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. *Mutat. Res.*, **392**, 19–30.
- Kirsch-Volders, M., Sofuni, T., Aardema, M. et al. (2003) Report from the in vitro micronucleus assay working group. Mutat. Res., 540, 153–163.
- Decordier, I., Dillen, L., Cundari, E. and Kirsch-Volders, M. (2002) Elimination of micronucleated cells by apoptosis after treatment with inhibitors of microtubules. *Mutagenesis*, 17, 337–344.
- Yujiri, T., Fanger, G.R., Garrington, T.P., Schlesinger, T.K., Gibson, S. and Johnson, G.L. (1999) MEK kinase 1 (MEKK1) transduces c-Jun NH₂terminal kinase activation in response to changes in the microtubule cytoskeleton. J. Biol. Chem., 274, 12605–12610.
- Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Nature*, 281, 1312–1316.
- Sun,X., Farlane,M., Zhuang,J., Wolf,B.B., Green,D.R. and Cohen,G.M. (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem., 274, 5053–5060.
- Elhajouji, A., Van Hummelen, P. and Kirsch-Volders, M. (1995) Indications for a threshold of chemically-induced aneuploidy *in vitro* in human lymphocytes. *Environ. Mol. Mutagen.*, 26, 292–304.
- Elhajouji,A., Tibaldi,F. and Kirsch-Volders,M. (1997) Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors *in vitro* in human lymphocytes. *Mutagenesis*, 12, 133–140.
- Meintières, S., Biola, A., Pallardy, M. and Marzin, D. (2003) Using CTLL-2 and CTLL-2 bcl2 cells to avoid interference by apoptosis in the *in vitro* micronucleus test. *Environ. Mol. Mutagen.*, 41, 14–27.
- Jänicke,R.U., Sprengart,M.L., Wati,M.R. and Porter,A.G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem., 273, 9357–9360.
- Kirsch-Volders, M., Sofuni, T., Aardema, M. *et al.* (2004) Corrigendum to 'Report from the *in vitro* micronucleus assay working group' [*Mutat. Res.*, 540 (2003) 153]. *Mutat. Res.*, 564, 97–100.
- Kim,D.K., Cho,E.S. and Um,H.-D. (2000) Caspase-dependent and -independent events in apoptosis induced by hydrogen peroxide. *Exp. Cell Res.*, 257, 82–88.
- Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W. and Thornberry, N.A. (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J. Biol. Chem., 273, 32608–32613.
- Slee,E.A., Harte,M.T., Kluck,R.M. *et al.* (1999) Ordening the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspase-2, -3, -6, -7, -8 and -10 in a caspase-dependent manner. *J. Biol. Chem.*, **144**, 281–292.
- Verdoodt,B., Decordier,I., Geleyns,K., Cunha,M., Cundari,E. and Kirsch-Volders,M. (1999) Induction of polyploidy and apoptosis after exposure to high concentrations of the spindle poison nocodazole. *Mutagenesis*, 14, 513–520.
- Jänicke,R.U., Engels,I.H., Dunkern,T., Kaina,B., Schulze-Osthoff,K. and Porter,A.G. (2001) Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. *Oncogene*, **20**, 5043–5053.
- Cummings, B.S., Kinsey, G.R., Bolchoz, L.J.C. and Schnellmann, R.G. (2004) Identification of capase-independent apoptosis in epithelial and cancer cells. J. Pharmacol. Exp. Ther., 310, 126–134.
- Abramsson-Zetterberg, L. (2003) The dose-response relationship at very low doses of acrylamide is linear in the flow cytometer-based mouse micronucleus assay. *Mutat. Res.*, 535, 215–222.
- Granetto, C., Ottaggio, L., Abbondandolo, A. and Bonatti, S. (1996) p53 accumulates in micronuclei after treatment with a DNA breaking chemical, methylnitrosourea, and with the spindle poison, vinblastine. *Mutat Res.*, 352, 61–64.
- Sablina,A.A., Ilyinskaya,G.V., Rubtsova,S.N., Agapova,L.S., Chumakov,P.M. and Kopnin,B.P. (1998) Activation of p53-mediated cell cycle checkpoint in response to micronuclei formation. *J. Cell Sci.*, **111**, 977–984.
- Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S. and Zeiger, E. HUman MicronNucleus project. (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat. Res.*, 534, 65–75.
- Shimizu,N., Shimura,T. and Tanaka,T. (2000) Selective elimination of acentric double minutes from cancer cells through the extrusion of micronuclei. *Mutat. Res.*, 448, 81–90.

- 23. Miossec, C., Dutilleul, V., Fassy, F. and Diu-Hercend, A. (1997) Evidence for CPP32 activation in the absence of apoptosis during *t* lymphocyte stimulation. *J. Biol. Chem.*, **272**, 13459–13462.
- Wilhelm,S., Wagner,H. and Häcker,G. (1998) Activation of caspase-3-like enzymes in non-apoptotic T cells. *Eur. J. Immunol.*, 28, 891–900.
- Zermati, Y., Garrido, C., Amsellem, S., Fishelson, S., Bouscary, D., Valensi, F., Varet, B., Solary, E. and Hermine, O. (2001) Caspase activation is required for terminal erythroid differentiation. *J. Exp. Med.*, **193**, 247–254.
- Carlile,G.W., Smith,D.H. and Wiedman,M. (2004) Caspase-3 has a nonapoptotic function in erythroid maturation. *Blood*, **103**, 4310–4316.
- Li,F.Z., Ackermann,E.J., Bennett,C.F., Rothermel,A.L., Plescia,J., Tognin,S., Villa,A., Marchisio,P.C. and Altieri,D.C. (1999) Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat. Cell Biol.*, 1, 461–466.
- Yan,X., Najbauer,J., Woo,C.C., Dashtipour,K., Ribak,C.E. and Leon,M. (2001) Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain. *J. Comp. Neurol.*, 433, 4–22.
- Hirata,H., Takahashi,A., Kobayashi,S., Yonehara,S., Sawai,H., Okazak.i, T., Yamamoto,K. and Sasada M. (1998) Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J. Exp. Med., 187, 587–600.
- Kottke, T.J., Blajeski, A.L., Meng, X.W. *et al.* (2002) Lack of correlation between caspase activation and caspase activity assays in paclitaxeltreated MCF-7 breast cancer cells. *J. Biol. Chem.*, 277, 804–815.
- Tanaka,T. and Shimizu,N. (2000) Induced detachment of acentric chromatin from mitotic chromosomes leads to their cytoplasmic localization at G₍₁₎ and the micronucleation by lamin reorganization at S phase. *J. Cell Sci.*, **113**, 697–707.

Received on July 30, 2004; revised and accepted on March 7, 2005