Caffeine effect on the mitotic delay induced by G2 treatment with UVC or mitomycin C

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It is well established that DNA lesions trigger cell cycle check-points causing a mitotic delay that is required for their repair before cells enter the mitotic phase. Caffeine, in some cases, can remove this delay and consequently potentiates the yield of induced chromosome aberrations. The objective of this study was to test the effect of a G2 treatment with S-dependent agents (UV light and mitomycin C) on the cell kinetics of a G2 cell population and evaluate whether post-treatments with caffeine could modulate removal of the expected cell cycle delay. Cell kinetics were monitored by analyzing the mitotic index (MI) values in combination with the 5-bromo-2′-deoxyuridine (BrdUrd) labelling technique. Chinese hamster fibroblast cultures were treated in G2 phase of the cell cycle with 8 and 15 J/m² UV light or 0.6 μg/ml mitomycin C for 1.5 h. Post-treatments with caffeine were performed at dose levels and recovery times where the mitotic indices were substantially reduced. The results obtained showed that both UV light and mitomycin C induced a G2 arrest, as indicated by MI values and the absence of BrdUrd-labelled metaphases. For UV light the G2 block was observed at lower and higher dose levels after 1.5 h, while for mitomycin C it was observed only at the higher dose level after 1 h. However, in both cases the block lasted ~1 h, after which, even though slowed down, the cell population entered mitosis, as indicated by increased MI values. This block was not removed by caffeine post-treatment. In contrast, caffeine G2 post-treatment was able to remove G2 arrest induced by G1→S treatments. Accordingly, our results suggest that both UV light- and mitomycin C-induced damage must be processed during S phase to allow caffeine to remove induced G2 blocks.

Introduction

It is known that treatments with mutagens induce DNA damage in living cells leading to the activation of cell cycle check-point control mechanisms in different phases of the cell cycle (Paulovich et al., 1997). Both prokaryotic and eukaryotic cells delay cell division following damage to DNA. The threat represented by damaged DNA is particularly serious during the two major cell cycle events: DNA replication and mitosis. Delays in progression from both G1 to S and from G2 to M occur in most organisms (Tolmach et al., 1977; Painter and Young, 1980; Lau and Pardee, 1982; Kaufmann et al., 1991; O’Connor et al., 1992).

It is thought that these cell cycle check-points permit repair of damaged DNA before replication or segregation of the affected chromosomes (Hartwell and Weinert, 1989) and should thus enhance cell survival and limit propagation of heritable genetic errors (Weinert and Hartwell, 1988). DNA damage may also induce apoptosis as an alternative strategy to prevent propagation of damaged cells. The tumour suppressor protein and transcription factor p53 is involved in both G1 arrest and nucleotide excision repair (Livingstone et al., 1992; Dulic et al., 1994; Abrahams et al., 1995). Through sequence-specific DNA binding p53 can modulate transcription of a variety of target genes following DNA damage (Cox and Lane, 1995); such genes are likely to be involved in G1 arrest or apoptotic cell death (Kastan et al., 1995).

In addition to this check-point, eukaryotic cells are able to arrest in the G2 phase of the cell cycle after DNA damage (Bernhard et al., 1994; Kaufmann, 1995; Murnane, 1995). Arrest in the G2 phase is accompanied by an increase in phosphorylation of Thr14 and Tyr15 of the catalytic p34 subunit of the cyclin-Cdk complex and by a decrease in Cdk 1 kinase activity in hamster cells as well as in human cells (Hain et al., 1993, 1994; Kharbanda et al., 1994). For Chinese hamster cells inhibition of phosphorylation has also been shown for p40, a Cdk 2-like protein (Hain et al., 1994).

The G2 phase arrest is temporary and can be released prematurely by caffeine, reducing the mitotic delay (Lücke-Hunle et al., 1983; Zampetti-Bossler and Scott, 1985; Darroudi and Natarajan, 1987; Hain et al., 1994; Russell et al., 1995).

During the mitotic delay some of the induced damage that could lead to chromosomal aberrations is repaired. Cancellation of delay reduces the time available for such repair, so that cells enter division with a higher proportion of DNA damage than would normally be the case (Painter, 1980).

The ability of caffeine to potentiate the induction of chromosome aberrations is due to inhibition of repair of DNA damage (Natarajan and Obe, 1983). However, it is also suggested that mitigated delay and enhanced aberration frequency are not necessarily causally related (Harvey and Savage, 1994). It is usual to distinguish between two main types of mechanisms for the production of chromatid-type aberrations; when induced by UV, alkylating agents and many other chemicals, aberrations arise by an S phase-dependent mechanism. This means that the DNA lesions caused by these agents do not give rise to chromatid-type aberrations before they have been replicated. Thus, DNA lesions induced by S phase-dependent agents in G2 do not result in chromatid aberrations until the second cell cycle after their induction. Chromatid aberrations may, however, be observed in the first mitosis after treatment when the lesions have been induced during G1 or S in unreplicated chromosomes or chromosome sites through 'misreplication' (Evans and Scott, 1969). A caffeine post-treatment potentiates the frequency of induced chromosome aberrations by S-phase dependent agents, provided that the treatment has been done in G1 or S phase of the cell cycle.

The aim of this work has been to test if a caffeine post-

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treatment is also able to remove the G2 arrest caused by a G2 treatment with S phase-dependent agents.

Materials and methods

Cell cultures

Chinese hamster fibroblasts (AA8), kindly provided by Prof. F. Cortés (University of Sevilla, Sevilla, Spain) were cultured in McCoy's 5 medium, supplemented with 15% newborn calf serum and 2% L-glutamine and incubated at 37°C in a 5% CO₂ atmosphere (100% humidity nominal). Cells were subcultured every 3-4 days at 3×10⁵ cells/25 cm² flask.

Approximately 18-20 h before treatment an appropriate number of 60 mm Petri dishes were prepared from a single pool of cells. Each Petri dish was seeded with 3×10⁵ cells.

UV and MMC G2 treatment

A scheme of the experimental procedure is shown in Figure 1. Immediately before treatment the cultures were shaken to remove mitotic cells to avoid any contamination of G2 cells with mitotic cells. Cultures were then exposed to 8 or 15 J/m² UV without any medium, after one wash with phosphate-buffered saline (PBS), or for 1.5 h to 0.1 or 0.6 µg/ml mitomycin C (MMC) in fresh complete medium with 30 µg/ml 5-bromo-2'-deoxyuridine (BrdUrd). The UV light was generated by a Spectroline UV lamp (254 nm).

At the end of treatment UV-treated cultures were resuspended in fresh complete medium containing 30 µg/ml BrdUrd, while MMC-treated cultures were washed twice and resuspended in fresh complete medium. In both treatments BrdUrd pulse labelling lasted 1.5 h.

Post-treatments with caffeine (1 h at a final concentration of 5 mM) were performed only at dose levels where the mitotic indices (MIs) were substantially reduced to check the sensitivity of the G2 block to caffeine. Colchicine (10 µM) was added to the cell cultures at the appropriate time before fixing as described in Figure 1. Cultures were incubated at intervals of 1-2 h over a maximum period of 8 h and cytological microscope slides prepared according to standard procedures.

Control cultures were handled under identical conditions. The experiments were repeated three times and the results are representative of these repeats.

Staining of slides

At least three slides per test point were prepared. One set was stained with 3% Giemsa for 5 min to be scored for MI. The remaining slides were processed using immunocytogenetic techniques with anti-BrdUrd antibodies conjugated with the fluorochrome FITC. The slides were denatured for 1 min in 10 mM NaOH, 70% ethanol, dehydrated in a 70, 90 and 100% ethanol series and air dried. The slides were then incubated in a moist chamber for 30 min with 100 µl/slide mouse anti-BrdUrd antibody (Boheringer-Mannheim) diluted 1:100 in immunological buffer (PBS, 0.5% bovine serum albumin, 0.5% Tween 20) under a 24X50 coverslip.

The slides were washed three times with PBS and subsequently incubated with 100 µl/slide goat anti-mouse IgG-FITC antibody (Boheringer-Mannheim) diluted 5:100 in immunological buffer for 30 min. After three washes in PBS and dehydration in ethanol, the slides were embedded in Vectashield mounting medium (Vector Laboratories) containing 0.3 µg/ml propidium iodide (PI).

Scoring of slides

Mitotic indices were determined from 1000 cells analysed with a 100X oil immersion objective.

BrdUrd labelling of mitotic cells was evaluated using a Zeiss (Axiophot) fluorescence microscope equipped with single and dual bandpass filters for FITC and PI and a CCD camera (Photometrix) operated by IPLab spectrum software. Images were captured through the above-mentioned system and analysed on a 21 inch high resolution monitor.

For each test point, from at least 100 metaphases, metaphases were classified as unlabelled (G2 cells) or early, mid or late replicating S phase cells according to the BrdUrd labelling pattern, as also described by Savage in human lymphocytes (Savage and Prasad, 1984). In our specific case we used chromosome X as a marker to allocate each metaphase to the three different S phase stages.

In Chinese hamster, chromosome X bears a short euchromatic arm which is early replicating and a long heterochromatic arm which is late replicating. According to these features, we allocated single metaphases to the different phases.

Fig. 1. Experimental scheme of the G2 treatments.
compartments as follows: (i) late S phase, the whole long arm of chromosome X is labelled (Figure 2a); (ii) mid S phase, chromosome X is uniformly labelled in both arms (Figure 2b); (iii) early S phase, the short arm of chromosome X is labelled (Figure 2c).

**Results**

**Analysis of mitotic indices**

The MI data have been plotted and reported in Figure 3a and b for UV- and MMC-treated cultures respectively. Analysis of the results shows that at the higher dose level for UV the MI was depressed to ~40% of the relevant control value, while at the lower dose level the decrease in MI started 1.5 h after treatment. The maximum inhibition of MI represented by a decrease of ~80% of the relevant control value was observed at both dose levels 2.5 h after treatment. The block lasted ~1 h, after which MI started to increase slowly. At the lower dose level, the MI values reached the relevant control value 5.5 h later, while at the higher dose level it reached only 50% of that value.

For MMC-treated cultures a marked depression in MI was only seen at the higher dose level, with a drop to 80% of the relevant control value 1 h later. This value increased slightly 1 h later, to 50% of the control value, after which it remained constant until the end of observation 8 h later, while control values progressively increased. At the lower dose level the MI values were in line with the control values up to 2 h, after which it dropped to ~50% of the control value in the next 2 h. This represented the maximum inhibition of MI at this dose level. From this point and until the end of observation (8 h) the MI values, even though slowed, followed the same trend as the control values. The fluctuations in MI in the UV and MMC controls almost certainly reflect perturbations introduced by the mitotic shake-off, the medium change and, in the case of the MMC experiment, the additional washes after a period corresponding to the treatment and the differences in the control frequencies between the UV and MMC experiments is due to different handling of the cultures in the two experiments. The increase in the MI with time in the MMC controls is, probably, due to a partial synchronization induced by the mitotic shake-off and by the vigorous washes used to remove MMC in the treated cultures. This trend in the MI values of MMC controls has been observed in all three repeats of the experiment and so the mitotic depression and the recovery seen following MMC and UVC treatments should not be considered an artefact.

**Analysis of BrdUrd labelling**

The percentages of labelled metaphases (early, mid and late replicating S phases) at different recovery times have been plotted and are presented in Figure 4a and b for UV- and MMC-treated cultures respectively. The results show that the appearance of labelled mitotic cells in the control was observed 2.5 h after pulse labelling, while for the UV-treated cells it was delayed to 5.5 h. At this time the percentage of labelled metaphases was slightly higher at 8 J/m² compared with 15 J/m², showing that this delay is dose dependent. After 5.5 h the ‘labelling index’ increases with time but does not reach the control values, at least in the time range analysed. Figure
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Fig. 3. Time course of the MI after G2 exposure to (a) UVC radiation and (b) MMC. After mitotic shake-off cells were treated with UVC or MMC, then challenged in fresh complete medium and fixed at various intervals to determine the percent of mitotic cells. For each test point 1000 cells were analysed.

Fig. 4. Percentage of labelled metaphases after different recovery times of (a) UVC- and (b) MMC-treated cultures. Cultures were pulse labelled with 30 µg/ml BrdUrd for 1.5 h after UV radiation or during the MMC treatment. Slides, prepared for each experimental point as described in Materials and methods, were scored and 100 metaphases were randomly analysed and subdivided into G2 (unlabelled metaphases) or S phase (labelled metaphases).

5a-c shows the distribution of labelled metaphases in the three classes according to the BrdUrd labelling pattern (early, mid and late S phase) following treatment with 8 and 15 J/m². The results show that early S phase is strongly delayed at both dose levels compared with the control culture, while the appearance of late S phase is slowed in a dose-dependent manner.

For the MMC treatment, when the pooled data are analysed (Figure 4b) a delay in the appearance of labelled metaphases was observed at the higher dose level only. This delay appears to be caused mainly by early S phase, which was markedly slowed (Figure 6b).

Caffeine effect on UV- and MMC-induced mitotic delay

In order to study the effect of caffeine on the prevention of the delay in G2 phase exit, we post-treated AA8 cells for 1 h with 5 mM caffeine after treatment with UV or MMC. The results obtained, reported in Table I, show that caffeine is unable to remove the G2 block caused by UV irradiation and MMC treatment when performed in the G2 phase, as indicated by MI values.

Discussion

In the present study we observed that both UVC and MMC G2 treatments induced a delay in the G2 phase of Chinese hamster fibroblasts (AA8). This delay has been also described in other cell lines after UVB treatment (Hain et al., 1996) and, to a lesser extent, after UVA treatment (Bannud et al., 1995). Furthermore, the length of the G2 block depends on the dose levels used, as previously described by Orren et al. (1995). For both UV and MMC treatments, the arrest of cells is
immediate at higher dose levels, while at lower doses only UV is able to induce G2 block, which is shifted by 1.5 h compared with the higher dose level. This different behaviour may be explained not only by different cytotoxic effects, but also by different modulation of the block of S phase cells. In fact, while for MMC the block at the higher dose level causes a shift of 2 h in the appearance of early S phase cells, followed by a rapid recovery, in the UV treatment at both dose levels, early S phase cells and, to a lesser extent, mid S phase, even though delayed as in MMC-treated cells, do not recover from the block. In this case late S phase cells were slowed in a dose-dependent manner. Nevertheless, the block was partially reversible in both treatments and our results show that cells were able to leave G2 phase gradually during the analysed recovery periods. This suggests that early S cells are most damaged by the treatment. Finally, our experimental data show that a 5 mM caffeine post-treatment is unable to remove the G2 delay caused by G2 treatment with MMC or UV. That this cell line is insensitive to caffeine can be excluded, as we have treated AA8 cells in G1–S phase with MMC or UV and post-treated with caffeine in the G2 phase. In this experiment (data not shown) it was confirmed that G2 arrest was removed by a G2 caffeine post-treatment. It is not still clear how caffeine removes the G2 arrest. Some authors believe that caffeine acts predominantly in two ways: increasing the activity of cdc2 kinase (Hain et al., 1994) or inhibiting DNA repair and consequently causing premature entry into mitosis (Link et al., 1995). Moreover, caffeine shows a high affinity for single-stranded DNA and might mask damaged sites by upstream blocking of the signalling pathway that links DNA repair to the G2 check-point (Weibezahn and Coquerelle, 1986). In fact, caffeine has been shown in vitro to alter the
ability of certain damage recognition proteins (Uvr A and photolyase) to find and bind sites of UV damage in DNA in bacterial extracts (Selby and Sancar, 1990).

Also, caffeine might interfere with DNA repair and the G2 check-point by inhibiting topoisomerases I and II (Shin et al., 1990). Indirect molecular involvement of topoisomerases in DNA repair has not yet been demonstrated (Stevner and Bohr, 1993), but it has recently been proved that topoisomerase II is involved in G2 check-point control (Downes et al., 1994).

Our data show that even if the G2 block is induced by G2 treatment with an S phase-dependent agent, its removal by caffeine is dependent on 'processing' of the lesions through S phase, in order that caffeine recognizes it. Actually, we cannot exclude the possibility that G2 arrest, induced by UVC or MMC, is not only dependent on DNA damage, but also on injury to other cellular structures (Schneidermann et al., 1996).

<table>
<thead>
<tr>
<th>G2 treatment</th>
<th>MI (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>4.9</td>
</tr>
<tr>
<td>0.1 μg/ml MMC</td>
<td>4.9</td>
</tr>
<tr>
<td>0.6 μg/ml MMC</td>
<td>2.7</td>
</tr>
<tr>
<td>Control + 5 mM caffeine</td>
<td>1.5</td>
</tr>
<tr>
<td>0.1 μg/ml MMC + 5 mM caffeine</td>
<td>2.0</td>
</tr>
<tr>
<td>0.6 μg/ml MMC + 5 mM caffeine</td>
<td>1.3</td>
</tr>
<tr>
<td>Control</td>
<td>3.3</td>
</tr>
<tr>
<td>8 J/m²</td>
<td>0.5</td>
</tr>
<tr>
<td>15 J/m²</td>
<td>0.7</td>
</tr>
<tr>
<td>Control + 5 mM caffeine</td>
<td>2.0</td>
</tr>
<tr>
<td>8 J/m² + 5 mM caffeine</td>
<td>0.1</td>
</tr>
<tr>
<td>15 J/m² + 5 mM caffeine</td>
<td>0.5</td>
</tr>
</tbody>
</table>
However, the UVC radiation doses used in this work essentially cause DNA damage.

In conclusion, these data show that G2 treatment with UVC or MMC is able to cause a G2 arrest and that caffeine does not remove this G2 block, whereas it is able to remove a G2 arrest caused by G1 or S treatment with the same agent. Therefore, it can be concluded that, in the case of the tested S phase-dependent agents, in order to show a caffeine effect the induced DNA lesions have to pass through S phase.

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References


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