A negative adaptive response is expressed in peripheral blood lymphocytes that are exposed to mitomycin C and cycloheximide

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Summary

**Purpose:** Mitomycin C (MMC) and cycloheximide (CHX) are known for their apoptotic and antitumor activity. CHX is also known for its property to inhibit protein synthesis and to reduce cytotoxicity of various antitumor drugs, i.e. inducing an adaptive survival response (ASR). The purpose of this study was to evaluate the effect of ASR induced by CHX in cells exposed to clastogenic doses of MMC.

**Materials and methods:** In all experiments we used human peripheral blood lymphocytes of 10 healthy male non-smokers, 25-35 years of age. Three groups were established. One control group or PBS-treated group. Two distinctive experimental groups were based on the induction or non-induction of ASR by CHX, i.e. one with MMC alone and a second one with CHX and MMC. The effect of ASR was induced by CHX at a dose of 10 µg/ml. MMC was used in 3 dose levels: 0.05 µM, 0.15 µM and 0.6 µM. To evaluate ASR induced by CHX in cells exposed to MMC we used the cytokinesis-blocked micronucleus test (CBMN) in vitro.

**Results:** CHX at a dose of 10 mg/ml induced an ASR in human peripheral blood lymphocytes of healthy subjects exposed to increasing doses of MMC. CHX induced statistically highly significant difference (p < 0.001) in the nuclear division index (NDI) compared to cells exposed to MMC alone. Genotoxicity of MMC measured by the percentage of micronuclei in binuclear (BN) cells was not elevated in the presence of CHX. Also, the increase in the NDI was correlated with the decrease in nuclear fragmentation (NF).

**Conclusion:** The observed differences in NF and the NDI between the two groups showed that ASR to MMC induced by CHX could be a consequence of inhibition of apoptosis. We argue that adaptation (pro-life processes) can overwhelm its positive aspects (antimutagenic and anticarcinogenic) by increasing the population of cells with chromosome aberrations (chromosome instability) by apoptotic inhibition. CHX disturbs the apoptotic signal. Understanding that ASR can act as a pro-survival process leading to inhibition of apoptosis shall enhance in the future our knowledge of anticarcinogenesis, thus utilizing new paths for better treatment of cancer.

**Key words:** adaptive survival response, apoptosis, cycloheximide, micronucleus test, mitomycin-C

Introduction

An adaptive response of the genome protection machinery occurs in cells exposed to genotoxic stress. ASR whereby cells demonstrate a survival advantage when exposed to very low doses of ionizing radiation (IR), 4-24 h prior to a high dose of IR (challenge dose), were first reported over 15 years ago [1]. These responses were linked to “hormesis”, which implied that exposure to low levels of IR may in one way be beneficial to the cell, but on the other hand increased survival does not necessarily mean that the treatment is “beneficial” [2]. ASR may also be a major effector in IR-induced carcinogenesis and the formation of secondary cancer cells from exposed normal cells, numerous cell generations post-IR treat-
ment [2]. Substantial data is accumulating regarding the interplay between genomic instability, delayed onset of apoptosis, the emergence of neoplastically transformed cells and the ASR phenomena induced by IR and chemical agents [3-6]. An “adaptive response” was also observed in cells exposed to reactive oxygen species (ROS) inducible by alkylating agents. After treatment with a sublethal level of an alkylating agent, cells developed transient resistance to a subsequent toxic dose of alkylating agents [7]. ROS react with most cellular components, and are invariably genotoxic because they react with both the deoxyribose and bases in DNA, thus generating a plethora of base lesions and strand breaks. Many of these lesions are cytotoxic and mutagenic. Most, if not all, of the lesions induced by ROS are repaired via the base excision repair (BER) pathway, in which the damaged bases are removed by specific DNA glycosylases, leaving abasic sites that are subsequently cleaved by apurinic/apyrimidinic endonucleases (APE). The major mammalian APE (called APE-1) contributes more than 80% of the total APE activity [7-9]. DNA repair machinery adaptively responds to oxidative and nitrosative stress, both in vitro and in vivo. Although pro-oxidant states [10] are pro-apoptotic, they may have deleterious effects on the cell by generating damage in key cancer genes and proteins. There has been a growing evidence that tumor suppressor proteins, when involved in ASR processes, lead to instability [6]. p53 has been shown to transactivate two proteins, when involved in ASR processes, lead to instability [6]. p53 has been shown to transactivate genes involved in freeradical formation [6,8,9]. In mammalian cells overexpression of specific BER enzymes can generate chromosomal instability and may contribute to tumorigenesis [6,11,12].

p21Waf1/Cip1/Sdi1 is a key protein involved in both G1 and G2 cell cycle arrest [13-15]. Wild-type p21Waf1/Cip1/Sdi1 can also inhibit apoptosis and stimulate transcription of secreted factors with mitogenic and anti-apoptotic activities [13]. These known facts highlight the pro-carcinogenic role for p21Waf1/Cip1/Sdi1 and a need to re-assess the role of this protein as a genome protector. This adaptive imbalance, resulting in cell survival, might have deleterious effects because this allows for a cell to accumulate genetic mutations and protein damage.

Two views can be elucidated by these investigations: the first or beneficial ASR, in which cells adapt to genotoxic stress without altering the structure of their genetic material; and the second or negative ASR, in which there is survival of cells with chromosomal and genetic damage, i.e. where apoptosis is inhibited, leading to initiation of pro-carcinogenic processes [6,16,17].

In cancer therapy, ASR should have an especially important role in risk evaluation, i.e. the synergistic or additive effect of a single chemotherapeutic agent with radiation therapy or combination therapies of various antitumor agents in the treatment of cancer can have a negative ASR effect, leading to the survival of damaged cells. The use of chemotherapy has become more extensive in recent years and has added to the success of treatments, while long term follow up has permitted the assessment of survivors for the development of second malignant tumors, which could also be a sequence of a negative ASR effect [18].

The phenomenon of ASR has been mostly studied with low IR doses [19], but alkylating compounds such as MMC, bleomycin, hydrogen peroxide and metals may also induce an adaptive response, used as challenging agents and showing properties of cross-adaptation [20-22].

Cross-adaptation could influence a negative adaptive response resulting in survival of damaged cells, i.e. a possible mechanism for the occurrence of secondary tumors. The protein synthesis inhibitor CHX was used to explore a possible negative adaptation response in cells exposed to challenging doses of MMC.

CHX can suppress the enhancing effect of sodium arsenite on the incidence of chromatid breaks and chromatid exchanges induced by ethylmethylsulfonate (EMS) in Chinese hamster ovary cells [23]. The suppressive effect of CHX to paclitaxel cytotoxicity was used in the human breast cell line MCF-7 to evaluate apoptosis induction, i.e. CHX-suppressed apoptosis [24]. CHX can induce chromosomal instability expressed as premature centromere separation [16,17,25]. These properties of CHX were used to estimate ASR response in human peripheral blood lymphocytes when challenged with increasing doses of MMC using the CBNM test. This test is used as a reliable endpoint for various cytotoxic and genotoxic agents [26]. ASR was assessed by the formation of micronuclei and by using the NDI in human peripheral blood lymphocytes. Here we argue that adaptation is a balance between apoptosis and survival processes in which ASR can lead to survival of damaged cells with possible pro-carcinogenic consequences.

Materials and methods

In all experiments we used material obtained from human peripheral blood of 10 non-smokers healthy male subjects, 25-35 years of age. The effect of ASR was induced by CHX. All chemicals used in our experiments were purchased from Sigma Chemicals Inc.
1. Induction of ASR

ASR was induced by 10 µg/ml of CHX with an 18 h incubation time without adding phytohemaglutinin (PHA) (G0-phase).

ASR induced by CHX in peripheral blood lymphocytes showed no changes in chromosomal structure and micronucleus formation compared to a negative control group treated with normal saline [16].

2. CBMN test in vitro

This test was carried out according to the method described by Fenech [27]. Peripheral blood samples from 10 healthy male subjects were taken in previously heparinized test tubes. Lymphocyte-rich plasma was cultivated at a concentration of 5×10⁵ cells/ml in RPMI medium supplemented with 10% fetal calf serum (FCS) and a standard solution of antibiotics (penicillin and streptomycin). Three groups were established. One control group or PBS-treated group. Two distinctive experimental groups were based on the induction or non-induction of ASR by CHX. In all groups lymphocytes were incubated for 18 h before adding PHA. After the incubation time, cells were rinsed and washed of remaining CHX with RPMI. Lymphocytes of all groups were stimulated by adding 2% of PHA (Falcon). Forty-eight hours after PHA stimulation, MMC was added to the cultures at a dose of 0.05 µM/ml, 0.15 µM/ml, and 0.6 µM/ml. Forty-four hours after PHA stimulation, cytochalasin B was added at a concentration of 4 µg/ml. After the last fixation cell suspension was dropped onto clean slides. After air drying (24 h), cells were stained with 2% Giemsa for 3 min. The presence of micronuclei was analysed in 1000 binuclear cells. Blood samples were coded with an identification number and slides were scored blind. For mononuclear cells analysis, at least 1000 binuclear cells per culture were analyzed, registering micronuclei according to the criteria of Fenech [27]. The ability of cells to proliferate in vitro was evaluated by counting the number of cells with 1, 2 and 4 micronuclei at the same slide using the NDI. This index was estimated using the formula:

\[ \text{NDI} = \frac{\text{MI} + 2\text{MII} + 3\text{MIII} + 4\text{MIV}}{10^3} \]

were MI to MIV represent the number of cells with 1 to 4 nuclei, respectively, and N is the number of cells scored.

Statistical analysis

For statistical analysis we used the Mann-Witney U-rank test performed by Windows compatible programme -Statistica 5, for establishing the frequency of micronuclei and the X² test for estimating the NDI index.

Results

ASR induced by 10 µg/ml CHX in peripheral blood lymphocytes and then challenged by increasing doses of MMC showed slightly decreased frequency of micronuclei and the presence of NF, but significantly increased proliferative index or NDI.

Table 1 shows a highly statistical significant difference (p <0.001) in the number of binuclear cells with micronuclei between the negative control group (5±3.03) and both experimental groups (MMC alone and MMC plus CHX) in all respective doses: 0.05 µM (13.5±5.1; 16.2±4.7); 0.15 µM (18.1±6.31; 23±6.3, respectively) and the dose of 0.6 µM MMC (27.4±8; 29.7±6.7). These results show that all doses of MMC are genotoxic, i.e. clastogenic, when compared to the control group.

When comparing both experimental groups our results showed no statistically significant difference between all challenging doses (Table 1). Still, CHX induced a low ASR effect based on the absolute values seen in Table 1. The more profound effect of ASR induced by CHX in peripheral blood lymphocytes challenged with increasing doses of MMC can be seen by the proliferative index or NDI and by the detection of NF which was decreased in the experimental group with CHX (Table 1, Figure 1).

Increase in the NDI shows decreased cytotoxicity of MMC. Changes in the presence of NF in the experimental group with CHX preincubation correlated with an increase in the NDI (Table 2).

NDI shows the proliferative capability of the peripheral blood lymphocytes. Table 2 shows the NDI as a measure of cytotoxicity. In the experimental group of MMC alone our results showed a decrease in the NDI (1.033, 1.020 and 1.015) between all doses of MMC (0.05 µM/ml, 0.15 µM/ml and 0.6 µM/ml, respectively) compared to the control group (1.707).

Comparing the NDI between doses in the experimental group with CHX and challenging doses of MMC (0.05 µM/ml and 0.15 µM/ml) an increase of the NDI (1.062 and 1.069) was noticed, showing a possible increase in the ASR effect, but in the highest
The decrease of NF in the CHX experimental group showed a connection of NF and the ASR effect. In the experimental group with CHX the decrease of NF corresponded to an increase of NDI (Table 2, Figure 1 and 2). Figure 1 and 2 show a clear difference in the presence of NF between experimental groups, i.e. MMC alone (Figure 2) and the group with CHX plus MMC (Figure 1) in the same dose of MMC (0.15 µM).

**Table 1.** Number of micronuclei in peripheral blood binuclear lymphocytes in control, challenged and experimental groups

<table>
<thead>
<tr>
<th>Concentration (µM/ml)</th>
<th>Number of MN&lt;sup&gt;+&lt;/sup&gt; and BN&lt;sup&gt;+&lt;/sup&gt; cells</th>
<th>% of BN cells</th>
<th>Number of MN in 10&lt;sup&gt;3&lt;/sup&gt; BN cells</th>
<th>NDI&lt;sup&gt;+&lt;/sup&gt; (1 × M1 + 2 × M2 + 3 × M3 + 4 × M4) × 10&lt;sup&gt;–3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/PBS(K)</td>
<td>1028</td>
<td>55.7</td>
<td>5.0</td>
<td>3.03</td>
</tr>
<tr>
<td>0.05 µM (A) Mitomycin C</td>
<td>1014</td>
<td>3.1</td>
<td>16.2</td>
<td>4.7</td>
</tr>
<tr>
<td>0.15 µM (B) Mitomycin C</td>
<td>1000</td>
<td>2.0</td>
<td>23.0</td>
<td>6.3</td>
</tr>
<tr>
<td>0.6 µM (C) Mitomycin C</td>
<td>1003</td>
<td>1.45</td>
<td>29.7</td>
<td>6.7</td>
</tr>
<tr>
<td>CHX/0.05 µM (D) Mitomycin C</td>
<td>1012</td>
<td>5.3</td>
<td>13.5</td>
<td>5.1</td>
</tr>
<tr>
<td>CHX/0.15 µM (E) Mitomycin C</td>
<td>1000</td>
<td>6.1</td>
<td>18.1</td>
<td>6.31</td>
</tr>
<tr>
<td>CHX/0.6 µM (F) Mitomycin C</td>
<td>1009</td>
<td>2.76</td>
<td>27.4</td>
<td>8.0</td>
</tr>
</tbody>
</table>

<sup>+</sup>MN=mononuclear, <sup>+</sup>B=binuclear, <sup>+</sup> nuclear division index, <sup>+</sup>cycloheximide

K:A, U=1.5; p < 0.0002*** A:B, U=8.5; p < 0.0051** A:D, U=35; p < 0.25 (NS)
K:B, U=000; p < 0.000157*** A:C, U=3; p < 0.00038*** A:E, U=28; p < 0.09 (NS)
K:C, U=000; p < 0.00015*** B:C, U=23.5; p < 0.045* C:F, U=41; p < 0.49 (NS)
K:D, U=6.5; p < 0.0010*** D:E, U=28; p < 0.099 (NS) NS= not significant
K:E, U=1.5; p < 0.0002*** D:F, U=6; p < 0.00082***
K:F, U=000; p < 0.000157*** E:F, U=17.5; p < 0.014*

*statistical difference (p < 0.05); **statistically significant difference (p < 0.01); ***statistically highly significant difference (p < 0.001)

**Figure 1.** Presentation of the effect of CHX on cells exposed to MMC at a dose of 0.15 µM. Binuclear cell with micronuclei (A), apoptotic cells (B) and nuclear fragmentation (NF). (alkaline Giemsa 2% × 1000).

**Figure 2.** Presentation of the effect of MMC alone at a dose of 0.15 µM. Binuclear cell with micronuclei (A), nuclear fragmentation (B), a three-nucleated cell (C), a necrotic cell (D) (alkaline Giemsa 2% × 1000).

Comment. Comparing Figures 1 and 2 over the presence of NF, i.e. with and without the induction of ASR by CHX, the presence of NF shows various degrees of the cytotoxic potential of MMC. Figure 1 displays a difference in the expression of NF. This shows that CHX has induced ASR or a cytoprotective effect to peripheral blood lymphocytes exposed to a challenge dose of MMC. However, this effect does not mean that it is beneficial to the cell.

A dose of 0.6 µM MMC a decrease in NDI (1.029) was noticed, showing that a correlation between the CHX dose and the concentration of the challenging dose existed (Table 2). Still, using the x² test, the proliferative index showed a statistically highly significant difference (p < 0.001) between the experimental group with CHX and the experimental group with MMC alone in all challenging doses, possibly implying that the ASR effect was still effective (Table 2).

NF indicates the existence of high cytotoxic effect of antitumor agents. It is a subjective measure of cytotoxicity but an important parameter to evaluate. The decrease of NF in the CHX experimental group showed a connection of NF and the ASR effect. In the experimental group with CHX the decrease of NF corresponded to an increase of NDI (Table 2, Figure 1 and 2). Figure 1 and 2 show a clear difference in the presence of NF between experimental groups, i.e. MMC alone (Figure 2) and the group with CHX plus MMC (Figure 1) in the same dose of MMC (0.15 µM).
Discussion

Complex pathways have evolved in the genome protection machinery in order to adapt to a genotoxic environment. When 15 years ago the first data on ASR came to public it was hoped that new treatment schedules could help protect the genome exposed to high doses of cytotoxic agents. Today, complex profiles of gene expression come into the open when cells are exposed to genotoxic stress, showing that the proteins believed to protect the genome are now the ones leading to the path of genome instability. This duality in protein action has received attention and is a matter of investigation and theory [6,17]. This is important to understand when assessing the use of molecules in current cancer chemoprevention and treatment strategies.

Although new protein and RNA synthesis appear to be required for ASR [28] and that CHX or actinomycin D treatments block ASR, activation of common transcription factors was not noticed during low dose IR priming reactions [29]. Alternatively, the induction of new proteins during ASR may be involved in cell cycle control or prevention of apoptosis [2,6]. CHX shows a dual role in expressing induced apoptosis, i.e. the capability to induce apoptosis or to prevent it is based on the cell line, synergistic effects with other agents and possibly on the dose [24,30-34]. CHX showed a high adaptive effect to paclitaxel, a microtubule poison to cancer and normal cells, seen as an increased level of the NDI, lowering NF. Still, this adaptive effect showed the presence of a number of cells with chromosome damage, suggesting abrogation of apoptosis by inhibition of bcl-2 phosphorylation [16,24,35].

The CHX dose of 10 µg/ml was chosen because it does not induce micronuclei formation in human lymphocytes [16,25], although it shows an antiproliferative property seen as a decrease in the percentage of binuclear cells and the NDI. MMC was chosen as a challenging agent since its genotoxic properties are very well known and documented, thus rendering the detection of an ASR easier. This challenging agent was also selected because of its mechanism of genotoxicity, which is different of other challenging agents used, i.e. MMC is a cross-linker and MMC genotoxicity can also be explained by various mechanisms, including a one-electron reduction to a semiquinone free radical [36] in which some reductases are involved. This reduction pathway is dependent on the presence of oxygen, with production of superoxide anion, hydrogen peroxide and hydroxyl radicals. This is an important fact as CHX has an inhibitory effect on apoptosis in the presence of ROS [31,33,35,37]. Also, pretreatment of cells with a low dose of X-rays followed by exposure to DNA damaging agents, such as bleomycin (which can induce double-strand breaks) or MMC (which can induce DNA cross-links), lead to cross-adaptation (i.e., reduced chromosomal aberrations). This adaptive response could be blocked by incubation of lymphocytes with the protein synthesis inhibitor, CHX [2,28]. Even though some authors [26,38] showed IR and chemicals can be used as adaptive agents towards MMC genotoxicity, we here

### Table 2. Changes of the nuclear division index (NDI) in control and experimental groups

<table>
<thead>
<tr>
<th>Concentration (µM/ml)</th>
<th>M1 %</th>
<th>M2 %</th>
<th>M3 %</th>
<th>M4 %</th>
<th>NDI</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (K) 377 38 557 55.7 48 4.8 18 1.8 1.707 –</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.05 µM Mitomycin C (A) 968 96.8 31 3.1 1 0.1 / / 1.033 +++</td>
<td></td>
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<tr>
<td>0.15 µM Mitomycin C (B) 979 97.9 19.45 2 0.55 0.05 / / 1.020 +++</td>
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<tr>
<td>0.6 µM Mitomycin C (C) 985 98.5 14.65 1.46 0.35 0.03 / / 1.015 +++</td>
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<tr>
<td>'CHX/0.05 µM Mitomycin C (D) 943 94.3 53 5.3 3 0.3 1 0.1 1.062 +/-</td>
<td></td>
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<tr>
<td>'CHX/0.15 µM Mitomycin C (E) 934 93.4 61 6.1 3 0.3 1 0.1 1.069 +/-</td>
<td></td>
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<tr>
<td>'CHX/0.6 µM Mitomycin C (F) 969 96.5 28 2.8 1 0.1 0.15 0.01 1.029 +/-</td>
<td></td>
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</table>

1nuclear fragmentation, 2cycloheximide

X²>1000; p < 0.000*** for K: (A,B,C,D,E,F )

A:B; X²=4.27; p < 0.23 (NS) D:E; X²=1.3; p < 0.7(NS) A:D; X²=20.25; p < 0.00015***

A:C; X²=9.5; p < 0.023* D:F; X²=49; p < 0.001***

B:C; X²=1.6; p < 0.2 (NS) E:F; X²=58.4; p < 0.000***

*statistical difference (p < 0.05); ** statistically significant difference (p <0.01); *** statistically highly significant difference (p<0.001)

NS=not significant
argue that adaptation could lead to survival of damaged cells, as presented in our results. CHX-induced adaptation revealed that cells exposed to MMC showed a non significant decrease in its genotoxic properties. MMC cytotoxicity is based on the activation of apoptosis. When looking at the results and the presence of NF, it seems that CHX suppresses apoptosis, but this is not the case. Based on flow-cytometric analysis [16], CHX induces a higher percentage of apoptosis in human peripheral blood lymphocytes exposed to MMC. So how can the presence of NF and the elevated proliferative index be interpreted? Not an easy answer, i.e. NF is seen by some authors as a process leading to apoptosis [39], while others [16,40] argue that NF is a stage of nuclear envelope disorganization that can also lead to survival. This was seen by the detection of whole chromosomes using centromere probes in the micronuclei of NF cells by using the FISH technique [16]. NF versus increased proliferative index shows that cells adapted to genotoxic stress. The explanation of the negative adaptation effect of CHX can possibly be explained by CHX ability to induce premature centromere separation, thus acting on the centromere proteins that control separation and segregation of centromeres in anaphase, inducing genome instability [16,25]. The adaptive response of cells induced by CHX shows that some molecular events are associated with anaphase onset and cell-cycle transition checkpoint control. The cellular surveillance mechanisms that function as checkpoint controls during the cell cycle produce inhibitors of cell cycle transition points following the detection of DNA damage and mistakes in earlier cell cycle events. Although the checkpoint controls function as a safeguard for the timely initiation of anaphase and the orderly segregation of chromosomes, the controls can be overridden. Several studies have shown that anaphase can still occur after exposure of cells to microtubular-disrupting drugs and in the absence of the spindle [41]. This model was presented to CHX-induced ASR in paclitaxel-exposed cells [35] and should be evaluated in future experiments as a possible mechanism for understanding the abrogation of the cell cycle control machinery in MMC-exposed cells.

Understanding the intrinsic and extrinsic process of apoptosis and survival seen in the ASR phenomenon can unravel known mechanisms of positive adaptation in order to develop better paths to cancer treatments. At present, these investigations can help us to better assess the risk of combined therapies and therefore eliminate a possible occurrence of secondary tumors.

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