Cytotoxicity, DNA Cross-Linking, and Single Strand Breaks Induced by Activated Cyclophosphamide and Acrolein in Human Leukemia Cells

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ABSTRACT

The in vitro cytotoxicity and mechanism of action of cyclophosphamide (CP) were studied in a dual cell culture system, using rat hepatocytes and K562 human chronic myeloid leukemia cells. Cytotoxicity and DNA damage were measurable in K562 cells using CP concentrations that are clinically attainable. Alkaline elution analysis of cellular DNA demonstrated the presence of concentration- and time-dependent DNA interstrand cross-links, DNA-protein cross-links, and DNA single strand breaks in K562 cells following a 1-h exposure to cyclophosphamide activated by hepatocytes.

Hepatocyte-activated CP was 3 to 4 times more potent than phosphoramide mustard with regard to cytotoxicity and induction of DNA interstrand cross-links. Exposure to phosphoramide mustard did not produce single strand breaks, but exposure of K562 cells to acrolein resulted in substantial levels of single strand breaks. The demonstration of acrolein-induced single strand breaks following exposure to activated CP is a novel finding and suggests that acrolein may have a role in the cytotoxicity of CP.

INTRODUCTION

CP remains an important antineoplastic agent, displaying activity against a number of human tumors (1, 2). Despite its wide usage, the precise basis for the oncotoxic activity of the drug remains obscure (3). The requirement for cytochrome P-450-mediated activation to cytotoxic metabolites has limited in vitro studies of the mechanism of action of CP, and previous work has utilized self-activating derivatives of the drug which spontaneously decompose to 4-OH-CP, the main product of activation of the parent drug (4-6). However, the pharmacological and pharmacokinetic properties of biologically activated CP may exhibit some differences from those of self-activating derivatives. We have, therefore, used an hepatic system to generate activated CP. CP is presumed to exert its cytotoxicity via the cross-linking of cellular DNA, and studies with self-activating derivatives have demonstrated the presence of interstrand and DNA-protein cross-links, but not single strand breaks, following drug exposure (4, 6). Acrolein is released when 4-OH-CP decomposes to yield PM, the putative ultimate alkylating metabolite of CP, and acrolein is thought to be the causative agent of CP-induced cytisits, but is generally considered unlikely to contribute to the cytotoxic activity of CP (7).

Using a dual culture system of rat hepatocytes and human leukemia cells, we have studied the cytotoxicity of CP at the cellular level. In this paper we present evidence which is consistent with the assertion that 4-OH-CP is the transport form of CP and that phosphoramide mustard is the ultimate cross-linking metabolite. However, we also show that acrolein, which is released intracellularly from 4-OH-CP, is a highly reactive compound which causes DNA single strand breaks, and although not directly responsible for the DNA cross-linking and hence cytotoxic effect of CP, it may have other effects which do have a role in the cytotoxicity of CP.

MATERIALS AND METHODS

Hepatocyte Preparation and Incubation. Isolated hepatocytes were prepared from phenobarbitone-pretreated male Wistar rats essentially as described by Devalia et al. (8) with some modifications. Briefly, the hepatic portal vein was cannulated in situ, and the liver was perfused with Ca2- and Mg2-free HBSS (Gibco, Uxbridge, United Kingdom). The liver was carefully dissected out from the abdomen and transferred to a plastic dish, and the perfusion was continued with HBSS containing 0.05% collagenase (from Clostridium histolyticum; Boehringer Mannheim, Federal Republic of Germany) for at least 10 min. The liver capsule was then incised, and hepatocytes were gently shaken into HBSS containing 2.5% bovine serum albumin (Sigma Chemical Co., Poole, United Kingdom). The crude cell suspension was filtered through a 125-μm nylon mesh to remove cell clumps, spun at 5 x g for 3 min, resuspended in HBSS, spun, resuspended in HBSS containing gentamicin (50 μg/ml), and filtered through a sterile 56-μm nylon mesh. The cell suspension was finally spun again and resuspended in incubation medium consisting of 125 mM NaCl, 6 mM KCl, 1 mM MgSO4, 0.44 mM KH2PO4, 3.5 mM Na2HPO4, and 1.3 mM CaCl2. This buffer was supplemented immediately before use with 5 mM sodium pyruvate, 1% bovine serum albumin (Sigma Chemical Co., United Kingdom), and gentamicin (50 μg/ml), and the pH was adjusted to 7.4. Hepatocytes isolated by this method had an average viability, assessed by trypan blue exclusion, of 90 ± 4% (SD; n = 42).

In Vitro Activation of CP. Liver cells (106/ml) were incubated in sterile 25-ml siliconized glass Erlenmeyer flasks at 37°C in a shaking water bath at 80 strokes/min. Cyclophosphamide was obtained from Sigma Chemical Co. as the monohydrate and dissolved in PBS immediately before use. After incubation with drug for 1 h, the cell suspension was transferred aseptically to a 15 ml conical tube (Becton Dickinson, Poole, United Kingdom) and spun at 50 x g for 1 min. Aliquots of the resulting cell-free supernatant were then either analyzed for alkylating activity or immediately added to cultures of K562 experimental cells. There was no loss of hepatocyte viability after 1-h incubation with cyclophosphamide.

4-(4-Nitrobenzyl)pyridine Assay for Alkylating Activity. The method of Friedman and Bolger (9), as described by Acosta and Mitchell (10), was used to quantify nonspecific alkylating activity in aliquots of the supernatant from hepatocyte incubations. Standard curves were established using nitrosonitrogen mustard.

Leukemia Cell Culture and Cytotoxicity Assays. The K562 human chronic myeloid leukemia cell line was maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). For assessment of cytotoxicity, log-phase cells were suspended at 2 x 106/ml in full medium in cell culture tubes. Aliquots of the supernatant from the incubations of drug with hepatocytes were then added to the cells so as to provide a final 1/10 dilution of the original supernatant. Phosphoramide mustard (cyclohexylamine salt, a gift from the Drug Development Branch, Institute of Cancer Research, Sutton, United Kingdom) and acrolein (Aldrich Chemical Co., Gillingham, United Kingdom) were dissolved in sterile PBS immediately before use. Acrolein was purified by distillation before use. Drugs were incubated with cells for the stated times, after which cells were centri...
fuged, washed twice in drug-free medium, and finally resuspended in medium. In all experiments, control cells received an appropriate volume of drug vehicle. In the case of experiments with hepatocyte-activated CP, control cells received an appropriate volume of supernatant from hepatocyte incubations without CP. For colony-forming assays, the washed cells were serially diluted in full medium, and an appropriate final cell number giving approximately 100 colonies was added to culture plates, the final dilution being made into medium containing 0.3% Seaplaque agarose (Miles Biological, United Kingdom). Colonies were counted after 10 days of incubation at 37°C in a humidified atmosphere of 5% CO₂/95% air. Control cells had a colony-forming efficiency of 75% in this system.

Alkaline Elution. The alkaline elution procedure was performed essentially as described by Kohn et al. (11), with minor modifications. K562 cells at a density of 2 x 10⁷/ml were labeled for 30 h with 0.02 μCi of [³²P]thymidine per ml (specific activity, 56 mCi/mmole; Amer-sham, United Kingdom), centrifuged, and resuspended at 2 x 10⁷/ml prior to drug exposure. Cells were exposed to activated CP in supernatant from hepatocyte incubations, as described above, for 1 h, washed, and resuspended in full medium, and they were either placed on ice and analyzed immediately or incubated at 37°C for varying periods of time prior to elution analysis.

For measurement of cross-linking, single strand breaks were introduced into cellular DNA by exposure to a ¹³¹I source, and the cells were held at ice-cold temperatures throughout. For measurement of interstrand cross-links, cells received 300 rads, and for DNA-protein cross-links, 3000 rads. Assays for interstrand cross-linking and for DNA single strand breaks were conducted using 0.8-μm polycarbonate filters (Biorad, Watford, United Kingdom). DNA-protein cross-links were measured with 2.0-μm polystyrene chloride filters (Millipore BSWP 02500). Cells were lysed in 5 ml of sodium dodecyl sarcosine/NaCl/EDTA (disodium salt), 0.2%/0.04 M. Under deproteinizing conditions this solution contained proteinase K (500 μg/ml) (BDH Chemicals, Dagenham, United Kingdom) and was allowed to remain in contact with the filter for 1 h. The native DNA was then washed with 3 ml of 0.02 M EDTA, pH 10, and eluted with 0.02 M EDTA (acid form), adjusted to pH 12.2 with 20% tetrapropyl ammonium hydroxide (Sigma).

³H-labeled L1210 cells were included in elution analyses as internal standards. These cells were maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum and were labeled for 24 h with [³H]thymidine (0.05 μCi/ml) in preparation for elution. These cells received 150 R on ice and were mixed with an approximately equal number of ³H-labeled K562 cells prior to deposition on filters. Elution of [³H]DNA was plotted against simultaneous elution of ³H-labeled internal standard DNA as described by Kohn et al. (11). Cross-linking coefficient, Kr, is calculated as

\[ K_r = \frac{1 - r}{1 - r_0}^{1/2} - 1 \]

where \( r_0 \) is the retention of control cell DNA, and \( r \) is the retention of DNA from drug-treated cells, at the point when retention of internal standard DNA is 0.5. Cross-linking coefficient is multiplied by X-ray dose in order to express cross-linking in rad-equivalents. It should be noted that \( K_r \) will be affected by drug-induced DNA strand breaks which lead to more rapid elution of DNA. \( K_r \) is therefore increased by cross-links and decreased by strand breaks.

DNA-protein cross-link frequency is given by the coefficient \( P_r \). This is calculated as

\[ P_r = \frac{1}{\sqrt{1 - r}} - \frac{1}{\sqrt{1 - r_0}} \times 3000 \]

where \( r \) and \( r_0 \) are the DNA retentions, at time zero, of drug-treated (r) and control (r₀) cells. Three thousand is the X-ray dose (rads).

RESULTS

Alkylation Activity and Cytotoxicity of Hepatocyte-activated CP and of Phosphoramide Mustard. The alkylation activity present in the extracellular medium following exposure of CP to hepatocytes is maximal 1 h after addition of drug. Measurement of free CP, by a sensitive gas chromatography technique, demonstrated that essentially all the original CP was metabolized within 1 h (data not shown). Following a 1-h exposure of hepatocytes to various concentrations of CP, alkylation activity is linearly proportional to original drug concentration. CP concentrations in the range of 0 to 1.4 μmol/ml result in the production of alkylation activity in the range of 0 to 35 nmol of normitrogen mustard equivalents per ml of supernatant, in 1 h.

The cytotoxicity of 1- and 2-h exposures to supernatant from the hepatocyte incubations and the cytotoxicity of 1- and 2-h exposures to phosphoramide mustard towards K562 cells are shown in Fig. 1. The indicated CP concentrations are calculated from the original concentration in the hepatocyte incubations, taking into account the 1/10 dilution of the supernatant on addition to the K562 cells. No allowance has been made for metabolism of CP into nontoxic derivatives, which is substantial. Two-h drug exposures were more toxic than 1-h exposures. Hepatocyte-activated CP was 3 to 4 times more cytotoxic than equimolar phosphoramide mustard. When conversion of CP into nontoxic metabolites is taken into account, activated CP is clearly substantially more cytotoxic than PM.

Alkaline Elution Analysis of DNA Damage. Total DNA cross-linking as expressed by \( K_r \) was maximal 6 h after a 1-h exposure to hepatocyte-activated CP, after which the level of cross-linking gradually decreased (Fig. 2A). Inclusion of proteinase K digestion in the elution procedure demonstrated that hepatocyte-activated CP produced a mixture of interstrand and DNA-protein cross-links 6 h after a 1-h drug exposure (Fig. 2A).
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Fig. 2. DNA cross-linking in K562 cells. A, total cross-linking at various times after a 1-h exposure to hepatocyte-activated CP. Cells were exposed to 100 μM activated CP for 1 h and analyzed for cross-linking, without a proteinase K digestion after the stated times (h) of drug-free incubation. B, effect of proteinase K on DNA cross-linking. Cells were exposed to 40 μM hepatocyte-activated CP for 1 h and analyzed for cross-linking after 6 h, either with (∆A) or without (∆B) proteinase K digestion (C, control cells). C, apparent interstrand cross-linking in relation to concentration of hepatocyte-activated CP. Cells were exposed to various concentrations of hepatocyte-activated CP for 1 h and analyzed for interstrand cross-linking after 6 h of drug-free incubation. D, control cells; E, 20 μM CP; F, 40 μM CP; G, 50 μM CP; H, 100 μM CP. I, interstrand cross-linking induced by hepatocyte-activated CP and PM. Cells were exposed to 50 μM hepatocyte-activated CP (∆A) or 200 μM PM (∆B) for 1 h and analyzed for interstrand cross-linking after 6 h of drug-free incubation. C, control cells.

Interstrand cross-linking was maximal 6 h after a 1-h exposure to hepatocyte-activated CP and declined thereafter to very low levels by 16 h (Fig. 3A). Kc increased in proportion to the concentration of hepatocyte-activated CP at concentrations up to 50 μM, but it decreased at concentrations above 50 μM, suggesting the presence of drug-induced DNA single strand breaks (Fig. 2C). Exposure of cells to phosphoramidemustard also resulted in the induction of a mixture of interstrand and DNA-protein cross-links, with time courses of formation and removal essentially similar to those observed with hepatocyte-activated CP. This is illustrated in Fig. 3A in which the time course of interstrand cross-linking is shown following 1-h exposures to hepatocyte-activated CP (50 μM) or PM (200 μM). It should also be noted from this figure that PM concentrations approximately 4 times greater than hepatocyte-activated CP were required to produce equivalent interstrand cross-linking.

The greater potency of hepatocyte-activated CP with regard to induction of interstrand cross-linking is further illustrated in Fig. 2D, in which cells exposed to 50 μM hepatocyte-activated CP for 1 h displayed substantial interstrand cross-linking, whereas little interstrand cross-linking was seen in cells exposed to 50 μM PM. In contrast to the reduction in Kc observed at concentrations of hepatocyte-activated CP greater than 50 μM, PM produced an increase in Kc, in linear proportion to drug concentration at all concentrations tested (Fig. 3B). DNA-protein cross-linking induced by hepatocyte-activated CP was maximal 6 h after a 1-h drug exposure and gradually decreased thereafter, although DNA-protein cross-links were still detectable 24 h after removal of drug (Fig. 4A). DNA-protein cross-linking was linearly proportional to concentration of hepatocyte-activated CP (Fig. 4B).

The reduction in Kc observed in cells treated with concentrations of hepatocyte-activated CP greater than 50 μM suggested the presence of drug-induced single strand breaks. Initial elution analyses for single strand breaks (in which K562 cells did not receive prior irradiation) were performed immediately after exposure to hepatocyte-activated CP, at which time the level of interstrand cross-links, which might act to obscure any strand breaks, was low. These analyses demonstrated the presence of drug-induced, concentration-dependent DNA single strand breaks (Fig. 5A). Concentrations of hepatocyte-activated CP below 50 μM did not produce measurable single strand breaks.
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Fig. 5. DNA single strand breaks in K562 cells. A, single strand breaks immediately following a 1-h exposure to hepatocyte-activated CP (C, control cells; □, 20 μM CP; ◆, 60 μM CP; ○, 100 μM CP). B, single strand breaks 4 h after a 1-h exposure to hepatocyte-activated CP (symbols same as in A). C, single strand breaks at various times (h) after a 1-h exposure to 100 μM hepatocyte-activated CP. D, single strand breaks immediately following various times (h) of exposure to 100 μM hepatocyte-activated CP.

In the absence of proteinase K digestion, single-strand breaks could not be detected due to the obscuring effect of DNA-protein cross-links. All subsequent analyses for single strand breaks therefore included a proteinase K digestion. DNA single strand breaks were maximal 4 h after a 1-h exposure to hepatocyte-activated CP and then declined to almost undetectable levels by 8 h (Fig. 5, B and C). Exposure of cells to hepatocyte-activated CP for 2 h resulted in greater single strand breakage than exposure for 1 h, but in cells exposed for 4 h, the level of single strand breakage had decreased, probably because of the obscuring effect of interstrand cross-links.

PM did not cause single strand breaks in the concentration range of 0 to 500 μM with 1- or 2-h drug exposures. In contrast, a 1-h exposure to acrolein (0 to 20 μM) did produce substantial single strand breaks in K562 cells (Fig. 6). Marked cytotoxic effects (reduction in colony-forming ability) were observed in K562 cells exposed to acrolein concentrations above 5 μM.*

Fig. 6. DNA single strand breaks following a 1-h exposure to various concentrations of acrolein (C, control cells; □, 5 μM; ■, 10 μM; ▲, 20 μM).

DISCUSSION

We have developed a dual culture system of rat hepatocytes and human leukemia cells to study the mechanism of action of CP. Rat hepatocytes have been shown to represent more accurately in vivo metabolism of CP than microsomal preparations (12) and have been used in dual culture and coculture systems in studies of the metabolism and mechanism of action of several drugs, including CP (10, 12–14). The system described in this paper allows recovery of the “target cell” component of the dual culture system. It thus permits subsequent analysis of CP-exposed cells for DNA damage.

A 1-h incubation of CP with hepatocytes allows essentially complete activation and permits, with appropriate dilution of the cell-free supernatant, exposure of proliferating cells to concentrations of alkylating activity and CP metabolites broadly comparable to clinically observed plasma values of alkylating activity and free CP (15, 16).

The kinetics of the formation of DNA cross-linking was found to be in general agreement with the findings of Erickson et al. in studies with self-activating sulfhydryl derivatives of CP (6). Hepatocyte-activated CP produced a mixture of DNA-protein and interstrand cross-links. DNA-protein cross-links are present immediately following a 1-h exposure to hepatocyte-activated CP, are maximal at 6 h, and are still detectable 24 h after removal of drug. In contrast, interstrand cross-links were barely detectable immediately after a 1-h drug exposure, but they gradually increased to a maximal level 6 h after drug removal. Erickson and coworkers (6) reported a similar time course of interstrand cross-link formation, but in a study with 4-hydroperoxycyclophosphamide, maximum interstrand cross-linking was reported 3.5 h after drug removal (4). The delay in appearance of interstrand cross-linking may be attributable to the time required to convert monoadducts into cross-links, as has been reported with melphalan and cis-platinum, but not nitrogen mustard (22, 23). Alternatively, the delay may be due

* Unpublished observations.
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in part to the time required for intracellular 4-OH-CP to decompose, possibly via a 4-OH-CP-thiol intermediate, to phosphoramide mustard, the putative cross-linking metabolite of CP (1, 17–21). In parallel studies with phosphoramide mustard, a similar delay in the formation of interstrand cross-links was observed, although substantially higher concentrations of phosphoramide mustard were required to produce equivalent cytotoxicity and cross-linking. The 3- to 4-fold superior potency of hepatocyte-activated CP over phosphoramide mustard is in close agreement with other in vitro studies (4, 6). The data presented here are compatible with the hypothesis, advanced by other workers, that phosphoramide mustard is the ultimate cross-linking metabolite of CP and that 4-OH-CP is the transport form which enters cells (3, 4, 18, 19), but these data leave open the question of why activated CP is so much more potent than PM.

Interstrand cross-links are removed more rapidly than DNA-protein cross-links and decrease to very low levels 12 h after a 1-h exposure to hepatocyte-activated CP. The observed elution profile at this time almost certainly represents removal of cross-links and is not due to fragmentation of DNA from acutely injured cells, since no DNA single strand breaks were detected at this time, and drug concentrations were used which resulted in less than 1 log of cell kill. The loss of cross-linking may, therefore, be attributable to DNA repair processes. These time courses of cross-link removal are essentially similar to those described by Erickson et al. (6).

At CP concentrations up to 50 μM, the level of interstrand cross-linking after 6 h of drug-free incubation is linearly proportional to drug concentration, but at higher concentrations, the level of interstrand cross-linking appears to decline sharply. The unusual drop in the value of K at higher concentrations of activated CP suggested the presence of DNA single strand breaks as well as cross-links. Separate elution assays, without irradiation of cells, were performed, and the presence of single strand breaks was unequivocally established. Measurement of strand breaks immediately following drug exposure minimized the obscuring effect of interstrand cross-links, the level of which was very low at this time.

Single strand breaks have not been reported in studies with self-activating derivatives of CP (4, 6) or with cisplatin, melphalan, and nitrogen mustard (22, 23). The origin and significance of the breaks observed in the present work are, therefore, of considerable interest. The breaks are not due to nonspecific scission of DNA released from dying cells, since they were detectable immediately following a 1-h drug exposure, at which time cell viability remained at 100%, and they were observed following exposure to drug concentrations which resulted in less than 1-log cell kill. We have not observed single strand breaks in cells exposed to cytotoxic concentrations of phosphoramide mustard, and the breaks are unlikely, therefore, to be attributable to the excision repair of monoadducts between phosphoramide mustard and deoxyguanosine which has been previously demonstrated (24). The time course of induction and removal of single strand breaks is consistent with the explanation that the breaks are due to intracellularly released acrolein.

This compound is a highly reactive and cytotoxic aldehyde (25), and it is released when 4-OH-CP decomposes to yield phosphoramide mustard (26). The induction of single strand breaks by acrolein, which we demonstrate in this paper, confirms the observations of Erickson et al. (6) who demonstrated single strand breaks following treatment of L1210 cells with acrolein.

The demonstration of acrolein-induced single strand breaks following exposure to activated CP raises the question of whether acrolein has a role in the cytotoxicity of CP. Previous work suggests that acrolein released intracellularly from oxazaphosphorines may not directly contribute to the cytotoxicity of cyclophosphamide (7). The most significant implication of the demonstrated single strand breaks is that they provide clear evidence of the cellular reactivity of acrolein and strongly suggest that other important cellular macromolecules may be damaged or inactivated by intracellularly released acrolein. The acrolein-induced depletion of cellular glutathione content and its possible relevance to the cytotoxicity of activated CP are discussed in the accompanying paper (27).

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