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Formation and disappearance of DNA interstrand cross-links in human colon tumor cell lines with different levels of resistance to chlorozotocin

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Abstract—Three human colon tumor (HCT) cell lines, designated C, Moser and 116, exhibiting a gradation of resistance to chlorozotocin, a glucose-linked chloroethylnitrosourea (1-, 2.9-, and 5.8-fold respectively) were examined to assess the determinants of drug sensitivity. Although the O^6-alkylguanine-DNA transferase content was relatively higher in the most resistant 116 cells than in the sensitive cell line C, its level in Moser cells did not correlate with the intermediate chlorozotocin sensitivity. Glutathione content in these tumor cell lines did not show a parallelism with drug resistance. The ethidium bromide fluorescence assay was used to quantitate the kinetics of DNA interstrand cross-link formation and its removal after drug exposure. The peak levels of DNA interstrand cross-links induced in HCT cells correlated with their resistance to chlorozotocin with cross-link indices of 0.03, 0.10 and 0.20, respectively, for 116, Moser and C cell lines. All three cell lines demonstrated DNA cross-link repair to different extents. While the smaller number of cross-links formed in resistant 116 and Moser cells were eliminated in a rapid phase of repair, the lesions formed at a much greater frequency in C cells remained largely unrepaired. These results draw attention to the role of increased DNA cross-link repair as a mechanism of nitrosourea resistance in the HCT cells studied.

The 2-chloroethylnitrosoureas (CENUs*) are powerful bifunctional alkylating agents which exert cytotoxicity mainly through the production of interstrand cross-links (ISCs) in DNA [1, 2]. Tumor cell resistance to these drugs is a major stumbling block leading to a less effective neoplastic containment in the chemotherapy of several human cancers [3, 4]. Much attention in our understanding of CENU resistance mechanisms has focused on the involvement of a cellular protein, namely, O^6-alkylguanine-DNA alkyltransferase (AGT) which is capable of removing the O^6-chloroethyl groups from guanine in DNA and thus effectively preventing the formation of cytotoxic ISCs in tumor cells exposed to CENUs [5, 6]. However, it is important to note that DNA-ISC production still occurs in tumor cells with higher AGT levels (designated Mer^+), although to a lesser extent than in the Mer^- cells [7, 8]. Since the drug-induced cytotoxicity is directly related to the net interstrand cross-linking of cellular DNA at any given time [9], elimination of DNA cross-links by means of excision repair could also contribute significantly to cell viability. Yet, except for two brief reports [10, 11], the enhanced removal of DNA-ISCs as a resistance mechanism has not been investigated for CENUs. To further understand the role of DNA cross-link repair in CENU resistance, in the present study, three human colon tumor (HCT) cell lines with different levels of resistance to chlorozotocin (CHLZ; a water-soluble CENU with negligible carbamoylating activity) were used and the repair of CHLZ-induced DNA-ISCs in the total genome was analyzed. AGT and glutathione levels in these cell lines were also quantitated to assess their contribution to CHLZ resistance.

Materials and Methods

CHLZ was obtained from the Drug Development Branch, National Cancer Institute and its stock solutions were prepared in ice-cold Hanks' Balanced Salt Solution just prior to use. HCT cell lines, 116, C and Moser, were provided by Dr. Michael Brattain of Baylor College of Medicine, Houston, TX. These cell lines were originally established from tumor specimens of untreated patients and have been well characterized by Brattain and his colleagues [12]. Cells were routinely maintained as monolayers in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Assay for drug sensitivity. Cell survival after CHLZ treatment was determined by the capillary tumor clonogenic cell assay [13]. CHLZ like other CENUs decomposes rapidly and spontaneously with a half-life of 15 min at physiological pH and 37° [14]. A short-term or a continuous exposure of tumor cells to this drug does not affect the extent of cell survival [15], because of its quick dissolution to the reactive alkylating moiety. In the present study, the HCT cells were treated continuously with CHLZ in determining the cell survival. Briefly, 0.3 mL of a cloning mixture was made up; the mixture consisted of 3×10^4 HCT cells, 0.2% agarose, drug and 20% FBS in DMEM. Thirty microliters of this mixture was drawn into sterile glass capillary tubes in triplicate, cooled, and then incubated at 37° in 5% CO_2 and a humidified atmosphere for 2 weeks. Colonies greater than 40 μm in diameter were counted under phase contrast microscopy and the surviving fractions were calculated [13].

Ethidium bromide fluorescence assay to quantitate the kinetics of DNA-ISC formation and its repair. This assay detects DNA-ISCs based on the differential binding of ethidium bromide to native and denatured DNA; cross-linked DNA upon boiling and cooling renatures quickly back into its duplex form with a corresponding increase in fluorescence, while the uncross-linked denatured DNA retains very little fluorescence. This method has been used to measure interstrand cross-linking of cellular DNA in several studies [16–18]. We used this procedure in view of its close similarity in sensitivity to that of alkaline elution, the simplicity of handling many samples simultaneously and non-interference from DNA breakage [16]. The protocol of Sriram and Ali-Osman [18] was followed without S_1 nuclease treatment. HCT cell monolayers (5×10^6 cells) were treated with 50 or 100 μM CHLZ in DMEM with 3% FBS for 90 min. Tumor cells were then resuspended in drug-free medium (DMEM–10% FBS) for different periods, up to 50 hr to allow cross-link repair.

* Abbreviations: CENU, chloroethylnitrosourea; ISC, interstrand cross-link; AGT, O^6-alkylguanine-DNA alkyltransferase; Mer, methylation excision repair; HCT, human colon tumor; GSH, glutathione; and CHLZ, chlorozotocin or (2-3-chloroethyl)-3-nitrosoureido-D-glucopyranose.

Cell viability was checked at each postincubation period in a separate flask by trypsin-EDTA treatment followed by trypan blue exclusion. Tumor cell monolayers were washed with ice-cold phosphate-buffered saline (PBS) and lysed *in situ* with a buffer (10 mM Tris–HCl, 1 mM EDTA, 0.2% Sarkosyl, pH 8.0) in the presence of proteinase K (100 μg/mL) for 12 hr at 37°. These samples were dialyzed against 10 mM Tris–HCl, 1 mM EDTA (pH 8.0) followed by RNase treatment (20 μg/mL) for 2 hr. DNA preparations were quantitated by spectrophotometry and 10 to 100 μg aliquots were mixed with 3 mL of a 20 mM potassium phosphate buffer (pH 12) containing 2 mM EDTA and 10 μg/mL of ethidium bromide. The fluorescence at 525 nm excitation and 590 nm emission was monitored before boiling the samples and 40 min after denaturation and cooling at room temperature. The extent of cross-linked DNA was calculated from measurements of the difference in fluorescence of DNA from control and CHLZ-treated cells according to Brent [19]. Assuming CHLZ cross-links are introduced randomly into the DNA population, the fraction of denaturable DNA molecules (those without cross-links) corresponds to the zero fraction of a Poisson distribution; conversely, the fraction of renaturable molecules (with cross-links) corresponds to the complement of the zero fraction. The mean number of cross-links per DNA molecule, N, is thus given by the formula, $N = -\ln x$, where x is fluorescence after denaturation divided by fluorescence before denaturation. DNA cross-link index (CLI) was calculated as previously described [18]: CLI = N (CHLZ-treated) − N (control)/N (control).

AGT assay. AGT activity was measured by the transfer of the O^6-methyl group from guanine in DNA to the AT protein using ^{3}H-methylated calf thymus DNA enriched for O^6-methylguanine [20]. Cell extracts were prepared by mechanical disruption of cells in 25 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA and 10% glycerol followed by centrifugation. Cell extracts with increasing protein concentrations were added to the [^{3}H]DNA substrate (10,000 cpm; 5 μg) at 37° and, after 40 min, the DNA was selectively hydrolyzed in 10% trichloroacetic acid at 80°C. The protein precipitate containing the AGT protein was collected by filtration on glass fiber filters [20] and radioactivity was quantitated by liquid scintillation counting. AGT activity in the linear part of the curve was taken to calculate the amount of the [^{3}H]methyl group transferred per milligram of protein.

Determination of glutathione content. GSH was measured in formic acid cell extracts by a fluorometric assay using *o*-pthalaldehyde [21]. Trypsinized cells were washed and resuspended in PBS. An aliquot was removed for the determination of cell number; the remainder was pelleted and cells were homogenized in 1 M formic acid. Different aliquots of the extracts recovered by centrifugation were reacted with HCHO to eliminate interference from histidyl compounds [21] and treated with *o*-pthalaldehyde. Fluorescence was read at 345 nm excitation and 425 nm emission, and GSH levels in extracts were determined by calibration with a standard curve.

Results and Discussion

In this study, we have characterized the sensitivity of three HCT cell lines to CHLZ with emphasis on demonstrating potential differences in their capacity to repair DNA-ISCs in relation to their drug resistance. Chlorozotocin, a glucose-linked CENU with relatively less myelotoxicity [22], can alkylate and cross-link DNA, but because its isocyanate hydrolysis product cyclizes immediately after it is formed, CHLZ does not form a reactive carbamoylating species; hence, it does not inhibit DNA repair unlike other carbamoylating CENUs [23]. Since the carbamoylation of proteins, involved in DNA repair may alter cross-link removal in a differential manner when comparing a panel of cell lines, CHLZ was chosen as the model CENU to avoid interference from carbamoylation. Figure 1 shows the CHLZ concentration–response curves for HCT cell lines 116, Moser and C. The drug concentration required to achieve a 0.5 log clonogenic cell kill was 75 μM for 116, 37 μM for Moser and 13 μM for C, thus demonstrating a varied spectrum of CHLZ sensitivity among these cell lines. These data suggest that compared to the most sensitive cell line C, Moser and 116 were approximately 2.9 and 5.8 times more resistant to CHLZ, respectively.

Tumor cell resistance to CENUs is likely to be multifactorial, that is, several mechanisms often acting together contribute to the resistance. Among these, a differential accumulation of CHLZ as a reason for the varying resistance seen in HCT cells can be excluded, because CHLZ, like other nitrosoureas has been shown to be transported by passive diffusion [24–26]. In the present study, the drug-induced interstrand cross-linking of DNA was evaluated and the biochemical parameters known to influence the cytotoxicity of CENUs in tumor cells were also quantitated. Table 1 summarizes these results. The maximum level of DNA cross-linking shown in Table 1 was derived 6 hr after 50 μM drug treatment for 116 and C cells and at 20 hr postincubation for Moser cells (also see Fig. 2 for similar kinetics of cross-linking after 100 μM CHLZ exposure). Although, CENU-induced interstrand cross-linking of DNA in most tumor cells is known to reach a peak at 6 hr following drug exposure [27], in brain tumor cells, we and others have found that a 12- to 24-hr postincubation period was necessary to achieve the highest degree of lesions [28], similar to the observation made in Moser cells. A prolonged existence and a differential rearrangement of the monofunctional lesions, aided by mechanisms yet unknown may explain this behavior. The cross-link index was highest for the C cell line and least for 116 with Moser being intermediate. These indices correlated in rank order with the decrease in surviving fractions of the HCT cell lines after exposure to 50 μM CHLZ (Table 1), indicating the importance of DNA-ISCs in the cytotoxicity elicited; however, the contribution of other lesions such as DNA–protein and DNA intrastrand cross-links to the overall lethality cannot be ignored. The extent of the CENU-induced DNA interstrand cross-linking can be affected by either the inactivation of the alkylating chloroethylcarbonium ion before its entry into

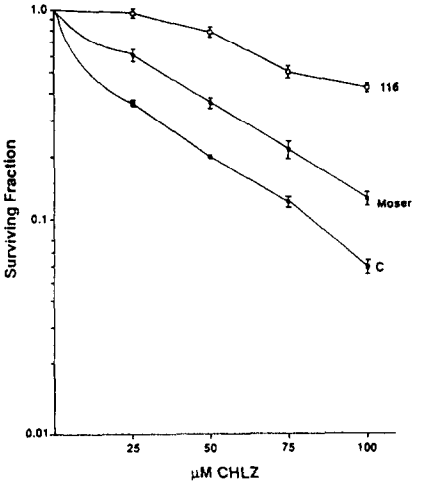

Fig. 1. Survival curves for the HCT cell lines 116, Moser and C following CHLZ treatment and capillary clonogenic cell assay. Values are means ± SD of three separate experiments.

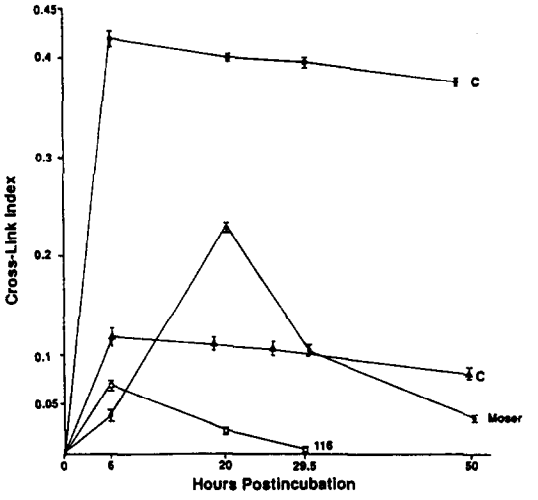

Fig. 2. Formation and elimination of DNA interstrand cross-links in HCT cells as a function of time after CHLZ treatment. Tumor cell monolayers were exposed to 100 μM CHLZ for 90 min and then resuspended in fresh medium. For cell line C, cross-link analysis was also performed after 25 μM CHLZ exposure. At the times specified, the ethidium bromide fluorescence assay was performed to quantitate DNA-ISCs. Key: (■), C cells (100 μM CHLZ); (▲), C cells (25 μM); (●), Moser cells (100 μM); and (□) 116 cells (100 μM). Data are means ± SD of three independent experiments.

the nucleus or the dealkylation of the monoadduct prior to the production of a cytotoxic ISC; two cellular constituents with the capacity to function in these roles and decrease CENU-induced cytotoxicity when present in increased amounts are glutathione and the AGT protein. *In vitro* inactivation of BCNU as well as the quenching of chloroethylated DNA by GSH were demonstrated recently [29, 30]. However, GSH content in HCT cells did not correlate with CHLZ sensitivity when expressed either per 10^6 cells (Table 1) or per mg protein (data not shown), with the most sensitive cell line C containing 1.25 times more GSH compared to the 5.8-fold resistant 116 cells. When levels of AGT protein, which can suppress cross-link formation by removing the O^6-chloroethyl monoadducts, were compared, 116 cells had four times more AGT activity than C cells, but Moser cells which were 2.9 times more resistant than the sensitive C cells had actually 3.75-fold less AGT than the latter (Table 1). From these results, it is clear that while AGT protein may contribute to CHLZ resistance to a marked extent in 116 cells, its content in Moser cells did not correlate with drug sensitivity. Despite a higher level of AGT present in 116 cells, it is significant that some chloroethyl monoadducts did escape removal to generate detectable amount of DNA cross-linking (Table 1 and Fig. 2).

An enhanced repair of DNA–DNA cross-links induced by *cis*-diamminedichloroplatinum and alkylating agents such as melphalan as a mechanism of tumor cell resistance has been described [31, 32]. The removal of a shorter ethylene bridge cross-link formed by CENUs in tumor cells has been reported and the role of cross-link repair in mediating CENU cytotoxicity has been alluded to [10, 11]. Here, we have further investigated if the drug resistance seen in our HCT cell lines is associated with higher cross-link repair. CHLZ produced concentration-dependent ISCs at 50 and 100 μM concentrations. Figure 2 depicts the kinetics of formation and removal of DNA-ISCs after 100 μM CHLZ treatment in HCT cells. Tumor cells remained largely viable (> 85%) at different times of repair analysis, suggesting that the decrease of ISCs seen took place in living cells. All three cell lines showed detectable cross-link repair to different extents. CHLZ formed fewer ISCs in the most resistant cell line 116, which were repaired almost completely in a rapid manner. The HCT cell line C showed a maximum amount of DNA-ISCs, 7.2 times more than 116 at 6 hr postincubation which were eliminated very slowly. Peak levels of ISCs accumulated at 20 hr in Moser cells were also removed quickly. While 67 and 93% of the ISCs formed at 6 hr were removed at 20 and 29.5 hr, respectively, in HCT 116 cells, only 3.6, 4.8 and 9.5% of cross-links were eliminated at 20-, 29.5- and 50-hr postincubation periods in the sensitive cell line C. In Moser cells, of the total ISCs formed at 20 hr, 54.8 and 83.8% were removed at 29.5 and 50 hr, respectively. The decrease of cross-links could not have been due to the dilution by DNA replication and cell division because cell number did not change appreciably in these experiments (data not shown). Additionally, it may be argued that the poor and efficient cross-link repair seen in C and 116 cells, respectively, was related to the heavy and lower amounts of DNA damage incurred rather than reflecting their inherent repair capacities. However, an examination of cross-link repair kinetics in C cells after 25 μM CHLZ treatment (yielding 37% cell survival) revealed a similar extent of DNA-ISC removal as observed after 100 μM CHLZ exposure (8% cell survival), despite a 3.5-fold decrease in ISC peak level (Fig. 2).

Two regulatory steps acting in concert appear to determine the net interstrand cross-linking of DNA by CENUs in tumor cells: (1) the removal of O^6-chloroethyl groups from guanine by the AGT protein in different parts of chromatin depending on its cellular content and accessibility to the cross-link precursors, thus controlling

Table 1. Quantitation of various parameters affecting CHLZ sensitivity in HCT cell lines

HCT cell lines	Fraction of colonic cell kill at 50 μM CHLZ	Peak levels of DNA interstrand cross-linking at 50 μM CHLZ (cross-link index)	AGT activity (pmol/mg protein)	Glutathione content (nmol/10^6 cells)
C	0.80 ± 0.07	0.20 ± 0.02	0.15 ± 0.04	4.56 ± 0.05
Moser	0.64 ± 0.06	0.10 ± 0.07	0.04 ± 0.01	3.04 ± 0.05
116	0.18 ± 0.03	0.03 ± 0.005	0.60 ± 0.07	3.65 ± 0.06

The extent of cytotoxicity was determined by the capillary clonogenic cell assay. DNA-ISC indices were derived at 6 hr postincubation for HCT 116 and C cells and at 20 hr for Moser cells. Procedures used for assaying AGT activity and GSH content are described in Materials and Methods. Results are the means ± SEM from three independent experiments.

the initial level of DNA-ISCs; and (2) the excision repair of ISCs by a set of repair enzymes to determine the extent and duration of persistence in DNA of the cytotoxic lesions, which in turn may dictate the inhibition/resumption of transcription, replication and also modulate the pathways that program cell death. It would be of interest to examine whether the accelerated DNA repair seen in drug-resistant cells is distributed evenly throughout the genome or if it is preferential to the functionally active chromatin as seen in some recent studies [33]. Work is underway to determine if resistance is accompanied by an elevation of other DNA repair enzymes such as glycosylase and polymerase. In conclusion, an increased DNA-ISC repair appears to be an important mechanism of CENU resistance and it merits further attention.

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Interleukin-1, platelet derived growth factor, free radicals and monocyte aryl hydrocarbon hydroxylase activity in liver disease. Role of cell communication

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Abstract—Monocytes were isolated from blood of human origin and cultured in supplemented Leibovitz (L-15) medium for 24 hr. The medium was then decanted and filtered, and all subsequent tests were done on monocyte conditioned medium (MCM). The monocytes of patients with liver disease spontaneously secrete temperature-sensitive arylhydrocarbon hydroxylase (AHH) inhibitory factors detectable in the MCM. Anti-interleukin-1 antibody (IL-1Ab) reduced the AHH inhibitory activity of the MCM, suggesting that part of the AHH inhibitory activity was due to interleukin-1 (IL-1). Platelet derived growth factor did not affect AHH activity. Interleukin-1β was detectable in MCM but did not differ significantly between patients and normal volunteers. A time course experiment indicated that interleukin-1β inhibited hepatocyte AHH activity after only 2 hr of incubation. Catalase partially blocked the AHH inhibitory activity of MCM suggesting that activated oxygen intermediates are partially involved in the AHH inhibitory activity of the MCM. Simultaneous incubation of interleukin-1β and catalase did not prevent or augment the inhibitory action of IL-1 on AHH activity. IL-1 stimulates collagen synthesis and elevates serum procollagen type 3 peptide (P-III-P). Results indicated that serum P-III-P was elevated in blood sources producing temperature-sensitive AHH inhibitory factor.

Arylhydrocarbon hydroxylase (AHH*) activity is mediated by cytochrome P450, is involved in the metabolism of many drugs, xenobiotics and endogenous substrates, and is usually associated with the liver but found in most tissues [1]. The activity of this enzyme is inhibited by compounds that stimulate the reticuloendothelial system [2]. The activity of this enzyme is decreased in patients with cirrhosis [3, 4] and in animal models of liver disease [5, 6]. This study will attempt to determine if interleukin-1 (IL-1), platelet derived growth factor (PDGF) and activated oxygen intermediates are involved in the depression of AHH activity in liver disease.

Materials and Methods

Patients (N = 12) had active chronic liver disease (primary biliary cirrhosis, N = 6, or alcohol-induced liver disease, N = 6) as reflected by histology. Patients had normal WBC counts and monocytes (%) within normal limits and no evidence of infection or systemic inflammation. Serum bilirubin was abnormal in 10 of 12 patients and 7 of the patients consumed alcohol. Normal volunteers (N = 12) were included for comparison.

Murine hepatocytes were isolated as previously described [2]. Peripheral blood monocytes, isolated under aseptic conditions using the method of Peterson [7], yielded 6×10^5 monocytes/10 mL. Endotoxin-free medium (< 0.04 ng/mL) was used for all cell preparations screened for endotoxin using the Limulus amoebocyte lysate assay (Sigma). Macrophages were identified by non-specific esterase staining [8]. Monocyte conditioned medium (MCM) was collected after 24 hr and filtered through 0.22 μm Millex filters; 1 mL of the medium was then incubated with hepatocytes (1.5×10^5 cells) in suspension for 2 hr at 37°. Hepatocytes were collected by centrifugation (50 *g* 1 min) and AHH activity was measured [4]. MCM did not directly neutralize hepatocyte AHH or interfere with its measurement nor did it affect cell viability. IL-1 is sensitive to heating at 70° for 1 hr [9]. Anti-human IL-1β polyclonal antiserum (25 μL) raised against the recombinant molecule (Cistron Biotechnology) was incubated overnight at 4° with 1 mL MCM (25 μL IL-1Ab neutralizes 12.5 half-maximal units of human recombinant IL-1 in the C3H thymocyte proliferation assay). Similar experiments were done using a non-specific antibody (anti-bovine albumin). MCM (1 mL) was also preincubated with 1870 U of catalase

* Abbreviations: AHH, arylhydrocarbon hydroxylase; IL-1, interleukin-1; IL-1Ab, anti-interleukin-1 antibody; 3-OHBP, 3-hydroxybenzo[α]pyrene; PDGF, platelet derived growth factor; P-III-P, procollagen type 3 peptide; TNF, tumor necrosis factor; and MCM, monocyte conditioned medium.