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Formation of Cyclophosphamide Specific DNA Adducts in Hematological Diseases

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Abstract

Background—Fanconi anemia (FA) patients are hypersensitive to DNA alkylating agents and require lower doses than non-FA patients to minimize serious toxicity. The mechanism by which hypersensitivity occurs is thought to be due to the inability of these individuals to effectively repair drug-induced interstrand DNA-DNA crosslinks. We recently developed a highly sensitive assay for cyclophosphamide specific interstrand DNA-DNA crosslinks (G-NOR-G) and are able to quantify and compare formation of these adducts in the blood of patients. Therefore we sought to determine whether FA patients have higher *in vivo* exposure to the cyclophosphamide specific interstrand DNA crosslink, G-NOR-G, relative to patients without FA.

Procedure—Cyclophosphamide interstrand DNA crosslinks were measured with the first dose of cyclophosphamide in FA and non-FA patients receiving a cyclophosphamide based preparative regimen prior to hematopoietic cell transplantation (HCT). FA patients received a lower cyclophosphamide dose than the non-FA patients (5–10 mg/kg/day vs. 50–60 mg/kg/day).

Results—Despite the lower cyclophosphamide dose and lower plasma concentrations in FA patients, they had G-NOR-G amounts similar to the non-FA patients (area under the curve (AUC)_{0-∞}, 99.8 vs. 144.9 G-NOR-G adducts/10⁶ nucleotides*hr, respectively, p=0.47). When G-NOR-G AUC was normalized for cyclophosphamide plasma concentrations, FA study subjects produced 15-fold higher adducts than non-FA patients (p=0.05).

Conclusions—FA patients are hypersensitive to DNA alkylating agents possibly as a result of greater formation of cyclophosphamide specific interstrand DNA crosslinks and/or diminished capacity for DNA repair. Identification and quantification of these adducts may be important determinant of cyclophosphamide related toxicity.

Keywords

Transplantation; Fanconi Anemia; cyclophosphamide; DNA adducts

Introduction

Fanconi anemia (FA) is a rare genetic disorder characterized by congenital malformations, predisposition to cancer and progressive bone marrow failure.(1) The FA genes encode for proteins which are involved in the regulation of the cellular response to DNA damage. Mutations in the FA genes lead to impaired DNA repair and presumably greater formation of interstrand DNA crosslinks or adducts after exposure to alkylating drugs (e.g. mitomycin C, diepoxybutane, melphalan, cyclophosphamide) and/or radiation.(2) As a result, FA patients are more sensitive to chemotherapy involving DNA alkylating agents, are at greater risk of toxicity and therefore lower doses are required. In the case of solid tumors, defects in FA genes with are often associated with greater tumor sensitivity to alkylating agents.(3,4)

Hematopoietic cell transplant (HCT) is the only curative therapy for FA patients with severe bone marrow failure. Pre-transplant conditioning chemotherapy usually includes the DNA alkylating agent, cyclophosphamide.(5) Because of excess toxicities observed, patients with FA receive substantially lower total doses of cyclophosphamide (20–40 mg/kg) compared to doses used in non-FA patients (100–120 mg/kg).(6)

Cyclophosphamide is metabolically activated by CYP2B6 enzyme and induces cellular death by forming DNA adducts, in particular, the interstrand DNA crosslink, [G-NOR-G], through formation of the active metabolite, phosphoramidate mustard (Figure 1). Cyclophosphamide primarily alkylates the N-7 position of guanine and forms 67% phosphotriester monoadducts, 26% N-7-guanine monoadducts, and 6.7% N-7-guanine-N-7 guanine interstrand crosslinks.(7) Even though interstrand crosslinks comprise only 5–10% of all adducts formed by alkylating agents, these adducts are the most physiologically relevant because of their ability to block DNA replication and their association with cytotoxicity.(8–11)

Several theories have been postulated as to the mechanism of cyclophosphamide induced hypersensitivity in FA. Such theories include genetic mutations in the DNA repair pathway thereby preventing the repair of damaged DNA induced by cyclophosphamide;(12–14) altered oxygen metabolism resulting in overproduction in reactive oxygen metabolites and; (15–17) altered drug metabolism resulting in high exposure of cyclophosphamide and/or its metabolites.(18) These theories speculate the accumulation of DNA adducts, in particular the interstrand cross links, in FA patients which induces a greater pharmacologic response. This study sought to verify whether FA patients have higher *in vivo* exposure to the interstrand DNA crosslink, G-NOR-G, relative to patients without FA.

Methods

Patients

Twenty pediatric and adult patients underwent HCT for a hematologic disorder (n=10 lympho-hematological malignancy and n=10 FA) at the University of Minnesota. All patients and/or guardians signed informed consent which was approved by the University of Minnesota IRB Board (IRB#0610M94328). Two patients were excluded from the final analysis due to missing time points or DNA extraction failure. Patient characteristics are shown in Table I. All subjects received intravenous cyclophosphamide over 2 hours at a constant infusion rate. FA study participants received one of the following conditioning

regimens: cyclophosphamide 5 mg/kg/dose IV daily given on days -6, -5, -4, -3, fludarabine 35 mg/m² daily IV on days -6, -5, -4, -3 plus antithymocyte globulin 30 mg/kg given daily on days -6, -5, -4, -3, -2 (n=2) for those with sibling donors; cyclophosphamide 10 mg/kg IV daily on days -5, -4, -3, -2, fludarabine 35 mg/m² IV daily on days -5, -4, -3, -2, antithymocyte globulin 30 mg/kg given daily on days -5, -4, -3, -2, -1, plus 300 cGy of total body irradiation (TBI) on day -6 (n=8) for those with unrelated donors. Non-FA patients received one of the following disease-specific regimens: cyclophosphamide 60 mg/kg/dose IV daily given on days -6 and -5 plus 165 cGy TBI twice daily on days -4, -3, -2, -1 (n=1); cyclophosphamide 50 mg/kg/dose IV on day -6 plus fludarabine 40 mg/m² IV daily for 5 days on day -6, -5, -4, -3, -2 and 200 cGy of TBI on day -1 (n=7); cyclophosphamide 50 mg/kg/dose IV on day -7 plus fludarabine 40 mg/m² IV daily for 5 days on day -6, -5, -4, -3, -2 plus Campath 0.2 mg/kg IV daily for 5 days on day -10, -9, -8, -7, -6 (n=1); cyclophosphamide 60 mg/kg daily on days -7 and -6, fludarabine 25 mg/m² daily IV on days -8, -7, -6 plus 165 cGy TBI twice daily on days -4, -3, -2, -1 (n=1).

Cyclophosphamide and DNA Adduct Quantification, and Pharmacokinetic Analysis

Pharmacokinetic sampling was conducted with the first dose of cyclophosphamide. Cyclophosphamide and G-NOR-G adducts concentrations were measured on each sample and were obtained at times 0 (prior to the start of infusion), 2, 6, 8 and 22 hours after the end of the 2 hour cyclophosphamide infusion. At each time point, 5 ml of blood was collected into a purple top tube and placed on wet ice. Plasma was separated by centrifugation and DNA was isolated from the remaining component. Plasma was frozen at -80 degrees C° until time of analysis. Plasma cyclophosphamide concentrations were measured by HPLC using a validated assay with UV detection as previously described.⁽¹⁹⁾ Briefly, ifosfamide was used as an internal standard. UV absorption data for the internal standard (ifosfamide) and cyclophosphamide was recorded at 195 nm and eluted at 19.2 and 22.2 minutes, respectively. The cyclophosphamide assay was linear from 0.01–20 ug/ml of cyclophosphamide and the lowest limit of quantification was 0.01 ug/ml. The accuracy and precision of the cyclophosphamide assay was 98.2% and 2.5%, respectively.

Cyclophosphamide specific DNA adduct (G-NOR-G) concentrations were measured in each blood sample after isolating DNA using Qiagen DNA extraction kits (Qiagen, Valencia, CA, USA) by the manufacturer's instructions. DNA amounts were estimated by UV spectrophotometry and G-NOR-G subsequently determined by HPLC-LSI-MS/MS analysis of deoxygaunine (dG) in enzymatic hydrolysates as described by Malayappan et al.⁽¹⁹⁾ Briefly, DNA samples were spiked with internal standard [¹⁵N₁₀]-G-NOR-G and subjected to neutral thermal hydrolysis (70°C for 1 hour) to release G-NOR-G as free base adducts. G-NOR-G and its internal standard were purified by solid phase extraction. They were eluted with 100% methanol (6 mL). Solid phase extraction recovery averaged between 90–95%. Fractions containing G-NOR-G and ¹⁵N₁₀- G-NOR-G were dried under nitrogen and the residues were reconstituted in 20 µL of 15 mM ammonium acetate buffer, pH 6.8 for capillary high pressure liquid chromatography electrospray ionization (ESI)⁺-tandem mass spectroscopy analysis. The lower limit of detection was 50 fmol with an accuracy and precision of 93% and 7.0%, respectively.

Plasma cyclophosphamide and G-NOR-G adduct concentrations were analyzed by noncompartmental analysis using standard software (WinNon Lin Professional 5.2, Pharsight Corp, Mountain View, California). Area under curve (AUC_{0-∞}), and C_{max} were determined for cyclophosphamide and DNA adducts. The AUC was estimated by the trapezoidal rule. C_{max} was the highest observed concentration. Because the FA and non-FA patients received different doses and consequently different plasma exposures of cyclophosphamide a ratio of G-NOR-G AUC_{0-∞} to cyclophosphamide AUC_{0-∞} was determined to normalize the number of adducts formed relative to the plasma exposure.

Statistical analysis

Comparisons of cyclophosphamide $AUC_{0-\infty}$, G-NOR-G adduct $AUC_{0-\infty}$, and concentration normalized DNA adduct exposure (DNA adduct $AUC_{0-\infty}$ /cyclophosphamide $AUC_{0-\infty}$) were assessed between FA and non-FA study subjects. Statistical comparisons between FA and non-FA groups, were made using two-way ANOVA.

Results

Patient Characteristics and Clinical Outcomes

Patient characteristics are given in Table I. FA patients were younger than the non-FA patients [median (range) 7 (1–9) vs 52 (25–56) years]. Time to donor stem cell engraftment was slightly longer in the FA patients. Non-FA patients engrafted at a median (range) of 6.5 (6–25) days posttransplant, whereas the FA patients engrafted at 11 (10–12) days. Treatment related mortality in the non-FA patients was 20% (95% CI: 0–45%) at day 100 and 58% (95% CI: 26–90%) at day 180 posttransplant. There was no treatment related mortality in the FA patients at day 100 or 180.

Cyclophosphamide Pharmacokinetics

Pharmacokinetic parameters are given in Table II. Non-FA patients received approximately ~ 10 fold higher daily cyclophosphamide dose (mg/kg) than FA patients ($p < 0.001$) and cyclophosphamide plasma exposure was significantly higher in the non-FA patients (Figure 2). Median (range) cyclophosphamide plasma concentrations in the non-FA patients at 2, 4, 8 and 22 hours after the end of the infusion were 60.9 (29.3–86.7), 36 (21.2–50.7), 31.2 (14.4–42.9), 27.3 (0.65–11.4) mcg/mL, respectively. Median (range) cyclophosphamide plasma concentrations in the FA patients at 2, 4, 8, and 22 hours after start of infusion were 5.8 (0.35–9.234), 2.89 (0.26–6.04), 2.0 (0.22–5.03) and 0.16 (0–2.8) mcg/mL, respectively. The median (range) cyclophosphamide $AUC_{0-\infty}$ was approximately 13-fold lower in the FA patients compared to non-FA (659.6 vs. 49.3 mcg/mL*hr) ($p < 0.001$).

Cyclophosphamide Interstrand DNA Crosslink (G-NOR-G) Pharmacokinetics

The absolute number of G-NOR-G adducts present at each time point post-dose in the non-FA and FA patients was similar (Figure 2). The median (range) concentrations at 2, 4, 8 and 22 hours after the end of cyclophosphamide infusion were 3.6 (0.1–58.7), 4.65 (0.4–26.5), 2.97 (0.42–26.4), 0.33 (0.03–4.05) G-NOR-G adducts/ 10^6 nucleotides, respectively in the non-FA patients. In FA subjects at 2, 4, 8, and 22 hours, concentrations were 8.45 (3.66–29.32), 5.45 (0.53–40.84), 2.19 (0.32–6.58), 0.58 (0–2.99) G-NOR-G adducts/ 10^6 nucleotides, respectively. As expected, no adducts were detected before cyclophosphamide administration in either group. Pharmacokinetic results are shown in Table II. The median G-NOR-G adduct $AUC_{0-\infty}$ was similar in the FA and non-FA patients (99.8 vs. 144.9, $p = 0.47$), despite the lower dose of cyclophosphamide in the FA patients. However, after normalization of G-NOR-G adducts for cyclophosphamide plasma concentrations FA patients had 15-fold more G-NOR-G adducts present than the non-FA patients (3.0 vs. 0.20 G-NOR-G to cyclophosphamide AUC ratio, $p = 0.05$). Maximum observed G-NOR-G concentrations occurred between 4 and 8 hours after completion of infusion. There was high (10–22 fold) interpatient variability in G-NOR-G AUC. Cyclophosphamide plasma exposure was poorly correlated with G-NOR-G adducts (Figure 3).

Discussion

To the best of our knowledge this is the first study to examine cyclophosphamide specific interstrand DNA crosslinks (G-NOR-G) in a FA pediatric population and an adult population with hematological malignancy.(20,21) Previous studies of interstrand crosslinks

have been conducted in cancer patients receiving platinum compounds and/or other alkylating agents.(22,23) However, regardless of the therapeutic agent, DNA adduct formation correlated with therapeutic responsiveness. In seven adults with multiple myeloma receiving high dose melphalan, individuals with the lowest adduct formation exhibited the poorest response to melphalan.(24) Therefore, quantification of *in vivo* adduct formation may be an important determinant of therapeutic efficacy and toxicity of cyclophosphamide.

Cyclophosphamide alkylates the N-7 position of guanine to form N-7-guanine monoadducts and N-7-guanine-N-7 guanine diadducts interstrand crosslinks.(7) Even though less abundant than the corresponding monoadducts, N-7-guanine-N-7 guanine diadducts interstrand crosslinks (G-NOR-G) are the most physiologically relevant because of their association with cytotoxicity.(8–11) In our study we demonstrated that we could measure the minor yet most physiologically relevant adduct (G-NOR-G) in two distinct populations receiving cyclophosphamide as part of their preparative regimen. Despite the significantly lower cyclophosphamide AUC_{0-∞} (Figure 2. and Table II) in the FA children, the absolute G-NOR-G exposures were not different from the non-FA group (p=0.47) (Figure 2B and Table II). After correcting the DNA adduct formation for cyclophosphamide exposure, FA patients produced 15-times more G-NOR-G adducts for every unit of cyclophosphamide present in the plasma relative to the non-FA patients (p=0.05, Table II). This supports current clinical practice of lower cyclophosphamide doses in patients with FA.

There are several issues related to cyclophosphamide adduct formation in these populations that deserve discussion. First FA patients may have DNA repair pathway deficiencies and genetic variants (e.g. BER, MMR, NHEJ) leading to the accumulation of G-NOR-G. Although variants may alter G-NOR-G formation no studies have evaluated their importance towards adduct formation. The sequencing of cyclophosphamide and radiation may also affect adduct formation. It is controversial if TBI should precede or follow chemotherapy in preparative regimens and no one clinical protocol has emerged as superior in this regard. TBI causes DNA damage and single strand DNA breaks; however, there are no data to date that suggest that radiation influences the formation of cyclophosphamide-specific DNA adducts in a negative or positive manner. (25) From a practical point, TBI is given first in most FA preparative regimens at our center so that patients are free from nausea and vomiting at the time of TBI administration. Cyclophosphamide is highly emetogenic and when given before TBI makes the delivery of TBI significantly more difficult. Cyclophosphamide is also not a known radiosensitizer in other cancer types. Two of the FA patients (no.'s 1 and 6 in Table 2) received no TBI in their preparative regimen. These two patients had DNA adduct AUC/cyclophosphamide AUC ratios higher than the median. However, should TBI affect adduct formation we would have expected these individuals to have the lowest cyclophosphamide adducts. Conclusions cannot be drawn from two subjects although it argues against TBI enhancement of cyclophosphamide adduct formation. Recent *in vitro* studies by Meybodi et al. demonstrated in leukocytes isolated from FA and non-FA subjects that initial radiation induced DNA damage was not different between the groups although the FA patients appeared to take longer to DNA repair(26). This study did not address the issue of combination chemotherapy and TBI sequencing. Finally, genetic polymorphisms in CYP2B6, and concomitant administration of drugs that induce or inhibit CYP enzyme activity may also affect adduct formation. Studies by Xie et al. demonstrated that genetic variants in the CYP2B6 enzyme resulted in an increase rate in 4-OH cyclophosphamide formation.(27) These issues should be addressed in future studies.

Despite the apparent differences in adduct formation between our two populations they displayed some commonalities. The maximum observed G-NOR-G concentrations occurred between 4 and 8 hours after the end of the cyclophosphamide infusion. This is consistent

with observations for melphalan N-ras and p53 gene specific interstrand crosslink adducts, whereby maximum melphalan induced adducts occurred at 8 hours post exposure.(24) Contradictory to our results, cyclophosphamide induced N-ras specific interstrand cross links occurred at 24 hours in one patient with poluarthritis nodosa.(24) In this study, G-NOR-G concentrations were poorly correlated with plasma cyclophosphamide concentrations (Figure 3). In patients with solid tumor receiving oxaliplatin, DNA adduct formation also did not correlate with platinum blood concentrations.(22) These findings support previous studies where no association was found between cyclophosphamide pharmacokinetics and clinical outcomes after HCT.(28) Cyclophosphamide is a prodrug that must be converted to active metabolites prior to its pharmacologic activity, therefore a better correlation may be observed between phosphoramidate mustard (an active metabolite) concentrations and G-NOR-G formation. Further studies are needed to examine the relationships between G-NOR-G, active metabolites, and clinical outcomes.

A limitation of our study is the lack of the ideal comparison group to the FA patients. The best control group is children with leukemia undergoing HCT of the same age who receives an identical preparative regimen and cyclophosphamide dose. This control group does not exist given the low doses of cyclophosphamide used in FA. Therefore, we chose to compare the FA children to an adult population receiving the most similar preparative regimens possible in our center. To directly address the known differences in metabolism rates between children and adults, and the varying cyclophosphamide doses used in FA vs non-FA protocols we measured parent cyclophosphamide blood levels concurrently with every cyclophosphamide DNA adduct measurement. From these data a cyclophosphamide DNA adduct measurement corrected for plasma concentration (DNA adduct/cyclophosphamide ratio) and indirectly for differences in metabolic rates was calculated.

In conclusion, we have shown for the first time that the interstrand DNA crosslink adduct, G-NOR-G, a minor but toxic adduct, is measurable in the blood of FA and non-FA patients using our highly sensitive and novel assay. FA patients formed significantly more G-NOR-G adducts relative to non-FA patients. Future studies are now feasible to determine the relationship between G-NOR-G formation and engraftment, treatment related mortality, toxicity and disease response in a larger number of subjects. Future studies should evaluate the association between DNA adduct formation and DNA repair variants, and the relationship between adduct formation and toxicity. Identification and quantification of other adducts (e.g. acrolien-DNA) may also be important towards the toxicity of cyclophosphamide.

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Figure. 1.
Cyclophosphamide metabolism and formation of guanine specific DNA adducts.

Figure. 2.

Time concentration profiles for cyclophosphamide (top panel) and G-NOR-G DNA adducts per 10^6 nucleotides (bottom panel). FA profiles are denoted with open squares ($\square$) and non-FA profiles are denoted with closed circles ($\bullet$). Times are hours after the end of a 2 hour infusion. Zero hour designates the time immediately before the start of infusion. Data are mean $\pm$ S.D.

Figure. 3. Correlation between cyclophosphamide exposure and G-NOR-G adduct formation in FA (top panel) and non-FA (bottom panel) study subjects.

Table I**Patient Characteristics**

	Non-Fanconi Anemia (n=9)	Fanconi Anemia (n=9)
Gender (M/F)	5/4	6/3
Age median (range)	52 (25–56)	7 (1–9)
Disease		
Aplastic anemia	1	-
Acute myelogenous leukemia	5	-
Chronic myelogenous leukemia	1	-
Multiple myeloma	1	-
NonHodgkin lymphoma	1	-
Race/ethnicity		
Caucasian	9	8
African American	0	1
FA Complementation Group		
A	-	5
D1/D2	-	1
Other	-	1
Not determined	-	2
Days to engraftment median (range)	6.5 (6–25)	11 (10–12)
Day 100 TRM (95% CI)	20% (0–45%)	0%
Day 180 TRM (95% CI)	58% (26–90%)	0%

TRM, treatment related mortality; engraftment is defined as ANC >500 cells μ L for three consecutive days

Table II

Patient	Total daily cyclophosphamide dose (mg)	Cyclophosphamide AUC (mcg/ mL * hr)	DNA adduct AUC (adducts/ 10 ⁶ nucleotides * hr)	Ratio of DNA adduct AUC /cyclophosphamide AUC
Non-FA n=9				
1	5640	515.3	96.7	0.19
2	4000	529.2	142.8	0.27
3	4600	659.6	425.3	0.64
4	3700	908.7	276.0	0.30
5	2750	868.0	19.0	0.02
6	3000	647.4	33.5	0.05
7	4450	463.0	50.8	0.11
8	3400	961.0	147.0	0.15
9	3000	300.6	177.4	0.59
Median (range)	3850 (2750–5640)	659.6 (300.6–908.7)	144.9 (19.0–425.3)	0.20 (0.02–0.64)
FA n=9				
1	115	4.2	64.3	15.16
2	195	8.1	36.1	4.43
3	210	82.3	26.3	0.32
4	206	167.5	171.3	1.02
5	100	134.9	148.7	1.10
6	95	39.7	141.2	3.55
7	165	81.6	271.5	3.33
8	93	49.3	72.4	1.47
9	190	33.6	99.8	2.97
Median (range)	140 (93–210)	49.3 (4.2–167.5)	99.8 (26.3–271.5)	3.0 (0.32–15.16)
p-value *	<0.001	<0.001	0.47	0.05

* P-values are comparison of non-FA and FA pharmacokinetic measures.