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Daily Variation in Non-protein Sulfhydryl Levels of Human Bone Marrow*

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Abstract—*There is increasing evidence that the dosing of certain antineoplastic agents based on a circadian type of schedule may have a beneficial effect on the outcome of patient therapy. Such regimens allow for a more intensive course of drugs to be administered due to a reduction in the toxicities associated with these agents. Since many of the antineoplastics in use today either form reactive intermediates or generate toxic free radical species within the cell, cellular thiols such as glutathione may play a role in their detoxification. Our studies were designed to investigate whether there exists a consistent daily fluctuation in the thiol content in human bone marrow samples. Five normal male volunteers, ages 26–32 years, underwent repeat bone marrow aspirations at approx. 8 a.m. and 8 p.m. Mean peak non-protein sulfhydryl (NPSH) levels of 47.7 nmole/mg protein occurred in morning bone marrow aspirates while evening aspirates showed markedly reduced mean levels of 7.9 nmole/mg protein. Reduced glutathione was used as a standard in these assays which measured sulfhydryls spectrophotometrically using Ellman's reagent. Our findings may help to explain the observed reduction in myelotoxicity in chemotherapy designed to take advantage of human circadian rhythms. These results support the proposition that the administration of certain cytotoxic drugs to match peak levels of thiols in the marrow may facilitate more intensive and active chemotherapy regimens.*

INTRODUCTION

DOSE INTENSITY, or the amount of drug administered per unit time, is of major importance in cancer chemotherapy and has been shown to correlate with treatment outcome in a variety of cancers including vincristine therapy of breast cancer, etoposide therapy in small cell carcinoma of the lung and cisplatin therapy in ovarian cancer [1]. Maintaining aggressive dose intensity is rarely possible, however, as dose levels and frequencies have to be adjusted downward due to toxic side-effects of the drugs being administered. Because many chemotherapeutic drugs have a low therapeutic index, attempts to improve drug tolerance in patients may greatly affect the outcome of therapy. An interesting method of reducing drug-induced toxicities is to alter the timing of drug administration and it is becoming clear that there are various chronobiologic rhythms which are important in cancer chemotherapy. Drugs whose efficacies appear to be associated with circadian rhythms include: ARA-C [2], cyclophosphamide [3], vincristine, melphalan, cisplatin, and the anthracyclines doxorubicin and daunomycin [4].

Animal studies with doxorubicin have indicated that the greatest toxicities are seen following its administration late in the animal's activity cycle while maximum tolerance is observed when the drug is given just before the transition from the resting to active state [4]. Cisplatin, on the other hand, was found to be tolerated better when given late in the animal's activity phase and was more toxic near awakening [5, 6]. Thus, the optimal dosing of these two agents in combination may be approx. 12 h apart with doxorubicin being administered at the beginning of the activity cycle and cisplatin given late in the period.

Human trials have been undertaken to assess such a dosing schedule in ovarian and transitional cell cancer of the urinary bladder [7, 8]. In these studies doxorubicin and cisplatin, two agents which are effective in these diseases [9], were administered on a circadian cycle with reduced toxicities observed when doxorubicin was administered at 6 a.m. followed 12 h later by cisplatin. Such a regimen

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allowed the patients to receive a higher dose intensity because there was less need for dose reduction due to drug toxicities. With a better understanding of the factors responsible for such a response, new regimens could be designed to take advantage of biological rhythms and thereby increase the effectiveness of many of the agents in use today.

Glutathione (GSH) may serve an important role in protecting the cell from toxic effects due to the reactive nature of many anticancer drugs. Concentrations of hepatic GSH in the rodent exhibit a circadian rhythmicity with peak levels occurring at the height of their feeding period [10]. Adams *et al.* [11] have shown that administration of a single dose of cyclophosphamide or ARA-C at therapeutic levels depleted GSH and glutathione *S*-transferase from mouse liver and bone marrow. These levels then rebounded to above normal levels approx. 5–7 days after being depleted. The animals were then able to survive a subsequent dose of cyclophosphamide (500 mg/kg) which was normally lethal. The time course of these changes clearly paralleled the changes in GSH and GST in normal murine cells, suggesting that there was a correlation between intracellular GSH levels and the alteration in drug toxicities.

This led to our interest in determining if there was a daily variation in the level of glutathione in the bone marrow of human subjects which might help account for the reduced clinical toxicities observed with doxorubicin and cisplatin when dosed according to circadian rhythms.

MATERIALS AND METHODS

Bone marrow studies were conducted in five healthy male volunteers, ages 26–32 years. Subjects were allowed to follow their normal daily routine and there was no restriction on food or fluid intake except that no drugs or alcohol were allowed prior to or during the sampling periods. Samples of bone marrow (8–10 ml) were obtained from either the iliac crest or the sternum at 8 a.m. and 8 p.m. and were assayed immediately. Nucleated cells were separated from erythrocytes using a 6% gentian dextran (Travenol Laboratories Inc., Deerfield, IL) gradient. The cells were washed with phosphate buffered saline, pH 7.4 and counted using a hemocytometer. An aliquot of 1×10^7 cells was used in an assay of non-protein sulfhydryl content performed in triplicate by the method of Sedlak and Lindsay [12]. Briefly, the cells were lyzed by sonication (model 250 sonifier, Branson Co, Danbury CT) and protein precipitated with 5% sulfosalylic acid. The protein-free lysate was then centrifuged at 12,000 ***g*** for 5 min and a 1 ml aliquot of the supernatant transferred to a tube containing 0.2 M Tris buffer and 0.02 M EDTA, pH 8.9. To each tube, 100 μl of 0.01 N 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) was added. The contents were mixed and the optical density measured at 412 nm. Reduced glutathione was used as a standard in these assays. Values are presented as nmoles NPSH per mg protein with protein determined by the method of Lowry *et al.* [13].

RESULTS

The NPSH levels found in the nucleated bone marrow cells are presented in Table 1. There was a statistically significant difference ($P < 0.001$, Student's *t*-test) between the morning and evening NPSH levels. NPSH levels were found to be at a maximum during the morning sampling with a mean value of 47.66 ± 5.73 and a range of 41.0–54.66 nmoles NPSH/mg protein. The apparent nadir was observed in the evening with a mean value of 7.89 ± 2.74 and a range from 4.43 to 10.30 nmoles NPSH/mg protein.

DISCUSSION

We have observed a daily variation in intracellular levels of bone marrow non-protein sulfhydryl levels in healthy human male volunteers. These values are elevated in the morning and subsequently decline through the day. Various animal models commonly demonstrate similar cycles. Circadian rhythms appear to play an important role in modulating anticancer drug toxicities. The timing of chemotherapy to match such circadian rhythms has been shown to be important for agents such as cyclophosphamide, ARA-C, doxorubicin, and cisplatin in murine tumors [4] and may also play a role in reducing doxorubicin and cisplatin toxicity in patients [7, 8].

In rodents, hepatic glutathione levels show a diurnal variation with a minimum value occurring toward the end of their active cycle and a peak just after their resting period. Such variations in glutathione are also seen with other tissues in the rat, differing in amplitude and frequency from the hepatic cycle [14]. A similar pattern appears to hold for the NPSH level, of which GSH is greater than 90% [15]. Studies have shown that toxicities of reactive compounds such as vinyldiene chloride are greatest if administered while GSH levels are at their lowest values [16]. Circadian rhythms also have been observed in animals for several enzymes as reported by North *et al.* [17]: In this study there was a cyclic variation in thirteen hepatic enzymes, including glutathione reductase, the enzyme responsible for maintaining the tripeptide GSH in the reduced, active state [18]. Among its many functions, glutathione acts to detoxify a wide variety of xenobiotics primarily by providing reducing equivalents, by scavenging free radicals and by enzymatically blocking peroxide mediated damage. It also

Table 1. *Bone marrow nucleated cell NPSH levels*

Subject	Age	NPSH (nmoles NPSH/mg protein) 8.00 a.m.	8.00 p.m.
1	26	41.00 ± 1.73*	10.30 ± 0.61*
2	27	48.33 ± 4.50	9.54 ± 0.80
3	28	48.00 ± 3.60	4.43 ± 0.84
4	32	45.33 ± 5.86	9.95 ± 0.98
5	26	54.66 ± 3.21	5.17 ± 1.13
	Overall mean:	47.46 ± 5.73†	7.89 ± 2.74

*Mean ± S.D. ($n = 3$).
†$P < 0.001$, Student's t-test.

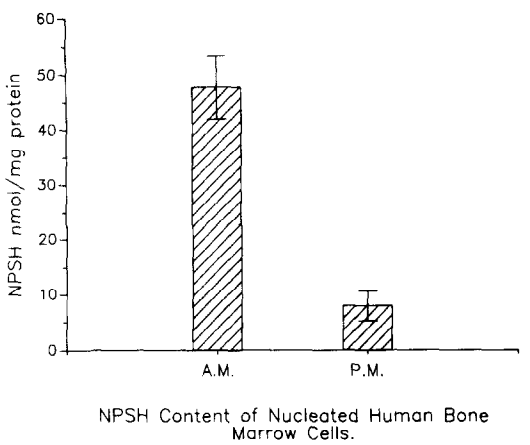

Fig. 1. *Non-protein sulfhydryl content of human bone marrow as a function of the time of day. Bars represent the mean ± S.D. NPSH level of five male volunteers.*

detoxifies electrophilic reactants by forming S-substituted conjugates which are more readily removed from the body [19] through the action of the enzyme glutathione *S*-transferase (GST).

When doxorubicin is administered at 6 a.m. followed 12 h later by cisplatin in patients, there appears to be a reduction in the toxicities associated with these two drugs. This allowed for a more intensive regimen to be administered [8]. The single acute dose limiting toxicity for doxorubicin is bone marrow suppression [20]. While cisplatin is minimally myelotoxic, its dose-limiting toxicities are renal tubular damage and, chronically, peripheral neuropathy [21]. Studies have shown [6, 22] that there is a decrease in both the gastrointestinal and nephrotoxicity of cisplatin when it is administered in the evening. This seems to be correlated with a circadian variation in urinary function with maximal excretory activity in the evening [23]. Thus, when cisplatin was administered in the evening there was a decrease in the peak urinary platinum levels, a greater urine output, and a smaller area under the curve (AUC) for cisplatin urinary concentration. Due to the electrophilic nature of the active species of cisplatin [24], GSH might be expected to play a role in reducing the cytotoxicity of such compounds. Andrews *et al.* [25] have shown that depletion of GSH has little effect on cytotoxicity due to cisplatin but causes a significant increase in the activity of *trans*-platinum. Glutathione appears to react with monofunctional adducts between *trans*-platinum and DNA thus preventing their transition to the more lethal bifunctional adducts [26].

Both human and animal studies have shown that doxorubicin is tolerated best when given shortly before awakening [4, 7, 8]. Our findings help to potentially explain such observations since doxorubicin may be detoxified by GSH-based enzymes. For instance, doxorubicin can undergo a redox cycling of its quinone function, leading to the formation of oxygen free radicals. These reactants, including hydroxyl and superoxide anions, may be responsible for its bone marrow and especially cardiac toxicity [27]. The maximum nucleated bone marrow cell NPSH values which we observed occurred during the morning samples and correspond to the period of greatest tolerance of doxorubicin seen in previous studies. This could help explain the reduction in toxicities observed with doxorubicin observed in the clinical studies using circadian scheduling because of the ability of glutathione to detoxify its reactive intermediates. Variations in bone marrow cellular levels of glutathione also could be important in protecting the body from the toxicities of other antineoplastic agents, such as cyclophosphamide which also seems to display circadian toxicities.

Maurer [28] has demonstrated that bone marrow from normal human subjects displays circadian rhythms in DNA synthesis and mitotic index. Our findings show that normal human bone marrow undergoes a 6-fold variation in NPSH content throughout the day. Whether this pattern is seen in patients or for prolonged periods is not known. However, the striking elevation in early morning sulfhydryls does suggest that dosing some drugs at this time may be indicated.

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