Chronopharmacology of Granulocyte Colony-Stimulating Factor in Mice

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ABSTRACT

The role of the sensitivity of bone marrow cells to, and the pharmacokinetics of granulocyte colony-stimulating factor (G-CSF) on the rhythm of leukocyte-increasing effect was investigated in ICR male mice housed under a standardized light-dark cycle (lights on at 0700, off at 1900). A significant circadian rhythm was demonstrated for leukocyte counts at 24 hr after G-CSF (250 μg/kg, s.c.) injection at six different circadian times (P < .01). The leukocyte counts of mice given G-CSF at 0500, 0900, 1300 or 1700 were significantly higher than those of mice given G-CSF at 2100 (P < .01, respectively). The rhythmic pattern resembled overall the rhythm occurring after saline injection. In the comparison between leukocyte counts after G-CSF injection at 0700 and 1900, the time when leukocyte counts are equal in nondrugged state, the leukocyte counts at 24 hr after G-CSF (250 μg/kg, i.v.) injection were approximately 50% higher in mice injected with the drug at 0700 than at 1900 (P < .01). Bone marrow cultures obtained at two times of day resulted in different numbers of myeloid colonies even when treated with the same concentrations of G-CSF in vitro. The colony-forming activity of G-CSF was significantly more potent at 0700 than at 1900 (P < .01). The plasma G-CSF concentrations after G-CSF (250 or 5 μg/kg, i.v.) injection were significantly higher in mice receiving injections with the drug at 0700 than at 1900 (P < .05, respectively). The area under the curve and mean residence time were significantly larger in mice injected with the drug at 0700 than at 1900 (P < .01, P < .05, respectively). Our suggests that the rhythm of G-CSF activity is caused by that of the sensitivity of bone marrow cells to, and the pharmacokinetics of the drug.

Bone marrow suppression is commonly associated with cytotoxic treatment of cancer (Evans, 1988). The cytotoxic effect on the bone marrow is due to a potentially irreversible damage of pluripotent stem cells, early committed progenitor cells and proliferating cells later in the maturation process, as well as to regulatory stroma cells in the bone marrow microenvironment (Hryniuk, 1987). Thus, the bone marrow-dependent side effects can lead to a suboptimal treatment of cancer patients (Frei and Canellos, 1980). However, a circadian dependence of antitumor drugs to bone marrow toxicity has been demonstrated, showing less dose reductions, less treatment related complications and less postponements of drug courses when drugs have been administered at certain times (Hrushesky, 1985; Hrushesky et al., 1989; Bjarnason and Hrushesky, 1994).

G-CSF is one of the hematopoietic growth factors that regulates the proliferation and differentiation of bone marrow progenitor cell populations (Clark and Kamen, 1987; Griffin, 1988). The rationale for the use of G-CSF in the treatment of cancer patients is 1) chemotherapy or radiotherapy results in neutropenia which compromises patients to morbidity and mortality due to bacterial and fungal infections; 2) frequently, the dose of chemotherapy must be reduced due to the myelosuppressive toxicity of anticancer agents and 3) it is often necessary to reduce doses, which in turn is thought to impair the antitumor response of effective therapeutic regimens. However, circadian variations in proliferative activity in bone marrow, both regarding CFU-GM and DNA synthesis, have been demonstrated (Smaaland et al., 1992; Perpoint et al., 1995). The results suggest that administration of G-CSF at the time of greatest responsiveness of the bone marrow relative to proliferative circadian rhythms may more effectively accelerate the regeneration of granulocyte/thrombocyte number. However, there is little information on the chronopharmacological aspect of G-CSF in vivo and the chronopharmacokinetics of G-CSF (Wood and Hrushesky, 1994).

This study was designed to examine the existence of circa-

ABBREVIATIONS: G-CSF, granulocyte colony-stimulating factor; CFU-GM, colony-forming unit granulocyte-macrophage, AUC; area under the curve, MRT, mean residence time VRT, variance of residence time.
dian rhythm in the pharmacological action of G-CSF in mice and to elucidate the mechanisms underlying the rhythm from the viewpoints of the sensitivity of bone marrow cells to, and the pharmacokinetics of the drug.

**Materials and Methods**

**Animals.** ICR male mice (5 wk old) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were housed 10 per cage under a standardized light-dark cycle (On at 0700, off at 1900) at a room temperature of 24 ± 1°C and humidity of 60 ± 10% with food and water available *ad libitum*.

**Influence of G-CSF dosing time on leukocyte counts in mice.** In general, s.c. or i.v. route is used for the therapy with G-CSF. Because s.c. route is technically convenient in rodents, it was used to investigate the rhythm of leukocyte counts after G-CSF injection at six different circadian times. Groups of 10 mice were injected s.c. with 250 µg/kg of G-CSF (Nartograstim, Neu-up, Kyowa Hakko Kogyo Co. Ltd. Tokyo, Japan) (Okabe *et al.*, 1990) or saline at 0900, 1300, 1700, 2100, 0100 or 0500. The dosage of G-CSF was determined by preliminary dose-response trials. Twenty µl blood samples were drawn by orbital sinus collection using micropipettes (Drummond Scientific, Broomall, PA) at 24 hr after G-CSF or saline injection. To observe the time course of leukocyte-increasing effect induced by G-CSF, groups of 10 mice were injected i.v. with 250 µg/kg of G-CSF or saline at 0700 or 1900. The dosing times were selected to make leukocyte counts equal in nondrugged state. Intravenous route was used to avoid a variation of G-CSF concentration at the absorption process. Blood samples were drawn before and at 12, 24, 36 and 48 hr after G-CSF or saline injection. Leukocyte counts were measured by Sysmex F-300 (Toa Iyou Denshi, Kobe, Japan).

**Influence of G-CSF dosing time on pharmacokinetics of G-CSF in mice.** The same dosage as that used in the study observing the pharmacological action of G-CSF was used to investigate the role of G-CSF concentration on the dosing time-dependent difference in the leukocyte-increasing effect of G-CSF. Groups of 8 mice were injected i.v. with 250 µg/kg of G-CSF at 0700 or 1900. Blood samples were drawn at 24 hr after G-CSF injection. Because the dosage of G-CSF (5 µg/kg) is used in humans, the dosage was used to investigate the dosing time-dependent difference in the pharmacokinetic parameters of G-CSF. Groups of 8 mice were injected i.v. with 5 µg/kg of G-CSF at 0700 or 1900. Blood samples (approximately 50 µl for each sample) were drawn at 10, 30 min, 1, 2, 3 and 4 hr after G-CSF injection. The samples were immediately centrifuged at 1000g for 5 min at 4°C. The plasma G-CSF concentrations were determined by G-CSF ELISA system (Amersham Life Science, Tokyo, Japan).

**Circadian stage-dependent change in granulocyte colony formation by G-CSF in bone marrow cells.** Groups of four mice were killed and their femurs were removed at 0700 or 1900. Thereafter, femurs were flushed with 5 ml of 0.9% NaCl solution (2.5 ml from each end of the bone). The cell suspension from both femurs was pooled and centrifuged at 400 × g for 10 min at 4°C. The pellets were washed twice with 10 ml of ice-cold 0.14 M NaCl and 0.01 M sodium phosphate (pH 7.4) and then resuspended in 2 ml of same buffer. The cells were resuspended at a density of 1 × 10⁶ cells/ml in α-minimum essential medium supplemented with 30% fetal bovine serum, 5 × 10⁻⁴M 2-mercaptoethanol, antibiotics (penicillin, kanamycin, streptomycin) and 0.35% agarose. The resuspended nucleated cells were then plated (0.9 ml/plate) onto 35-mm plastic gridded tissue culture dishes containing various concentrations of G-CSF (0.1 ml/plate). The cells were incubated for 7 days at 37°C in a humidified atmosphere of 5% CO₂ in air. Colonies containing 50 or more cells were counted blindly by the same investigator using an inverted microscope.

**Statistical analysis.** Statistical moment analysis was employed to calculate the pharmacokinetic parameters such as AUC, MRT and VRT. The statistical significance of differences between groups was validated by analysis of variance, Bonferroni method and Student's *t* test. Data analyses for circadian rhythms were done by the cosinor method to yield evidence of 24-hr rhythmicity and to obtain indices of the mesor (rhythm-determined average), amplitude (measure of the extent of a rhythmic change) and acrophase (peak time of a phase reference). *P* < .05 was considered to be significant.

**Results**

**Influence of G-CSF dosing time on leukocyte counts in mice.** The leukocyte counts of mice given saline showed a significant circadian rhythm dependence (*P* < .01, fig. 1). The leukocyte counts of mice given saline at 0900, 1300 or 1700 were significantly higher than those of mice given saline at 2100 (*P* < .01, respectively). In cosinor analysis, the leukocyte counts of mice given saline also showed a significant circadian rhythm dependence (*P* < .01) with mesor of 7481 ± 284 (mean ± S.E., *n* = 10) counts/mm³, amplitude of 2015 ± 402 counts/mm³ and acrophase of 12.40 ± 0.46 hr.min. A significant circadian rhythm dependence was demonstrated for leukocyte counts at 24 hr after G-CSF (250 µg/kg, s.c.) injection (*P* < .01). The leukocyte counts of mice given G-CSF at 0500, 0900, 1300 or 1700 were significantly higher than those of mice given G-CSF at 2100 (*P* < .01, respectively). In cosinor analysis, the leukocyte counts of mice given G-CSF also showed a significant circadian rhythm dependence (*P* < .05) with mesor of 15464 ± 488 counts/mm³, amplitude of 2013 ± 690 counts/mm³ and acrophase of 10.23 ± 1.19 hr.min. The leukocyte counts at 24 hr after G-CSF injection at six different circadian times were significantly higher when compared with those after saline injection at corresponding dosing time (*P* < .01, respectively). The rhythm pattern resembled overall the rhythm occurring after saline

![Fig. 1. Circadian rhythm of leukocyte counts at 24 hr after G-CSF (250 µg/kg, s.c.) or saline (□) injection at six different circadian times in mice. Each value is the mean of 10 observations with S.E. The leukocyte counts of mice given saline at 0900, 1300 or 1700 were significantly higher than those of mice given saline at 2100 (*P* < .01, respectively). The leukocyte counts of mice given G-CSF at 0500, 0900, 1300 or 1700 were significantly higher than those of mice given G-CSF at 2100 (*P* < .01, respectively). The leukocyte counts at 24 hr after G-CSF injection at six different circadian times were significantly higher when compared with those after saline injection at corresponding dosing time (*P* < .01, respectively).
injection. Injection of G-CSF resulted in a parallel increase in leukocyte counts.

The leukocyte counts at 12 hr after G-CSF (250 μg/kg, i.v.) injection at 0700 or 1900 were significantly higher as compared with those after saline injection at corresponding dosing time (P < .01, respectively) (fig. 2). The leukocyte counts at 24 hr after G-CSF injection at 0700 showed a peak. The high levels of leukocyte counts were maintained for 12 to 48 hr. The leukocyte counts after G-CSF injection at 1900 showed high levels for 12 to 36 hr and decreased thereafter. The leukocyte counts at 24 hr after G-CSF injection were approximately 50% higher in mice injected with the drug at 0700 than at 1900 (P < .01). The leukocyte counts at 12, 36 and 48 hr after G-CSF injection showed no significant difference between the two dosing times.

Influence of G-CSF dosing time on pharmacokinetics of G-CSF in mice. The plasma G-CSF concentrations at 24 hr after G-CSF (250 μg/kg, i.v.) injection were significantly higher in mice injected with the drug at 0700 than at 1900 (183.8 ± 37.2, 76.0 ± 22.8 pg/ml, mean ± S.E., n = 8, P < .05). There was a significant dosing time-dependent difference in plasma G-CSF concentrations after G-CSF (5 μg/kg, i.v.) injection (fig. 3). The plasma G-CSF concentrations at 10 min, 1, 2, 3 and 4 hr after G-CSF injection were significantly higher in mice injected with the drug at 0700 than at 1900 (P < .05, respectively). Table 1 showed the influence of dosing time on G-CSF pharmacokinetic parameters after G-CSF (5 μg/kg, i.v.) injection. The AUC and MRT were significantly larger in mice injected with the drug at 0700 than at 1900 (P < .01, P < .05, respectively). However, there was no significant dosing time-dependent difference in VRT.

Circadian stage-dependent change in granulocyte colony formation by G-CSF in bone marrow cells. There was a significant time-dependent difference in the granulocyte colony formation stimulated by G-CSF (fig. 4). The colony-forming activity of G-CSF at concentrations of 25, 50 and 100 ng/ml was significantly more potent in bone marrow cells obtained from mice at 0700 than at 1900 (P < .01, respectively).
Discussion

The leukocyte-increasing effect of G-CSF is considered to reflect the increase in neutrophil counts and neutrophil granulopoiesis (Okabe et al., 1990). Namely, no significant changes in erythrocyte, lymphocyte or monocyte counts are observed during the treatment of G-CSF, although neutrophil counts show a significant increase. Therefore total leukocyte counts instead of differential blood cell counts have been measured as an index of pharmacological effect of G-CSF in our study.

The leukocyte counts at 24 hr after G-CSF (250 μg/kg, s.c.) injection showed a significant circadian rhythm dependence with higher levels in the late dark phase and the light phase and lower ones in the early dark phase. The rhythmic pattern resembled overall the rhythms occurring in nondrugged state. These results indicate clearly that leukocyte counts after G-CSF injection show a circadian rhythm associated with the rhythm occurring in nondrugged state. The mechanisms underlying circadian rhythm of leukocyte counts in nondrugged state may be associated with the rhythmicity in the proliferation and differentiation of bone marrow progenitor cells, the mobilization of preformed mature cells from the bone marrow cavity or other pooling organs such as blood vessels, the destruction or removal of cells (Haus, 1994).

Because there is a significant circadian rhythm in leukocyte counts in nondrugged state, it is important to determine the circadian rhythm of G-CSF action itself, namely the difference between responses in drugged and nondrugged states. It is possible to calculate the increment in leukocyte counts in each G-CSF-treated mouse at each time of day over the mean leukocyte count in saline-treated mice matched for each time of day. However, the kinds of summarized data were not determined in the present study, because different mice for each group were used. Actual leukocyte counts would be more informative and important, because actual leukocyte counts were important indices in the therapy with G-CSF and the clinical evaluation of the drug. Therefore the comparison between leukocyte counts after G-CSF injection at 0700 and 1900, the time when leukocyte counts are equal in nondrugged state, was performed to examine the dosing time-dependent difference of G-CSF leukocyte-increasing effect itself. The dosing time of 0700 is the time the leukocyte counts in nondrugged state are increasing. The dosing time of 1900 is the time when leukocyte counts in nondrugged state are decreasing.

The leukocyte counts at 12 hr after G-CSF (250 μg/kg, i.v.) injection at 0700 or 1900 were significantly higher as compared with those after saline injection at corresponding dosing time. The high levels of leukocyte counts were maintained for 12 to 48 hr. G-CSF regulates the proliferation and differentiation of bone marrow progenitor cell populations (Clark and Kamen, 1987; Griffin, 1988) and also stimulates the mobilization of preformed mature neutrophils from the bone marrow cavity or other pooling organs such as blood vessels (Okabe et al., 1990). The elevation of leukocyte counts in the early period of injection reflects the mobilization of preformed mature neutrophils. However, the elevation of leukocyte counts in the later period of injection reflects the proliferation and differentiation of bone marrow progenitor cell. The leukocyte-increasing effect at 24 hr after G-CSF (250 μg/kg, i.v.) injection was significantly more potent in mice receiving injections with the drug at 0700 than at 1900. The dosing time-dependent difference in leukocyte counts seems to reflect real G-CSF activity such as the proliferation and differentiation of bone marrow progenitor cell. In general, the circadian change of drug susceptibility could be caused by that in the sensitivity of living organisms to, and/or the pharmacokinetics of drugs (Ohdo et al., 1988, 1991, 1995, 1996, 1997; Song et al., 1993a; Ogawa et al., 1997).

The colony-forming activity of G-CSF was more potent in bone marrow cells obtained from mice at 0700 than at 1900. The result is in good agreement with that published for bone marrow cells in mice (Perpoint et al., 1995). Namely, the highest colony-forming activity of G-CSF was observed in the early light period and the lowest in the late light period. Bone marrow cultures obtained at two times of day resulted in different numbers of myeloid colonies even when treated with the same concentrations of G-CSF in vitro. The result suggests that the sensitivity of bone marrow cells to G-CSF varies depending on the time of a day. It seems to reflect the time-dependent difference in the response of bone marrow cells to G-CSF not with mobilization but with proliferation and differentiation in vitro system. The time-dependent change in the sensitivity of bone marrow cells to G-CSF seems to contribute to, at least in part, that in the leukocyte-increasing effect of the drug in vivo.

A significant dosing time-dependent change was demonstrated for plasma G-CSF concentrations irrespective of dosage. The higher concentration of G-CSF was observed at a time when the leukocyte increasing-effect of the drug increased and the lower concentration observed at a time when it decreased. The rhythmicity seems to contribute to, at least in part, that in the activity of the drug. The time-dependent change of G-CSF concentration is closely related to that in the AUC and MRT. Kidney plays a major role in the elimination of G-CSF, although bone marrow cells contribute, to some extent, to it (Kuwabara et al., 1995a, b). The elimination of drug from kidney is restricted mainly by the rate which it can be transported from blood to kidneys, i.e., kidney blood flow (Benet and Sheiner, 1985). Both renal blood flow and glomerular filtration rate have been found to follow a circadian rhythm, with a maximum during the active period of animals (Cal et al., 1986; Labrecque et al., 1988). The rhythm of G-CSF elimination in our study corresponds nicely to the rhythms of renal blood flow and renal filtration. It seems that the higher blood flow and filtration during the active period of animals may contribute to the increased rate of renal drug clearance. Similar pattern of rhythms have been noted in the clearance of gentamicin, lithium and amikacin which are mainly eliminated by glomerular filtration (Song et al., 1993b; Shito et al., 1992; Hosokawa et al., 1993).

Our study suggests that the rhythm of G-CSF activity is caused by that of the sensitivity of bone marrow cells to, and the pharmacokinetics of, the drug. A significant circadian rhythm dependence has been demonstrated for leukocyte counts in nondrugged state in humans (Haus, 1994). There is a significant dosing time-dependent difference in plasma G-CSF concentrations and pharmacokinetic parameters of G-CSF after an intravenous injection of G-CSF (5 μg/kg), the dosage being used in humans. Therefore, the choice of dosing time associated with the rhythmicity of the response of bone marrow cells to, and the pharmacokinetics of G-CSF may
help to achieve a rational chronotherapeutic strategy, increasing the therapeutic effects of G-CSF.

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References


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