To the Editor:

Okabe et al. recently reported the biologic and physicochemical properties of recombinant human granulocyte colony-stimulating factor (rhG-CSF) and its mutant (KW-2228) in which amino acids were replaced at five positions in the N-terminal region of rhG-CSF by gene-mutation techniques. Based on comparative studies on the pharmacokinetics, they concluded that KW-2228 seems to be more biologically stable in plasma than intact rhG-CSF after intravenous injection into male C3H mice. However, it must be pointed out that some data and the explanation presented by them are questionable. First, despite intravenous dosing, the plasma concentration of KW-2228 "maintained" a higher level for 60 minutes compared with intact rhG-CSF and an "up-and-down" plasma concentration profile of intact rhG-CSF was observed. In their results, they failed to apply pharmacokinetic analysis to the observed plasma concentration-time data. Second, they claimed that superior in vitro stability of KW-2228 in plasma affected the pharmacokinetic difference, but the in vivo half-lives of both rhG-CSF were markedly shorter than in vitro half-lives. Therefore, the contribution of plasma proteases to the in vivo rhG-CSF elimination may be negligible.

We also investigated the pharmacokinetics of intact rhG-CSF in male C3H mice to determine whether the observations by Okabe et al. are real or artifacts. Male C3H mice were obtained from Japan SLC Co, Ltd (Shizuoka, Japan). The animals were housed for at least 1 week before experimentation with free access to food and water. All animals were used when they were between 9 and 11 weeks of age. Animals were assigned randomly to groups corresponding to the serum sampling times. This design provided four animals per sampling time. A dose of 100 μg/kg (2.59 ± 0.15 μg/head, mean ± SD) rhG-CSF produced by Kirin Brewery Co, Ltd was administered through the tail vein of the animal. The dose described above was almost identical to the dose in the previous experiment of Okabe et al. Under light ether anesthesia, blood samples were serially twofold diluted and [3H]thymidine incorporation over 5 hours by murine bone marrow cells was measured after 67 hours of incubation. The standard was composed of 400 ng/mL rhG-CSF in serum. (V) Standard; (○) 10 minutes; (●) 20 minutes; (△) 40 minutes; (△) 1 hour; (□) 2 hours; (■) 4 hours; (◇) 6 hours; (◇) 8 hours.

Fig 1. rhG-CSF serum activity after intravenous injection to mice. rhG-CSF (100 μg/kg) was injected and blood samples were collected. Serum samples were serially twofold diluted and [3H]thymidine incorporation over 5 hours by murine bone marrow cells was measured after 67 hours of incubation. The standard was composed of 400 ng/mL rhG-CSF in serum. (V) Standard; (○) 10 minutes; (●) 20 minutes; (△) 40 minutes; (△) 1 hour; (□) 2 hours; (■) 4 hours; (◇) 6 hours; (◇) 8 hours.
was obtained from a heart puncture at 10 minutes, 20 minutes, 40 minutes, 1 hour, 2 hours, 4 hours, 6 hours, and 8 hours after dosing and the obtained blood samples were centrifuged at 18,000g for 5 minutes to separate the serum. The serum was then frozen in liquid nitrogen and stored at -80°C until use. We used serum instead of plasma because anticoagulants may influence the in vitro activity of rhG-CSF. The rhG-CSF concentrations in the serum samples were measured by proliferation assay established previously using bone marrow cells obtained from male C3H mice femur. 24

A typical experiment to assay the in vitro biologic activity in serum samples is shown in Fig 1. In contrast to the report of Okabe et al., Fig 1 shows a steady decrease in activity with time. The average rhG-CSF concentration-time data after intravenous administration show a biexponential elimination profile (Fig 2). Pharmacokinetic parameters were estimated by fitting the serum concentration-time data to a two-compartment model equation using a nonlinear least-squares program (NONLIN84; Statistical Consultants, Inc, Lexington, KY) operated on a VAX 8350. The half-lives were 5.2 (α) and 122 minutes (β) and these values were similar to those obtained from other species, such as rat2 and human. 5 However, the serum concentrations described above were 20-fold higher than those of the previous experiment by Okabe et al through the experimental period.

The very different data of Okabe et al. 1 for rhG-CSF pharmacokinetics may result from their use of an inappropriate assay method that led to a failure to take into account the true pharmacokinetic properties of rhG-CSF. A more reliable assay method must be used to determine whether the differences in pharmacokinetic properties between KW-2228 and intact rhG-CSF exist and affect in vivo hematopoietic activities.

RESPONSE

As Tanaka and Kaneko pointed out, the pharmacokinetic analysis of KW-2228, a mutant recombinant human granulocyte colony-stimulating factor (rhG-CSF), in mice was not performed because the plasma concentration of intact rhG-CSF was "up-and-down." The experiment, however, was set up carefully and performed under the same experimental conditions, so that the data would be believed to be true and comparable with each other. We, in addition, have performed more detailed and quantitative experiments of pharmacokinetic study of KW-2228 in mice, rats, and monkeys by some improved methods. In response to Tanaka and Kaneko, we show here the pharmacokinetic data of KW-2228 in mice comparing with intact rhG-CSF. Male BALB/c mice obtained from Japan Charles River (Atsugi, Japan) (6 to 8 weeks old, 21 to 26 g of body weight) were maintained and used under pathogen-free conditions with free access to food and water. The mice were randomized and five animals were provided for each blood sampling. All animals were not given food and water during the 8 hours after the drug administration. Fifty micrograms per kilogram (1.05 to 1.25 µg/mouse) of KW-2228 or rhG-CSF was injected into the mice.
Fig 1. Correlation between plasma concentration determined by the bioassay and ELISA after intravenous or subcutaneous administration of KW-2228, into mice (50 μg/kg). (A) Intravenous administration. The line indicates the linear regression curve, Y = 0.984 X + 15.059, r = .978 (n = 34). (B) Subcutaneous administration. The line indicates the linear regression curve, Y = 1.046 X + 2.642, r = .960 (n = 35).

Fig 2. Plasma levels of KW-2228 and rhG-CSF after (A) intravenous or (B) subcutaneous administration of 50 μg/kg KW-2228 (○) or rhG-CSF (●) into mice. Each point with a bar represents the mean ± SD of five mice. *P < .05 versus rhG-CSF by Student's t-test.

through the tail vein or back shoulder subcutaneously, and blood was obtained by surgical dislocation at 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours after the dosing. The blood samples were collected in heparinized tubes and centrifuged at 1,500g for 10 minutes at 4°C to obtain plasma, and then frozen at −20°C until assay. The plasma concentrations of KW-2228 and rhG-CSF were measured by a sandwich enzyme-linked immunosorbent assay (ELISA). This ELISA has been confirmed to highly correlate to the bioassay using NFS-60 leukemic cells in several experiments using the plasma of humans, monkeys, rats, and mice, as shown in Fig 1. This ELISA has some advantages of high sensitivity (detection limit, 0.2 ng/mL) compared with the bioassay (detection limit, 2 ng/mL), and can avoid interference and/or stimulation effects of unknown components in the plasma. Pharmacokinetic parameters were estimated by fitting the plasma concentration-time data using a feathering method. The statistical analysis between KW-2228 and rhG-CSF was performed by Student's t-test (two-tailed).

As shown in Fig 2A, the mean plasma levels of KW-2228 and rhG-CSF after the intravenous administration showed biexponential elimination profiles similar to the data of Tanaka and Kaneko. However, the concentration of KW-2228 was significantly higher than that of rhG-CSF at all time points during the first 60 minutes, eg, 581.0 ± 39.1 ng/mL for KW-2228 and 473.2 ± 47.6 ng/mL for rhG-CSF at 5 minutes, and 289.0 ± 37.1 ng/mL and 190.6 ± 14.3 ng/mL at 60 minutes, respectively. This result suggests that the bioactivity of KW-2228 in the plasma retained higher level than
that of rhG-CSF, which confirmed our previous experimental results, in a summary, although the previous data were all underestimated. The distribution volume (1/kg) and total clearance (1/h/kg) of rhG-CSF were a little higher (about 1.4 and 1.3 times, respectively) than those of KW-2228, although there was no difference in the half-lives between KW-2228 and rhG-CSF, in which T1/2a was 0.17 hours for KW-2228 and 0.18 hours for rhG-CSF, and T1/2b was 1.46 hours for KW-2228 and 1.69 hours for rhG-CSF. The pharmacokinetic difference was confirmed by other experiments using monkeys.

Additionally, in the experiment of subcutaneous administration of KW-2228 (50 μg/kg) to mice (Fig 2B), the plasma level of KW-2228 maintained a significantly higher level than rhG-CSF. The plasma concentration of KW-2228 was twofold to 2.5-fold that of rhG-CSF during the 6 hours after the dosing, and total clearance of rhG-CSF was about twofold that of KW-2228. This difference in the plasma concentration between both G-CSFs was thought to derive from the difference in stability in the plasma and in the absorption from the tissue between both G-CSFs. Thus, a plasma concentration of KW-2228 higher than that of rhG-CSF may explain, in part, the superior in vivo biologic activity of KW-2228.

Concerning the stability in the plasma, the resistance to protease degradation of KW-2228 was confirmed in other experiments against some commercially available proteases such as trypsin and plasmin, although it could be only one of the possible explanations, at the moment. We are now investigating the factor(s) that might explain the difference in the stability between KW-2228 and rhG-CSF.

REFERENCES

Pharmacokinetics of recombinant human granulocyte colony-stimulating factor in mice [letter; comment]

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