Cancer Chemother Pharmacol (1996) 38 (Suppl): S74–S77

ORIGINAL ARTICLE

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Cloning of thrombopoietin and its therapeutic potential

Abstract Recently, four groups reported the cloning of thrombopoietin (TPO), also called c-Mpl ligand, from various species. In this study, we examined the in vitro and in vivo biological activity of TPO and its therapeutic efficacy in experimental animal models. Recombinant human TPO (rhTPO) supported the formation of only megakaryocyte (MK) colonies from rat marrow MK progenitor cells [colony-forming units-megakaryocyte (CFU-MK)] and predominantly acted on GpIIb/IIIa$^+$ CFU-MK at the late stage of differentiation. MKs generated from rat GpIIb/IIIa$^+$ CFU-MK after 3 days of liquid culture in the presence of rhTPO had mature characteristics. rhTPO stimulated an increase in the size of TPO-induced cultured rat MKs and in the number of elongated cytoplasmic processes, also called proplatelets, from these MKs in a dose-dependent manner. Administration of rhTPO to normal BALB/c mice daily for 5 days caused dose-dependent thrombocytosis. Treatment with rhTPO induced an increase in the size and number of marrow MKs and an expansion of the marrow CFU-MK pool. We further examined the effects of rhTPO on chemotherapy-induced thrombocytopenia in animal models. Following treatment with mitomycin C, mice received daily injections of various doses of rhTPO. Administration of rhTPO reduced the severity of thrombocytopenia and accelerated the recovery of platelets in a dose-dependent fashion: there was a significant reduction in the decrease in numbers of marrow MKs and CFU-MK with rhTPO treatment. Treatment with rhTPO also significantly improved neutropenia in mitomycin C-treated mice. Similar therapeutic efficacy was observed in cynomolgus monkeys with thrombocytopenia induced by nimustine. In addition, there was no significant change in several serum-chemistry parameters, in C-reactive protein, an acute phase protein, or in some variables involved in the blood-coagulation system. Furthermore, platelets from mice made thrombocytotic by repeated administration of rhTPO showed normal aggregation function. These results strongly suggest the clinical usefulness of rhTPO for the treatment of thrombocytopenia.

Key words Thrombopoietin · CFU-MK · Megakaryocyte · Platelet · Thrombocytopenia

Work presented at the 11th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium "Cytokines and New Anticancer Agents on the Horizon of Oncology", 24–25 November 1995, Nagoya, Japan

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Introduction

The process of megakaryocytopoiesis leading to platelet production includes (1) proliferation and differentiation of the committed megakaryocyte (MK) progenitor cells [colony-forming units-megakaryocyte (CFU-MK)] into MKs; (2) MK development, characterized by nuclear polyploidization and cytoplasmic maturation; and (3) cytoplasmic fragmentation of MKs and platelet release. This process has long been thought to be regulated by a lineage-specific humoral factor called thrombopoietin (TPO).

Recently, we and several other groups reported the identification, purification, and cloning of TPO, also called c-Mpl ligand, from various species [2, 6, 9, 12, 13, 21]. Using a quantitative in vitro assay to measure the production of MKs from rat GpIIb/IIIa$^+$ CFU-MK [15], we purified rat TPO from the plasma of irradiated rats using multiple fractionation steps and determined its partial amino acid sequences [9]. On the basis of the sequence information, we isolated rat and human TPO cDNAs [9, 17] and human genomic DNA [19]. Among the various rat tissues tested, TPO mRNA expression was greatest in the liver, and TPO activity was observed in some rat hepatoma-derived cell lines [18].

Since the cloning of the TPO gene, the recombinant molecule has become available and its physiologic role in

the regulation of megakaryocytopoiesis and thrombopoiesis has started to be clarified [1, 4, 7, 8, 10, 11, 13, 16, 20, 22]. In this study we investigated the in vitro and in vivo effects of recombinant human TPO (rhTPO) on MK development and platelet production and its therapeutic efficacy in myelosuppressed animal models.

Materials and methods

Preparation of CFU-MK GpIIb/IIIa$^+$ and GpIIb/IIIa$^-$ cell fractions from normal rat bone marrow

The GpIIb/IIIa$^+$ fraction of rat bone marrow cells, highly enriched for late CFU-MK, was obtained as described elsewhere [14]. Briefly, marrow cells were fractionated by Percoll density-gradient centrifugation, adherence depletion, and positive selection by immunoadsorption on a plastic dish precoated with a monoclonal antibody against rat GpIIb/IIIa (P55 antibody). For preparation of the GpIIb/IIIa$^-$ fraction, cells obtained after the adherence-depletion step were subjected to two 1-h periods of incubation on a P55 antibody-coated dish and unadsorbed cells were collected.

MK colony-forming assay

The MK colony-forming assay was performed in soft agar in a 35-mm tissue-culture dish as described elsewhere [14].

Quantification of proplatelet formation

Rat marrow GpIIb/IIIa$^+$ cells highly enriched for CFU-MK were cultured in 200 µl of Iscove's modified Dulbecco's medium containing 10% fetal calf serum and various concentrations of rhTPO in a 96-well tissue-culture plate. The quantity of proplatelets per culture was determined at different times. To examine the effects of rhTPO on rat MKs, cultured MKs grown from the GpIIb/IIIa$^+$ fraction of CFU-MK in the presence of rhTPO for 3 days were collected, washed, and recultured with or without rhTPO in a 96-well tissue-culture plate. The MK size and the number of proplatelets per culture were measured on day 1 and day 2 of reculture.

Platelet aggregation

Male BALB/c mice were made thrombocytotic by administration of repeated rhTPO injections, and blood was taken at a platelet peak. Platelet-rich plasma prepared from normal and thrombocytotic mice was used in an aggregation test. Platelet aggregation was performed according to Born's method [3].

rhTPO administration to normal mice and to animals with chemotherapy-induced thrombocytopenia

Various doses of rhTPO were given subcutaneously to male BALB/c mice once daily for 5 days, and blood was taken at different times for measurement of platelet and neutrophil counts and of hemoglobin concentration. The numbers of marrow MKs per six randomly selected 400× fields in histological sections of the femur were counted under light microscopy. The size of MKs was determined using a Vidas Plus image analyzer (Carl Zeiss, Oberkochen, Germany) consisting of a light microscopy unit. An MK colony-forming assay was performed to measure the numbers of CFU-MK in the femur. Briefly, marrow cells were cultured with murine interleukin 3 (IL-3) and, after 7 days of incubation, MK colonies were counted as described elsewhere [14]. In a murine chemotherapy model, male BALB/c mice were pretreated with mitomycin C before repeated administration of rhTPO. In a nonhuman primate model, male cynomolgus monkeys were pretreated with the chemotherapeutic agent nimustine (ACNU) before treatment with rhTPO. Serum from monkeys treated with rhTPO was used for serum chemistry, C-reactive protein (CRP) determination, and analysis of some variables related to the blood-coagulation system, such as the prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration.

Results and discussion

Responsiveness of GpIIb/IIIa$^+$ and GpIIb/IIIa$^-$ fractions of rat CFU-MK to rhTPO

To evaluate the target cell population of TPO at the progenitor cell level, GpIIb/IIIa$^+$ and GpIIb/IIIa$^-$ fractions of rat CFU-MK were tested for their ability to produce MK colonies in response to rhTPO and recombinant rat IL-3 (rrIL-3; Kato et al., unpublished results). rhTPO supported only MK colony growth from both fractions in a dose-dependent fashion, showing that TPO serves as an MK colony-stimulating factor (Meg-CSF), as has been reported for murine and human CFU-MK [1, 4, 10, 16]. The numbers of MK colonies obtained from the GpIIb/IIIa$^+$ fraction were similar at the optimal concentrations of rhTPO and rrIL-3. In contrast, the maximal numbers of MK colonies obtained from the GpIIb/IIIa$^-$ fraction stimulated with rhTPO were considerably lower than those obtained with rrIL-3. These data indicate that rhTPO predominantly acts on GpIIb/IIIa$^+$ late CFU-MK; this is supported by a previous report by Debili et al. [5], who have shown that c-Mpl is predominantly expressed on CD34$^+$/CD41$^+$ human marrow CFU-MK. The MK colony size (the number of MKs comprising individual MK colonies) from rat GpIIb/IIIa$^+$ CFU-MK was enhanced with increasing concentrations of rhTPO. rhTPO induced smaller MK colonies than IL-3, which induced larger MK colonies of heterogeneous size. These data indicate that TPO serves as a potent MK maturation factor and a Meg-CSF.

Development of MKs and proplatelets from rat GpIIb/IIIa$^+$ CFU-MK in liquid cultures containing rhTPO

The addition of rhTPO to liquid cultures containing rat GpIIb/IIIa$^+$ CFU-MK resulted in the generation of big, round MKs on day 3 (Horie et al., unpublished results). Interestingly, on day 4, some MKs underwent a dramatic morphological change, becoming long, extended cytoplasmic processes called proplatelets. The time course and TPO dose dependency of proplatelet formation from rat GpIIb/IIIa$^+$ CFU-MK were examined in 96-well tissue-culture plates. Formation of proplatelets did not occur until day 4. The number of proplatelets peaked on day 5 and thereafter decreased, probably due to fragmentation into platelets; a number of platelet-sized particles could be seen after 5 days of culture. Microscopic examination revealed that the larger MKs formed the larger proplatelets.

Effects of rhTPO on rhTPO-induced cultured rat MKs

Cultured MKs grown from rat GpIIb/IIIa$^+$ CFU-MK in the presence of rhTPO for 3 days showed markedly increased nuclear ploidy and well-developed demarcation membranes, indicating their maturity (Horie et al., unpublished results). These data are consistent with the findings reported by Kaushansky et al. [10]. Cultured rat MKs were then used to investigate the effects of rhTPO on mature MKs. Cultured MKs were incubated with various concentrations of rhTPO, and the size of MKs and proplatelet formation were measured. On both day 1 and day 2 of reculture, some megakaryocytes produced proplatelets in the absence of rhTPO. TPO addition did not produce a significant increase in the number of proplatelets. In contrast, rhTPO induced a dose-dependent increase in the mean size of megakaryocytes that did not produce proplatelets. These data suggest that fully mature megakaryocytes, which are competent to produce proplatelets, do not respond to rhTPO.

Effects of in vivo administration of rhTPO to normal mice

Normal male BALB/c mice receiving various doses of rhTPO daily for 5 days showed a dose-dependent increase in platelet counts with a peak on day 8 (Kabaya et al., unpublished results). In contrast, rhTPO administration did not affect neutrophil counts and slightly decreased hemoglobin concentrations in a dose-dependent fashion. The size of MKs in the femur significantly increased by day 3 in rhTPO-treated mice as compared with those in vehicle-treated mice, returning to normal by day 12. In rhTPO-treated mice, the number of MKs in the femur began to increase by day 3, peaked on day 6, and returned to normal levels by day 12. Moreover, the numbers of CFU-MK in the femur significantly increased by day 3, peaked on day 8, and decreased thereafter.

Effects of rhTPO on aggregation of murine platelets

We compared adenosine diphosphate (ADP)-induced aggregation of platelets from normal mice and mice rendered thrombocytotic using repeated injections of rhTPO (Torii et al., unpublished results). rhTPO did not induce aggregation of platelets from either normal or thrombocytotic mice in vitro. Preincubation with rhTPO significantly enhanced ADP-induced aggregation of platelets from both groups of mice. These data suggest that murine platelets produced by stimulation by rhTPO have normal aggregatory function.

Therapeutic efficacy of rhTPO in animal models with thrombocytopenia associated with myelosuppression

We first tested the effects of rhTPO in a murine chemotherapy model (Akahori et al., unpublished results). After treatment with mitomycin C, various doses of rhTPO were given to mice subcutaneously for different numbers of consecutive days; the duration of administration was dependent on the rhTPO dose. In vehicle-treated control mice, there was a platelet nadir on day 14, after which platelet counts returned to normal values by day 26. Treatment with rhTPO reduced the severity of thrombocytopenia and facilitated platelet recovery; at high rhTPO doses there was no decrease in platelet counts. There was no significant decrease in the numbers of CFU-MK in the femur of rhTPO-treated mice, and the numbers of MKs returned to normal more quickly in rhTPO-treated mice than in vehicle-treated mice.

Mice treated with mitomycin C also became neutropenic and slightly anemic. rhTPO administration also significantly improved neutropenia and anemia. Similar therapeutic efficacy has been shown in myelosuppressed mice treated with carboplatin [20].

We further tested the therapeutic efficacy of rhTPO in cynomolgus monkeys treated with the chemotherapeutic agent nimustine. After nimustine treatment, monkeys were given various intravenous doses of rhTPO daily for 28 days. In vehicle-treated animals, platelet counts decreased to a nadir on day 18 and returned to normal by day 30. rhTPO treatment decreased the severity of thrombocytopenia, accelerated platelet recovery to baseline values, and shortened the duration of thrombocytopenia. rhTPO administration also significantly improved neutropenia in a dose-dependent fashion. The serum-chemistry parameters measured and the C-reactive protein concentrations were unchanged by rhTPO administration. There was no difference in the PT, APTT, or fibrinogen concentrations measured between rhTPO-treated and vehicle-treated monkeys. These results suggest that rhTPO has therapeutic potential in the treatment of thrombocytopenic patients.

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