Volume 260, number 2, 176–178 FEB 08038 January 1990

Thrombopoietic activity of human interleukin-6

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Received 1 December 1989

Thrombopoietin (TPO), a regulatory factor in platelet production, was purified from the conditioned medium of TNK-01 cells cultured in the presence of human interleukin-1. The N-terminal sequence of purified TPO was determined to be VPPGEDSKDVAAPHRQPLT, identical to that of the N-terminal region of human interleukin-6 (IL-6). Two forms of TPO with molecular masses of 24 and 27 kDa were identified as IL-6 by Western analysis using an anti-IL-6 antibody. Commercial recombinant human IL-6 produced in *Escherichia coli*, stimulated megakaryocyte colony formation in the presence of mouse interleukin-3 and increased the number of peripheral platelets in mice in a dose-dependent manner. From these results, it is concluded that human IL-6 has thrombopoietic activity.

Thrombopoietin; Interleukin-6; Platelet production

1. INTRODUCTION

Thrombopoietin (TPO) has been thought to be a humoral regulatory factor in thrombopoiesis. The molecular nature of TPO has not yet been clarified, although several attempts at purification have been reported [1,2]. We established a cell line, TNK-01, derived from liposarcoma tissue of a patient who had shown a marked thrombocytosis and megakaryocytosis. After removal of the tumor, the platelet count returned to normal, indicating secondary thrombocytosis due to liposarcoma. We found the thrombopoietic activity in vivo and in vitro in the conditioned medium of TNK-01 cells cultured in the presence of human interleukin-1. In this paper, we describe some physicochemical properties of the purified TPO, human interleukin-6 (IL-6), and its thrombopoietic activity.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The cell line TNK-01 established from the tumor was maintained in Dulbecco's modified Eagle medium (DMEM, Gibco Laboratories) containing 10% fetal calf serum (FCS, Hyclone Laboratories) and antibiotics at 37°C in humidified 5% CO_2 atmosphere. TNK-01 cells were cultured in DMEM containing 2% FCS, antibiotics and 10 units/ml of interleukin-1 (IL-1, Boehringer Mannheim) for 4–7 days to obtain the conditioned medium.

2.2. *Bioassay for TPO*

Female BDF_1 mice (5–8 weeks old) were subcutaneously injected with 0.5–1 ml of samples or the vehicle for five days. Blood samples were taken from the vena cava inferior under anesthesia on day 5, and platelets were counted in a Micro Cell Counter (CC-180, Toa Medical Electronics).

The megakaryocyte colony stimulating activity of samples was determined using mouse bone marrow cells by the method of Sakaguchi et al. [3] in the presence or absence of 100 units/ml of mouse interleukin-3 (IL-3).

2.3. *Purification of TPO*

Conditioned medium (3 l) was concentrated to 120 ml by ultrafiltration, and the concentrate was applied to a column (5.0 × 38 cm) of DEAE-Sepharose Cl-6B equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl. The column was washed with the same buffer, and the concentrated pass-through fraction was subjected to TSK G3000SW (2.5 × 60 cm) gel-permeation HPLC. Fractions containing TPO were pooled, concentrated, and subjected to a reverse phase column (Vydac Protein C4, 4.6 × 250 mm) equilibrated with 5% acetonitrile in 0.1% trifluoroacetic acid (TFA). The proteins absorbed were eluted with a linear gradient of acetonitrile from 5% to 80% in 0.1% TFA. Pooled TPO fractions were then subjected to a second reverse phase HPLC in the same manner as described above to obtain 20 μg of purified TPO.

2.4. *Amino acid sequence analysis*

The amino acid sequence analysis was carried out using an Applied Biosystems 470A protein sequencer equipped with a model 120A on-line PTH-analyzer.

2.5. *Electrophoresis*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [4] on 13.5% gels.

3. RESULTS AND DISCUSSION

Cytochemical and electron microscopic studies of TNK-01 cells showed an accumulation of lipid droplets in the cytoplasm. The cell markers of TNK-01 were determined immunohistochemically using monoclonal

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

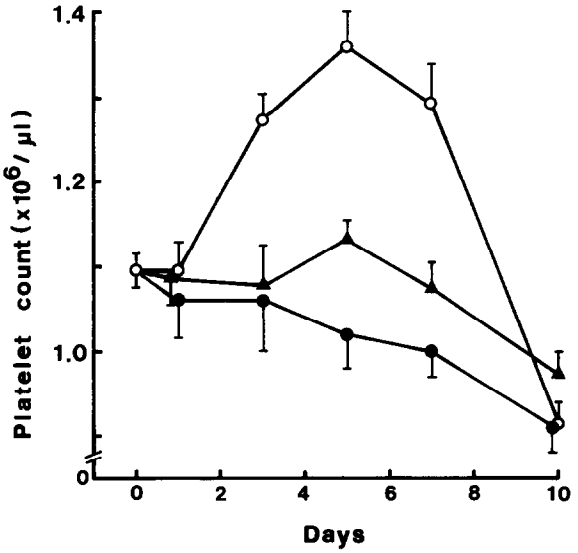

Fig.1. Thrombopoietic activity of the TNK-01-conditioned medium. Female BDF1 mice (5–8 weeks old, n = 4) were subcutaneously injected with 1 ml of a TNK-01-conditioned medium or vehicle for five days. Blood samples were taken from the vena cava inferior under anesthesia on days 0, 1, 3, 5, 7 and 10, and platelets were counted in a Micro Cell Counter (CC-180, Toa Medical Electronics). (○) Conditioned medium of TNK-01 cultured in the presence of 10 units/ml of human IL-1α; (▲) medium containing 10 units/ml of human IL-1α; (●) medium alone.

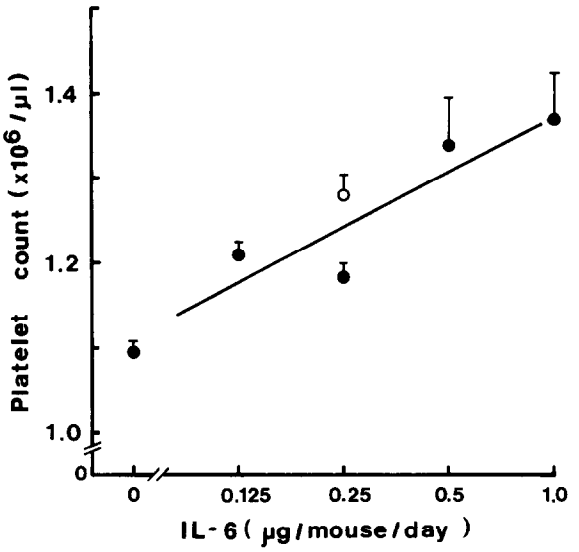

Fig.3. Thrombopoietic activity of human IL-6 in vivo. Thrombopoietic activity of recombinant human IL-6 and the TPO purified from the TNK-01-conditioned medium were compared by the method described in the legend of fig.1. Mice were injected with 0.5 ml of samples diluted with DMEM supplemented with 2% FCS, and the blood samples were taken on day 5. (●) Recombinant human IL-6; (○) TPO purified from TNK-01-conditioned medium.

antibodies, and were consistent with those of the original tumor. The thrombopoietic activity in the conditioned medium of TNK-01 cells cultured in the presence of IL-1 is shown in fig.1. IL-1 did not show the activity, and the number of erythrocytes and leukocytes was not changed during the examination.

Purified TPO showed a major protein band of an estimated molecular mass of 24 kDa and a minor band of 27 kDa on SDS-PAGE as shown in fig.2A. The N-terminal amino acid sequence of purified TPO was identical to that of the N-terminal region of human

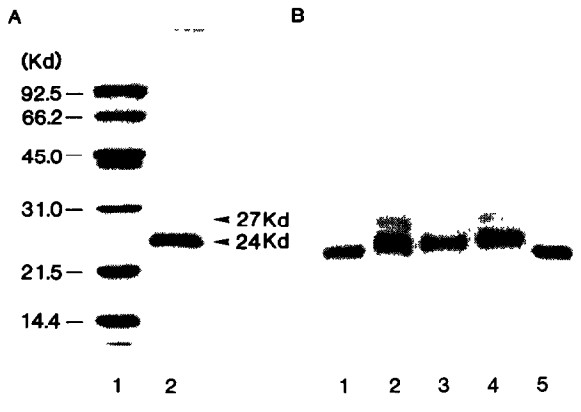

Fig.2. SDS-PAGE analysis of the purified TPO. (A) Molecular mass estimation of the purified TPO. Marker proteins (Bio-Rad) and 0.5 μg of the TPO were electrophoresed and stained with Coomassie brilliant blue R-250. (B) Western analysis. Recombinant human IL-6 (Amersham International, lanes 1 and 5), 150 ng, non-treated TPO (lane 2), 200 ng, N-glycanase-treated TPO (lane 3), 200 ng and neuraminidase and O-glycanase-treated TPO (lane 4), 200 ng, were electrophoresed and subjected to Western analysis using an anti-IL-6 antibody (Genzyme).

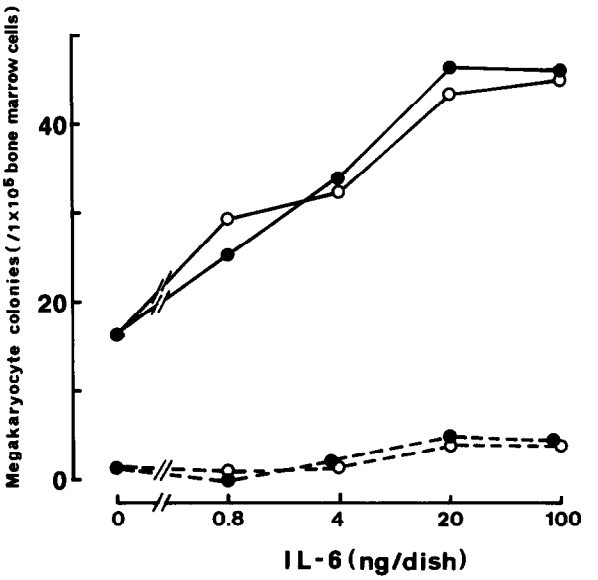

Fig.4. Megakaryocyte colony-stimulating activity of human IL-6. Bone marrow cells obtained from female BDF1 mice were cultured in the presence (solid line) or absence (broken line) of 100 units/ml of mouse IL-3 for 6 days at 37°C in humidified 5% CO_2 atmosphere. Cultures were mounted on glass slides, fixed with 5% glutaraldehyde and stained for acetylcholinesterase. A megakaryocyte colony was counted as a cluster of three or more acetylcholinesterase-positive cells. (●) Recombinant human IL-6; (○) TPO purified from a TNK-01-conditioned medium.

IL-6 [5,6]. The amino acid composition of TPO was in accordance with that previously reported, except for proline (data not shown) [7]. The two forms of TPO were also identified as IL-6 by Western analysis using an anti-IL-6 antibody (fig.2B, lane 2). The 27 kDa form was converted to the 24 kDa form by *N*-glycanase digestion (fig.2B, lane 3), indicating that the former carries an N-linked carbohydrate chain. The 24 kDa form may contain a different type of the O-linked carbohydrate chain which Gross et al. have reported [8], because no molecular mass change was observed by successive digestion of the TPO with neuraminidase and *O*-glycanase (fig.2B, lane 4). Further evidence that IL-1 stimulated TNK-01 cells produce IL-6 was obtained by Northern analysis (data not shown).

Fig.3 shows that the purified TPO and human IL-6 produced in *Escherichia coli* increased the number of peripheral platelets in mice. Both TPO and IL-6 stimulated the megakaryocyte colony formation in vitro in the presence of mouse IL-3 in a dose-dependent manner (fig.4). IL-6 is a multifunctional cytokine known to be a B cell-stimulating factor [9], a hybridoma-plasmacytoma growth factor [10], a hepatocyte-stimulating factor 1 [11] and an interferon-β_2 [12]. In the hemopoietic system, Ikebuchi et al. [13] have reported that IL-6 stimulated the blast colony formation with IL-3 synergistically in cultured bone marrow cells. Recently, Ishibashi et al. have reported that human IL-6 is a promotor of the maturation of murine megakaryocytes [14]. In addition to these reports, the results shown in this paper reveal that IL-6 acts as a regulator of thrombopoiesis in vivo.

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