RAPID COMMUNICATION

In Vivo Adenovirus Vector–Mediated Transfer of the Human Thrombopoietin cDNA Maintains Platelet Levels During Radiation- and Chemotherapy-Induced Bone Marrow Suppression

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Thrombopoietin (TPO, c-mpl ligand) has emerged as a major hematopoietic cytokine stimulating megakaryocyte proliferation, endomitosis, and platelet production. This study shows that a single administration of an adenovirus (Ad) vector encoding TPO (AdCMV.TPO) abrogates thrombocytopenia induced in mice by carboplatin and irradiation. Normal Balb/c mice receiving the vector had increased platelet counts peaking at 7 days and returning to baseline by day 15. Mice rendered pancytopenic with 500 rads and 1.2 mg of carboplatin had a nadir platelet count of five percent of the baseline. Mice receiving AdCMV.TPO 3 days before receiving irradiation and chemotherapy achieved a platelet nadir fourfold higher, and had significant reduction in duration of thrombocytopenia, than mice receiving the control Ad vector. Introduction of AdCMV.TPO the same day of chemother-

apy and irradiation was equally effective in acceleration of platelet recovery, but administration of AdCMV.TPO 3 days after chemotheraphy-radiation had little effect on platelet recovery. At 30 days after therapy bone marrow and spleen of mice treated with AdCMV.TPO were populated with a large number of polyploid megakaryocytes, but there was no evidence of circulating megakaryocytes in the liver or lungs and no pathologic bone abnormalities such as osteosclerosis or myelofibrosis. These observations suggest that an Ad vector may be an excellent delivery system to provide adequate TPO production to maintain platelet levels in circumstances associated with life-threatening thrombocytopenia.

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MATERIALS AND METHODS

Adenovirus vectors. AdCMV.TPO is an Elα-, partial E1b-, partial E3-, Ad5-based vector with an expression cassette in the E1 region containing a 1.7-kb human TPO cDNA driven by the cytomegalovirus major immediate/early promoter/enhancer.13,14 The TPO cDNA was a gift from D. Eaton (Genentech, South San Francisco, CA). The control vector AdCMV.null is similar in design, except it contains no gene in the expression cassette.15 All vectors were amplified, purified and titered as previously described.15

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Animals. Balb/c mice (6 to 20 weeks of age) were from Charles River Laboratories (Wilmington, MA). The age, sex, and body weight (>20 g) of mice were matched for each experiment. Each experiment included at least 6 animals for each data point.

*In vitro expression of the TPO cDNA.* To evaluate the ability of the AdCMV.TPO vector to express the TPO cDNA and direct the production of functional TPO, the A549 lung carcinoma cell line (American Type Culture Collection, ATCC CCL 185, Rockville, MD) was grown in minimal essential media (GIBCO-BRL, Gaithersberg, MD) with 10% fetal bovine serum, 50 units/mL penicillin G, and 50 mg/mL streptomycin. The A549 cells (2 × 10^6 cells) were plated in a 10 cm culture dish (Falcon, Franklin Lakes, NJ). After 24 hours, the cells were infected with AdCMV.TPO or AdCMV.null (multiplicity of infection [MOI] 20) in serum free medium. After 90 minutes, the culture medium was replaced with fresh serum-containing medium, and the culture continued for 48 hours. The culture medium was then replaced with 5 mL of serum-free medium for 24 hours, the “conditioned medium” was removed and centrifuged at 1,000 rpm, 4°C to remove floating cells. The resultant supernatant was passed through a 0.22-μm syringe filter and stored at −70°C. The cells were recovered from the culture dish and RNA extracted as previously described.13,14

To evaluate the cell RNA for the presence of TPO mRNA, Northern analysis was performed using total RNA (5 μg) hybridized with a 32P-labeled 1,089-bp full-length cDNA human thrombopoietin probe. The membrane was then stripped and probed with a 32P-labeled γ-actin probe as a positive control.

To assess the biological activity of TPO present in the A549 conditioned medium, 0.5 mL of conditioned medium was injected subcutaneously in Balb/c mice (Charles River Laboratories), once daily for 3 days, and the platelet count was quantified from blood drawn from the tail vein. To insure that there was no AdCMV.TPO vector in the conditioned medium, the titer of AdCMV.TP0 was determined using a Coulter counter (Coulter, Hialeah, FL). The number of platelets increased from pretreatment levels to a sevenfold peak level at day 7 with the highest dose (10^9 pfu) of the Ad vector. Increases in platelet levels were detected with doses of ≥10^7 pfu (P < .05, all comparisons to controls), but not at 10^6 pfu. At all doses, the platelet count gradually decreased to pretreatment level, by day 17 at 10^6 pfu, day 15 at 10^7 pfu, and by day 10 at 10^8 pfu. No increase in platelet levels was observed with administration of 10^9 pfu of the AdCMV.null vector nor with administration of the vehicle.

*AdenoVirus administration in association with myeloablative therapy.* Balb/c mice were rendered parapneoplastic using a combination of 500 μL sublethally total body irradiation and a single subcutaneous injection of 1.2 mg carboplatin (Bristol-Myers, Princeton, NJ), a regimen designed to produce severe thrombocytopenia.16 Mice were injected (intraperitoneal, single dose, 10^9 pfu) with AdCMV.TPO (n = 6, for each data point), AdCMV.null (n = 6) or vehicle (n = 6) 3 days before, simultaneously, or 3 days after induction of myeloablative therapy.

*Quantification of hematologic parameters.* Blood samples for platelet count were drawn from the tail vein with a capillary pipette (Unopette; Fisher Scientific, Springfield, MA). The platelet number was determined using a Neubauer hemocytometer (Fisher Scientific) under phase contrast microscope. The white blood count (WBC) was determined using a Coulter counter (Coulter, Hialeah, FL). The hematocrit was measured using heparinized micro-hematocrit capillary tubes (Fisher Scientific).

Bone marrow (BM) and spleen histology. To evaluate the BM from animals receiving the AdCMV.TPO vector and myeloablative therapy, femurs, spleen, lung, lymph nodes, and liver were removed from mice 30 days after myeloablative therapy and administration of AdCMV.TPO (10^9 pfu) or AdCMV.null (10^9 pfu) vectors. Tissue was fixed in 4% paraformaldehyde in PBS and stained with hematoxylin and eosin. The bone sections were analyzed histochemically using Gomori’s reticulin stain.30

**RESULTS**

*In vitro evaluation of the AdCMV.TPO vector.* Two lines of evidence showed the AdCMV.TPO vector functioned to express the TPO cDNA as expected. First, Northern blot analysis revealed 1.8-kb mRNA transcripts of the thrombopoietin cDNA in A549 cells infected with AdCMV.TPO, but not in cells infected with the control vector (Fig 1A).

Second, Balb/c mice injected with conditioned medium from A549 cells infected with AdCMV.TPO showed an increased platelet count on days 3, 7, and 10 (P < .05, all comparisons to controls). At day 7, the platelet count was approximately fivefold above baseline, returning to the normal range by day 17. There was no significant increase in the platelet count in the mice treated with AdCMV.null conditioned medium nor in those treated with vehicle (Fig 1B).

*In vivo administration of AdCMV.TPO to normal mice.* The subcutaneous administration of AdCMV.TPO to Balb/c mice induced a significant rise in the platelet count (Fig 2). In general, the magnitude and duration of the increase of platelet counts correlated with the dose of virus administered. The number of platelets increased from pretreatment levels to a sevenfold peak level at day 7 with the highest dose (10^9 pfu) of the Ad vector. Increases in platelet levels were detected with doses of ≥10^7 pfu (P < .05, all comparisons to controls), but not at 10^6 pfu. At all doses, the platelet count gradually decreased to pretreatment level, by day 17 at 10^6 pfu, day 15 at 10^7 pfu, and by day 10 at 10^8 pfu. No increase in platelet levels was observed with administration of 10^9 pfu of the AdCMV.null vector nor with administration of the vehicle.

*AdenoVirus administration in association with irradiation and chemotherapy.* Administration of the AdCMV.TPO vector maintained the platelet levels in mice receiving radiation and carboplatin, but the effectiveness of the therapy depended on when the vector was administered in relation to the induction of the myeloablative therapy (Figs 3 through 5). In animals receiving the AdCMV.null vector or the vehicle control, the radiation and carboplatin induced a profound thrombocytopenia, with approximately 10- to fivefold reduction in the platelet count with a nadir at 7 to 17 days after myeloablation (Figs 3A, 4A, and 5A). In contrast, when the AdCMV.TPO vector was administered 3 days before the myeloablation, the platelet levels were sustained, dropping only two to threefold at days 9 to 11 (P < .03, compared to controls at nadir; Fig 3A). Further, the recovery to normal levels and above was accelerated by approximately 1 week. An equally dramatic protection of platelet levels was achieved with administration of the AdCMV.TPO vector at the same time as the radiation plus carboplatin (P < .05, all compared to controls at nadir; Fig 4A). However, when the AdCMV.TPO vector was administered 3 days after myeloablation, although there was some protection of the platelet levels in the second week after myeloablation, the protection was not maintained, reaching
Fig 1. In vitro evaluation of AdCMV.TPO-mediated transfer and expression of the TPO expression cassette by Northern blot analysis and by in vivo quantification of platelet number in mice following administration to mice of conditioned medium from cells infected with AdCMV.TPO in vitro. (A) Expression of TPO mRNA transcripts in the A549 human lung carcinoma cell line 3 days after in vitro infection with AdCMV.TPO or the control vector AdCMV.null. Shown is a Northern blot of total RNA extracted from A549 cells and hybridized with a 32P-labeled TPO cDNA probe (lanes 1 and 2) or γ-actin cDNA probe as a control (lanes 3 and 4). Lanes 1 and 3—AdCMV.TPO (20 moi); lanes 2 and 4—AdCMV.null (20 moi). The size of the transcripts is indicated. (B) Platelet number in blood of Balb/c mice following administration of conditioned medium (0.5 mL) from AdCMV.TPO infected cells. The medium was administered subcutaneously on three consecutive days, starting at day 0. Shown is the platelet count for administration of medium of AdCMV.TPO infected cells (TPO-CM, □), AdCMV.null infected cells (Null-CM, ◦), or vehicle (○).

Fig 2. Increase in the platelet count in blood following administration of AdCMV.TPO to normal Balb/c mice. Shown is the platelet count of mice receiving (single administration, subcutaneously at 0 time) AdCMV.TPO (10⁸ pfu [□], 10⁷ pfu [○], 10⁶ pfu [△], or 10⁵ pfu [▲]), AdCMV.null (10⁶ pfu [○]) or vehicle (□).
Fig 3. Evaluation of the ability of AdCMV.TPO to maintain platelet levels in mice receiving myeloablative therapy 3 days after administration of the vector. Myeloablative therapy was induced in Balb/c mice using radiation plus carboplatin 3 days after the intraperitoneal administration of AdCMV.TPO (10⁶ pfu/1, AdCMV.null (10⁷ pfu) or vehicle (PBS). The date of ablative therapy is considered to be day 0. (A) Platelet levels. (B) WBC. (C) Hematocrit. Number of platelets (A) and WBC (B) were plotted on log scale. Hematocrit (C) is presented by %. The arrows indicate the date of radiation (RT) and chemotherapy (CT) and the timing of administration of the adenovirus vector (Ad).

Fig 4. Evaluation of the ability of AdCMV.TPO to maintain platelet levels in mice receiving myeloablative therapy on the same day as administration as in Fig 3, except for the timing of vector administration. (A) Platelet levels. (B) WBC. (C) Hematocrit.

The data show that a single injection of the AdCMV.TPO vector ameliorates the severe thrombocytopenia associated with myeloablation.

Adenovirus-mediated delivery of TPO. From the available data, it appears that TPO can protect platelet levels from...
myeloablation, but that there is a "window" of dose and time that is necessary to protect, yet not lead to adverse effects. Ad vectors may be an ideal delivery system for this application.

First, by delivering the TPO cDNA rather than the protein, the recipient becomes the TPO protein "factory," eliminating the need to purify the recombinant protein. A similar strategy has been used to deliver the fibroblast growth factor-4 (HST-1) gene and maintain platelet levels using an adenovirus vector.23 Second, as with the protein, the Ad vector can be given in various doses, thus controlling the peak platelet levels. Third, the Ad vector can be administered through various parenteral routes.5-8 Finally, the single administration of the first generation Ad vector used in this report provides transient expression, thus providing more TPO (integrated over time) than a single administration of the TPO protein, but not an excessive amount of TPO that might be associated with adverse effects. Unlike the requirement for persistent expression of the erythropoietin cDNA to treat the anemia of chronic renal failure,24 transient expression of the TPO cDNA during the initial phases of myeloablative therapy is likely the optimal strategy to prevent life threatening thrombocytopenia, without rebound megakaryocytopoiesis and thrombocytosis. Importantly, not only did platelet levels return to normal in the Ad vector recipients, but histological analysis of the mice receiving AdCMV.TPO showed predominance of polyploid megakaryocytes in the spleen and BM, no megakaryocytes in the liver, lung, or circulation, and no evidence of osteosclerosis or myelofibrosis within the bone marrow microenvironment. In contrast, Yan et al22 have shown that chronic overexpression of TPO by ex vivo retroviral introduction of the TPO cDNA into BM progenitors, and transplantation of the modified cells into mice, results in persistent elevation of platelets, osteosclerosis, and myelofibrosis, suggesting that prolonged overexpression of TPO maybe deleterious to the BM microenvironment.

Effects on other progenitors. In mice receiving AdCMV.TPO before and immediately after institution of myeloablative therapy, there was a decrease in duration and/or the severity of leukopenia and anemia. Although it is possible that the recovery of myeloid and erythroid series was directly related to TPO,22,27 it may be secondary to a decrease in bleeding and general well-being in the TPO treated mice resulting in improved hematocrit and WBC. Alternatively, because megakaryocytes are capable of elaborating cytokines such as interleukin-6 (IL-6), IL-1, and IL-3,9 the rapid recovery of the other lineages seen in TPO treated mice may be due to rapid increase in the megakaryocyte mass and increase in cytokine production by megakaryocytes within the BM microenvironment.

In summary, adenoviral transfer of the TPO gene into mice treated with sublethal doses of chemotherapy/irradiation results in a decrease in the severity and duration of thrombocytopenia with smooth recovery of the platelet count to baseline level. In contrast to retroviral gene therapy, where chronic exposure of TPO may produce myelofibrosis, a single injection of adenoviral vector expressing transgene for TPO may provide an ideal and safe alternative to daily injection of TPO for the treatment of chemotherapy/radiation induced thrombocytopenia. Direct introduction of AdCMV.TPO into the BM microenvironment may provide a novel approach for therapy of thrombocytopenic conditions that are amenable to cytokine therapy.

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Fig 6. Evaluation of BM and spleen of mice after administration of AdCMV.TPO. Evaluation of megakaryocytes in the bone marrow and spleen of mice at 30 days after subcutaneous administration of AdCMV.TPO. Hematoxylin stain of the BM (A, B, and C) and spleen (D, E, and F) of mice after subcutaneous administration of AdCMV.null (A and D, original magnification × 100) and AdCMV.TPO (B, C, E, and F, original magnification × 400). AdCMV.TPO treated mice show a predominance of polypoid megakaryocytes both in spleen and BM. Reticulin stain (G) of day 28 BM of trephine biopsy of the mice treated with AdCMV.TPO (original magnification × 400).
REFERENCES


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