Effective Prevention of Thrombocytopenia in Mice Using Adenovirus-mediated Transfer of *HST-1 (FGF-4)* Gene

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Abstract

***HST-1 (FGF-4)* gene product is a member of the fibroblast growth factor family with a signal peptide and plays a crucial role in limb development. We showed previously that an intraperitoneal injection of replication-deficient adenovirus containing the *HST-1* gene (Adex1*HST-1*) into normal mice caused a twofold increase in peripheral platelet count. To investigate whether Adex1*HST-1* could effectively prevent experimentally induced thrombocytopenia in mice, we injected Adex1*HST-1* intraperitoneally into thrombocytopenic mice induced by administration of a chemotherapeutic agent and/or by irradiation. A single Adex1*HST-1* injection caused continuously increased levels of serum HST-1 protein for at least 30 d and increased the count of large megakaryocytes in bone marrow, which specifically recovered platelet counts and more efficiently diminished the extent and duration of thrombocytopenia than any other reported cytokine or any combination of cytokines so far. In the other peripheral hematological parameters, no discernible differences were detected. No other apparent side effects were observed. Therefore, this method could be useful for treatment and/or prevention of thrombocytopenia induced by chemotherapy and/or irradiation for cancer treatment. (*J. Clin. Invest.* 1995. 96:1125–1130.) Key words: fibroblast growth factor 4 • platelet • megakaryocyte • adenovirus vector • gene transfer**

Introduction

The fibroblast growth factor (FGF) family consists of at least nine related heparin-binding growth factors: acidic and basic FGFs, INT-2 protein, HST-1 protein/FGF-4/K-FGF, FGF-5, HST-2 protein/FGF-6, KGF (keratinocyte growth factor), AIGF (androgen-induced growth factor) (1), and FGF-9 (2). Recently, FGFs have been shown to be mitogenic toward a broad spectrum of mesodermal, ectodermal, and endodermal cells, inducing cell proliferation and differentiation in vitro (3). Acidic and basic FGFs are best characterized and are well known for their versatility as well as ubiquitous tissue distribution. Additionally, acidic and basic FGFs are involved in megakaryocytopoiesis in vitro, as we and others have reported (4–6).

FGFs exert their biological action through binding and activating high-affinity cell surface FGF receptors (FGFRs)[1] that have an intrinsic tyrosine kinase activity (7). The binding of FGF to the receptor induces receptor dimerization, transphosphorylation, and subsequent association with cytoplasmic signaling molecules leading to DNA synthesis or differentiation (8). Four receptors, flg or FGFR-1, bek, K-*sam* or FGFR-2, FGFR-3, and FGFR-4, have been identified (9–14), and HST-1 protein binds both FGFR-2 and FGFR-1 (15, 16).

The *HST-1 (FGF-4)* gene, encoding HST-1 protein or FGF-4, was originally identified in our laboratory as a transforming gene from human gastric tumor and is located on chromosome 11q13 (17–19). Unlike acidic and basic FGFs, the HST-1 protein is easily secreted out of the cells since it contains a signal peptide (18). Amplified in a variety of human tumors (20, 21), the *HST-1* gene is not expressed even in tumors with the gene amplification. In contrast to the acidic and basic FGF genes, the *HST-1* gene is normally dormant in adult tissues, and its expression is particularly restricted in the apical ectodermal ridge of embryonal limb bud (22), which suggests that the HST-1 protein plays a crucial role in limb development (23).

Replication-deficient recombinant adenoviruses have been successfully used to transfer foreign DNA into a variety of cells and to express the gene in vivo (24, 25). To explore the biological function of the *HST-1* gene in vivo, we injected the adenovirus vector containing human *HST-1* cDNA (Adex1*HST-1*) intraperitoneally into normal mice. We previously discovered and reported that a single injection of Adex1*HST-1* resulted in a twofold increase in peripheral platelet count for ~ 30 d (26).

Here, we report that a single injection of Adex1*HST-1* into mice caused a continuous increase in serum HST-1 protein for ~ 30 d and thus effectively prevented experimentally induced thrombocytopenia by a chemotherapeutic agent and/or whole body irradiation without any detectable side effects. In addition,

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1. *Abbreviations used in this paper:* Adex1*HST-1*, Adex1*HST-1*L in reference 26; Adex1*LacZ*, Adex1SR*LacZ*L in reference 26; FGFR, fibroblast growth factor receptor.

the Adex1*HST-1* injection resulted in an increase in the count and the size of megakaryocytes in bone marrow, which specifically recovered platelet count without any other hematological abnormality including platelet sensitivity to aggregation.

Methods

Materials. ICR mice (male and female, 6 wk of age) were purchased from Charles River Japan (Yokohama, Japan). Cisplatin/*cis*-diamminedichloroplatinum was obtained from Nippon Kayaku Co. (Tokyo, Japan). Carboplatin/*cis*-diammine [1,1-cyclobutanedicarboxylato] platinum, ADP, collagen, and thrombin were from Sigma Chemical Co. (St. Louis, MO). Unopette was from Becton Dickinson Co. (Franklin Lakes, NJ). Mouse anti-human FGF-4 monoclonal IgG was from R&D Systems (Minneapolis, MN).

Adenovirus construction and preparation. The construct of the recombinant adenovirus was reported previously (27). In brief, this replication-deficient adenovirus is based on adenovirus type 5 which lacks the E1A, E1B, and E3 regions of the virus and contains the SRα promoter, human *HST-1* cDNA, and SV40 poly(A) signal sequences inserted into the E1-deleted region. Recombinant *lacZ* adenovirus (Adex1*LacZ*), used as a control, contains the SRα promoter, *LacZ* gene, and SV40 poly (A) signal. Purified virus stocks were prepared through CsCl step gradient centrifugation as described (28).

Adenovirus injection into mice. In each thrombocytopenic experiment, mice were injected intraperitoneally with Adex1*HST-1* (n = 8), or Adex1*LacZ* (n = 8) used as a control, at a dose of 1×10^9 plaque-forming units per mouse 3 d before the thrombocytopenic regimen, and the other mice (n = 8) were not treated with adenoviruses.

Thrombocytopenic regimens. We used three types of thrombocytopenic regimens: cisplatin administration, 3.0 Gy irradiation, and a combination of 5.0 Gy sublethal irradiation and carboplatin administration (29). Since these regimens produce modest to severe thrombocytopenia in mice with the platelet nadir occurring 7–11 d after myelosuppression as preliminary experiments, and 10–12 d were required to obtain the increase in platelet count after the Adex1*HST-1* injection, we administered these regimens on day 0, which was 3 d after the adenovirus injection. In each regimen, mice received the chemotherapeutic drug cisplatin at a dose of 8 mg/kg body wt as a single intraperitoneal injection, or 3.0 Gy irradiation from a ^{60}Co source, or 5.0 Gy sublethal irradiation followed by a single intraperitoneal injection of 1.2 mg/mouse of the chemotherapeutic drug carboplatin.

Peripheral hematology. Blood samples for platelet count were drawn from the retro-orbital vein with a capillary pipette of Unopette on various days just before and after the virus injection. Platelet number was counted with a Neubauer hemacytometer (Kayagaki Irika Kogyo Ltd., Tokyo, Japan) under microscope. For the other peripheral hematological parameters, total blood was drawn from the heart of the anesthetized mice on day 7. The other peripheral hematological parameters were counted with a hematology analyzer (Cysmex NE8000; Toa Medical Electronics Co. Ltd., Tokyo, Japan).

Marrow megakaryocytes. For a megakaryocyte survey, femurs were removed from the anesthetized mice 7 d after the cisplatin administration or the 3.0 Gy irradiation, fixed with 20% formalin, dehydrated, embedded in paraffin, cut in thin sections, and stained with hematoxylin and eosin. Morphologically identifiable megakaryocytes of which the nucleus was recognized were counted per arbitrary microscopic area of one square millimeter. Megakaryocyte diameters were also measured by determining the average of two perpendicular diameters in each specimen of 100 random megakaryocytes of which the nucleus was recognized in the section.

ELISA of HST-1 protein. Blood samples were drawn from the retro-orbital vein or from the heart of the anesthetized mice. The amount of serum HST-1 protein was detected with indirect ELISA using mouse anti-human FGF-4 monoclonal IgG as described (30). The optical density of 492 nm was measured with a kinetic microplate reader (model 3550; Bio Rad Laboratories, Hercules, CA).

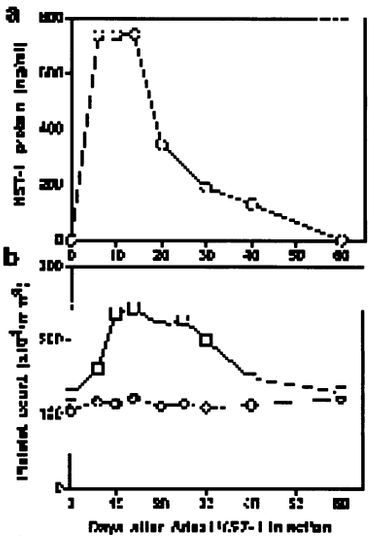

Figure 1. Kinetic analysis of the serum HST-1 protein concentration and the peripheral platelet count in a normal mouse injected with Adex1*HST-1*. (*a*) The values for the serum HST-1 protein concentration in one representative Adex1*HST-1*–injected mouse. (*b*) The values for platelet counts in the same Adex1*HST-1*–injected mouse (*boxes*) and one Adex1*LacZ*-injected mouse (*diamonds*). Normal ICR mice were injected intraperitoneally with Adex1*HST-1* or Adex1*LacZ* at the same dose of the virus per mouse, and the serum HST-1 protein concentration and platelet counts were determined.

Platelet aggregation. Platelet aggregation was performed as described (31, 32) with minor modifications. Briefly, total blood was anticoagulated with 3.8% sodium citrate (1/10 vol/vol), and centrifuged at 100 *g* for 10 min to obtain platelet-rich plasma. Some amount of the platelet-rich plasma was further centrifuged at 1,500 *g* for 20 min to obtain platelet-poor plasma. Platelet numbers of the platelet-rich plasma were then adjusted to 30×10^4/mm^3 by diluting with the platelet-poor plasma for assays with ADP or collagen. For an assay with thrombin, platelets were washed and then suspended at the same final concentration with Tyrode's buffer (33). All agonists were prepared in serial twofold physiologic saline dilutions: 2^{-5} through 2^{-16} mg/ml of ADP or collagen, and 2^0 through 2^{-11} U/ml of thrombin. In each assay, 20 μl each of platelet-rich plasma and agonist solution was mixed, agitated for 1 min, and then observed microscopically. The absolute value of the exponent of the minimum agonist concentration that was required to induce aggregation masses of more than five platelets was finally determined. All the procedures were carried out at room temperature.

Statistical analysis. The results are expressed as means±SD. The means of groups were compared using unpaired Student's t test. $P < 0.05$ is considered statistically significant.

Results

Adex1HST-1 increased the serum HST-1 protein in normal mice. We first examined the serum concentration of HST-1 protein by ELISA after the Adex1*HST-1* injection into normal mice. On day 12, the serum HST-1 protein in the Adex1*HST-1*- injected mice (n = 5) was 640±138 ng/ml. In one Adex1*HST-1*- injected mouse, representative kinetic data of the HST-1 protein and peripheral platelet count were shown in Fig. 1. The HST-1 protein concentration reached the highest level of ~ 740 ng/ml on day 6 after the virus injection (Fig. 1 *a*) and the platelet count increased up to day 10 (Fig. 1 *b*). The high level of the serum HST-1 protein continued for 10 d and the platelet count remained over a twofold increase for 20 d. As the serum HST-1 protein decreased after day 30, the platelet count gradually returned to a normal level. After day 60 the protein was no longer detected, indicating the protein concentration went below the detection limit of 12 pg/ml. This result indicates that the transfer and expression of *HST-1* gene suc-

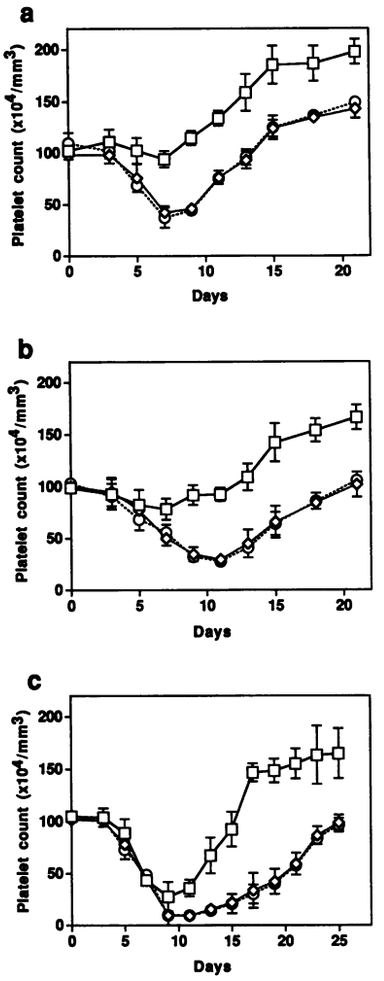

Figure 2. Kinetic analysis of peripheral platelet count in thrombocytopenic mice after the Adex1*HST-1* injection. (*a*) Cisplatin-treated mice, (*b*) 3.0 Gy irradiated mice, and (*c*) 5.0 Gy irradiated and carboplatin-treated mice. In each experiment, we injected Adex1*HST-1* into the first group of mice (n = 8, *boxes*) and Adex1*LacZ* into the second group (n = 8, *diamonds*) 3 d before the thrombocytopenic regimen. The third group (n = 8, *circles*) was not injected with adenoviruses. The mean values for platelet counts with SD are presented.

cessfully caused the production of the serum HST-1 protein, accompanied by the continuous increase in platelet count.

Adex1HST-1 effectively recovered platelet count in thrombocytopenic mice. To explore the therapeutic effects of Adex1*HST-1* on thrombocytopenia, we injected Adex1*HST-1* into mice and induced thrombocytopenia 3 d later by cisplatin administration, or by 3.0 Gy irradiation, or by the combination of 5.0 Gy irradiation and carboplatin administration. In the cisplatin experiment, the platelet count of the Adex1*HST-1*–injected group showed only a 6% decrease ($94.1 \times 10^4/mm^3$) on day 7 and severe thrombocytopenia was not observed during the experiment, while the platelet counts of the other two groups reached a nadir of a 60% decrease ($40.4 \times 10^4/mm^3$) on day 7, with their platelet counts returning to the pretreatment levels by day 15 (Fig. 2 *a*). The Adex1*HST-1*–injected mice showed significantly higher platelet counts than the other two groups on days 5–21. In the 3.0 Gy irradiation experiment, the platelet count of the Adex1*HST-1*–injected group showed only a 20% decrease ($79.5 \times 10^4/mm^3$) on day 7 and the platelet count rapidly recovered to a normal level by day 9, while the platelet counts of the other two groups reached a nadir of a 72% decrease ($27.9 \times 10^4/mm^3$) on day 11, and their platelet counts remained reduced for an additional 7 d, returning to the pretreatment levels by day 21 (Fig. 2 *b*). The Adex1*HST-1*–injected mice showed significantly higher platelet counts than the other two groups on days 7–21.

To further investigate the therapeutic effect of Adex1*HST-1* on more severe thrombocytopenia, we used the combined regimen of sublethal 5.0 Gy irradiation and carboplatin administration, which induced a > 90% decrease in platelet count and a more prolonged period of thrombocytopenia in mice. The platelet count of the Adex1*HST-1*–injected group showed a 72% decrease ($28.0 \times 10^4/mm^3$) on day 9 and rapidly recovered to a normal level by day 15, while the platelet counts of the other two groups reached a nadir of a 91% decrease ($9.3 \times 10^4/mm^3$) on day 11 and remained reduced for an additional 12 d, returning to the pretreatment levels by day 25 (Fig. 2 *c*). Thus the Adex1*HST-1* injection significantly diminished the platelet nadir and effectively decreased the duration of thrombocytopenia, with peripheral platelet counts remaining significantly higher than those of the other two groups on days 9–25. In these three experiments, the gene transfer of *HST-1* effectively prevented experimentally induced thrombocytopenia in mice.

In these thrombocytopenic regimens, cisplatin caused a moderate thrombocytopenia but a marked and prolonged rebound thrombocytosis in mice. This is in contrast to the effect of the other two regimens, which caused a more severe thrombocytopenia and no rebound thrombocytosis. The rebound thrombocytosis after the cisplatin regimen is probably due to the proliferation of immature megakaryocyte precursors that survive cisplatin treatment by virtue of their low proliferative state, and these precursors are probably released from negative feedback mechanisms normally mediated by more mature populations within the megakaryocytic lineage, in the same way as the 5-FU regimen (34).

Adex1HST-1 increased mature marrow megakaryocytes in thrombocytopenic mice without change of other peripheral hematology. To detect the effects of Adex1*HST-1* on the other peripheral hematological parameters and the cells in bone marrow, peripheral blood samples and histological sections of bone marrow were investigated. In the other peripheral hematological parameters such as red blood cell count, hemoglobin concentration, white blood cell count, and white blood cell differentials, no discernible differences were detected among the three groups of mice 7 d after these thrombocytopenic regimens (data not shown). Histological sections of bone marrow on day 7 (Fig. 3) indicated that the Adex1*HST-1*–injected groups showed a significant increase in the count and the size of megakaryocyte in comparison with the other two groups (Table I). Consequently, these results indicate that the transfer of the *HST-1* gene into thrombocytopenic mice increased mature megakaryocytes in bone marrow without any changes of the other peripheral hematological parameters and thus specifically increased the peripheral platelet count.

Adex1HST-1 produced as much serum HST-1 protein in thrombocytopenic mice as in normal mice. We also assessed the serum HST-1 protein concentration of thrombocytopenic mice 20 d after the virus injection. Each Adex1*HST-1*–injected mouse treated with cisplatin or with 3.0 Gy irradiation showed a protein level of 490 and 370 ng/ml, respectively, which corresponds to approximately the same concentration as that in the normal mice 20 d after the Adex1*HST-1* injection.

Adex1HST-1 increased platelets with normal aggregation in thrombocytopenic mice. To investigate the physiological function of the increased platelets above the normal level after thrombocytopenia in the Adex1*HST-1*–injected mice and to detect any thromboembolic complications caused by the Adex1*HST-1* injection, we analyzed platelet sensitivity to ag-

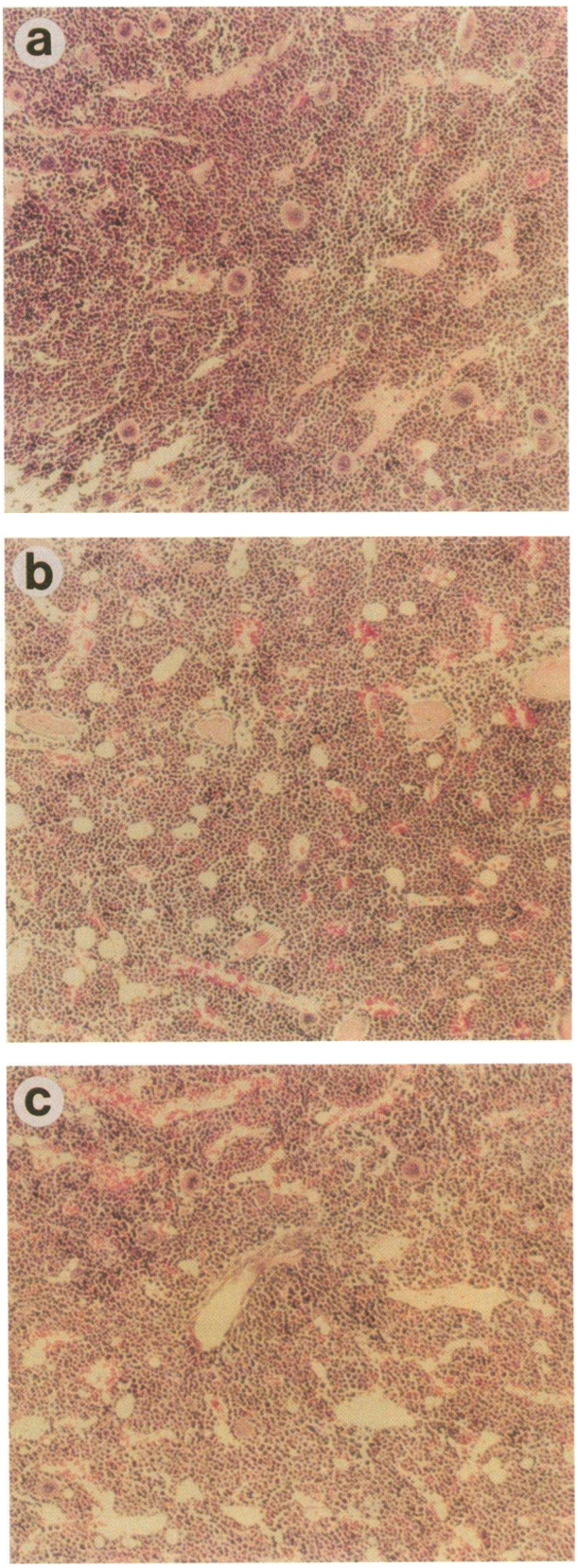

Figure 3. Microscopic findings in bone marrow of 3.0 Gy irradiated mice on day 7. Representative femoral histological sections (× 100) are demonstrated; (*a*) Adex1*HST-1*–injected mouse, (*b*) Adex1*LacZ* injected mouse, and (*c*) mouse without administration of adenovirus. It should be noted that the Adex1*HST-1*–injected mouse showed an increased number of large megakaryocytes.

Table I. Increased Megakaryocyte Count and Size in Bone Marrow of Adex1HST-1–injected Mice on Day 7

Treatment	Adenovirus	Megakaryocyte in bone marrow	
		Cell count (per mm^2)	Cell size
			μm
Cisplatin	Adex1*HST-1*	48.5±5.2	26.1±6.6
	Adex1*LacZ*	17.9±2.9	18.9±5.9
	None	18.3±2.9	18.1±4.7
3.0 Gy irradiation	Adex1*HST-1*	50.0±10.1	29.0±4.1
	Adex1*LacZ*	18.5±5.4	17.4±5.8
	None	17.5±3.1	18.7±5.9

Megakaryocyte count and size in bone marrow in mice 7 d after cisplatin injection or 3.0 Gy irradiation are shown. The megakaryocyte counts represent the mean±SD of 20 microscopic areas. Megakaryocyte diameters represent the mean±SD of 100 cells.

gregation in the Adex1*HST-1*–injected mice treated with the combination regimen on day 30 ($n = 6$) in comparison with untreated normal mice with the same age ($n = 6$). Each absolute value of the exponent of the minimum agonist concentration that was required to induce aggregation masses was the same between the Adex1*HST-1*–injected mice and the normal mice; the final concentration of each agonist to induce aggregation was 2^{-11} mg/ml of ADP, 2^{-11} mg/ml of collagen, and 2^{-4} U/ml of thrombin in both groups of mice. Thus, the increased platelets after thrombocytopenia in the Adex1*HST-1*–injected mice functioned as well as those in the normal untreated mice at least in aggregation, and the platelet sensitivity to agonist-induced aggregation was not as high as that causing thromboembolic complications.

The eight Adex1*HST-1*–injected mice treated with a chemotherapeutic agent or whole body irradiation were killed 6 mo after the virus injection, and no tumor formation was detected macroscopically. No other side effects such as inflammation, severe weight loss, or thromboembolic complications were detected in the entire course of the experiments.

Discussion

We showed previously that HST-1 protein itself has an ability to increase the platelet count significantly in normal mice, on the ground that several subcutaneous injections of recombinant HST-1 protein into mice resulted in an increased platelet count with a sharp peak on day 10 (26). Therefore, in the Adex1-*HST-1*–injected thrombocytopenic mice, the continuously increased circulating serum HST-1 protein, secreted from the Adex1-*HST-1*–infected cells, contributed to the increased count of large megakaryocytes, which appeared to be mature enough to release platelets, and resulted in a specific recovery of platelet count, although the sites of production of the HST-1 protein after the Adex1*HST-1* injection are yet to be elucidated. More-

over, the extent that HST-1 protein acted on megakaryocytopoiesis and thrombocytopoiesis in these thrombocytopenic experiments may have been enhanced by a synergistic action of other cytokines including thrombopoietin as a feedback mechanism during the recovery from thrombocytopenia.

Other various cytokines and growth factors as affecting megakaryocytopoiesis and thrombocytopoiesis in vitro and in vivo have been reported such as IL-1 (35), IL-3 (36–38), IL-6 (39–41), IL-11 (42, 43), c-*kit* ligand (stem cell factor) (44), erythropoietin (45), GM-CSF, and acidic and basic FGFs (6). However, injections of these cytokines or combinations of them for several consecutive days caused only a 30–70% increase in platelet count in normal animals and did not show apparently effective therapeutic actions on thrombocytopenia (29, 34, 46). In contrast to this, our results showed that a single injection of Adex1*HST-1* into normal mice caused over a twofold increase in platelet count for a period of 30 d and prevented severe thrombocytopenia more effectively.

In addition, these cytokines were injected into animals many times over several consecutive days, probably due to the short half-life of the proteins. On the other hand, one advantage of the recombinant adenovirus vector is a continuous gene expression for at least 30 d with only a single injection, and thus Adex1*HST-1* worked effectively to prevent thrombocytopenia induced by chemotherapy and/or irradiation for cancer treatment. Our results also have made adenovirus a general model of gene delivery for a short-term therapy of acute disorders.

However, a few modifications of the method are required before practical applications. Firstly, some modifications of adenovirus vector construction will be needed. Although no apparent inflammation after a high-dose injection of adenovirus (47) was observed through our experiments, an inactivation of the E2a gene on the virus, which attains a longer gene expression and less inflammation (48), or an insertion of another promoter to give a stronger gene expression, which decreases the amount of the virus used to obtain the same effect, is required. Since it is also necessary to control the therapy interval so as to avoid the potential risk of an increased likelihood of thromboembolic complications when the platelet count rises substantially after thrombocytopenia, an insertion of such a promoter will be important in enabling us to induce and reduce the gene expression as we like. Secondly, it is known that repeated treatment of adenovirus on animals may cause the production of antibodies to the virus and may prevent a repeated therapeutic use for humans. Further studies are needed to evaluate the host immune response to virus. Finally, although no tumor formations were observed in the Adex1*HST-1*–injected mice for at least 12 mo, further careful observation is required for any possible carcinogenic activity of the *HST-1* gene in vivo, because the *HST-1* gene induced meningeal tumors and soft tissue fibrosarcomas in nude mice after the injection of recombinant retroviruses carrying *env* and *HST-1/K-FGF* genes (49) and the HST-1 protein has in vitro and in vivo angiogenic activity as we showed previously (50, 51).

Here arises a question: is the action of the HST-1 protein on megakaryocytopoiesis mediated by direct or indirect effect on megakaryocytes? Since we and others reported that some megakaryocytic cell lines and normal megakaryocytes contain mRNAs of FGF receptors type 1 and type 2 (4, 6), the HST-1 protein may bind those FGF receptors on megakaryocyte progenitors, directly inducing the proliferation and/or maturation of megakaryocytes. However, there is still a possibility that the HST-1 protein may indirectly lead to increased megakaryocytopoiesis by acting on fibroblasts and endothelial cells in marrow stromal cells, because the HST-1 protein was initially characterized as a growth factor for mesenchymal cells and is mitogenic for both fibroblasts and endothelial cells (50). Further studies are required to understand the mechanism responsible for megakaryocytopoietic and thrombocytopoietic action of the HST-1 protein.

Recently, during preparation of this paper, c-*Mpl* ligand, which is thought of as thrombopoietin itself, was cloned and its administration to normal mice caused a fourfold increase in platelet count (52). Although its administration to thrombocytopenia has not been published yet, it may be interesting to test its synergistic action with the HST-1 protein on thrombocytopenia.

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