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Sodium orthovanadate (vanadate), a potent mitigator of radiation-induced damage to the hematopoietic system in mice

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Previous *in vitro* and *in vivo* studies have shown that sodium orthovanadate (vanadate), an inorganic vanadium compound, could effectively suppress radiation-induced p53-mediated apoptosis via both transcription-dependent and transcription-independent pathways. As a potent radiation protector administered at a dose of 20 mg/kg body weight (20 mg/kg) prior to total body irradiation (TBI) by intra-peritoneal (ip) injection, it completely protected mice from hematopoietic syndrome and partially from gastrointestinal syndrome. In the present study, radiation mitigation effects from vanadate were investigated by ip injection of vanadate after TBI in mice. Results showed that a single administration of vanadate at a dose of 20 mg/kg markedly improved the 30-day survival rate and the peripheral blood hemogram, relieved bone marrow aplasia and decreased occurrence of the bone marrow micronucleated erythrocytes in the surviving animals. The dose reduction factor was 1.2 when a single dose of 20 mg/kg was administered 15 min after TBI in mice using the 30-day survival test as the endpoint. Results also showed that either doubling the vanadate dose (40 mg/kg) in a single administration or continuing the vanadate treatment (after a single administration at 20 mg/kg) from the following day at a dose of 5 mg/kg per day for 4 consecutive days further significantly improved the efficacy for rescuing bone marrow failure in the 30-day survival test. Taken together, these findings indicate that vanadate would be a potent mitigator suppressing the acute lethality (hematopoietic syndrome) and minimizing the detrimental effects (anhematopoiesis and delayed genotoxic effects) induced by TBI in mice.

Keywords: Vanadate; mitigator; radiation-induced hematopoietic syndrome; bone marrow micronucleated erythrocytes; mice

INTRODUCTION

Total body irradiation (TBI) at high doses induces organ failure and even death. The hematopoietic system is one of the most radio-sensitive tissues in the body. The tragedy of the atomic bombs detonated in 1945 in Japan caused almost 200,000 fatalities, approximately 10% of the fatalities were caused by TBI-induced bone marrow aplasia. Nowadays, there are ten of thousands of people all over the world who are dealing with radiation daily at hospitals, laboratories, military units and nuclear power plants. Uncontrolled exposure to radiation from nuclear accidents or potential terrorism incidents will present challenges unlike those encountered in radiotherapy or space radiobiology [1–4].

For radiation protection, various mechanisms such as free radical scavenging, calcium channel blocking, inhibition of lipid peroxidation, enhancement of DNA repair and stimulation of stem cell proliferation are considered important [5]. Thiols dominated the fields of radiation protection

from 1950s through to the 1980s. Later, studies on radio-protectors appeared to be at a turning point: only a few new candidates have been proposed and non-thiol protectors, including protease inhibitors, vitamins, metalloelements and calcium antagonists are actually playing a large role in radio-protection. In the 1990s, interest increased in endogenous protective systems as opposed to chemical radio-protectors [6]. Since the late 20th century efforts have been made to identity and develop novel agents for radiation mitigation and therapeutic treatment regimens. With the successful development of radiation protection agents, there is also a shift in emphasis from radio-protectors that need to be administered before radiation exposure to radiation mitigators that could be administered after radiation exposure. Though radiological protection has evolved internationally in the past 50 years, at present there remain a limited number of post-exposure therapeutic agents, and currently there is no satisfactory mitigation agent for rescuing patients from acute lethality [7]. It is unlikely that the documented prophylactic radio-protectors will have a great effect as most of these agents must be administered in advance of exposure to intercept or immediately repair damage or enhance repair mechanisms. Antioxidant radio-protectors, such as Amifostine®, the only drug qualified for clinical application, are prophylactic rather than mitigative, as the benefit of antioxidants is minimal unless present in the blood stream at the time of exposure to irradiation [8]. From a practical point of view it is important to develop radiation mitigators and therapeutic agents. There is a need for research to identify additional biological targets and effective treatments [3]. Mitigation and treatment of radiation injuries require accurate and rapid application of appropriate therapy. In contrast to radio-protectors, radiation mitigation agents would be administered after exposure to target the post-irradiation events, thus apoptosis inhibitors become an important choice especially as radiation mitigators that are intended to minimize the apoptosis-inducing sensitivity of target organs. In addition to the development and application of new and potent antioxidants, growth factors, cytokines and immunomodulators, also based on non-free radical scavenging mechanisms, thrombopoietin receptor agonist, somatostatin analog, metal or metalloid compounds inhibitors of cyclin-dependent kinase 4 (CDK4) and CDK6 and p38 inhibitor were tested as mitigators in animal models [9–23]. Next came strategies to develop mitochondrial targeted radiation damage mitigators, such as p53/mdm2/mdm4 protein complex inhibitors and p53-upregulated modulators of apoptosis (PUMA) inhibitors [24]. TBI at high doses induces massive apoptosis in the target organs and agents capable of minimizing the apoptosis-inducing sensitivity by inhibition of either pro-apoptotic components or activation of anti-apoptotic ones would be candidates for potent radio-protectors. Application of apoptosis inhibitors is a new approach to the development of agents intended for the prophylaxis, mitigation and treatment of radiation injuries. p53 is a well-known key molecule in the machinery responsible for the radiation sensitivity of target organs, and thus it is considered a good target for migitative and therapeutic treatments. Among five chemical p53 inhibitors reported [25–29], vanadate has a more potent anti-apoptotic activity than the others (pifithrinα, pifithrinμ, sodium salicylate, cadmium chloride). In fact, agents that inhibit the pro-apoptotic functions of p53 [25–27], mimic the anti-apoptotic Bcl-2 family proteins [30], or enhance anti-apoptotic pathways by activation of the Toll-like receptor 5 signal [31] were verified recently. As radiation-induced cell death is mainly a p53-dependent event, p53 is a critical target for radio-protection to escape the apoptotic fate. A short-term inhibition of p53 would be the key to safe radio-protection [26] as it could effectively protect against radiation damage without alteration in radiation late effects such as increased carcinogenesis. p53 exerts its tumor-suppressive function primarily through the oncogenic-stress pathway rather than the DNA-damage pathway, so temporary systemic inactivation of p53 would be a relatively safe approach to protect from cell death resulting from radiation-induced DNA damage [32–34].

Vanadate has insulin-like effects on the glycolytic pathway via increasing basal fructose-2,6-bisphosphate levels, counteracting the glucagon effect and stimulating glycolytic flux [35]. Vanadate activates adenylate cyclase [36], acts as growth factor-mimetic compounds and regulates differentiation of osteoblast-like cells [37]. Vanadium has antitumorigenic potential, a stimulative action on hematopoiesis [38] and increases the percentages of reticulocytes and polychromatophilic erythrocytes in the peripheral blood [39]. For acute toxicity of vanadate, the LD_{50} values in rats were 36.3 mg/kg and 330 mg/kg for oral and intraperitoneal (ip) administration, respectively. In our previous studies the LD_{50} was about 60 mg/kg in imprinting control region mice for ip administration. According to the Occupational Safety and Health Administration, and the National Institute for Occupational Safety and Health in the United States, vanadate is classified as having no carcinogenic effects. In a series of previous investigations, the effects of vanadate as a radio-protector against apoptosis *in vitro* in cultured cells and against bone marrow death *in vivo* in mice have been studied [40, 41]. As a bifunctional inhibitor of p53, vanadate functions by underlying the mechanisms that suppress p53-dependent cell death through inhibition of the transcription-dependent and transcription-independent pathways. Consequently, vanadate is superior to other reported single-pathway inhibitors of p53 [26, 27]. As onset of apoptosis is a late event compared with radiation-induced DNA damage, this suggested a possibility for vanadate as a candidate to be a radiation mitigator applied post-irradiation. In the present study, the efficacy of vanadate in mitigating radiation-induced damage in terms of acute lethality and residual damage in the

hematopoietic system was further investigated. By verifying the efficacy for rescue from acute death and residual damage in the hematopoietic system in mouse survivors from the vanadate-treated group (receiving both the sublethal dose of TBI and post-irradiation vanadate administration) and the non-vanadate-treated group (receiving only the sublethal TBI), the present investigation aimed to study whether post-TBI administration of vanadate, as a radiation mitigator, could rescue the acute killing effect, namely, bone marrow death, and relieve late detrimental consequences of radiation such as residual anhematopoiesis and delayed genotoxic effects in mice.

MATERIALS AND METHODS

Animals

Imprinting control region (ICR) strain female mice aged 7 weeks old were purchased from SLC, Inc. (Japan). The mice were maintained in a conventional animal facility under a 12-h light/12-h dark photoperiod (lights on from 8:00 a.m. to 8:00 p.m.). Animals were housed in autoclaved cages with sterilized wood chips and allowed free access to standard laboratory chow (MB-1; Funabashi Farm Co., Japan) and acidified water ($pH = 3.0 + 0.2$) *ad libitum*. Animals were acclimatized to the laboratory conditions for 1 week as an adaptation period before use. To avoid possible effects from the developmental condition of the animals, 8-week-old mice with a significantly different body weight (more or less than the mean + 2 SD) were omitted from this study. On the basis of our previous studies, at least 30 mice in the present study were used in each experimental group, and all experiments were repeated at least once. All experimental protocols involving mice were reviewed and approved (Experimental Animal Research Plan Nos. 09-1042 and 09-1042-1) by The Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS). The experiments were performed in strict accordance with the NIRS *Guidelines for the Care and Use of Laboratory Animals*.

Irradiation

X-rays were generated with an X-ray machine (Pantak-320S; Shimadzu, Japan) operated at 200 kVp and 20 mA, using a 0.50-mm Al + 0.50-mm Cu filter. An exposure rate meter (AE-1321M, Applied Engineering Inc., Japan) was used for the dosimetry. The dose rate for delivering the TBI was about 0.70 Gy/min. The mice were held in acryl containers and were exposed to TBI at room temperature.

Sodium orthovanadate (vanadate)

Vanadate was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The physiological normal saline (Otsuka Pharmaceutical Co., Ltd, Naruto, Tokushima, Japan) was used as a solvent to make vanadate solution for ip injection.

Mouse model for induction of bone marrow failure

The same model for radiation-induced bone marrow failure established in our previous work [41] was applied to the present study. In brief, ICR strain female mice aged 8 weeks screened out by body weight were finally used for TBI at a dose of 7.5 Gy. The pathological morphology showed serious bone marrow aplasia and hemorrhage in femur. Most of the deaths occurred around 2 weeks after TBI and all animals died within 30 days under the experimental setup.

Timing for post-TBI vanadate administration

Mice were exposed to TBI at a dose of 7.5 Gy and then either left with no treatment (but immediately given a normal saline ip injection), or they received a vanadate ip injection at a dose of 20 mg/kg body weight (20 mg/kg) immediately or at various times from 15 min to 240 min post-TBI. Good timing for post-TBI vanadate administration was determined by comparing the efficacy of the mitigation effect from vanadate ip injection at different times after TBI using mouse 30-day survival rates as the endpoint.

Biological endpoints

The 30-day survival test

The number of deaths occurring within the 30-day period after TBI at a dose of 7.5 Gy was recorded. During the investigation when any mouse lost 20% of its body weight or appeared moribund, it was euthanized by overdose CO_2 inhalation and counted as a death. When the post-TBI vanadate administration induced a significant suppression of mortality caused by the TBI alone, the efficacy of vanadate was considered successfully approved.

Determination of dose reduction factor (DRF)

In the present study, DRF referred to the fold change in radiation dose to produce a given level of lethality, and it was calculated from the ratio of the 50% lethal dose (LD_{50}) value of vanadate-treated group to the normal saline-treated group after TBI. The mice were randomly divided into four groups, namely, the control group receiving a physiological saline ip injection, the irradiation group receiving TBI and a physiological saline ip injection, the group receiving TBI and a vanadate ip injection at a dose of 20 mg/kg, and the group receiving TBI and a vanadate ip injection at a dose of 40 mg/kg. Except for the control group, each of the groups was further divided into five subgroups, receiving, respectively, a TBI dose of 5.5 Gy, 6.0 Gy, 6.5 Gy, 7.0 Gy and 7.5 Gy. All 20 mice were used either in the control group or in each subgroup of the other three groups receiving both TBI and vanadate administration.

Peripheral blood hemogram

Animals surviving the 30-day survival test were anesthetized by CO_2 inhalation on the 31st day after TBI. The peripheral blood was collected from a femoral artery and the animals were killed by cervical dislocation. A differential blood cell count was done using a blood cell differential automatic analyzer (SYSMEX K-4500; Sysmex Corporation, Japan). The data for each experimental group were from at least five mouse survivors.

Micronucleus test

A bone marrow micronucleus test was carried out according to Schmid [42] with minor modifications [43, 44]. Bone marrow smears prepared from both femurs were processed for the enumeration of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs). The slides were coded to avoid any observer bias. The micronuclei were scored using a light microscope at a magnification of 1000 ×. At least 5000 cells per mouse were counted, and the data for each experimental point were from at least five mice.

Bone marrow pathological examination

Pathological samples of mouse femurs obtained in the preliminary trials in our previous study [41] were selected and used in the present study. The selected samples were from mice that were given a TBI at a dose of 8.0 Gy and with or without post-irradiation vanadate treatment. These animals were anesthetized by CO_2 inhalation 7 days post-TBI, and then immediately killed by cervical dislocation. The femurs were collected, formalin-fixed, decalcified and paraffin-embedded. The sagittal sections of the femurs were stained with the routine hematoxylin and eosin (H&E) staining method for histopathologic evaluation. Light microscopic assessment of bone marrow cellularity was performed. The representative images were used as the supporting evidence for the pathological conditions of bone marrow cells after TBI with or without vanadate treatment.

Noxa expression in bone marrow cells

The *Noxa* gene is a direct transcriptional target of p53, noxa protein is a pro-apoptotic BH3-only protein that belongs to one of the distinct three subgroups of the Bcl-2 family of proteins, being critical for p53-mediated apoptosis. The *Noxa* gene showed a significant transcriptional increase in bone marrow cells after mouse TBI [41]. In the present study *Noxa* gene expression was used to evaluate the effect of vanadate on p53 transcription. Mouse bone marrow cells were isolated from surgically resected femurs 4 h after TBI. Total RNA was extracted from bone marrow cells using the Ultraspec RNA isolation system kit (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription of 2 μg of total RNA with oligo (dT) primer (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out by using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City CA, USA) on an Applied Biosystems 7400 real-time PCR system (Applied Biosystems). The specificity of the PCR products was confirmed by melting curve analysis with 7500 system v1.4 software. Relative expression levels were calculated based on the difference in C_T values between the test samples and control (unirradiated bone marrow cells). This was normalized with expression levels of β-actin by using the equation E_{Noxa} $(C_{Ttest}{}^{Noxa} - C_{Tcontrol}{}^{Noxa})/E_{\beta\text{-actin}}$ $(C_{Ttest}{}^{\beta\text{-actin}} - C_{Tcontrol}{}^{\beta\text{-actin}})$ as described [45]. Primers used in these analyses were as follows: Noxa, 5′-GGTGGCC AGCAGATACGTGA-3′ (forward primer) and 5′-GCTTCC AGTAACAGGCAAACCTAGA-3′ (reverse primer); β-actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ (forward primer) and 5′-ATGGAGCCACCGATCCACA-3′ (reverse primer).

Statistical analysis

For dose reduction factor determination, curvilinear regression of second degree was applied to the survival data using the programs embedded in KaleidaGraph Software (Version 4.1.2; Synergy Software, Hulinks Inc., Tokyo, Japan). Statistical evaluation of the data was carried out with the χ^2 test and Student's *t*-test, as appropriate. Statistical significance was assigned to $P < 0.05$.

RESULTS

Determination of timing for post-TBI vanadate administration

Mouse 30-day survival test was used for determination of the best timing for post-TBI vanadate administration. Mice were exposed to TBI at a dose of 7.5 Gy and then either left without treatment (but immediately given a normal saline ip injection), or they received a vanadate ip injection immediately or at various times from 15 min to 240 min post-TBI. Vanadate at a dose of 20 mg/kg, approximately one third of the dosage of its LD_{50} value in acute toxicological testing, was used. Results showed that vanadate administration at timings from just after exposure to 60 min later significantly suppressed animal death ($P < 0.01$), and the highest rescue effect (43.3% survival) was observed when it was administered immediately after TBI (Fig. 1). The rescue efficacy decreased then and totally disappeared when vanadate was given 240 min post-TBI. No animals survived in the group receiving only a normal saline injection after TBI. No deaths occurred in the animals that received only vanadate or only normal saline injection (data not shown). As the purpose of this work was to investigate the possible mitigation effect of vanadate, 15 min post-TBI,

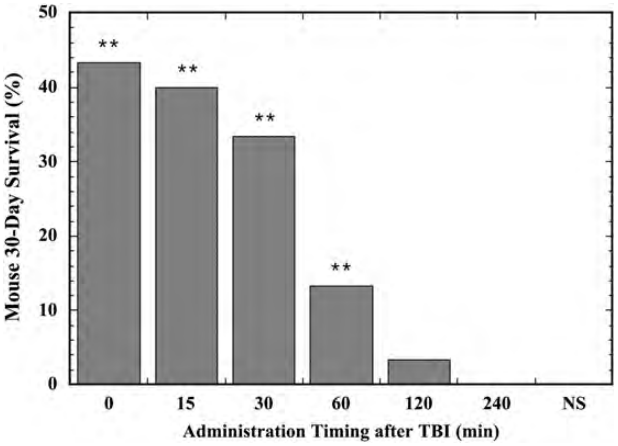

Fig. 1. Rescue effect of vanadate treatment at different administration timings on 30-day survival of mice. Mice were exposed to total body irradiation (TBI, 7.5 Gy) and then either received a vanadate ip injection (20 mg/kg body weight) immediately or at various times from 15 to 240 min post-TBI, or were left vanadate-untreated but immediately given a normal saline (NS) ip injection. ** indicate statistically significant differences ($P < 0.01$) compared with the vanadate-untreated group.

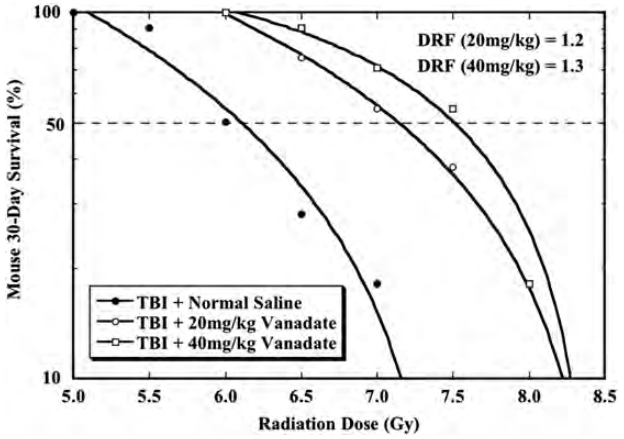

Fig. 2. Determination of dose reduction factor (DRF) for post-TBI vanadate treatment. Mice were exposed to TBI at a dose from 5.5 to 8.0 Gy and then given either vanadate (20 or 40 mg/kg body weight) or a normal saline ip injection at 15 min post-TBI. Curvilinear regression of second degree was applied to the analysis of the 30-day survival results. DRF was calculated from the ratio of $LD_{50/30}$ of the vanadate-treated group to that of the NS-treated group.

which resulted in a 40.0% survival, was selected and used as the timing for vanadate administration in the following studies.

Determination of dose reduction factor (DRF)

When curvilinear regression of second degree was applied to the data analysis, the survival curve for each group fitted a quadratic polynomial expression well (Fig. 2). The regression analysis yielded $LD_{50/30}$ as about 6.1 Gy, 7.2 Gy and 7.7 Gy, respectively, for the group receiving TBI and normal saline (TBI + Normal Saline), the group receiving TBI and vanadate at a dose of 20 mg/kg (TBI + 20 mg/kg Vanadate) and the group receiving TBI and vanadate at a dose of 40 mg/kg (TBI + 40 mg/kg Vanadate). The DRF, calculated from the ratio of $LD_{50/30}$ of vanadate-treated to NS-treated, was 1.2 and 1.3 for 20 mg/kg and 40 mg/kg doses, respectively.

Efficacy for multiple administration of vanadate

A single administration of vanadate at a dose of 20 mg/kg significantly improved survival, rescuing 40.0% of the exposed animals from otherwise a 100% lethality in the 30-day survival test study ($P < 0.01$) (Fig. 3). When the dose was doubled to 40 mg/kg, the survival markedly increased to 54.5% ($P < 0.05$) compared with the efficacy for 20 mg/kg administration. When further continuing the vanadate treatment from the second day at a dose of 5 mg/kg daily for 4 consecutive days after a single administration (20 mg/kg) on the first day, the efficacy was further improved to 56.7% ($P < 0.025$). No lethality was observed in the

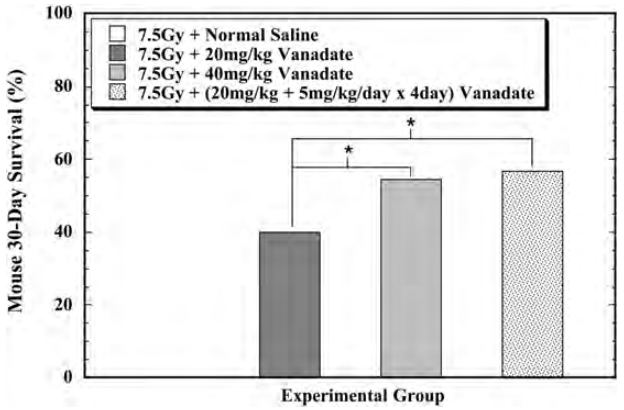

Fig. 3. Efficacy of multiple administrations of vanadate in terms of rescue effect on 30-day survival rate of mice. Mice were exposed to TBI (7.5 Gy) and then received either vanadate (20 or 40 mg/kg body weight) or a normal saline ip injection at 15 min post-TBI, or received multiple vanadate treatments at a dose of 5 mg/kg per day for 4 consecutive days after a single administration (20 mg/kg) on the first day. The open bar standing for the group receiving TBI and normal saline was not displayable in the figure as survival was 0% in this group. * indicates statistically significant differences ($P < 0.05$) between the two groups that were compared.

animals receiving the vanadate treatment alone (data not shown). These results indicated that in the 30-day survival test the efficacy of post-TBI vanadate administration for rescuing bone marrow failure could be significantly improved by either doubling the injection dose or continuing the vanadate treatment.

Attenuation of residual damage in the peripheral blood hemogram

Residual damage in the hematopoietic system was studied in the peripheral blood hemogram in the surviving animals 1 day after the 30-day survival test. Post-TBI administration of vanadate significantly reduced the decrease in peripheral leukocyte count and blood platelet count induced by the radiation exposure (Fig. 4). When doubling the dose to 40 mg/kg, the efficacy for improvement of leukocyte count was further markedly increased ($P < 0.01$) compared with the efficacy for 20 mg/kg administration. A tendency for improved ratio of PCEs to the sum of PCEs and NCEs was observed in the surviving animals receiving the vanadate treatment post-TBI compared with their counterparts that received only TBI, it was not statistically significant (data not shown). These data indicated that post-TBI administration of vanadate could relieve the detrimental effects from TBI in the hematopoietic system.

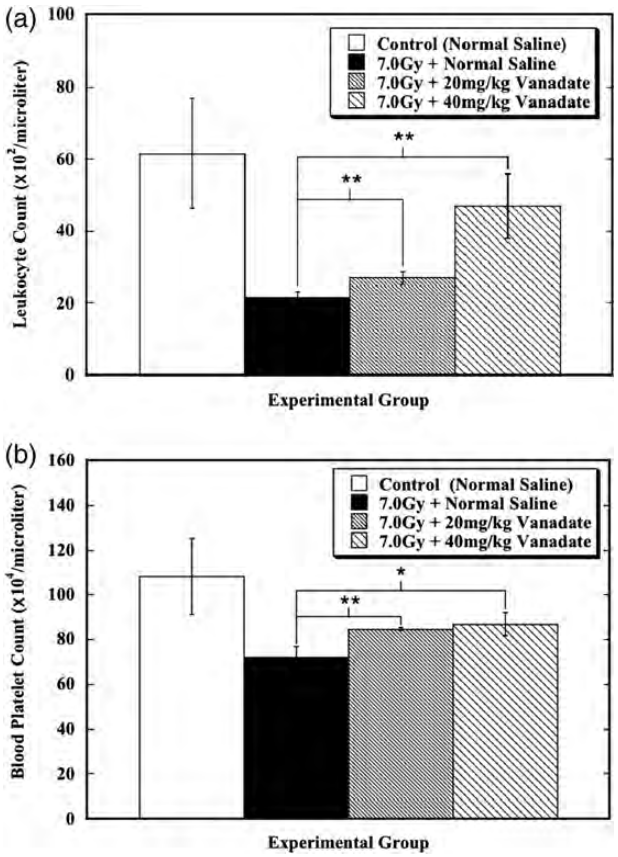

Fig. 4. Attenuation by vanadate treatment of residual damage in the peripheral blood hemogram in the surviving animals. Mice were exposed to TBI (7.0 Gy) and then treated with either vanadate (20 or 40 mg/kg body weight) or a normal saline ip injection at 15 min post-TBI. The Control group received only a normal saline ip injection without TBI. Peripheral blood was collected in the surviving animals 1 day after the 30-day survival test. Results of the leukocyte count and blood platelet count are shown in (a) and (b), respectively. * and ** indicate statistically significant differences at, respectively, $P < 0.05$ and $P < 0.01$ between the two groups that were compared.

Reduction of residual damage in bone marrow erythrocytes

The micronucleus test is a tool for genotoxic assessment. Residual damage, MNPCE and MNNCE were measured in the bone marrow cells of surviving animals 1 day after the 30-day survival test. To obtain more survivors and save animals as well, TBI was delivered at a dose of 7.0 Gy.

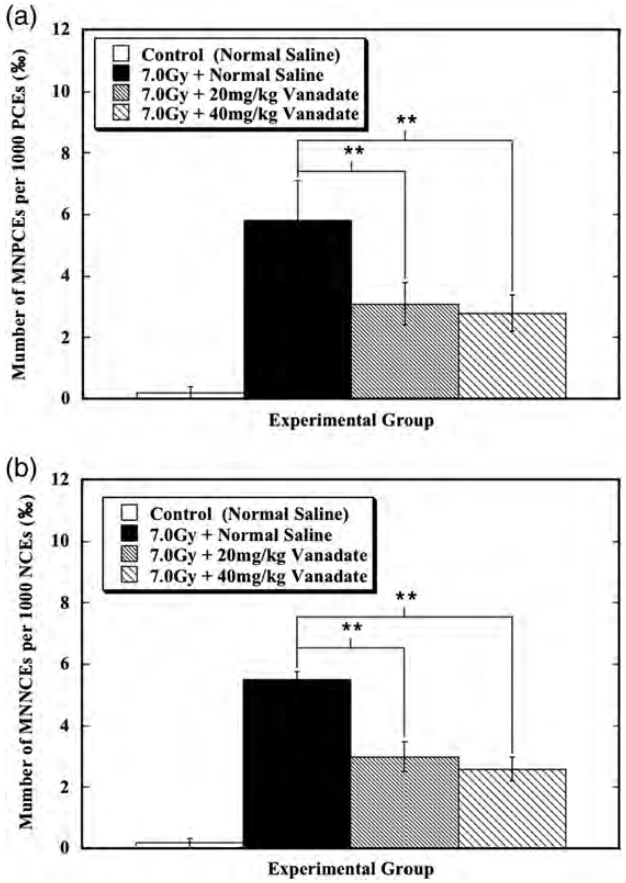

Fig. 5. Reduction of residual damage in bone marrow erythrocytes in the surviving animals. Mice were exposed to TBI (7.0 Gy) and then received either vanadate (20 or 40 mg/kg body weight) or a normal saline ip injection at 15 min post-TBI. The Control group received only a normal saline ip injection without TBI. Micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs) were measured in the bone marrow cells of surviving animals 1 day after the 30-day survival test. Fig. 5 illustrates the incidence as permille of MNPCEs to polychromatic erythrocytes (PCEs) (A) and MNNCEs to normochromatic erythrocytes (NCEs) (B), respectively. ** indicates statistically significant differences ($P < 0.01$) between the two groups that were compared.

Results obtained in surviving animals from the control group (receiving normal saline injection alone), the irradiation group (receiving TBI alone) and two irradiation plus vanadate treatment groups (receiving both TBI and vanadate injection at either 20 mg/kg or 40 mg/kg) are illustrated in Fig. 5. The micronucleus test study showed that post-TBI vanadate administration markedly reduced the occurrences of both MNPCEs per 1000 PCEs (Fig. 5a) and MNNCEs per 1000 NCEs (Fig. 5b) in the femur bone marrow when compared with that receiving TBI alone. A tendency was observed in the efficacy of doubling the dose for vanadate administration, but the improvement was not statistically significant. These results indicated that post-TBI administration could reduce the genotoxic effect of TBI in the hematopoietic system.

Relief of bone marrow cell aplasia

Because bone marrow failure was the main cause of animal death, pathological examinations of bone marrow cells were studied. The representative pathological images of femurs processed by H&E staining were shown in Fig. 6 as the supporting evidence for the pathological conditions of bone marrow after TBI with or without vanadate treatment. It was clearly shown that vanadate administration efficiently ameliorated the reduction of bone marrow cells (aplasia) induced by TBI, indicating strong correlations with the survival results. Doubling the vanadate dose further inhibited the reduction of bone marrow cells in the femurs in mice.

Inhibition of Noxa expression in bone marrow cells

As a p53-target gene, *Noxa* responded well to TBI in bone marrow cells [41]. It was chosen again in the present work as an indicator of transcriptional suppression of p53 by vanadate administration in the bone marrow cells. RT-PCR results showed that TBI at a dose of 5.0 Gy significantly increased expression of *Noxa* by 10.6-fold ($P < 0.01$) compared with its non-irradiated counterpart at 5 h after irradiation, while administration of vanadate at a dose of 20 mg/kg 15 min post-TBI markedly suppressed the increase from 10.6- to 2.4-fold ($P > 0.05$) (Fig. 7). These results confirmed the transcriptional inhibitory effect of post-TBI administration of vanadate on the transactivation of p53 in bone marrow cells in mice.

DISCUSSION

There are a number of situations in which humans are accidentally exposed to radiation. In many of these cases, the persons at risk would be accessible to topical application of a radiation mitigator. Vanadate is the first p53 inhibitor

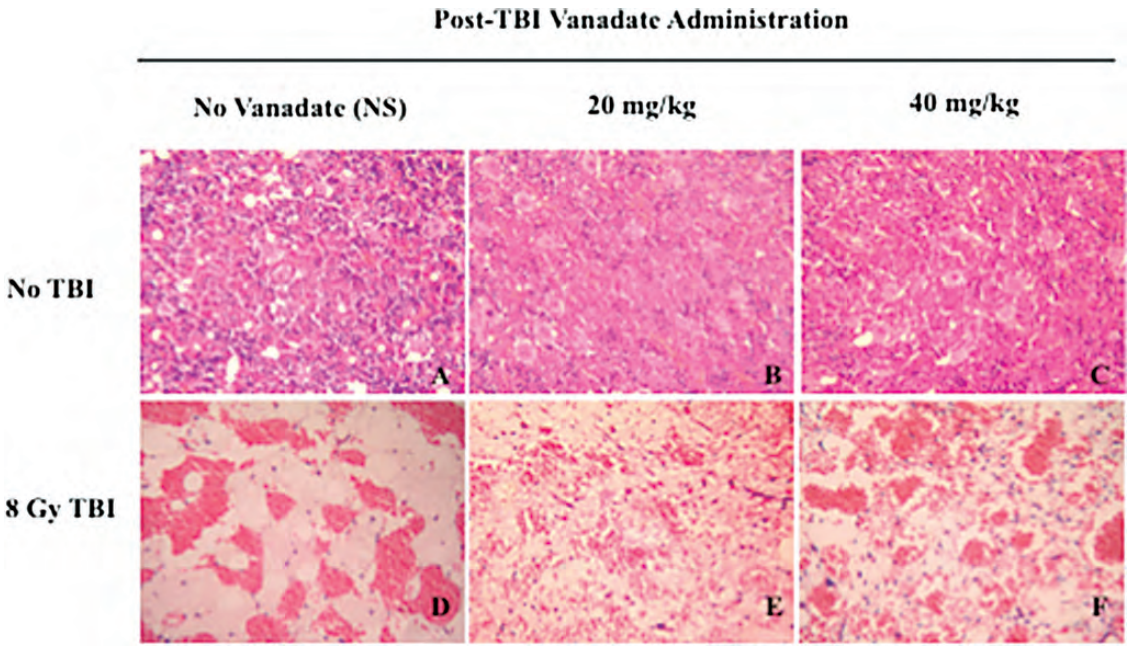

Fig. 6. Relief by vanadate treatment of bone marrow cell aplasia. Mice were exposed to TBI (8.0 Gy) and then given either vanadate (20 or 40 mg/kg body weight) or normal saline ip injection at 15 min post-TBI. The animals were sacrificed 7 days after irradiation and femurs were collected for pathological examination of bone marrow with H&E staining. Histological characteristics in images A, B and C (×300 magnification) show normal appearance of mouse bone marrow. TBI eradicates most of the nucleated cells, inducing hipocellular change with a clear alteration in the relationship to adipose tissue and hemorrhage (D). Post-TBI vanadate treatment significantly relieves the decrease in cellularity of nucleated cells and attenuates hemorrhage in femur bone marrow in mice (E and F).

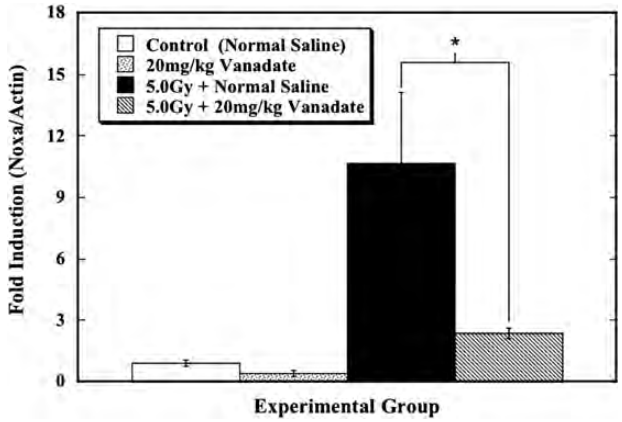

Fig. 7. Inhibition of *Noxa* expression in bone marrow cells. Mice were exposed to TBI (5.0 Gy) and then received either vanadate (20 mg/kg body weight) or normal saline ip injection at 15 min post-TBI. The unirradiated animals were given either normal saline or vanadate (20 mg/kg body weight) ip injection. The animals were sacrificed 5 h after irradiation and bone marrow cells were collected. * indicates statistically significant differences ($P < 0.05$) between the two groups that were compared.

that could inhibit both the transcription-dependent and transcription-independent p53 apoptotic pathways, and our previous study demonstrated that vanadate was a potent radio-protector against bone marrow failure in mice [41]. As a potent p53 inhibitor, it is capable of inducing p53 protein denaturation [40, 41] and effective against pro-apoptotic events post-irradiation. This theoretically makes vanadate possible as not only a candidate for a prophylactic agent, but also a mitigator or even a therapeutic agent in combination with other therapeutic agents. In the present study, we used rescuing acute lethality (mouse killing due to bone marrow death) and relieving later detrimental consequences (residual anhematopoiesis and delayed genotoxic effects) as parameters relevant to mitigation of radiation-induced damage to the hematopoiesis system. Effects from post-TBI administration of vanadate as a mitigator were investigated in the mouse model of TBI. Results showed that a single post-TBI administration of vanadate (20 mg/kg) efficiently suppressed the acute killing effect. As an effective radio-protector, in the previous work vanadate was administered 30 min before TBI and its DRF was 1.5 to 1.6 [41]. Tested as a possible radiation mitigator, in the present work vanadate was administered 15 min post-TBI and its DRF was 1.2 to 1.3. These values indicated that vanadate was both a potent radio-protector and a radiation mitigator. In the animals surviving a sublethal dose of TBI, decrease of leukocyte count and blood platelet counts were significantly relieved. As the recovery of blood platelet counts is one of the most important factors for restoration from bone marrow death [46], the blood hemogram data were consistent with the survival data. Results also showed that in the surviving animals rescued by vanadate administration, the incidences of MNPCEs and MNNCEs were markedly lower than those in the survivors that received the TBI alone. These findings indicated that post-TBI administration significantly relieved myelosuppression and reduced residual damage in bone marrow cells induced by the TBI. In addition, the advantage of consecutive administration was also investigated and confirmed based on the results on rescue of acute lethality from hematopoietic syndrome. Results also confirmed the transcriptional inhibitory effect of post-TBI administration of vanadate on the expression of p53 target gene *Noxa* in bone marrow cells in mice. Thus our findings indicate that vanadate could afford an opportunity for radio-protection as well as for therapeutic intervention following accidental radiation exposure.

In vivo studies in experimental animals have included protection against radiation-induced lethality due to hematopoietic or gastrointestinal injury, or other specific tissue damage, apoptosis, mutagenesis and carcinogenesis [47]. The most informative and useful preclinical studies relate protective effects to the toxicity of the candidate agent in the same animal model, as toxicity often parallels protective effects in general, and protective efficacy of the candidate agent with low or non-toxicity is needed. As the dose-limiting toxicity of vanadate was carefully considered, a dose of vanadate at 20 mg/kg, which was substantially lower than the efficient dose against apoptosis in cultured cells [40, 41], was used in most of trials in our *in vivo* investigations to avoid possible toxicity from vanadate itself. As to the mitigation efficacy, the pharmaceutical kinetics of absorption and distribution of the candidate reagent should be also taken into account. In this context, the high potency of vanadate could confer considerable advantages over those existing radio-protectors with macromolecules because it could be absorbed into the blood circulation quickly (i.e. via ip injection) and distributed uniformly in the body, and a low dosage is required for it to achieve the desired level of radiation mitigation. In considering the use of radio-protectors, mitigators or therapeutic agents, it is necessary to distinguish the potential applications. The timing of administration relative to radiation exposure is critical, and efficacy of the compounds is strongly related to pharmacokinetic considerations as well as to radiochemical considerations. Like all the characteristics an ideal radiation protector [6] should possess, as a precondition a good radiation mitigator should offer a good mitigation effect against both acute and late radiation damage and it should be suitable for easy administration, it should be rapidly absorbed and distributed throughout the body,with no significant toxicological effects. In addition, it should be readily available and not too expensive and be chemically stable to permit easy handling and storage. In fact, vanadate, as both a radiation protector and a radiation mitigator

against radiation-induced bone marrow syndrome, matches well most of the characteristics.

On the other hand, results obtained in our series of studies on vanadate also provide theoretically and practically an important hint for the development of novel kinds of p53 inhibitors and radiation mitigators. The zinc-binding site in p53 protein molecule is essential for DNA transcription and thus metal exchange (i.e. by Cd^{2+}) and Zn^{2+} chelator (i.e. N,N,N′,N′-Tetrakis(2-pyridinylmethyl)-1,2-ethanediamine) could cause its structural change resulting in inactivation of p53 protein [29]. In fact, treatment with metalloelement chelators was reported to facilitate tissue repair processes required for recovery from radiation injury including survival of lethally irradiated mice and rats [48]. Thus, mild zinc chelators that target the zinc binding site of p53 protein, being highly membrane-permeable hybrid compounds with low toxicity, would be important candidates for development of novel kinds of p53 inhibitors and potent radio-protectors and radiation mitigators as well. To develop novel mitigators and therapeutic agents against radiation, it is time to systemically establish a framework of an evolving radiation protection, which is based conceptually on anti-apoptotic but non-free radical scavenging mechanisms that modify radiation responses.

Taken together, the findings in the present study and our previous work indicate that vanadate is not only a potent radio-protector but also a promising mitigator to radiation damage in the hematopoietic system of mice. The high efficacy for post-irradiation application, the low toxicity and high chemical stability and user-friendly usability of vanadate support further potential evaluation of vanadate as a possible candidate to be a clinical mitigator of relevant radiation damage.

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