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Review

Radioprotective/Mitigative Effects of Thrombopoietin Receptor Agonists

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Since the discovery of X-rays in 1895, radiation has been widely used in medicine and industry, but its biological effects on health have also been a problem. A group of researchers in the United States discovered in 1948 that large doses of cysteine administered prior to radiation exposure could protect mice exposed to whole-body X-rays from radiation damage. Around the same time, a group in Belgium also reported a similar effect on cysteamine, a breakdown product of cysteine. Currently, the International Atomic Energy Agency recommends either granulocyte colony-stimulating factor (G-CSF) or granulocyte macrophage colony-stimulating factor (GM-CSF) for acute radiation syndrome (ARS) due to moderate to severe exposure of 2 - 6 Gy and interleukin-3 in combination with G-CSF, GM-CSF, erythropoietin and thrombopoietin (TPO) for more severe or lethal doses (>6 Gy). In addition, the U.S. Food and Drug Administration approved G-CSF, pegylated-CSF, and GM-CSF for hematopoietic ARS. There have been many reports on the radioprotective/mitigative agents, and several excellent reviews have been published. This review focuses on TPO and its receptor agonists, which are expected to be utilized in the future, and outlines the process from its discovery to its approval as a pharmaceutical drug, action, and future prospects.

Key words: c-Mpl, thrombopoietin, thrombopoietin receptor agonist, acute radiation syndrome, radiomitigator, total body irradiation

1. Introduction

Difficulties in treating radiation casualties are attributed to rapid and simultaneous organ failure, so-called acute radiation syndrome (ARS). Hematopoietic disorders are a well-known ARS and are life-threatening disorders due to high-dose radiation exposure. Radiation-induced hematopoietic damage appears in the hematopoietic organs, such as the bone marrow, spleen, and thymus, and induces secondary anemia, thrombocytopenia, and lymphopenia. The International Atomic Energy Agency (IAEA) has considered prevention of bone-marrow depression as a priority target of ARS therapy [1]. When radiation exposure exceeds the lethal dose, hematopoietic detriments are shown in not only mature cells but also in immature hematopoietic stem cells (HSCs). Since severe decreases in HSCs are irreversible, it is necessary to immediately maintain and recover the number of HSCs. However, restorations and maintains of mature haemocytes in first-aid treatment are essential for avoiding acute radiation death. The IAEA recommends transfusion and the administration of granulocyte colony-

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stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) administrations for mature haemocyte reductions and bone marrow transplantation for HSC insufficiency.

The development of radioprotective agents started from research conducted by Pat and Bucq *et al.* around 1950[2, 3], who reported for the first time the radioprotective effects of SH-containing cysteine and cysteamine. Subsequently, in a development program at the Walter Reed Research Institute of the U.S. Army, WR-2721 (amifostine) was found to be a clinically usable radioprotective agent from among more than 4,000 compounds[4]. At present, amifostine is the only radioprotective drug approved by the U.S. Food and Drug Administration (FDA) for head and neck cancer[5, 6].

There have been many reports on the radioprotective/mitigative agents, and several excellent reviews have been published by VJ Singh *et al.*[7-9] The present review focuses on thrombopoietin (TPO) and its receptor agonists with regard to its radioprotective/mitigative effects and provides an overview of the process leading to the discovery of TPO and its future prospects.

2. Discovery of TPO and TPO receptor agonists

Many extremely important discoveries concerning megakaryocytes and platelets were made in the early 20th century[10]. A number of researchers were involved in discovering the mechanism underlying platelet coagulation[11]. Wright discovered that platelets were detached portions of the cytoplasm of megakaryocytes [12]. Kelemen *et al.* first reported the term "thrombopoietin" to describe the humoral substance responsible for the production of platelets[13]. In the 1960s, several groups attempted to purify TPO from the plasma of thrombocytopenic animals. However, the existence of TPO could not be clearly demonstrated due to insufficient sensitivity of the method for evaluating biological activity. In the 1980s, an *in vitro* assay for megakaryocyte differentiation was developed. Although further purification was attempted and some of its biological activity was confirmed, attempts to generate TPO cDNA indicating the presence of the protein also failed[14].

A major step forward in this situation has been the discovery and characterization of myeloproliferative leukemia virus, which causes acute myeloproliferative syndrome in mice[14]. In 1990, the responsible oncogene (v-mpl, now termed Mpl) was cloned, and the protooncogene (c-mpl, also now termed Mpl) was obtained two years later[15, 16]. C-Mpl encodes a member of the haematopoietic cytokine receptor family[17], which includes the receptors for erythropoietin, interleukin (IL)-3, G-CSF, GM-CSF, IL-5, IL-7, IL-9, IL-11 and multiple lymphokines.

In 1994, three U.S. groups and a Japanese group each succeeded in cloning TPO by their own method as shown in the review of Hitchcock and Kaushansky[14]. Using the c-Mpl proto-oncogene product coupled to affinity matrices, scientists at Genetech and Amgen obtained sufficient purified porcine and canine TPO, respectively, to allow amino acid sequencing and cDNA cloning[18, 19]. In contrast to the biochemical purifications utilized by these groups, an expression cloning strategy was used by Lok and Kaushansky to obtain cDNA for murine and then human TPO[20]. Using an *in vitro* megakaryocyte-based assay, scientists at Kirin Pharmaceuticals also devised a 12-step conventional purification scheme and obtained sufficient purified TPO from the plasma of thrombocytopenic rats craft an amino acid sequence. They then cloned cDNA for rat TPO followed by multiple species of the protein, including the human hormone[21]. Initial *in vitro* experiments using the corresponding recombinant proteins demonstrated the effect of TPO on megakaryocyte maturation, and injections into normal mice resulted in impressive increases in peripheral blood platelet counts and marrow megakaryocytes.

The clinical development of recombinant TPOs was fiercely competitive from 1995 to 2000, as they were expected to prove useful as breakthrough drugs for thrombocytopenia. Two molecular forms exist for the formulation that was entered into clinical trials: the full-length glycosylation molecule recombinant human TPO (rhTPO) expressed in the CHO (Chinese hamster ovary) cell line, which has a circulating half-life of 20 to 40 h, and pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF). Administered subcutaneously, PEG-rHuMGDF has similar effects to rhTPO. Both drugs were confirmed to have platelet-enhancing effects in humans, and extensive clinical development was carried out[22]. However, TPO-recognizing antibodies appeared in the subjects of both drugs in overseas clinical trials.

After the development of the first-generation recombinant TPO preparation containing the natural molecular structure, the second-generation drug development began immediately following the discontinuation of the first-generation clinical development. TPO mimetics, such as artificial peptides, non-peptidic small molecules, and agonist monoclonal antibodies, have emerged one after another. This is truly a demonstration of accumulated knowledge in modern drug discovery. Drugs that bind to the receptor Mpl are collectively referred to as TPO receptor (TPOR) agonists[23, 24].

In March 2008, romiplostim (AMG-531, RP; subcutaneous injection) was approved by the U.S. FDA as an rhTPO for the treatment of adult chronic idiopathic thrombocytopenic purpura (ITP)[25]. It was subsequently approved in Japan in 2011. RP can reportedly double

platelet counts from 1 - 6 weeks with a once-weekly subcutaneous dose or increase them to levels above 50,000/μl. It also has the advantage of having a peptide fragment without endogenous TPO and sequence homology, which makes it difficult for neutralizing antibodies to form. Several oral, low-molecular-weight agonists of TPOR were subsequently developed, including eltrombopag (approved in the United States in November 2008 and in Japan in October 2010), avatrombopag (approved in the United States in May 2018 and not yet approved in Japan), and lusutrombopag (approved in the United States in July 2018 and in Japan in September 2015)[26] for the treatment of ITP and aplastic anemia. These agents are administered orally and result in significant increases in platelet counts in normal subjects as well as patients with thrombocytopenia due to hematologic and liver diseases.

3. Characteristics of TPOR agonists

TPO is constantly generated by the liver, which is the main producing organ, and is not regulated at the transcription level. Blood TPO concentrations are regulated by the thrombopoietin receptor (Mpl) expressed on the plasma membrane of platelets and megakaryocytes, and when platelets and megakaryocytes decrease, TPO trapped by Mpl decreases and the blood TPO level rises. However, a mechanism is considered to exist wherein when the platelet and megakaryocyte counts increase, the TPO captured by Mpl increases, and the blood TPO level decreases. The concentration of TPO in normal steady state human plasma is in the range of 22 - 256 pg/ml, 81.5 ± 5 pg/ml (n=97)[27].

Romiplostim is a dimer Fc-peptide fusion protein (peptibody). The peptibody molecule has two identical single-chain subunits, each one is made up of 269 amino acid residues. Each subunit consists of an IgG_1 Fc carrier domain that is covalently attached to a polypeptide sequence that contains two binding domains to interact with TPOR. Each domain consists of 14 amino acids. Interestingly, romiplostim's amino acid sequence is not similar to that of endogenous TPO[28].

Since then, three low-molecular-weight compounds have been approved as pharmaceuticals. Eltrombopag is a novel, orally bioavailable, small-molecule TPOR agonist that induces the differentiation and proliferation of megakaryocytes. The compound was approved for the treatment of ITP. The chemical formula of the compound is $C_{25}H_{22}N_4O_4$, and the molecular weight is 442.467[29, 30]. Avatrombopag was the second oral TPOR agonist approved for use as therapy of thrombocytopenia in adults with chronic liver disease undergoing surgical, radiologic, or medically invasive procedures. Like eltrombopag, avatrombopag is a low-molecular-weight peptide-like molecule that binds to the TPOR and causes its activation and the proliferation and differentiation of megakaryocytes, with a resultant increase in the synthesis and release of platelets. The chemical formula of the compound is $C_{29}H_{34}Cl_2N_6O_3S_2$, and the molecular weight is 649.65. Lusutrombopag was the third oral TPOR agonist approved for the treatment of thrombocytopenia in adults with chronic liver disease who are scheduled to undergo an invasive procedure. Like eltrombopag and avatrombopag, lusutrombopag is a low-molecular-weight peptide-like molecule that binds to the TPOR and causes its activation and the proliferation and differentiation of megakaryocytes, with a resultant increase in the

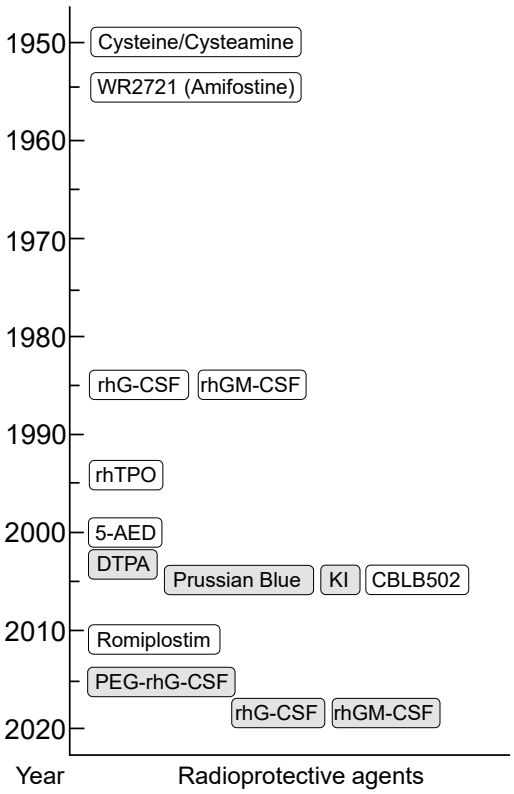

Fig. 1. Discovery and development of major radioprotective agents. Cysteine and cysteamine are SH compounds, which have historically been reported to have a radioprotective effect in the early stages. Amifostine is an organic thiophosphate prodrug which is hydrolysed in vivo by alkaline phosphatase to become an SH compound with active cytoprotective properties. In the 1980s, the discovery and progress of genetic modification technology led to the production of many cytokines such as rhG-CSF, rhGM-CSF and TPO. DTPA (zinc/calcium diethylenetriamine pentaacetate), prussian blue and KI (potassium iodide) are radionuclide removal compounds currently licensed or under investigation in the United States. 5-AED (5-androstenediol) is a natural adrenocortical steroid hormone. CBLB502 is a truncated flagellin polypeptide and an agonist of Toll-like receptor 5. The radioprotective efficacy of 5-AED and CBLB502 were confirmed in studies conducted on irradiated mice and NHPs[7-9]. The gray column shows the year of FDA approval.

synthesis and release of platelets. The chemical formula of the compound is $C_{29}H_{32}Cl_2N_2O_5S_2$, and the molecular weight is 591.54.

TPOR (c-Mpl) is a member of the type I cytokine receptor family along with receptors for a number of interleukins, growth hormone, prolactin, colony stimulating factors and erythropoietin. The extracellular domain consists of two cytokine receptor motifs with WSXWS sequences and shows the highest homology with erythropoietin receptors. TPOR itself has no kinase activity, yet after dimerization[31, 32]. Binding of TPO causes phosphorylation of Janus kinase 2 (JAK2) and signals from the phosphorylated molecules to the JAK2-Signal transducer and activator of transcription 3 (STAT3)/ STAT5 pathway, the Mitogen-activated protein kinase (MAPK) pathway (extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and p38 MAPK pathway), and the phosphoinositide 3-kinase (PI3K)-AKT pathway (Fig. 1)[32~34]. RP is a peptibody composed of 2 binding sequences of 14 amino acids each separated by 8 glycine residues and fused to a human IgG1 Fc domain. These 2 set of 14 amino acids bind at the extracellular part of the TPOR, similar to human TPO, despite having no amino acid sequence similarity. RP stimulates the three main downstream signaling pathways as shown above without a preference for any of the pathways. Very recently, Rommel *et al.* reported that RP differs from TPO in the phosphorylation intensity, reduction of signaling after RP stimulation caused by differential internalization kinetics and megakaryocyte maturation (poly ploidy)[35].

4. Rreduction effect of TPOR agonists on radiation damage

Several TPOR agonists were also reported to have radiation protective/mitigative effects. However, since small-molecule TPOR agonists have high specificity for human TPOR, these agents act on human TPOR, not murine TPOR[36]. Yoshida *et al.* explained that for these reagents to exert their pharmacological action, the 499th histidine (H499) of the TPOR transmembrane region, a species-specific amino acid sequence in humans and chimpanzees, is essential[37, 38]. Therefore, rodent models cannot be used to evaluate the radioprotective/mitigative activity of these small-molecule TPOR agonists. Previous studies on the reduction of radiation damage by rhTPO, its derivatives, and RP are described in this section.

Mouthon *et al.* showed that the administration of recombinant full-length murine TPO (rmTPO, 0.3 μg/kg) 2 h after irradiation induced a 30-day survival of approximately 90% in 8-Gy-irradiated C57BL/6J mice; this total body irradiation (TBI) dose resulted in 100% mortality within 30 days in the placebo-treated mice[39]. Subsequently, this group reported the efficacy of different schedules of rmTPO administration at doses ranging from 7 to 10 Gy in mice[40]. The results showed that the administration of TPO should be performed shortly after irradiation to obtain the optimal effect of TPO on the individual survival. In addition, Wang *et al.* showed that injections of 25 μg/kg rhTPO for 14 consecutive days after lethal irradiation of 8 Gy resulted in a survival rate of 50%-60% at day 28[41]. Very recently, Xing *et al.* reported the effect of rhTPO on the hematopoietic response and survival of mice and nonhuman primates (NHPs) exposed to TBI. They concluded that a single administration of rhTPO might represent a promising and effective radiomitigative strategy for victims of radiation disasters [42]. Regarding other types of TPOR agonists bearing TPO mimetic peptides, Satyamitra *et al.* reported that a single dose of 2 mg/kg ALXN4100TPO-VL+H administered 24 h prior to radiation exposure resulted in a 94% survival, while a single dose administered 6 h after 9 Gy resulted in a 44% survival[43]. Although there are some countries have approved rhTPO for clinical use, rhTPO is known to have side effects caused by neutralizing antibodies[44]. Unfortunately, the clinical application of these candidate compounds is currently impossible, even though they have a substantial mitigating effect on radiation damage.

In contrast, RP is a clinically approved drug that can be used following emergency and high-dose radiation exposure without delay. Bunin *et al.* analyzed the effects of RP in a mice model as a medical countermeasure to improve the survival and platelet recovery following acute radiation[45]. They concluded that a single injection of RP administered 24 h after TBI was a promising radiation medical countermeasure that dramatically increased the survival, with or without pegfilgrastim, and hastened the platelet recovery in mice. Wong *et al.* evaluated the pharmacodynamics and pharmacokinetics of RP alone and in combination with pegfilgrastim in an NHPs model of ARS caused by 5.5 Gy γ-radiation and found that RP did indeed improve the hematological parameters in this model[46]. Our group has also been investigating the effectiveness of PR in reducing radiation damage since shortly after its approval. A single administration of RP to C57BL/6J mice exposed to a lethal dose (7 Gy) of γ-radiation resulted in a 100% complete survival[47, 48]. Our previous studies clarified that PR promoted the recovery of pan-cytopen in the bone marrow, spleen and lung[48], improvement of DNA repair[47], reduction of apoptotic hematopoietic cells[47], the reactive oxygen species (ROS) removal function[48], a significant increase in mesenchymal stem cells[48], the release of extracellular vesicles containing functional microRNA[48], regulation of nuclear factor-erythroid-2-related factor 2 target genes[49], and inhibition of liver-damaging proteins[50], suggesting that RP reduces disability and promotes the regeneration of

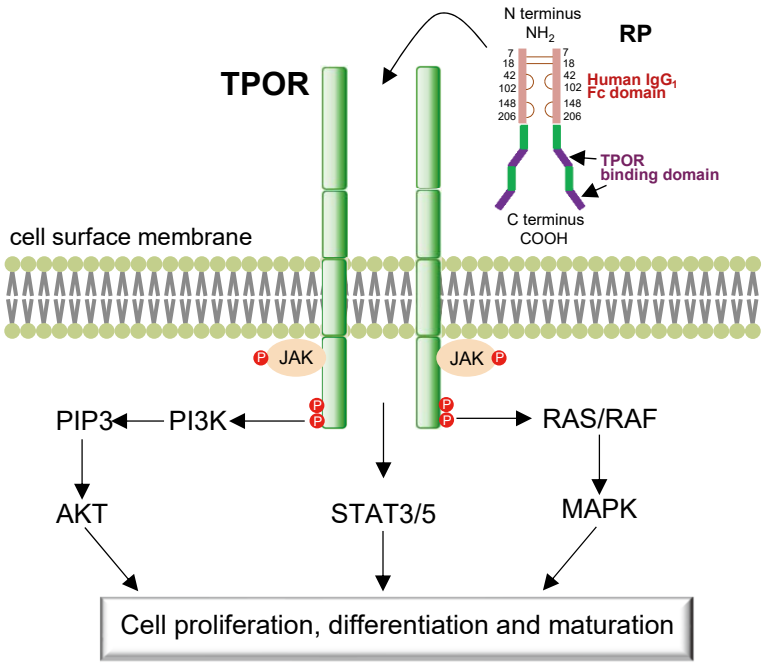

Fig. 2. Overview of the structure and mechanism of action of Romiplostim.
Cellular mechanisms of action of romiplostim to the TPOR on the megakaryocyte causes conformational change in the receptor, resulting in downstream activation of the various signaling pathways including JAK2/STAT3/5, PI3K/AKT, MAPK, ultimately resulting in increased platelet production. P, phosphorylation; RAF, rapidly accelerated fibrosarcoma kinase; RAS, rat sarcoma GTPase; RP, romiplostim; PIP3, phosphatidylinositol (3, 4, 5) triphosphate.

radiation-induced multiple organ failure through a variety of actions.

TPOR agonists have also been reported to promote DNA repair in hematopoietic stem/progenitor cell (HSPC) populations by modulating the efficiency of the DNA-dependent protein kinase catalytic subunit-dependent non-homologous end joining pathway[51, 52] and inhibiting apoptosis in HSPCs (Fig. 2)[53]. Recently, Vlachodimitropoulou *et al*. reported that eltrombopag, a small-molecule oral TPOR agonist, is a powerful iron chelator that mobilizes iron and ferritin, reduces ROS independently of eltrombopag's TPOR effect, and restores insulin production to clinically achievable levels[54]. Stickney *et al*. reported that the duration of severe thrombocytopenia appeared to correlate with death to a greater extent than the duration of severe neutropenia[55]. Thrombocytopenia appears to be more clinically relevant to the survival in case of ARS than has been previously recognized. Various effects of RP, which has already been approved as a drug, on the reduction of radiation damage have been demonstrated. Although a more detailed study of RP is necessary, RP is expected to be a recognized as a promising and effective radioprotective/mitigative strategy for victims of radiation disasters in the near future.

5. Outlook for the future

This article has focused on the effects of various TPOR agonists for reducing radiation damage. However, for rapid and effective medication, it is extremely important to obtain the accurate information concerning the dose (i.e. how much radiation the victim has received) and damage (i.e. how much biological damage was suffered). Chromosome aberration analyses are used as the most reliable international standard method for dose evaluations, but there are issues with rapidity, as these analyses requires a high level of expertise and several days to complete. Our research group has examined the relationship between the radiation dose and changes in the expression of messenger RNA and microRNA detected in the blood of irradiated mice. We have found that some messenger RNA increases 24 h after irradiation in a dose-dependent manner[56, 57]. Furthermore, it was discovered that the expression of some blood proteins fluctuated in response to TBI and that amino acids of specific serum proteins were chemically modified (unpublished data); further research on the utility of radiation exposure biosensors is underway. Finally, although the exact dose should be confirmed by a "chromosomal aberration analysis", identifying

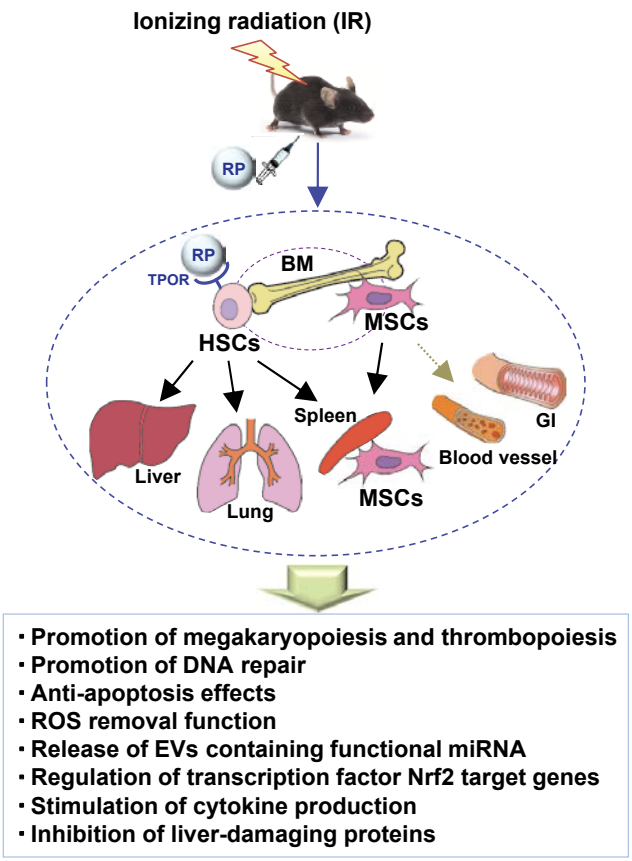

Fig. 3. Estimated mechanism of action of Romiplostim.
The administration of RP to lethally irradiated mice induce numerous downstream signalling pathways involving JAK, STAT, AKT-, and ERK1/2 through TPOR expressed on HSCs, resulting in the promotion of haematopoiesis, especially megakaryopoiesis and thrombopoiesis, in the bone marrow, spleen, lung and liver. The various actions of RP, such as promotion of DNA repair, anti-apoptosis effects and ROS removal functions, may cause the growth of MSCs, leading to the release of EVs containing functional miRNA and regulation of transcription factor Nrf2 target genes, stimulation of cytokine production and inhibition of liver-damaging proteins. BM, bone marrow; EVs, extracellular vesicles; GI, gastrointestinal tract; HSCs, haematopoietic stem cells; MSCs, mesenchymal stem cells; Nrf2, nuclear factor-erythroid-2-related factor 2; ROS, reactive oxygen species; RP, romiplostim; TPOR, thrombopoietin receptor.

biomarkers may lead to the development of a simple kit for estimating the approximate exposure doses at accident sites.

For the practical application of radiomitigative agents in humans, a variety of issues still need to be addressed, such as the safety and efficacy of their application in humans, the optimal doses of the drugs, the optimal duration and timing of administration and the applicable range of radiation doses that can be effectively countered. Furthermore, long-term adverse risks such as carcinogenesis and leukemia in survivors should be considered, even if acute radiation damage is avoided using such drugs. However, efficacy testing in humans is not possible. Singh *et al*. mentioned in their review manuscript[58] that in order to expedite the development of radiation countermeasures for life-threatening situations, where human efficacy trials are neither feasible nor ethical, the FDA has implemented the 'Animal Rule'. Under such conditions, where human efficacy testing is not possible, the Animal Rule applied to the development and evaluation of drugs and biologics to reduce or prevent life threatening conditions caused by exposure to lethal or permanently disabling agents[59].

Before March 11, 2011, when the Great East Japan Earthquake struck, 54 nuclear reactors were operating in Japan, supplying about 30% of domestic electricity. However, following the Fukushima nuclear accident caused by the earthquake, new regulatory standards for safety measures at nuclear power plants have come into effect, and it has become necessary to clear the strict safety standards for earthquakes and tsunamis. As a result, all reactors have been shut down, with only 5 nuclear power plants including 9 nuclear reactors currently operating in compliance with the new standards and another 24 nuclear reactors under consideration or schedules for decommissioning (as of June 23, 2020). However, there are 442 nuclear reactors operating in the world in 31 countries around the world, and the amount of power generated by these reactors has increased for the sixth consecutive year (as of January 1, 2019). Furthermore, there are 55 nuclear reactors currently under construction in 19 countries (China, India, and South Korea) (as of January 1, 2020), and construction plans are expected to exceed 139 nuclear reactors in 31 countries (as of January 1, 2019)[60]. From a global perspective at least, the risk of radiation accidents and exposure due to nuclear power plants has not yet decreased. Of course, even if safety can be assured by the improvement of safety countermeasures adequate preparation is indispensable. A large portion of Japanese has only ever lived in modern society that cannot exist without a stable supply of electricity, and in addition to safety measures and emergency measures that are not usually noticed, establishing effective and safe medical measures has become a social issue. Establishment of drug therapy in emergency medicine for radiation exposure is an important issue in crisis management.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Appendix

Acronyms and abbreviations

ARS, acute radiation syndrome; BM, bone marrow; BMNC, BM nucleated cell; EVs, extracellular vesicles; CHO, Chinese hamster ovary; DTPA, zinc/calcium diethylenetriamine pentaacetate; ERK1/2, extracellular signal-regulated kinase 1/2; 5-AED, 5-androstenediol; FDA, U.S. Food and Drug Administration; G-CSF, granulocyte colony-stimulating factor; GI, gastrointestinal tract; GM-CSF, granulocyte macrophage-colony stimulating factor; HSC, haematopoietic stem cells; IAEA, International Atomic Energy Agency; IL-3, interleukin-3; IR, ionizing radiation; ITP, idiopathic thrombocytopenic purpura; JAK, janus kinase; KI, potassium iodide; MAPK, mitogen-activated protein kinase; Mpl, thrombopoietin receptor; MSC, mesenchymal stromal and stem cells; NHPs, nonhuman primates; Nrf2, nuclear factor-erythroid-2-related factor 2; PAI-1, plasminogen activator inhibitor 1; PEG-rHuMGDF, pegylated recombinant human megakaryocyte growth and development factor; PI3K, phosphatidylinositol 3-kinases; PIP3, phosphatidylinositol (3, 4, 5) triphosphate; RAF, rapidly accelerated fibrosarcoma kinase; RAS, rat sarcoma GTPase; ROS, reactive oxygen species; RP, romiplostim; STAT, signal transducer and activator of transcription 3; TBI, total-body irradiation; TPO, thrombopoietin; TPOR, thrombopoietin receptor; WR-2721, amifostine.