Shimane J. Med. Sci., Vol.36 pp.1-10, 2019

Clinical Application to Hematological Diseases of Mesenchymal Stem Cells

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（Received January 5, 2019; Accepted May 23, 2019）

Mesenchymal stem cells（MSCs）have many function, including immunomodulatory activity, tissue-repair capability and regeneration of bone, cartilage and fatty tisssues. MSC culture methods have been established, and safety can be ensured, so MSCs have been applied clinically to treatment of numerous diseases. With respect to hematological diseases, MSC treatment is expected to be effective for therapy-resistant acute graft-versus-host disease（GVHD）. In addition, MSCs have been applied to areas such as treatment of chronic GVHD, prevention of GVHD, promotion of hematopoietic stem cell engraftment, and treatment of refractory aplastic anemia. However, on the basis of the cellular characteristics of MSCs, the potential for problems such as increase in relapse, exacerbation of infection, and cancer development must be borne in mind. This article review the current status of, issues with clinical applications to hematological diseases of MSCs.

Key words: mesenchymal stem cell, hematological disease, graft versus host disease

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INTRODUCTION

Mesenchymal stem cells（MSCs）are stem cells that are present in tissues such as the bone marrow（BM）, fatty tissues, cartilage, tooth pulp, and placenta, and, as stem cells, have self-renewality, and the capacity to differentiate into mesodermal cells, including bone, cartilage, and fat. MSCs have numerous different functions, including immunomodulatory activities and tissue-repair activities, as well as tissue regeneration, and there have been reports of those function being made use of, resulting in efficacy against various diseases. Furthermore, in addition to it being relatively easy to collect tissues containing MSCs, the culture operations are simple, and no severe adverse events have been reported in clinical use, so it is expected that safe and readily usable cell therapies based on MSCs as stem cells will be developed. This review is focused on treatment of hematological diseases using MSCs.

WHAT ARE MSCS?

MSCs were originally reported as a fibroblast-like osteogenic cell population isolated from BM, and, in addition to forming a stromal microenvironment in which hematopoietic stem cells（HSCs）are present, they are characterized as spindle-shaped adherent cells that have the capacity to differentiate into bone, fatty tissue, and cartilage cells［1］. In recent years, there have been reports about the feasibility of culturing MSCs from a wide range of tissues, including fatty tissues, cartilage, muscles, tendons, ligaments, synovium, dental pulp, umbilical blood, umbilical cord（UC）, and placenta, as well as BM［2］. However, a controversial issue is whether the MSCs in each of these tissues are the same, or whether MSCs from each tissue have similar characteristic properties［2, 3］. In addition, it has clearly been shown *in vitro* that these cells do not differen-

tiate only into osteoblasts, adipocytes and cartilage cells, but also into other cells of the mesodermal lineage, such as myocytes, cardiomyocytes, and vascular cells, and cells of the ectodermal and endodermal lineages, including cells of the nervous system, skin, retinal pigment epithelium, lungs, liver, and renal tubules [2]. However, *in vivo*, although differentiation to form bone, cartilage and fat has been shown, no differentiation into other cells or tissues has, and it is therefore considered that MSCs, rather than being true stem cells, are pluripotent cells of a mesenchymal lineage. On this basis, as the definition of "MSC", the International Society for Cellular Therapy has proposed that the following criteria must be met [4]:

- Cells that adhere to a plastic dish under standard culture conditions, that is, culture in α minimal essential medium (αMEM) + 20% fetal bovine serum (FBS).
- Cells that express CD105, CD90, CD73 and CD44.
- Cells that do not express CD45, CD34, CD14, CD11b, CD79, CD19 and human leukocyte antigen (HLA)-DR.
- Cells that in vitro differentiate into Osteoblasts, adipocytes and chondroblasts.

MSCS HAVE MULTIPLE FUNCTIONS AND ARE CLINICALLY APPLIED TO MANY DISEASES

In addition to MSCs having the capacity to differentiate into bone, cartilage, and fat, they have various other functions, including immunomodulatory, and tissue repair effect [5-7]. Their activities can be classified by two main function, such as cell replacement and trophic activities. Cell replacement involves recovery of organ function, that is, tissue homeostasis, regeneration and repair, due to MSCs or cells differentiated from MSCs homing on target organs and tissues, undergoing engraftment, and thus replacing damaged cells, whereas trophic action consists of contribution to recovery of organ function, that is, normalization of immunological abnormalities, and tissue homeostasis and repair, due to MSCs or cells stimulated by MSCs producing nutritional factors, cytokines, extracellular matrix components, extracellular vesicles (microvesicles and exosomes), and nanotubes [5, 6] (Fig. 1). By cell replacement and trophic effect, MSCs have malti-potential abilities such as differentiation, immunomodulating, anti-inflammatory, anti-bacterial, antioxidant and antiapoptotic actions, resulting in inducing many efficacies such as tissue regeneration, tissue homeostasis, normalization of immunologic abnormality, and tissue repair. In addition to their having these multiple functions, it is expected, for the following reasons, that MSCs will offer safe and readily usable stem cells for cell therapy: (i) they can be collected relatively easily and at low cost from BM, fatty tissues, placenta, and UC; (ii) the ethical considerations are minor and have been resolved; (iii) the culture methods needed for MSC proliferation are simple, and have been fully established; and (iv) no severe adverse effects, such as death or tumor formation, due to MSC administration have been reported in any of the numerous clinical studies that have been carried out [7] (Table 1). Approximately 1,000 clinical studies have been carried out with MSCs in Japan and overseas (MSC clinical trials were charted by region based on search results sourced from https://ClinicalTrials.gov (retrieved May 1, 2018)). It is therefore expected that still more clinical studies on this field will be carried out in future.

MSC THERAPY FOR HEMATOLOGICAL DISEASES

1) Hematopoietic stem cell transplantation

(1) Treatment of acute graft-versus-host disease (GVHD)

The clinical application of MSCs that is most widespread is their use for treating GVHD. GVHD is the most common complication of allogeneic hematopoietic stem-cell transplantation, and has major effects on the mortality rate [8, 9]. In the case of steroid-resistant acute GVHD in particular, no treatment has been established. The first report of alleviation of GVHD was published in 2004, having been achieved by administration of MSCs to a 9-year-old male with acute GVHD with which steroids and cyclosporin were ineffective [10]. This is considered to have been a result of the immunomodula-

tory activities of MSCs (Fig. 2). In other words, in connection with the adaptive immune response, MSCs suppress the proliferation and differentiation of B cells, suppress the proliferation and cytotoxicity of effector T cells, and promote the mobilization of regulatory T cells [11, 12]. In addition, in terms of innate immunity, MSCs have roles in suppressing the cytotoxicity of natural killer cells, reducing the numbers of inflammatory M1 macrophages, mobilizing M2 macrophages, which have anti-inflammatory activities, and suppressing the maturation of dendritic cells, which are essential for antigen-specific T-cell responses [11, 12]. Acute GVHD involves inducing the activation of donor T-cells against host antigens, delete and release of inflammatory cytokines, such as IL1 and tumor necrosis factor α (TNFα), which causes damage to host tissues such as skin, liver and intestines. It is therefore considered that MSCs are primarily involved in suppression of T-cell activation [8, 11, 12].

Numerous clinical studies on acute GVHD have been carried out in Japan and overseas, and several systematic reviews have been published. Chen *et al.* have summarized the effects on steroid-resistant acute GVHD [13]. With respect to clinical studies of MSC therapy for steroid-resistant acute GVHD, reference was made to 6,963 publications in the PubMed and EMBASE databases, and 13 studies, covering 301 subjects, were reviewed, as they met criteria such as that MSCs were used to treat refractory acute GVHD, and factors affecting the efficacy of MSC treatment of steroid-resistant acute GVHD were reported. The responses to treatment were classified as (i) complete response (CR), with full recovery from all symptoms; (ii) partial response (PR), with the severity of GVHD reduced, but full recovery not occurring; and (iii) mixed response (MR), with the severity in one organ reduced, but the symptoms showing no change in other organs. On this basis, the overall response was defined as the sum of CR, PR and MR The result was that the number of patients showing CR was 136 (45.2%), and the total number showing PR or MR was 69 (22.9%). The response to treatment with dermal steroid-resistant acute GVHD was greater than when this condition was in the intestines [CR: odds ratio (OR): 1.93; 95% confidence interval (CI): 1.05

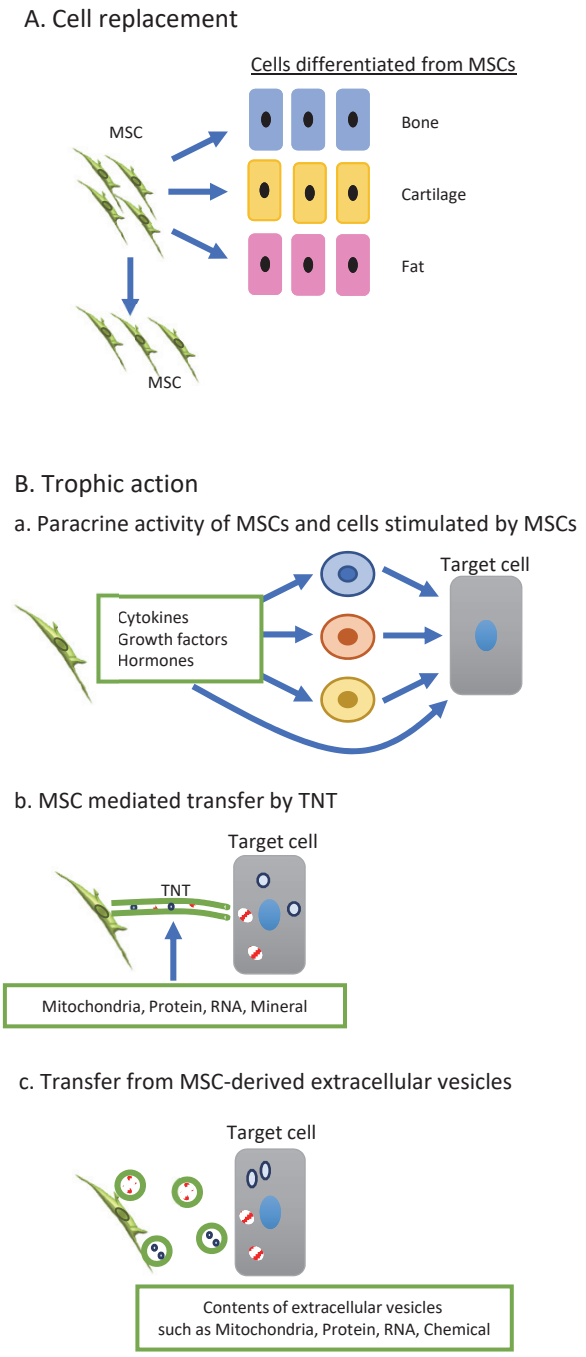

Fig. 1. MSCs have two main functions by different mechanisms
A, Cell replacement. MSC and/or cells differentiated from MSC homes to the targeted tissues/organs, engrafts, and replaces the damaged cells.; B, Trophic effect. MSC and/or cells stimulated by MSC produce cytokines, growth factors, extracellular matrix, intracellular microvesicles, exosomes, and/or nanotubes.; a, Paracrine activity of MSCs and cells stimulated by MSCs. MSCs secrete paracrine factors such as growth factors, cytokines, and/or hormones.; b, MSC mediated transfer by tunneling nanotubes (TNTs). MSCs transfer such as mitochondria, and/or small molecules such as mineral, RNA, or protein) through TNTs; c, Transfer from MSC-derived exosomes or microvesicles. MSCs transfer mitochondria and/or small molecules such as chemical, RNA, protein by extracellular vesicles.

Table 1. Many clinical application for MSCs

Diseases	Source	Function of MSCs		
		Immunomodulation	Tissue repair	Tissue regeneration
GVHD	Allo, Auto	+	+	
Engraftment of HSCs in HSCT	Allo, Auto	+		
Multiple sclerosis	Auto>Allo	+	+	
Neural stroke	Auto	+	+	
Myocardial infarction	Auto	+	+	
Spinal cord injury	Auto	+	+	
Crohn's disease	Auto	+	+	
Liver cirrhosis	Auto	+	+	
Acute lung injury	Auto	+	+	
Wound healing (diabetic foot, ASO, radiation injury)	Auto	+	+	
Osteoarthritis	Auto			+
Cartilage defect	Auto			+
Hunter syndrome	Allo			+
Osteogenesis imperfecta	Allo			+
Hypophosphatasia	Allo			+

Using many and diverse role of MSC, there are many clinical trials in world wide. The famous clinical use is graft versus host disease (GVHD). These clinical trial indicate that safety is confirmed, but clinical efficacy is variable. HSC, hematopoietic stem cell; HSCT, HSC transplantation; ASO, Atherosclerosis obliterans. Allo and auto mean allogenic and autologous MSCs, respectively. '+' indicates MSC function to make a effect on each disease.

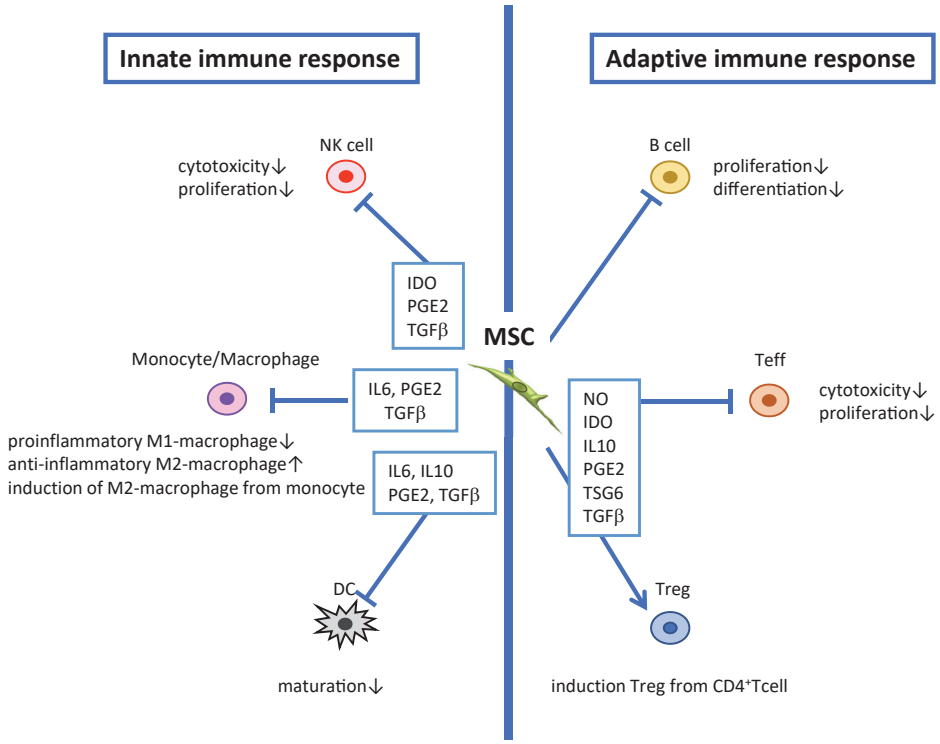

Fig. 2. Immunomodulatory functions of MSCs
MSCs can regulate various innate and adaptive immune cells, such as monocytes, macrophages, dendritic cells, NK cells, T cells, B cells. Interestingly, the immunosuppressive function of MSCs, which is induced by inflammatory cytokines, such as IFNγ and TNFα or IL-1, is not inherent. MSCs produce various immunosuppressive molecules, such as nitric oxide (NO), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), transforming growth factor beta (TGFβ), and inter- leukin-6 (IL-6), TNFα–induced gene/protein 6 (TSG-6), and chemokines. Teff, effector T cell; Treg, regulatory T cell.

to 3.57; $p < 0.05$) or liver (CR: OR: 2.30; 95% CI: 1.12 to 4.69; $p < 0.05$; and overall response: OR: 2.93; 95% CI: 1.06 to 8.08; $p < 0.05$). In addition, the MSC efficacy was higher with grade-II than grade-III/-IV steroid-resistant acute GVHD (CR: OR: 3.22; 95% CI: 1.24 to 8.34; $p < 0.05$). Furthermore, the responsiveness of children tended to be higher than that of adults (CR: OR: 2.41; 95% CI: 1.01 to 5.73; $p = 0.05$). With respect to toxicity and the number of organs damaged by GVHD, no differences in clinical efficacy depending on number of MSC doses were found, and no toxicity due to MSC administration was found. In a review of steroid-resistant acute GVHD restricted to adult patients, in addition to the above findings, a positive correlation was found between CR and overall survival rate, but no such correlation was found with PR [14]. Furthermore, as the maximum malignancy recurrence rate after MSC administration was 17%, this cannot be said to be high in comparison with the recurrence rates predicted on the basis of previous data [14].

Notwithstanding the above, numerous factors, including the following, have the potential to affect MSC therapeutic efficacy: age, disease type, origin of HSCs (BM, peripheral blood, or umbilical cord blood), factors relating to the HSC donor [sex, related or unrelated, HLA compatibility], pre-treatment regimen (myeloablative or non-myeloablative transplantation), complications at treatment initiation, origin of MSCs (BM, UC, or fatty tissue), type of medium used for MSC culture (α MEM, or Dulbecco's modified Eagle's medium), serum added to the medium (FBS, or human platelet lysate), MSC cell culture passage number, MSC storage conditions until use (stored frozen; or not frozen, but isolated when fresh, and then cultured), MSC dose, and number of MSC doses. On the basis of numerous previous clinical studies, it cannot be said that MSC is effective against all types of steroid-resistant GVHD independently of these factors, and it is therefore important to clarify the patient subgroups with which MSC treatment is effective.

Research has recently been carried out on biomarkers for predicting MSC efficacy, and prognosis after MSC administration. Boome *et al*. have clearly shown that, when the following six soluble biomarkers, put forward by Levine *et al*. in 2012 [15], were measured before MSC administration, they were correlated with one year-survival after HSC administration (hazard ratio (HR): 2.924; CI: 1.485 to 5.758): IL2Rα, TNF receptor 1, hepatocyte growth factor, IL-8, elafin, and regenerating islet-derived protein 3α [16]. In addition, increase in soluble suppression of tumorigenicity 2 (ST2), measured 2 weeks after the initial MSC administration, was found to be correlated with increased mortality risk (HR: 2.389; CI: 1.144 to 4.989), and increase in the number of immature myeloid dendritic cells was associated with decreased mortality rate (HR 0.554; CI: 0.389 to 0.790) [16]. In studies of biomarkers predicting MSC efficacy, when MSC cytotoxicity due to peripheral blood mononuclear cells (PBMCs) from acute GVHD patients was investigated, MSCs showed more apoptosis with PBMCs from patients with whom MSC therapy was effective against grade-III/-IV steroid-resistant acute GVHD than with PBMCs from patients with whom no efficacy was shown, and analysis using a receiver operating characteristics curve showed that, in the group with whom MSCs were effective, the proportion of MSCs that underwent apoptosis due to PBMC was at least 14.85% [17]. It is hoped that more and larger-scale studies will be carried out, so that more precise biomarkers can be identified.

(2) Treatment of chronic GVHD

The frequency of chronic GVHD has increased in recent years, but almost no progress has been achieved in treatment of severe chronic GVHD [18]. In addition, adverse effects due to long-term exposure to immunosuppressive agents have become problematic. The pathology of chronic GVHD involves inflammatory T-cells, and leads to tissue damage with associated qualitative and quantitative decreases in regulatory B-cells, regulatory T-cells, and IL10 [19]. It is therefore hoped that, as with acute GVHD, MSCs will show therapeutic efficacy against chronic GVHD by means of suppression of inflammatory T-cells and mobilization of regulatory T-cells.

A number of reports have been published on treatment of chronic GVHD using MSCs [20-27]. MSCs have been used to treat numerous cases of

immunosuppressive-resistant GVHD, with administration to at least 100 patients to date. MSCs have been used more often with adults than with children. In the study in one report, the origin of MSCs was fatty tissues, whereas in all others it was BM, and, as with acute GVHD, the number of MSCs administered was approximately 1 million per body weight. There was a wide range of number of administrations, from 1 to 11, and it has been reported several times that, unlike the case with acute GVHD, administration was at intervals of approximately 1 month. When publications that report therapeutic efficacy for each of the affected tissues are collated, the total proportions of subjects showing CR or PR were 83.2%, 83.3%, 91.0%, 81.2%, 88.3%, 57.1% and 63.1% for lesions in the skin, buccal cavity, eyes, intestines, liver, lungs, and joints/muscles, respectively. No serious adverse reactions due to MSC administration were found. However, for the following reasons, the efficacy of MSCs against chronic GVHD must be investigated more carefully and in more detail in future: (i) as in the above clinical studies of acute GVHD, various different factors affect therapeutic efficacy; (ii) some subjects showed exacerbation of lesions after MSC administration; and (iii) the evaluations of symptom severity and alleviation rate were not consistent between published reports.

In exploratory research to identify biomarkers for predicting the efficacy of MSCs against chronic GVHD, the numbers of CD27-positive memory B-cells were found to increase significantly in the CR and PR groups, whereas the number decreased in the group showing absolutely no response [25]. In addition, the plasma levels of B-cell-activating factor (BAFF) in the CR and PR groups decreased, and the expression of BAFF-receptor increased in peripheral B-cells, whereas in the no-response group no changes in B-cell plasma BAFF level or BAFF-receptor expression were found [25]. Furthermore, in the CR and PR groups, the numbers of IL10-producing, CD5-positive, regulatory B-cells increased significantly after MSC administration [26]. These results suggest the potential usefulness of B-cell subset analysis for identification of chronic GVHD biomarkers.

(3) Prevention of GVHD and promotion of hematopoietic stem cell engraftment

Due to factors such as the decreasing birth rate leading to a shortage of HLA-compatible related donors, it has become difficult to transplant from HLA-compatible donors. In umbilical cord blood transplantation, the number of T-cells is small, so, even if the HLA is incompatible, the risk of GVHD is lower than with BM transplantation, but there is a problem with incomplete engraftment, due to the number of transplanted cells being small. There have been reports of favorable results with semi-HLA-compatible (haploidentical) transplantation, due to progress with pre-transplantation treatment and immunomodulatory agents, but the theory behind these methods has not been established.

As detailed above, it is hoped that MSCs will offer an effective method for treating GVHD. However, it has been reported that, in animal studies using MSCs, simultaneous transplantation of HSCs and MSCs not only inhibits GVHD onset, but also promotes HSC engraftment [28]. Clinical studies of simultaneous transplantation of MSCs and HSCs have also been carried out in humans [29-39]. In these studies, the target diseases were divided into hematological malignancies and aplastic anemia, and no clear findings relating to post-transplantation engraftment promotion or GVHD prevention were made with either. However, in each case, although transplantation was from non-HLA-compatible donors, which is typical of haploidentical transplantation, no increases in incomplete engraftment, GVHD onset rate or severity, or rates of occurrence or recurrence of infections or other post-transplantation complications were found. Although most previous studies were retrospective, nonrandomized, comparative studies, Gao *et al*. did carry out a randomized, double-blind, placebo-controlled study, in which it was clearly shown that repeated MSC administration after HSC transplantation reduced the frequencies of chronic GVHD and severe pulmonary lesions [40]. It has also been reported that blood cell recovery by immunosuppressive-resistant aplastic anemia can be achieved solely by MSC administration [41]. MSCs in the BM of aplastic anemia patients are phenotypically identical to those in healthy people, but have been reported to have lower proliferative

capacity, and to show increased tendency to proliferate into adipocytes [41, 42]. In addition, MSCs not only increase production of hematopoiesis-inhibiting factors such as TNFα and interferon γ (IFNγ), but also reduces the level of Transforming growth factor β (TGFβ), thus inhibiting the proliferation of regulatory T-cells. There is therefore the potential for the MSCs from healthy people, which promote proliferation of hematopoietic cells, and thus maintain BM homeostasis, and regulate immunity (Fig. 2), to offer a novel therapeutic option for aplastic anemia.

(4) Effects on other post-transplantation complications

It has been reported that MSCs administered to treat GVHD migrate into injured tissues, leading to repair of those tissues [43]. There have also been reports about the possibility of MSCs preventing onset of thrombotic microangiopathy and veno-occlusive disease / sinusoidal obstruction syndrome [44, 45]. However, the numbers of cases in these reports were small, and there is therefore a need for accumulation of more cases before further evaluation.

(5) Concerns relating to MSC administration

(i) Suppression of graft-versus-tumor effects

There are two main pillars to treatment by allogeneic transplantation:

(a) Complete eradication of malignant tumors by pre-treatment.

(b) Allogeneic immunity, which consists of graft-versus-tumor effects against the residual tumor.

As shown in Fig. 2, MSCs are involved in suppressing cytotoxic T-cells and natural killer cells, and there are therefore concerns about suppression of graft-versus-tumor effects, and thus increased recurrence. Ning *et al*. have reported that, when HSC transplantation is carried out concomitantly with MSC administration, whereas the GVHD occurrence rate is low, the recurrence rate is high, and the disease-free survival rate is therefore low [46]. However, no significant increases in recurrence have been reported in any other of the numerous studies that have been carried out.

(ii) Exacerbation of infection

MSCs suppress immune reactions, and there are therefore concerns about MSC therapy exacerbating infectious diseases after HSC transplantation. However, due to decrease in the number of neutrophils, treatment with immunosuppressive agents, etc., HSC transplantation itself involves a high rate of infection, and it is therefore difficult to make judgments about the effects of MSC treatment. However, it has been reported that the frequency of cytomegalovirus, Epstein-Barr virus (EBV), and adenovirus infection is not increased by MSC treatment [47]. In addition, even *in vitro*, no decreases in cytomegalovirus- or EBV-specific T-cells occurred when MSCs were administered [16].

(iii) MSC transformation and cancer development

When pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells, are injected into immunocompromised mice, this leads to teratoma formation. In relation to HSC transplantation, it has been reported that donor-derived HSCs develop leukemia. MSCs are similarly a type of pluripotent stem cell, so there are concerns about malignant transformation and cancer development was in fact found after long-term culture of murine MSCs. However, there have been no reports of cancer development with clinical application of human MSCs. In order to obtain the number of MSCs needed for therapeutic use, they are cultured *ex vivo*, but, depending upon the culture conditions, cytogenetic abnormalities that are factors in MSC malignant transformation may be acquired, and cytogenetic abnormalities have in fact been found, in late passages, not in early passages, indeed, after three or four passages. However, these cells have not been found to form tumors in *in vivo* models, or to undergo malignant transformation *in vitro*. It has been reported that this is due to cultured MSCs having short telomeres, and thus becoming senescent, irrespective of cytogenetic abnormalities [48]. An additional cause is that the administered MSCs do not survive in the donor's tissue for an extended time. Therefore, no evidence for malignant transformation or cancer development was found, but it is nevertheless essential to be aware about the possibility of MSC-related cancer development in all clini-

cal studies using MSCs.

(iv) FBS used in culture

The number of MSCs present in BM, UC, etc., is very small in relation to the number needed for clinical use, and MSCs must therefore be cultured *in vitro*. Culture of the MSCs used in most previously reported clinical studies involved use of FBS as a growth factor. However, for several reasons, including the risk of contamination with pathogens (bacteria, viruses, mycoplasmas and prions), and the FBS composition not being consistent, and varying even between different lots of the same FBS formulation, it cannot be said that the qualitative consistency of cultured MSCs can be ensured. In addition, there is the potential for the proteins contained in FBS to give rise to allogeneic immunity, and for repeated administration to induce an allergic reaction. Recently, there has been an increase in the number of clinical studies carried out using MSCs cultured using platelet lysate instead of FBS, but the problems of infection risk and quality variation between lots have also not been resolved with platelet lysate. It is hoped that non-serum medium that can be guaranteed as being of consistent quality will be developed in future.

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