banks around the world. Thus, if researchers would use only the cell lines that have been tested by STR polymorphism analysis in cell banks, misidentification of human cell lines will be eradicated in the future.

On the other hand, virus infection is another critical issue in the use of immortalized cell lines. First, biohazard of cell lines should be excluded. Thus, in relation to the cell lines derived from liver cells, we routinely evaluate the infection of hepatitis B and C viruses (HBV and HCV). In relation to the cell lines derived from hematopoietic cells, we routinely evaluate the infection of human immunodeficiency virus type 1 (HIV1) and human T cell leukemia virus type 1 (HTLV-1). Second, the effects of virus infection on the characteristics of the infected cell lines must be taken into account, since it is clear that alteration of cell characteristics by virus infection leads to erroneous interpretation of the experimental results.

The AGS cell line infected with PIV5 is a sub-clone that has been deposited in a cell bank; the parental AGS cell line was not infected with PIV5.[2] Thus, the PIV5 infection of the AGS sub-line appears to have occurred during the culture prior to the deposition of the sub-line in the cell bank. Since PIV5 can infect the cells of various animal species,[1] PIV5 may be present in some of the materials typically used for cell culture, such as serum. Although the infection of AGS cells appears to be a secondary event during culture, it is still possible that primary cancer cells may be infected with PIV5 and that this infection produces resistance to IFN in the clinic.

Although the cell lines deposited in our cell bank and tested in this study were free of PIV5 infection, we want to emphasize the importance of testing PIV5 infection for immortalized cell lines, especially when they are used to analyze the mechanism of signal transduction of IFN (Fig. 2). In addition, it is important for the scientific community to be aware that cells may be persistently infected with viruses that significantly alter cellular physiology. Since many viruses able to infect cell materials are present and the effects of such infection on cellular physiology are largely unknown, the authentication of immortalized cell lines regarding virus infection is necessary and should be continued enthusiastically in cell banks around the world as one of the most pivotal missions.

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In vitro production of transfusable red blood cells

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Background The supply of transfusable red blood cells (RBCs) is not sufficient in many countries. If transfusable RBCs could be produced abundantly from certain resources it would be very useful. We have developed a method to produce enucleated RBCs efficiently from haematopoietic stem cells present in umbilical cord blood (Nat Biotechnol 2006; 24:1255). More recently, it was reported that enucleated RBCs could be abundantly produced from human embryonic stem (ES) cells (Blood 2008; 112:4475). The common obstacle for application of these methods is that these methods require very high cost to produce sufficient number of RBCs that are applicable in the clinic.

Aims If erythroid cell lines (immortalized cell lines) able to produce transfusable RBCs *in vitro* were established, they would be valuable resources. However, such cell lines have not been established. To evaluate the feasibility of establishing useful erythroid cell lines, we attempted to establish such cell lines from mouse ES cells.

Methods We developed a robust method to obtain differentiated cell lines following the induction of haematopoietic differentiation of mouse ES cells. Briefly, we have used feeder cells and several kinds of humoral factor so as to induce haematopoietic differentiation of mouse ES cells efficiently.

Results We established five independent haematopoietic cell lines using the method. Three of these lines exhibited characteristics of erythroid cells. Although their precise characteristics varied, each of these lines could differentiate *in vitro* into more mature erythroid cells, including enucleated RBCs. Following transplantation of these erythroid cells into mice suffering from acute anaemia, the cells proliferated transiently, subsequently differentiated into functional RBCs, and significantly ameliorated the acute anaemia. In addition, we did not observe formation of any tumours following transplantation of these cells (PLoS ONE 2008; 3:e1544).

Conclusions To the best of our knowledge, this is the first report to show the feasibility of establishing erythroid cell lines ablility to produce mature RBCs. Considering the number of human ES cell lines that have been established so far and the number of induced pluripotent stem cell lines that will be established in future, the intensive testing of a number of these lines for erythroid potential may allow the establishment of human erythroid cell lines similar to the mouse erythroid cell lines.

Introduction

Transfusion therapies involving red blood cells (RBCs), platelets and neutrophils depend on the donation of these cells from healthy volunteers. However, unpredictable adverse results can ensue from transfusion therapies

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because of the donation of cells from a very large number of anonymous volunteers. For example, transfusion of blood products that include hazardous viruses or prions is difficult to prevent completely because, occasionally, tests to detect them yield pseudo-negative results. This comment is, of course, not intended as a criticism of the volunteers who donate the haematopoietic cells. However, there is little doubt that RBCs, platelets and neutrophils produce *in vitro* will be candidate materials to replace cells donated from such a large group of anonymous individuals. To date, the use of haematopoietic cells produced *in vitro* has not proved practical for routine therapeutic applications.

RBC transfusion

Red blood cells transfusion was the first transplantation procedure to be established and is now routine and indispensable for many clinical purposes. However, in many countries the supply of transfusable materials is not always sufficient. In Japan, for example, the supply of RBCs with an AB/RhD(−) phenotype is always lacking because individuals with this RBC phenotype are rare. This problem of inequalities in the supply and demand for RBCs has stimulated interest in the development of *in vitro* procedures for the generation of functional RBCs from haematopoietic stem cells or progenitor cells present in bone marrow or umbilical cord blood (Fig. 1) [1–4].

Risk of RBC transfusion

It is important to realize that clinical risk factors associated with RBC transfusions have not been entirely excluded. One notable and very severe complication of transfusion is transfusion-related acute lung injury (TRALI), which has only recently been recognized and has not yet been eliminated. One of the possible causes of TRALI may be a factor in the transfused materials, such as antibodies against antigens on the leucocytes of the recipient. As mentioned above, this type of adverse outcome results from the dependence of blood transfusion on the supply of blood from many individuals. Problems may arise if this donated blood is utilized without sufficient preliminary trials being carried out on each sample. The use of RBCs derived from selected human resources may help to alleviate these problems because they can be intensively tested for pathogens before clinical use. Trial transfusions of a minimal amount of material into each recipient could also be performed to determine if there are unexpected complications. Therefore, the establishment of resources for *in vitro* production of RBCs (Fig. 1) will provide a means to alleviate many problems associated with RBC transfusion.

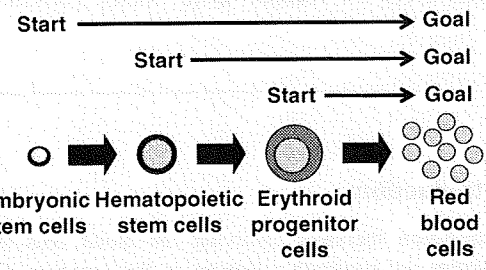

Fig. 1 *In vitro* production of red blood cells from embryonic stem cells, haematopoietic stem cells and erythroid progenitor cells.

RBC production from haematopoietic stem cells

The haematopoietic stem cells that are present in bone marrow and umbilical cord blood are promising materials for *in vitro* production of RBCs. In particular, umbilical cord blood cells are readily available as they are usually discarded. Provided the mother of a neonate consents the use of the umbilical cord, this material can provide a useful resource without any further complicating critical or ethical concerns.

Neildez-Nguyen *et al.* [1] reported that human erythroid cells (nucleated cells) produced on a large scale *ex vivo* could differentiate *in vivo* into enucleated RBCs. This study demonstrated that erythroid progenitor cells produced *in vitro* from haematopoietic stem and progenitor cells could have a clinical application as an alternative method for transfusing terminally differentiated RBCs. More recently, the same group described an *ex vivo* methodology for producing fully mature human RBCs from haematopoietic stem cells [2]. The enucleated RBCs produced by this approach are potentially even more valuable as they should be functional immediately after transfusion without requiring time for enucleation as is necessary with the erythroid cells.

In vitro expansion of erythroid progenitor cells

Many factors that act on haematopoietic stem and progenitor cells have been identified and analysed [5,6] and can be utilized for attempts to expand the numbers of these cells *in vitro*. Neildez-Nguyen *et al.* [1] developed a culture protocol to expand $CD34^+$ erythroid progenitor cells based on a 3-step expansion of cells by sequential supply of specific combinations of cytokines to the culture medium [7]. Their procedure allowed the *ex vivo* expansion of $CD34^+$ haematopoietic stem and progenitor cells into a pure erythroid precursor population. When these erythroid precursor cells were injected into non-obese diabetic, severe combined immunodeficient mice, the erythroid cells proliferated and

underwent terminal differentiation into mature enucleated RBCs.

Enucleation of erythroid progenitor cells

The mechanism of erythroblast enucleation, a critical step in RBC production, has not yet been fully elucidated [8,9]. The role of the interaction of erythroblasts with other cells, such as macrophages, is a controversial topic in this process [10–14]. Macrophages in retinoblastoma (Rb) gene-deficient embryos are unable to physically interact with erythroblasts and RBC production is impaired in these embryos [13]. In addition, *in vitro* production of enucleated RBCs from immature haematopoietic progenitor cells proceeds efficiently in the presence [2] but not in the absence [1] of feeder cells.

However, enucleation can apparently be initiated *in vitro* in erythroblasts that have been induced to differentiate *in vivo* to a developmental stage that is competent for nuclear self-extrusion [14,15]. We developed a method to produce enucleated RBCs efficiently *in vitro* without use of feeder cells [3]. Our system for expanding erythroid progenitor cells and inducing efficient enucleation of those progenitor cells is shown in Fig. 2. The increase in cell numbers observed in our method [3] was similar to that obtained by Neildez-Nguyen *et al.* [1] with a method that avoided the use of feeder cells.

The method we developed [3] included vascular endothelial growth factor and insulin-like growth factor-II (IGF-II) in the culture medium. These two factors have been reported to promote the survival, proliferation and/or differentiation of haematopoietic progenitors [16–18]. Consistent with these findings, these factors promoted the expansion of erythroid progenitors [3]. However, a much more important feature of our culture system is that it allowed erythroid cells to differentiate to a developmental stage competent for nuclear self-extrusion [3]. It has generally been thought that efficient enucleation of erythroblasts is largely dependent on signals mediated by cells in their local environment [10–13]. However, our data demonstrate that the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation [3]. Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation. In addition, as culture without the use of feeder cells is technically easier and less expensive, the method we developed has the potential to be a cost-effective means of producing transfusable RBCs on a large scale from immature haematopoietic progenitor cells.

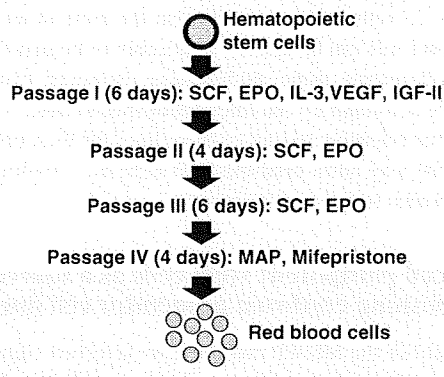

Fig. 2 Culture protocol developed by Miharada *et al.* [3] for the efficient production of enucleated red blood cells without feeder cells from haematopoietic stem cells. SCF, stem cell factor; EPO, erythropoietin; IL-3, interleukin-3; VEGF, vascular endothelial growth factor; IGF-II, insulin-like growth factor-II; MAP, mixture of D-mannitol, adenine and disodium hydrogen phosphate dodecahydrate.

RBC production from embryonic stem cells

The induction of haematopoietic cells from mouse ES cells is well established [19–25]. The stromal cell line, OP9 [26], has proved to be a useful feeder cell line for haematopoietic cell induction from mouse ES cells [27]. OP9 cells enable induction of both primitive and definitive erythropoiesis from mouse ES cells [28]. However, mass culture of mouse ES cells to produce pure erythroid progenitor cells and embryoid bodies can also be achieved through the addition of exogenous growth factors [29]. Similarly, the induction of haematopoietic cells from non-human primate ES cells [30,31] and human ES cells [32–41] has been reported recently.

Before any cells derived from human ES cells can be used in the clinic, it will be necessary to carry out preclinical studies, possibly in experimental primates [42]. Attempts have been made to induce haematopoietic cells from primate ES cells [18,31] using the CMK-6 line that was derived from the cynomolgus monkey [43]. We modified a previously described method [31] to induce differentiation in CMK-6 cells on OP9 stromal cells without embryoid body formation, and observed that haematopoietic cells could be induced from CMK-6 cells [18]. However, OP9 cells are very sensitive to variations in maintenance conditions, including medium source and serum lot, and this sensitivity can influence the ability of OP9 cells to support haematopoiesis [37]. To avoid any possible difficulties posed by this characteristic, we sought to identify an alternative feeder cell line and also searched for specific humoral factors that improved the efficacy of the method. As a result, we found that use of the well-known cell line, C3H10T1/2 along with IGF-II had considerable and beneficial effects on induction of haematopoietic cells from primate ES cells [18]. Furthermore, the C3H10T1/2 cells were capable of long-lasting *in vitro* production of terminally differentiated blood cells including RBCs [18].

Given that the ultimate goal of developing RBCs from human ES cells is for clinical application, then the use of feeder cells derived from non-human species should be avoided. It has been reported that human ES cells express an immunogenic non-human sialic acid when they are cultured with non-human-derived materials [44]. This observation is very critical and merits further intensive investigation. Before clinical applications of blood cells derived from human ES cells are undertaken, preclinical *in vivo* studies using experimental primates are likely to provide essential safety information. With regard to RBCs, the method we developed [18] may enable a preclinical study in primates as it is able to produce abundant RBCs from ES cells. If *in vivo* immunological reactions originating from non-self antigens peak at negligible levels in experimental primates, then RBCs obtained from human ES cells using a method involving non-human feeder cells may still be suitable for clinical applications.

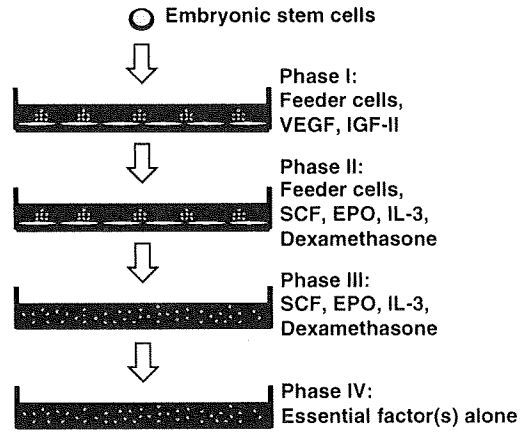

Fig. 3 Culture protocol developed by Hiroyama *et al.* [46] to establish an erythroid progenitor cell line from embryonic stem cells. VEGF, vascular endothelial growth factor; IGF-II, insulin-like growth factor-II; SCF, stem cell factor; EPO, erythropoietin; IL-3, interleukin-3.

Establishment of erythroid progenitor cell lines able to produce transfusable RBCs

It is notable that the efficiency of generation of erythroid progenitor cells and RBCs varies depending on the culture methods employed and the ES cell lines used. However, even with optimal experimental procedures and the most appropriate ES cell line, the generation of abundant RBCs directly from primate ES cells is a time-consuming process [18]. If human erythroid progenitor cell lines that efficiently produce transfusable and functional RBCs can be established, they would represent a much more valuable resource for producing RBCs than ES cell lines (Fig. 1).

Several mouse and human erythroid cell lines have been established. However, to the best of our knowledge, there is no cell line that can efficiently differentiate into enucleated RBCs. It is generally difficult to establish haematopoietic cell lines from adult haematopoietic stem and progenitor cells as both are sensitive to DNA damage and are unable to maintain the lengths of telomere repeats on serial passage [45]. In contrast, ES cells are relatively resistant to DNA damage and maintain telomere lengths on serial passage [45]. Therefore, these characteristics of ES cells may be advantageous for the establishment of cell lines as differentiated cells derived from ES cells may retain them.

Recently, we developed a robust method to obtain differentiated cell lines following the induction of haematopoietic differentiation of mouse ES cells (Fig. 3), and established five independent haematopoietic cell lines using this method [(46]. Three of these lines exhibited characteristics of erythroid cells, and they were designated mouse ES cell-derived erythroid progenitor (MEDEP) cell lines. Although their precise characteristics varied, each of the MEDEP lines could differentiate *in vitro* into more mature erythroid cells, including enucleated RBCs. Following transplantation into mice suffering from acute anaemia, MEDEP cells proliferated transiently and subsequently differentiated into functional RBCs. Treated mice showed a significant amelioration of acute anaemia. In addition, MEDEP cells did not form tumours following transplantation into mice. This report was the first to demonstrate the feasibility of establishing erythroid cell lines ability to produce mature RBCs.

At present, the mechanism underlying the establishment of differentiated cell lines from ES cells has not been elucidated. Nevertheless, our data clearly indicate that useful erythroid cell lines can be reproducibly obtained from mouse ES cells. Given that differentiation strategies developed for mouse ES cells often differ from those applied to human ES cells [47], it is likely that the method we developed [46] will not be directly applicable to human ES cells and will require some modification. However, given the number of human ES cell lines established to date, it is possible that intensive testing of these lines for their erythroid potential may allow establishment of human erythroid cell lines similar to those of the mouse.

Induced pluripotent stem cells as a source for establishing erythroid progenitor cell lines

To establish the MEDEP cell lines, we screened eight types of mouse ES cell line and succeeded in establishing MEDEP cell lines from three of these [46]. By extrapolation from this result, it may be that many more human ES cell lines than currently available worldwide will be necessary to establish usable erythroid cell lines. In this context, the establishment of human induced pluripotent stem (iPS) cell

lines [48–52] should help to solve the problem of a potential shortfall as human iPS cells have very similar characteristics as human ES cells.

Clinical application of erythroid progenitor cell lines

We reported that MEDEP cells did not exhibit tumorigenicity *in vivo* [46]. Nevertheless, the tumorigenic potential of any human erythroid cell line will need to be thoroughly analysed prior to clinical use [53,54]. In addition, it may be advisable to engineer these cells in such a way that they are eliminated if a malignant phenotype arises for any reason [55]. Alternatively, the use of terminally differentiated cells that no longer has the capability of proliferating should allow clinical applications of ES cell derivatives without the associated risk of tumorigenicity. Thus, for example, RBCs lack nuclei following terminal differentiation, and are highly unlikely to exhibit tumorigenicity *in vivo*. As such, even if the original ES cells and/or their derivatives possessed abnormal karyotypes and/or genetic mutations, they might nonetheless be useful for clinical applications, provided that they could produce functional RBCs. Indeed, the MEDEP lines included many cells possessing abnormal karyotypes, however, the vast majority of the cells in each cell line nevertheless differentiated into mature erythroid cells and transplantation of these cells significantly ameliorated anaemia [46]. In general, immortalized cell lines are not necessarily homogenous in karyotype even after cloning. The emergence of cells possessing abnormal karyotypes is often observed following continuous culture of immortalized cell lines. Hence, periodical recloning and selection of cell lines is recommended to maintain their genotype.

As described above, various methods have been developed that enable the production of enucleated RBCs from human haematopoietic stem and progenitor cells [1–3]. Therefore, once appropriate erythroid cell lines have been established, it should be possible to use these methods to produce enucleated RBCs *in vitro*. As RBCs are much smaller than normal nucleated cells, RBCs produced *in vitro* could be selected by size prior to use in the clinic so as to exclude nucleated cells, for example, by filtration. In addition, X-ray irradiation might be useful for eradicating any contaminating nucleated cells without affecting the RBCs.

Another potential obstacle to the clinical use of ES cell derivatives is that of immunogenicity [56,57]. Transplanted MEDEP cells could not ameliorate acute anaemia in mouse strains other than those from which each individual cell line was derived or in immunodeficient mice [46], suggesting immunological rejection in heterologous strains. Hence, the clinical application of erythroid cell lines will require use of many cell lines that express different major histocompatibility antigens. However, *in vitro* generated RBCs need to be compatible with ABO and RhD antigens alone (eight types in total), meaning that eight types of erythroid cell line would suffice to generate RBCs of all the different blood types required for clinical application. Recently, a technique was developed for the removal of the A and B antigens from RBCs [58]. Thus, removal of the antigens from RBCs that initially expressed A or B antigens, may allow transfusion of these RBCs into individuals that possess antibodies against A or B antigens. Such a technique might also be useful for transfusion of RBCs generated from erythroid cell lines *in vitro*. Of note, the establishment of a human erythroid cell line lacking the genes to produce A, B and RhD antigens would be a very useful resource for clinical application because such a cell line would produce O/RhD(−) RBCs, which would in theory, be transfusable into all individuals.

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