BRC5 cells appeared to be less effective for amelioration of anemia than MEDEP-E14 cells (Hiroyama et al., 2008). Given that the in vitro proliferation activity of MEDEP-BRC5 cells was lower than that of MEDEP-E14 cells, the in vivo proliferation activity of MEDEP-BRC5 cells might have also been lower than that of MEDEP-E14 cells (Hiroyama et al., 2008). In addition, hemoglobin synthesis in MEDEP-BRC5 might have been less efficient than in MEDEP-E14 (Hiroyama et al., 2008).

Immunogenicity of human ES cell derivatives is one of the potential obstacles to their clinical use (Drukker and Benvenisty, 2004; Boyd et al., 2005). Indeed, transplanted MEDEP cells do not ameliorate acute anemia in mouse strains other than those from which the individual lines were derived or in immuno-deficient mice, suggesting immunological rejection by the heterologous strains. Hence, if human erythroid cell lines are to be established, the clinical application of these cells might involve application of a number of lines that express different major histo-compatibility (MHC) antigens.

4.5 Lack of tumorigenicity of MEDEP

Approximately three months after transplantation, Venus-positive cells were absent from the bone marrow and spleen of mice transplanted with MEDEP-E14-Venus cells (Hiroyama et al., 2008). In addition, although we examined all other transplanted mice up to 6 months after transplantation, no tumors were observed in MEDEP-transplanted mice or MEDMC-transplanted control mice (Hiroyama et al., 2008). Furthermore, subcutaneous transplantation of MEDEP cells ($2x10^7$ cells/injection site) did not give rise to any tumors, whereas subcutaneous transplantation of the same number of parent ES cells led to the formation of a teratoma (Hiroyama et al., 2008).

What mechanism underlies the lack of tumorigenicity of MEDEP? MEDEP cells can only be successfully cultured in the presence of excess growth factor(s) and cannot proliferate or survive without such growth factor(s). Therefore, MEDEP cells cannot proliferate or survive in vivo in the presence of the normal range of growth factors. Indeed, when MEDEP-E14-Venus cells were transplanted into mice that were not in an anemic condition, an increase in RBC numbers in the peripheral blood was not observed and Venus-positive cells were not detected in the bone marrow or spleen by flow cytometry a few days after transplantation. By contrast, when MEDEP cells were transplanted into mice suffering from acute anemia, the concentration of growth factors was upregulated in the mice due to anemia and thus MEDEP cells proliferated. Following recovery from anemia, there was a reduction in growth factors in the serum and MEDEP cells no longer proliferated or survived. We suggest that the concentration of growth factors determines whether or not MEDEP cells can proliferate with the potential for tumorigenicity.

Therefore, establishment of growth factor-dependent erythroid cell lines may be of particular value for clinical applications. Nevertheless, when human erythroid cell lines are established, the tumorigenic potential of these lines will still need to be exhaustively analyzed prior to their use in the clinic (Vogel, 2005; Hentze et al., 2007). It may also be advisable to engineer such cells so that they can be eliminated should a malignant phenotype arise for any reason (Schuldiner et al., 2003).

4.6 RBCs derived from MEDEP are functional in vivo

To confirm that the RBCs derived from the transplanted MEDEP cells are functional in vivo, we monitored the response of transplanted mice to a second induction of hemolysis.

Hemolysis was induced and followed by cell transplantation; a second induction of hemolysis was performed five days after cell transplantation (Figure 7) (Hiroyama et al., 2008). Analysis of blood counts was not performed at any time point in this experiment, because collection of peripheral blood would affect the results. We observed that one of the eight mice in the group transplanted with MEDEP-E14 cells died, while seven of the eight mice in the group transplanted with control cells (MEDMC-NT2) died (Figure 7). Mice that did not receive any transplanted cells showed a mortality rate similar to that of mice transplanted with control cells. This result is consistent with observed increase in RBC numbers five days after cell transplantation (Figure 6). In other words, this result indicated that RBCs derived from MEDEP cells were functional in vivo and that mice transplanted with MEDEP cells could survive a severe acute anemia caused by a second induction of hemolysis.

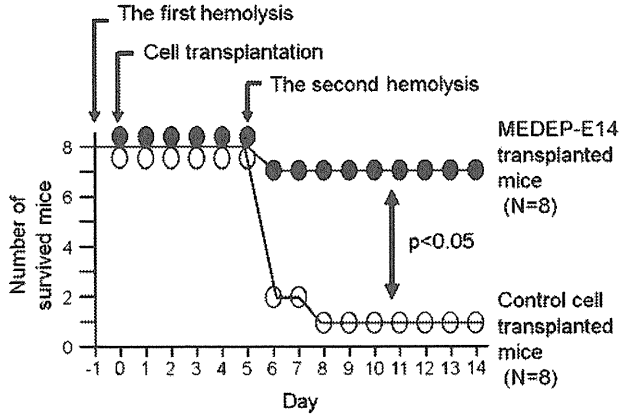

Fig. 7. Survival curves of mice transplanted with MEDEP or control cells following severe acute anemia.

5. Strategy for clinical use of hematopoietic cells produced in vitro

The most critical obstacle to use of ES cell-derived cells is the potential for tumorigenicity. First, there is a risk that the transplanted cells include ES cells and that such contaminant cells could be tumorigenic. Second, even if ES cells can be completely excluded from the transplanted sample by some method, the transplanted cells may revert to an ES-like state and could be tumorigenic. Therefore, when we consider the possibility of clinical application of ES cell-derived cells, thorough preclinical studies need to be carried out.

Establishment of erythroid progenitor cell lines from human ES cells would provide a valuable source of material for further utilization. However, the risk of tumorigenicity of such cells is similar to that of ES cells since they are immortalized. As mentioned above, growth factor dependent cell lines that can proliferate only in the presence of excess growth factor(s) in vitro might offer candidate cell lines for use in the clinic. Those cells could be transplanted with a simultaneous injection of the requisite growth factors and, after achievement of their clinical purpose, the cells would be eliminated due to eventual deprivation of growth factor(s). In any case, the risk of tumorigenicity must be taken into account in the transplantation of nucleated cells derived from immortalized cells, since we cannot predict at present how these cells will behave after transplantation.

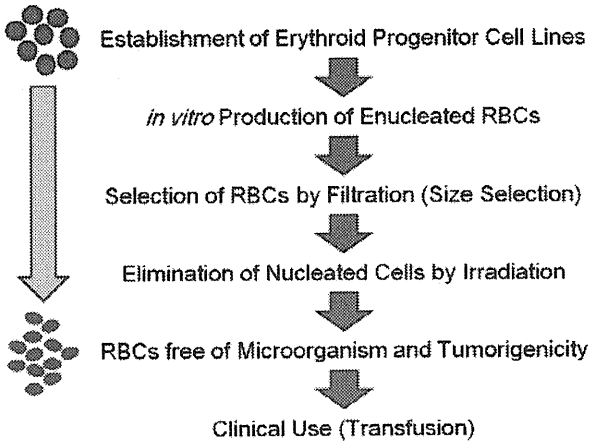

Fig. 8. Strategy for production of transfusable RBCs from immortalized erythroid cell lines.

On the other hand, RBCs and platelets are very specific cells in the body that lack nuclei following terminal differentiation. These anuclear cells cannot form tumors in vivo. Therefore, RBCs and platelets, even if they are derived from immortalized cells, could be transfused without concerns about possible tumorigenicity. Following production of RBCs or platelets in vitro, pure populations can be selected by size using filtration, since they are much smaller than normal nucleated cells. In addition, any nucleated cells, which are still present in the sample after size selection, could be eliminated by irradiation. Irradiation of RBC samples is already routinely performed to eliminate lymphocytes in some clinical protocols. Therefore, if we can establish an in vitro culture system that enables abundant production of RBCs or platelets, then the cells could be applied in the clinic using the procedures described above (Figure 8). To establish such a culture system, progenitor cell lines able to produce enucleated cells in vitro will be essential.

6. Establishment of RBC progenitor cell lines from human ES cells or human iPS cells

The reproducible establishment of MEDEP cell lines described above strongly suggests that similar erythroid cell lines could also be established from human ES cells. We, therefore, sought to establish human erythroid progenitor cell lines. The methods used to induce hematopoietic cells from ES cells and to culture the induced hematopoietic cells are similar to those established for MEDEP cell lines (Figure 3), with the exception that the corresponding human factors were applied and IL-3 was not used at all. Exclusion of IL-3 was based on our finding that the compound was not necessary for establishment of MEDEP cell lines (see above).

Initially, we used three human ES cell lines, KhES-1, KhES-2 and KhES-3, that had been established in Japan. However, we were unable to induce hematopoietic cells from all three lines and, compared to mouse ES cells, the efficiency of production of hematopoietic cells was extremely low. As a result, we have yet been successful in establishing immortalized cell lines from the three ES cell lines.

During the course of the experiments using these human ES cell lines, a breakthrough discovery in the field of regenerative medicine was reported, namely, the establishment of human iPS cells (Takahashi et al., 2007) following that of mouse iPS cells (Takahashi and Yamanaka, 2006). This discovery prompted us to establish human iPS cells, since the characteristics of pluripotent stem cells, such as ES cells, differ among cell lines. In other words, we speculated that we could obtain iPS cell lines that could have the ability to differentiate into hematopoietic cells. We were able to establish a number of human iPS cell lines using fibroblast-like cells derived from neonatal tissues (Fujioka et al., in press). Fortunately, we were able to induce abundant numbers of hematopoietic cells from some of these iPS cell lines and also to establish immortalized hematopoietic cell lines from the induced hematopoietic cells. Currently, we are investigating the characteristics of these immortalized hematopoietic cell lines. Some seem to be erythroid cell lines.

7. Concluding remarks

We propose that by utilizing ES cells or iPS cells it will be possible to establish human erythroid progenitor cell lines able to produce enucleated RBCs. RBCs produced by in vitro culture of such erythroid cell lines could be applied in the clinic following size selection and elimination of nucleated cells by irradiation.

Once an erythroid cell line able to produce O/RhD(-) RBCs is established, it could be used as necessary to produce RBCs for transfusion into patients around the world. Obviously, there would be a few patients for whom this approach would not be practicable, such as those possessing Rh-null RBCs.

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Review Article

Plasticity of Cells and *Ex Vivo* Production of Red Blood Cells

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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. Our group has developed a method to produce enucleated RBCs efficiently from hematopoietic stem/progenitor cells present in umbilical cord blood. More recently, it was reported that enucleated RBCs could be abundantly produced from human embryonic stem (ES) cells. The common obstacle for application of these methods is that they require very high cost to produce sufficient number of RBCs that are applicable in the clinic. If erythroid cell lines (immortalized cell lines) able to produce transfusable RBCs *ex vivo* were established, they would be valuable resources. Our group developed a robust method to obtain immortalized erythroid cell lines able to produce mature RBCs. To the best of our knowledge, this was the first paper to show the feasibility of establishing immortalized erythroid progenitor cell lines able to produce enucleated RBCs *ex vivo*. This result strongly suggests that immortalized human erythroid progenitor cell lines able to produce mature RBCs *ex vivo* can also be established.

1. Introduction

Transfusion therapies involving RBCs, platelets, and neutrophils depend on the donation of these cells from healthy volunteers. However, unpredictable adverse results can ensue from transfusion therapies 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. There is little doubt that RBCs, platelets, and neutrophils produced *ex vivo* would be candidate materials to replace cells donated from such a large group of anonymous individuals.

The development of technologies such as PCR and gene knockout that enable the manipulation of an organism's genetic material contributed tremendously to progress in the life sciences in the final decades of the last century. This century looks to continue this progress through the development of further new technologies relating to cell manipulation.

2. Discovery of Plasticity in Terminally Differentiated Cells

It was believed for a long time that epigenetic modifications in differentiated somatic cells were irreversible. This meant that terminally differentiated cells could never return to being immature cells. However, in 1962, it was reported that the nuclei of somatic cells of an amphibian (frog) were reprogrammed following transfer into enucleated unfertilized eggs [1]. Following transfer of a somatic cell nucleus, the egg could undergo cell division and differentiate to produce an adult frog. This result clearly indicated that epigenetic modifications in terminally differentiated somatic cells were reversible. Dr. John Gurdon, who performed this groundbreaking study, received the Albert Lasker Basic Medical Research Award in 2009.

Initially, many biologists believed that this reversibility of epigenetic modifications in terminally differentiated cells was restricted to amphibian somatic cells and did not occur in mammalian somatic cells. However, in 1997, a nuclear transfer experiment in sheep in which somatic nuclei were

transferred into unfertilized eggs showed that epigenetic modifications in terminally differentiated mammalian somatic cells were also reversible [2]. This experiment famously resulted in the birth of the first live cloned sheep, named "Dolly".

3. Immortalization of ES Cells

The methodology for isolating and culturing mouse ES cells was first developed in 1981 [3] and has aided research in a wide range of biological studies. Dr. Martin Evans, who developed the technology for establishing mouse ES cell lines, was awarded a Nobel Prize in 2007 together with Dr. Mario Capecchi and Dr. Oliver Smithies, who developed homologous recombination technology in mouse ES cells. As a result of these technical advances, functional analysis of genes has progressed considerably using mice with gene knockouts or other genetic modifications.

It is well known that mouse cells can be immortalized simply by continuous *in vitro* culture, for example, using the so-called "3T3 protocol". One widely exploited example of an immortalized cell line is NIH3T3, which continues to be used in a wide range of experiments. In contrast, it is not possible to immortalize human somatic cells in a similar manner and this difficulty gave rise to the widespread assumption that it would not be possible to establish human ES cell lines. However, in 1998, 17 years after the first establishment of mouse ES cell lines, it was reported that human ES cell lines could also be produced by continuous *in vitro* culture [4].

4. Therapeutic Cloning

The ability to reprogram mammalian somatic cells by nuclear transfer and to establish human ES cell lines stimulated medical scientists to investigate the creation of ES cell lines using nuclear transfer as a potential means of achieving "therapeutic cloning". If this technology could be established as a viable therapy, then patients who would benefit from somatic cell transplantation could be treated with nuclear-transferred ES cells produced using their own somatic cells, which would avoid the possibility of transplant rejection as the cells possess the same major histocompatibility (MHC) antigens as host tissue.

Although an earlier report of successful therapeutic cloning by a group in Korea proved false, it was recently reported that primate ES cell lines have been established by nuclear transfer technology [5]. Since unfertilized primate eggs are much more fragile than those of rodents, it may still take some time to establish the technology for use in human therapeutic cloning. However, the prospect of using such therapy no longer seems to be so distant.

5. A Search for Alternative Technologies to Therapeutic Cloning

An important limitation to the use of therapeutic cloning is that it requires unfertilized eggs. Human eggs are very difficult to obtain, and, moreover, their use for this purpose also raises serious ethical issues. For these reasons, a search has been initiated for alternative methodologies that avoid nuclear transfer. One approach has been to search for factors in unfertilized eggs that may be required for the reprogramming of transferred somatic nuclei. Another avenue of research has been to elucidate which genes specifically function in ES cells, since these genes may maintain the undifferentiated state of ES cells, and thus might be able to induce reprogramming of nuclei in terminally differentiated somatic cells.

The research group led by Dr. Shinya Yamanaka reported the first success in the latter approach. They were able to induce differentiated mouse somatic cells to become pluripotent stem cells by the application of four defined factors [6]. The enforced expression of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc in terminally differentiated somatic cells induced cellular reprogramming and changed the cells into ES-like pluripotent stem cells. These reprogrammed cells were named "induced pluripotent stem (iPS) cells". Subsequently, in the year after establishment of human iPS cell lines was first reported, several other groups also succeeded with this methodology [7–10]. Dr. Shinya Yamanaka, who developed the method, was given the Albert Lasker Basic Medical Research Award in 2009 together with Dr. John Gurdon.

The mechanisms underlying the reprogramming of terminally differentiated somatic cells following the enforced expression of the four factors remain to be elucidated. It is now known that expression of these factors after exogenous introduction is completely suppressed in established iPS cells. Thus, the factors seem to be required only for the reprogramming process but not for maintenance of pluripotency. Regardless of the mechanisms involved, this discovery clearly indicated that terminally differentiated somatic cells could be reprogrammed without nuclear transfer into unfertilized eggs and opened a new dawn for therapeutic cloning [11–13].

6. *Ex Vivo* RBC Production from Hematopoietic Stem/Progenitor Cells

The rapid progress relating to cell manipulation technology described above prompted many scientists in various fields to consider cell therapy using the cells produced and/or manipulated *ex vivo*. The scientists in the field of hematology are naturally aiming to produce the terminally differentiated blood cells able to use in the clinic. RBC 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 *ex vivo* procedures for the generation of functional RBCs from hematopoietic stem cells or progenitor cells.

The hematopoietic stem cells that are present in bone marrow and umbilical cord blood are promising materials

for *ex vivo* production of RBCs. In particular, umbilical cord blood cells are readily available, as they are usually discarded. Provided the mother of a neonate consents to use of the umbilical cord, this material can provide a useful resource without any further complicating critical or ethical concerns.

Neildez-Nguyen et al. reported that human erythroid cells (nucleated cells) produced on a large scale *ex vivo* could differentiate *in vivo* into enucleated RBCs [14]. They 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 [14]. This study demonstrated that erythroid progenitor cells produced *ex vivo* from hematopoietic stem and/or progenitor cells could have a clinical application as an alternative method for transfusing terminally differentiated RBCs. Later, the same group described an *ex vivo* methodology for producing fully mature human RBCs from hematopoietic stem/progenitor cells [15]. 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.

7. Enucleation of Erythroid Progenitor Cells

The mechanism of erythroblast enucleation, a critical step in RBC production, has not yet been fully elucidated [16, 17]. The role of the interaction of erythroblasts with other cells, such as macrophages, is a controversial topic in this process [18–22]. Macrophages in retinoblastoma gene- (*Rb*-) deficient embryos are unable to physically interact with erythroblasts, and RBC production is impaired in these embryos [21]. In addition, *in vitro* production of enucleated RBCs from immature hematopoietic progenitor cells proceeds efficiently in the presence [15] but not in the absence [14] of feeder cells.

However, enucleation can apparently be initiated *ex vivo* in erythroblasts that have been induced to differentiate *in vivo* to a developmental stage that is competent for nuclear self-extrusion [22, 23]. Consistent with these findings, our group discovered a method to produce enucleated RBCs efficiently *ex vivo* without use of feeder cells [24]. Our system for expanding erythroid progenitor cells and inducing efficient enucleation of those progenitor cells is shown in Figure 1.

The method we developed included VEGF and IGF-II in the culture medium [24]. These two factors have been reported to promote the survival, proliferation, and/or differentiation of hematopoietic progenitors [25–27]. Consistent with these findings, these factors promoted the expansion of erythroid progenitors [24]. 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 [24]. It has generally been thought that efficient enucleation of erythroblasts is largely dependent on signals mediated by cells in their local environment [18–21]. However, the data we reported demonstrate that the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation [24]. Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation. In addition, since 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 hematopoietic stem/progenitor cells.

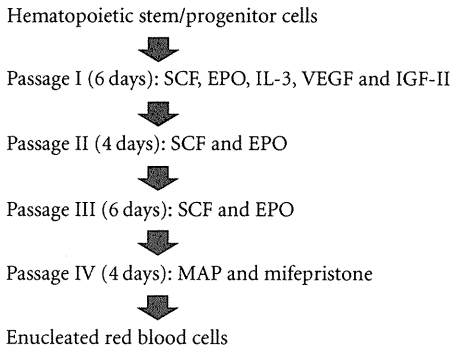

FIGURE 1: Culture protocol for the efficient production of enucleated red blood cells without feeder cells from hematopoietic stem/progenitor cells. Passage I~III are the steps to expand erythroid progenitor cells. Passage IV is the step to induce enucleation of progenitor cells. SCF, stem cell factor; 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.

8. RBC Production from ES/iPS Cells

ES/iPS cells possess the potential to produce various differentiated cells able to function *in vivo*, and thus represent another promising resource for RBC production *ex vivo*. Furthermore, since ES/iPS cell lines are immortalized, they can be used repeatedly and have potential to produce abundant differentiated cells in the quantities required for clinical use. However, it will be important to carry out routing screening of the ES/iPS cell lines for *de novo* chromosomal aberrations and/or genetic mutations that may arise during culture, before these long-term cell cultures are applied in the clinic. Unsurprisingly, there is now a widespread and enthusiastic debate on standardization of the characteristics of ES/iPS cells for regenerative medicine protocols that exploit these cell lines. In our opinion, since chromosomal aberrations and genetic mutations are inevitable in long-term cell cultures, only ES/iPS cell lines that have been cultured for a limited period, for example, less than 30 passages, should be selected for clinical use.

Hematopoietic cells, including those in the erythroid lineage, have been generated from mouse ES cells [28–31], nonhuman primate ES cells [32–34], and human ES cells [35–41]. Our group has also established a long term *in vitro* method for culturing hematopoietic cells derived from ES cells of the nonhuman primate, the cynomolgus monkey [27]. Recently, abundant productions of mature RBCs from human ES cells [42] and human iPS cells [43] were also reported.

9. Establishment of Immortalized Erythroid Progenitor Cell Lines Able to Produce Enucleated RBCs

As described above, we can now produce mature RBCs by *in vitro* culture of ES/iPS cells or the hematopoietic stem/progenitor cells present in umbilical cord blood. In practice, however, the efficiency of RBC generation varies with the quality of the ES/iPS cell line, or the umbilical cord blood sample. Since ES/iPS cell lines can be utilized repeatedly, derivation of RBCs from ES/iPS cells appears to be more practical. However, even with optimal experimental procedures and the most appropriate ES/iPS cell line the generation of abundant RBCs directly from ES/iPS cells is a costly and time-consuming process. If immortalized human erythroid progenitor cell lines can be established that have efficient production of mature RBCs, they would provide a much more useful resource than ES/iPS cell lines.

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 hematopoietic cell lines from adult hematopoietic stem and progenitor cells, as both are sensitive to DNA damage and are unable to maintain the lengths of telomere repeats on serial passage [43]. In contrast, ES cells are relatively resistant to DNA damage and maintain telomere lengths on serial passage [44]. Therefore, these characteristics of ES/iPS cells may be advantageous for the establishment of cell lines, since differentiated cells derived from ES/iPS cells may retain them. In fact, an erythroid cell line has been established from *in vitro*-differentiated GATA-1-deficient mouse ES cells [45].

Recently, we developed a robust method to obtain differentiated cell lines following the induction of hematopoietic differentiation of mouse ES cells (Figure 2) and established five independent hematopoietic cell lines using this method [46]. Three of these lines exhibited characteristics of erythroid cells, and they were designated **m**ouse **E**S cell-**d**erived **e**rythroid **p**rogenitor (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 anemia, MEDEP cells proliferated transiently and subsequently differentiated into functional RBCs. Treated mice showed a significant amelioration of acute anemia. In addition, MEDEP cells did not form tumors following transplantation into mice. This paper was the first to demonstrate the feasibility of establishing immortalized erythroid cell lines able to produce mature RBCs.

After the work above, we have continuously cultured the established MEDEP cell lines so as to observe whether the characteristics of them were stable. After long-term cultures for more than one and a half year, all MEDEP cell lines maintained the characteristics able to differentiate into mature erythroid cells producing hemoglobin abundantly (Figure 3). Of note, the characteristics of one of the MEDEP cell lines, MEDEP-BRC5, have changed to that able to produce enucleated RBCs very efficiently; that is, more

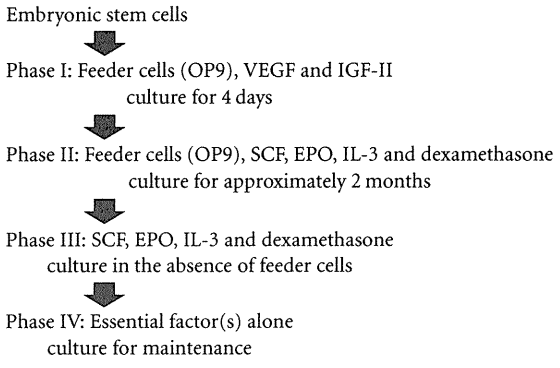

FIGURE 2: Culture protocol to establish erythroid progenitor cell lines from embryonic stem cells. In most cases, the cells failed to proliferate within two months of the initial induction of differentiation from ES cells. Induced cells that could proliferate continuously for approximately two months (60 days) were subsequently cultured in the absence of OP9 cells and in the presence of hematopoietic humoral factors. Cells that could proliferate in the absence of OP9 cells were cultured further. Approximately four months after the initial induction of differentiation of the cells, we evaluated the factors that were essential for the proliferation of each cell line. VEGF, vascular endothelial growth factor; IGF-II, insulin-like growth factor-II; SCF, stem cell factor; EPO, erythropoietin; IL-3, interleukin-3.

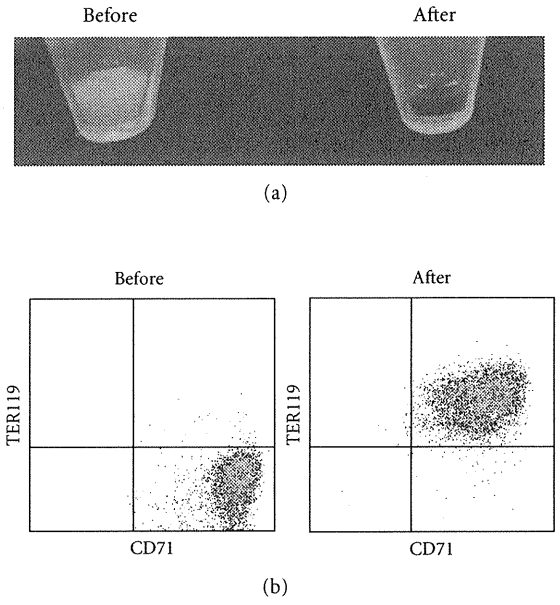

FIGURE 3: *In vitro* differentiation of a MEDEP cell line, MEDEP-BRC5. MEDEP-BRC5 cells cultured continuously for more than one and a half year was analyzed. The *in vitro* differentiation of MEDEP-BRC5 was performed by culture for four days after deprivation of stem cell factor and addition of erythropoietin. (a) Cell pellets before and after *in vitro* differentiation. Red cell pellet indicates abundant hemoglobin production in the cells. (b) Flow cytometric analyses. Before and After, the cells before and after *in vitro* differentiation; CD71, transferrin receptor; TER119, a cell surface antigen specific for mature erythroid cells.

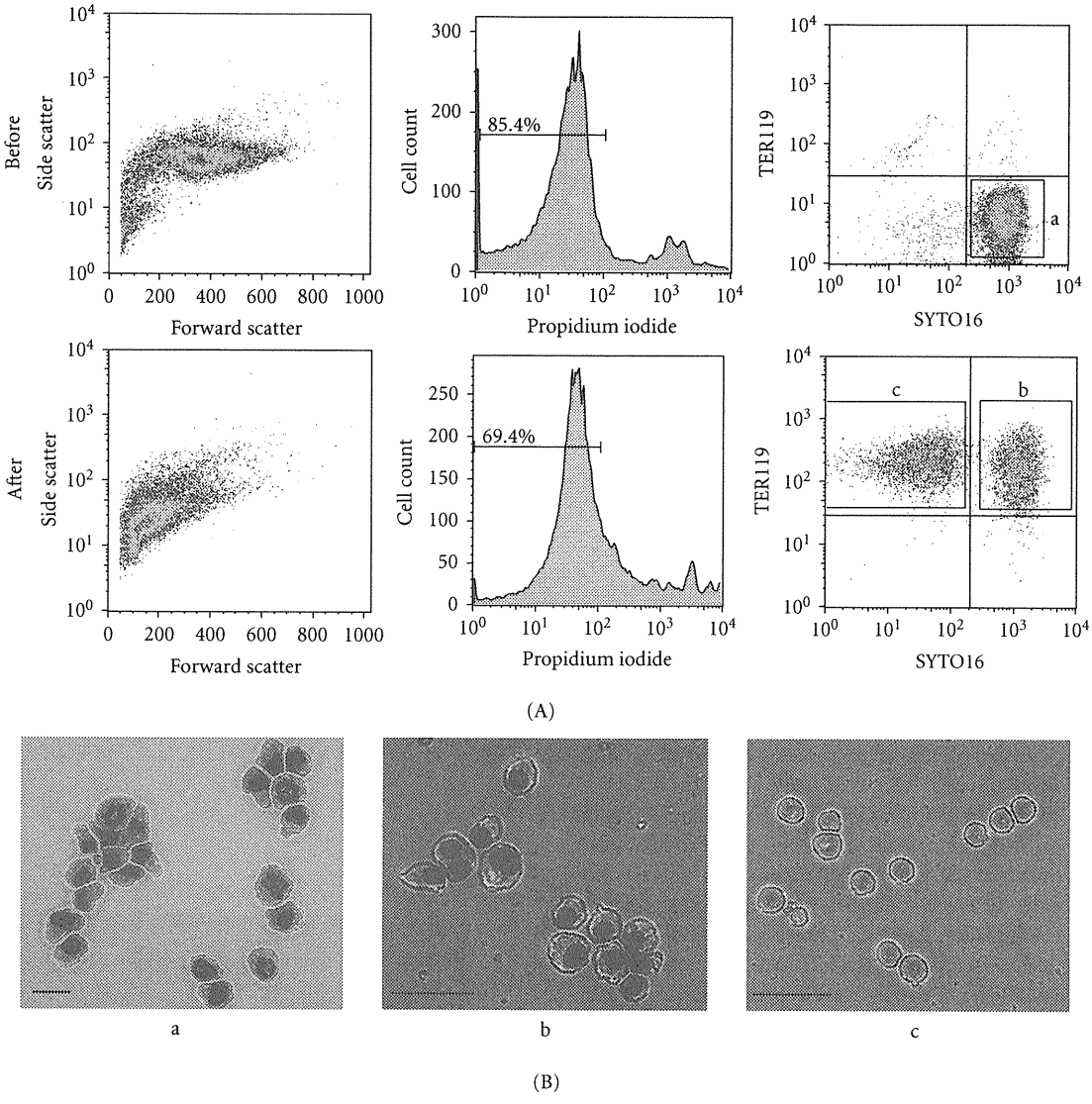

FIGURE 4: *In vitro* enucleation of a MEDEP cell line, MEDEP-BRC5. The *in vitro* differentiation of MEDEP-BRC5 was performed as described in Figure 3. (A) Flow cytometric analyses. Before and After, the cells before and after *in vitro* differentiation. Percentages of propidium iodide-negative viable cells are shown. TER119, see Figure 3. SYTO16, a cell membrane-permeable fluorochrome dye to stain nucleic acids. Following *in vitro* differentiation, 52% of the cells were TER119-positive and SYTO16-negative cells, that is, the cells lacking nuclei. (B) Morphology of cells collected from the a, b, and c fractions shown in (A). Scale bars indicate 20 μm.

than 50% of the cells were the enucleated RBCs following the induction of differentiation into mature erythroid cells (Figure 4). This result demonstrates that the interaction of erythroid progenitor cells with other cells is not necessary for efficient enucleation.

At present, the mechanism underlying the establishment of differentiated cell lines from ES cells has not been elucidated. Nevertheless, the data we reported 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], the method we developed [46] may not be directly applicable to human ES cells and will require some modification.

10. iPS Cells as a Source for Establishing Immortalized 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 iPS cell lines [7–10] should help to solve the problem of a potential shortfall, since human iPS cells have very similar characteristics as human ES cells.

Therefore, we attempted to establish human iPS cell lines and were able to establish a number of human iPS cell lines using fibroblast-like cells derived from neonatal tissues [48]. Fortunately, we were able to induce abundant numbers of hematopoietic cells from some of these iPS cell lines and also to establish immortalized hematopoietic cell lines from the induced hematopoietic cells (unpublished results). Currently, we are investigating the characteristics of these immortalized hematopoietic cell lines. Some seem to be erythroid cell lines.

11. 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 analyzed prior to clinical use [49, 50]. 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 [51].

Alternatively, the use of terminally differentiated cells that no longer have the capability of proliferating should allow clinical applications of ES/iPS 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/iPS cells and/or their derivatives possessed abnormal karyotypes and/or genetic mutations, they might, nonetheless, be useful for clinical applications, provided that they can produce enucleated 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 anemia [46].

As described in this paper, various methods have been developed that enable the *ex vivo* production of enucleated RBCs from human hematopoietic stem/progenitor cells [14, 15, 24] and ES/iPS cells [42, 43]. Therefore, once appropriate erythroid progenitor cell lines have been established, it should be possible to apply these methods for producing enucleated RBCs *ex vivo*. Since RBCs are much smaller than normal nucleated cells, RBCs produced *ex vivo* 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/iPS cell derivatives is that of immunogenicity [52, 53]. Transplanted MEDEP cells could not ameliorate acute anemia 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 (MHC) antigens. However, *ex vivo*-generated RBCs need to be compatible with ABO and RhD antigens alone. Furthermore, the establishment of an immortalized human erythroid cell line lacking the genes to produce A, B, and RhD antigens would be a very useful resource for clinical application, since such a cell line would produce O/RhD(−) RBCs, which would, in theory, be transfusable into all individuals.

12. Conclusions

It is now highly likely that immortalized human erythroid progenitor cell lines able to produce enucleated RBCs can be established in the near future. We believe that the transfusion of RBCs produced *ex vivo* from such cell lines will become a standard procedure in the clinic.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Gastric acid induces mitochondrial superoxide production and lipid peroxidation in gastric epithelial cells

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Abstract
Background Gastric hydrochloric acid (HCl) has been regarded as an inciting factor in gastric mucosal injuries and has been reported to induce lipid peroxidation in vitro. However, because HCl is not an oxidant per se, the exact mechanism by which the acid induces lipid peroxidation is unknown. We hypothesized that gastric acid may disrupt mitochondrial transmembrane potential and induce the production of superoxide in mitochondria, which subsequently may induce lipid peroxidation and apoptosis in gastric mucosal cells.
Methods Firstly we treated gastric epithelial RGM1 cells with solutions containing various concentrations of HCl (i.e., of varying pH), and examined cellular injury, lipid peroxidation, and apoptosis with specific fluorescent dyes. Secondly, we performed electron paramagnetic resonance (EPR) spectroscopy of isolated, acid-exposed mitochondria from the cells, using a spin-trapping reagent for superoxide, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO). Finally, we established novel RGM1 cells that overexpressed manganese superoxide dismutase (MnSOD), which removes superoxide from mitochondria, and examined the effect of acid treatment on cellular membrane lipid peroxidation.
Results The results indicated that the exposure to acid indeed induced cellular injury, cellular lipid peroxidation, apoptosis, and the demonstration of the exact superoxide spectra on EPR spectroscopy in gastric epithelial cells, and that overexpression of MnSOD decreased superoxide production and prevented cellular lipid peroxidation.
Conclusion These results suggested that gastric acid, like nonsteroidal anti-inflammatory drugs (NSAIDs), induces mitochondrial superoxide production, which induces gastric cellular injury by triggering cellular lipid peroxidation and apoptosis.

Keywords Gastric acid · Lipid peroxidation · Superoxide ($O_2^{\cdot-}$) · Mitochondria · Manganese superoxide dismutase (MnSOD)

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Introduction

Gastric acid consists primarily of hydrochloric acid (HCl) and is secreted from gastric parietal cells in response to various stimuli, including food ingestion. Gastric acid has been regarded as an inciting factor in gastric mucosal injuries such as peptic ulcer and nonsteroidal

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anti-inflammatory drug (NSAID)-induced gastropathy, because strong acids produce coagulation necrosis resulting from the desiccating action of the acid on proteins in exposed tissues [1]. Therefore, in clinical practice, reducing gastric acid secretion with histamine-2 receptor antagonists (H2RAs) or proton pump inhibitors (PPIs) has been the mainstay of treatment for peptic ulcers and other gastric mucosal injuries—as the old dictum states, "no acid, no ulcer [2]"—even after the discovery of the involvement of *Helicobacter pylori* in peptic ulcer disease [3]. However, the pathophysiology of gastric acid-induced gastric injuries other than necrosis has not been well elucidated.

Lipid peroxidation was reported to be involved in gastric acid-induced gastric mucosal injury [4, 5]. A recent study demonstrated that treatment with exogenous HCl enhanced the amount of lipid peroxidation in an ischemia/reperfusion model of the rat stomach [4], suggesting that lipid peroxidation was induced by the acid. However, because HCl is not an oxidant per se, the exact mechanism by which gastric acid induces lipid peroxidation in gastric mucosal cells is unknown.

Recent reports have demonstrated that NSAIDs, some of the most potent inciting factors of gastric mucosal injury, induced the dissipation of mitochondrial transmembrane potentials and the production of reactive oxygen species (ROS) by mitochondria, thereby causing lipid peroxidation and apoptosis [6, 7]. Reactive oxygen species from mitochondria; in particular, superoxide ($O_2^{\cdot-}$), are a major cause of cellular oxidative damage [8]. Nonsteroidal anti-inflammatory drugs uncouple oxidative phosphorylation, and this process dissipates the mitochondrial transmembrane potential [9] or opens the mitochondrial permeability transition pores [10], leading to the liberation of cytochrome *c* from the mitochondrial intermembranous space into the cytosol and to the production of ROS by the mitochondria, thereby causing lipid peroxidation and apoptosis [6, 7].

We hypothesized that gastric acid, like NSAIDs, may disrupt the mitochondrial transmembrane potential and induce the production of mitochondrial superoxide, which subsequently may induce lipid peroxidation and apoptosis in gastric epithelial cells. We treated gastric epithelial RGM1 cells with solutions of various pH values and examined the cells for the intracellular generation of ROS, lipid peroxidation, and apoptosis. The generation of superoxide by mitochondria upon treatment with low-pH solutions was confirmed by electron paramagnetic resonance (EPR) spectroscopy, using a spin-trapping reagent for superoxide, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) [11]. We further examined the diminished effects under low pH, using clones that stably overexpressed manganese superoxide dismutase (MnSOD). The results indicated that the exposure to the acid indeed induced cellular injury, lipid peroxidation, and apoptosis; that EPR spectroscopy demonstrated exact superoxide spectra in gastric epithelial cells; and that overexpression of MnSOD decreased the amount of lipid peroxidation and prevented acid-induced cellular injury. These results suggest that MnSOD in mitochondria is efficient in protecting against low pH-induced cellular injuries and that mitochondria are the primary sites of low pH-induced cellular oxidative injuries.

Methods

Cell culture

A gastric epithelial cell line previously established at our laboratory, RGM1 [12], was reobtained from RIKEN BioResource Center (Tsukuba, Japan). Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12; Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY, USA) and 2 mM glutamine at 37°C in a humidified incubator with 5% CO_2.

Solutions and reagents

To prepare each acidic solution, 0.1 N HCl was added to deionized distilled water. To maintain the osmolarity of the solutions at a physiological level, NaCl was added to yield a chloride concentration of 154 mEq/L. Hanks' balanced salt solution (Wako Pure Chemical Industries, Osaka, Japan) was used as the pH 7 solution. A MITO-ISO2 mitochondrial isolation kit was purchased from Sigma Chemical (St. Louis, MO, USA); a Tetra Color One (TC-1) cell proliferation assay kit was purchased from Seikagaku (Tokyo, Japan); a Cell Death Detection ELISA kit was purchased from Roche Diagnostics (Basel, Switzerland); diphenyl-1-pyrenyl-phosphine (DPPP) and 3,6-Bis(diethylamino)-9-[2-(4-methylcoumarin-7yl-oxycarbonyl)phenyl]-3,6-bis (diethylamine) xanthylium chloride (MitoRed) and 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole, trihydrochloride (Hoechst 33258) were purchased from Dojindo (Kumamoto, Japan); and CYPMPO was purchased from Radical Research (Tokyo, Japan). All other chemicals were reagent grade.

MnSOD c-DNA transfection

The pCR3.1-Uni plasmid (Invitrogen, Carlsbad, CA, USA) containing a sense human MnSOD cDNA insert was kindly supplied by Dr. Akashi (National Institute of Radiological

Sciences, Chiba, Japan) [13, 14]. The RGM1 cells were transfected using the Lipofectamine 2000 reagent transfection system (Invitrogen) in accordance with the manufacturer's instructions. Briefly, cells were plated onto 60-mm dishes and incubated for 24 h before transfection at 80% confluence. The cells were stably transfected with 5 μg pCR3.1-Uni plasmids containing a sense human MnSOD cDNA insert and linearized with *Sca*I in serum-free DMEM/F12, and 5 h later, the medium was changed to DMEM/F12 containing 10% fetal bovine serum. The controls were transfected with pCR3.1-Uni plasmids without a human MnSOD cDNA (vector alone) insert and linearized with *Sca*I. Stable clones of both the MnSOD and control plasmid transfectants were selected with Geneticin (Life Technologies, Carlsbad, CA, USA) at a final concentration of 500 μg/mL. Selected cellular clones that expressed MnSOD (MnSOD-6 and -8), the selectable marker alone (vector), and the parental cell (RGM1) were used in all of the experiments. Selected clones were routinely maintained in DMEM containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and 500 μg/mL Geneticin at 37°C in humidified air containing 5% CO_2.

Cytotoxicity assay

Cellular injury was examined with the TC-1 assay kit according to the manufacturer's instructions. Cells at a concentration of 10^5 cells/mL were placed in the wells of a microtiter plate. Twenty-four hours later, the cells were washed with phosphate-buffered saline (PBS; Gibco) and incubated in pH 1, 2, 3, 4, and 7 solutions for 30 min, and then washed with PBS and incubated with serum-free media containing 10 μL TC-1 for 30 min. Each well's absorbance at 450 nm was measured using a multimode plate reader (DTX 880; Beckman Coulter, Fullerton, CA, USA).

SOD activity gel assay

Cells were sonicated in 50 mM potassium phosphate buffer (pH 7.8). A total of 60 μg protein/lane was electrophoresed through a nondissociating riboflavin gel consisting of 5% stacking gel (pH 6.8) and 12% running gel (pH 8.8) at 4°C. To visualize the SOD activity, the gels were first incubated in 2.43 mM nitroblue tetrazolium (Wako Pure Chemical Industries, Osaka, Japan) in deionized water for 20 min and then incubated in 0.028 mM riboflavin (Wako Pure Chemical Industries) and 280 mM N,N,N′,N′-tetramethylethylenediamine (Wako Pure Chemical Industries) in 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the dark. The gels were then washed in deionized water and illuminated under fluorescent light until clear zones of SOD activity were evident. Gel photographs were taken using an Alpha Imager (Cell Biosciences, Santa Clara, CA, USA). The MnSOD bands were quantified using ImageJ 1.38, which is available on the Internet via a file-transfer protocol from http://rsb.info.nih.gov/ij/. The MnSOD activity of the parental cell was normalized to 1, and the relative MnSOD activities of the other cells were calculated. The mean of the integrated density obtained from the 3 independent files was used as a representative value for the experiment.

Cellular microscopic fluorescence analysis

RGM1 cells were incubated on a Lab-Tek II slide chamber (Nalge Nunc International, Naperville, IL, USA) at a concentration of 10^5 cells/mL per well. Lipid peroxidation was investigated using DPPP, mitochondrial activity was investigated using MitoRed, and apoptotic change was investigated using Hoechst 33258. Cellular fluorescence images of the cells were observed, and their intensities were measured using a chilled charge-coupled device (CCD) camera (AxioCam color; ZEISS, Oberkochen, Germany)-mounted epifluorescence microscope (Axiovert135M; ZEISS) connected to an image analyzing system (Axio Vision; ZEISS).

Apoptosis analysis

DNA fragmentation was examined using the Cell Death Detection ELISA, which is a photometric enzyme immunoassay for qualitative and quantitative in vitro determination of the cytoplasmic histone-associated DNA fragments of induced cell death. Cells incubated in DMEM/F12 on a slide chamber were exposed to each acidic solution for 30 min. After the cells were washed with PBS, they were collected and fractionated into a cytoplasmic fraction. After treatment with ELISA solutions according to the manufacturer's instructions, the fluorescence intensities of the DNA fragments were measured at 405 nm using a multimode plate reader. Apoptosis was examined using Hoechst 33258 solution, according to the manufacturer's instructions. This solution stains damaged DNA to emit an intense blue fluorescence. The excitation and emission wavelengths of the Hoechst-DNA complex are 352 and 461 nm, respectively. Cells incubated in DMEM/F12 on a slide chamber were exposed to each acidic solution for 30 min. After being washed with PBS, the cells were incubated in DMEM/F12 containing 50 μM of the Hoechst 33258 solution for 30 min. Fluorescence images of the cells were observed using the above-mentioned system, and the fluorescence intensities were analyzed using NIH ImageJ 1.42q.

Detection of lipid peroxidation

Diphenyl-1-pyrenyl-phosphine (DPPP) reacts specifically with hydroperoxides and hydrogen peroxide generated

within cell membranes to give fluorescent diphenyl-1-pyrenyl-phosphine oxide (DPPP oxide), which emits fluorescence with excitation and emission wavelengths of 351 and 380 nm, respectively [15]. Cells incubated in medium on a slide chamber were exposed to each acidic solution for 30 min. After being washed with PBS and incubated in DMEM/F12 containing 10 μM DPPP for 30 min, the fluorescence intensities were measured using the above-mentioned system. Lipid peroxidation in the acid-treated cells was also examined by immunohistochemical staining using 4-hydroxy-2-nonenal (4-HNE)-modified protein, which is a highly toxic aldehyde product of lipid peroxidation and a sensitive marker of oxidative damage and lipid peroxidation [16]. RGM1 cells were incubated in 2-well glass chamber slides (Lab-Tek II Chamber Slide; Nalge Nunc International, Rochester, NY, USA) at a cell density of 10^5 cells/mL per well. The cells were treated with each acidic solution for 30 min and then washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. After being washed with PBS and blocked with bovine serum albumin, the cells were incubated with anti-4-hydroxy-2-nonenal monoclonal antibody (JaICA, Shizuoka, Japan) for 1 h at room temperature. The immunohistochemical staining was developed using VECTASTAIN ABC kits and Vector Red (Vector Laboratories, Torrance, CA, USA).

Preparation of mitochondrial fractions and detection of lipid peroxide

Mitochondrial fractions were prepared using the MITOISO2 mitochondrial isolation kit according to the manufacturer's instructions [17]. Briefly, confluent RGM1 cells were trypsinized and centrifuged for 5 min at 600*g*. After the cells were washed with PBS, they were resuspended in ice-cold PBS containing the prepared lysis buffer and incubated on ice for 5 min. The extraction buffer was added, and the cells were then centrifuged for 10 min at 600*g* at 4°C. The supernatant was centrifuged for 10 min at 10,000*g* at 4°C. The pellet was resuspended in each acidic solution containing DPPP on a slide chamber. After 30 min, the fluorescence intensities were measured using the above-mentioned cellular microscopic fluorescence analysis system, with 352- and 461-nm bandpass filters for excitation and emission, respectively.

Measurement of mitochondrial transmembrane potential

Mitochondrial membrane potentials were measured with a cell-membrane-permeable rhodamine-based dye, MitoRed. The fluorescence intensity of this dye depends on the mitochondrial membrane potential; thus, it can be used as an indicator of mitochondrial activity [18]. The excitation and emission wavelengths of MitoRed are 559 and 588 nm, respectively. The RGM1 cells incubated on a slide chamber were exposed to each acidic solution for 30 min. After the cells were washed with PBS and incubated with DMEM/F12 containing 200 nM MitoRed for 30 min, the fluorescence intensities were measured using the above-mentioned system.

EPR spectroscopy

The RGM1 cells were treated with pH 1, 2, 3, 4, and 7 solutions for 30 min. The mitochondria of the cells were then isolated using the MITOISO2 mitochondrial isolation kit, according to the manufacturer's instructions. The mitochondrial pellet was resuspended with 5 mM respiratory substrates (succinate, glutamate, and malate), 5 mM nicotinamide adenine dinucleotide (NADH), and 10 mM CYPMPO [19]. The reaction mixture was immediately transferred to a quartz flat cell (RDC-60, 60 × 6 × 0.3 mm; Radical Research). The concentration of proteins in the final reaction mixture was 250 μg/mL, as evaluated according to a previously described method (Bio-Rad Laboratories, Hercules, CA, USA) [20]. The EPR spectra were recorded by using a JEOL-TE X-band spectrometer (JEOL, Tokyo, Japan). All EPR spectra were obtained under the following conditions: 10 mW incident microwave power, 100 kHz modulation frequency, 0.1 mT field modulation amplitude, and 15 mT scan range. The hyperfine splitting constants (hfsc) and spectral computer simulation were analyzed using a Win-Rad Radical Analyzer System (Radical Research). All EPR spectra shown are representative of at least 3 independent experiments.

Statistical analysis

The statistical significance of differences in the data was evaluated using analysis of variance (ANOVA) followed by the Duncan multiple range test. Statistical comparisons were made using the Scheffé test. A P value of <0.05 was considered significant.

Results

Gastric epithelial cell injury due to acid exposure

To examine whether gastric acid treatment induced injury in gastric epithelial cells, we treated RGM1 cells with low-pH solutions and examined them for cellular injury. We treated the cells with the acidic solutions for 15, 30, and 60 min, and the extent of the cellular damage was examined using the TC-1 assay. The cells treated with solutions

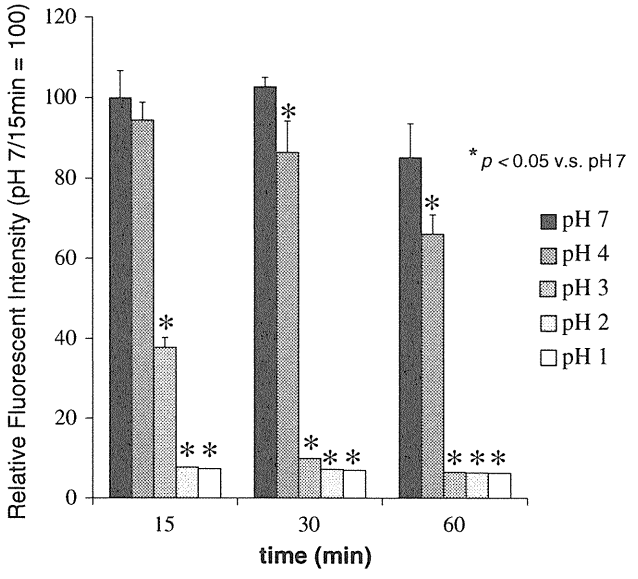

Fig. 1 Cellular injuries examined using the Tetra Color One (TC-1) assay in RGM1 cells treated with solutions of various pH values (pH 1, 2, 3, 4, and 7) for 15, 30, and 60 min. Data are expressed as percentages of pH 7 and 15 min (mean ± SD). Cellular viabilities were decreased in a time- and pH-dependent manner. Cells treated with strong acid (pH 1 and pH 2) were significantly more injured than cells treated with milder acid (pH 3 and pH 4) and the control cells (pH 7). Most of the cells treated with stronger acid (pH 1 and pH 2) were considered necrotic, and this was confirmed by microscopic analysis (data not shown). $*P < 0.05$ versus pH 7

that had a pH of less than 6 indeed underwent injury in a pH-dependent manner (Fig. 1).

Lipid peroxidation due to acid exposure

To examine whether gastric acid exposure induced lipid peroxidation in gastric epithelial cells, we treated RGM1 cells with acidic solutions and examined them for lipid peroxidation using DPPP-oxide fluorescence and using an antibody for 4-HNE-modified protein. The fluorescence analysis using DPPP-oxide, a specific marker of lipid peroxidation [15], indicated that lipid peroxidation was induced in a time-dependent manner in the cells treated with pH 3 solution, compared with the cells treated with pH 7 solution (Fig. 2a). We also evaluated, by immunohistochemical staining, the acid-exposed cells using 4-HNE-modified protein, a highly toxic aldehyde product of lipid peroxidation and a sensitive marker of lipid peroxidation [16]. The fluorescence intensity of anti-4-HNE-modified protein antibody was significantly increased in a time-dependent manner in the cells treated with pH 3 solution (Fig. 2b), indicating that lipid peroxidation was induced in the acid-treated cells. These results clearly demonstrated that lipid peroxidation was induced by the acid exposure.

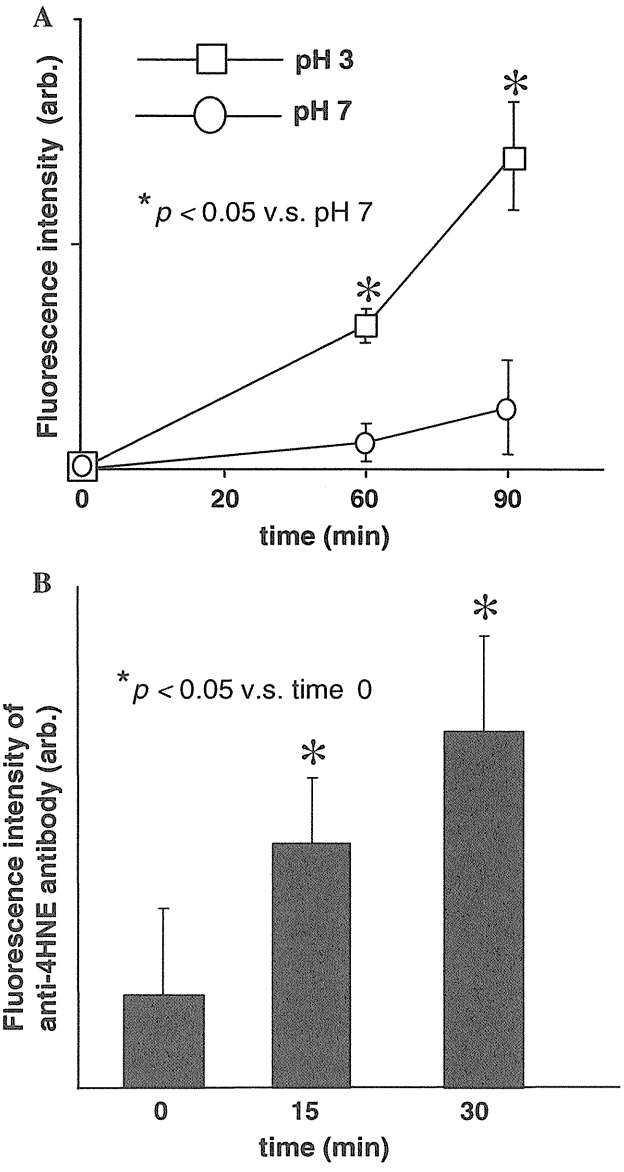

Fig. 2 Lipid peroxidation of acid-treated RGM1 cells measured with diphenyl-1-pyrenyl-phosphine (DPPP) fluorescence and 4-hydroxy-2-nonenal (*4-HNE*)-modified protein. **a** The DPPP fluorescence intensities of the cells treated with pH 3 solution were significantly higher than those of the control cells at 60 and 90 min, indicating that lipid peroxidation was induced by the acid. $*P < 0.05$ versus time 0. **b** Cells with acid-induced lipid peroxidation were immunohistochemically stained and lipid peroxidation was measured with 4-HNE-modified protein, a sensitive marker of this phenomenon. The fluorescence intensity of anti-4HNE-modified protein antibody in the acid-treated cells was significantly increased in a time-dependent manner. $*P < 0.05$ vs. time 0. *arb.* arbitrary unit

Apoptosis induced in cells treated with pH 3 and pH 4 solutions

Apoptosis was investigated using the Cell Death Detection ELISA, a photometric enzyme immunoassay for qualitative and quantitative in vitro determination of cytoplasmic