cell cultures. As hESF9 medium consists of minimum essential components, improper handling greatly affects cell viability and culture outcome.

Second, there are variations in the characteristics of hES/iPS cell lines as most of the researchers working on hES/iPS cells have come to realize. hES cell lines, Shef1, Shef5, and HUES1 cultured in the University of Sheffield were able to attach to type I collagen and grow well in hESF9 medium (Furue et al. 2008). However, we found that attachment activity of the KhES-1 line (Nakatsuji 2005), which was established in Kyoto University, to type I collagen seemed low, suggesting that there is a difference between the cell lines in their attachment ability. Owing to regulatory issues for hES cell importation into Japan, we were unable to directly compare KhES-1 cells with Shef1 or HUES1. We have examined the attachment and growth activity of other hES lines (KhES-3 from Kyoto University, Kyoto, Japan; H9 from National Stem Cell Bank, WiCell, Madison, WI) and the MRC-5-derived iPS cell line Tic (JCRB 1331, JCRB Cell Bank, Osaka, Japan), which was established in the National Center for Child Health and Development, on type I collagen (Nitta Gelatin, Osaka, Japan) in hESF9 medium (Cell Science & Technology Institute, Sendai, Japan). The results show that there is a difference between hES/iPS cell lines in attachment activity to type I collagen. This result suggests that there is a difference among cell lines in integrin signaling (Fig. 1).

Based on these findings, we have modified the culture protocol and tried to culture KhES-1 and KhES-3 cell lines on fibronectin (Sigma, St. Louis, MO) in hESF9 medium without feeders (Fig. 2). Matrigel, a basement membrane preparation from the Engelbreth–Holm–Swarm mouse tumor, is often used for feeder-free culture for hES/iPS cells with MEF-conditioned medium (Draper et al. 2004). However, it contains a complex and ill-defined mixture of fibronectin, laminin, type IV collagen, entactin, and heparan sulfate proteoglycans, and various growth factors such as FGF-2, EGF, PDGF, and NGF (Yang et al. 2003). Ludwig et al. (2006) have reported that in place of matrigel, a combination of collagen IV, fibronectin, laminin, and vitronectin supported robust, long-term proliferation of human ES cells in their chemically defined medium TeSR1. We have previously reported by using defined serum-free culture conditions for mouse embryonic stem (mES) cell that integrins regulate mES cell self-renewal. mES cells remained undifferentiated when cultured on type I and type IV collagen or poly-D-lysine whereas they differentiated when cultured on laminin or fibronectin where LIF-induced self-renewal signaling was decreased (Hayashi et al. 2007). Now, we are investigating the role of integrins in the pluripotency of hES/iPS cells.

For robust cultures, we have further modified the culture protocol. We have used 1 U/ml dispase (Roche Applied Science, Indianapolis, IN) to dissociate the cell colonies and washed the dispase with the medium supplemented with recombinant human albumin (1 mg/ml, Millipore, Bedford, MA). If differentiated cells appear in the culture, addition of low concentration of activin (2~10 ng/ml, R&D Systems, Minneapolis, MN) or middle concentration of noggin (10~20 ng/ml, R&D Systems) seems also to inhibit the differentiated cell growth as previously reported (Beattie et al. 2005; James et al. 2005; Vallier et al. 2005; Wang et al. 2005). However, addition of these growth factors confounds the analysis of the actions of other exogenous factors. We are using hESF9 medium to develop a drug screening test.

It would be convenient if the cell culture novice could propagate and passage any type of cell without difficulty. Unfortunately, this is currently not the case for undifferentiated hES/iPS cell lines. Although serum has proved to be a universal medium supplement that allowed the isolation and characterization of a few normal diploid cell lines and numerous abnormal transformed cell lines over the years, the use of serum or other undefined medium components impedes our ability to understand cell responses to controlled environmental stimuli. There are advantages and disadvantages to culturing hES/iPS cells under defined serum-free culture conditions, and the suitability of any particular medium depends on the purpose of the experiment.

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References

Adewumi O.; Aflatoonian B.; Ahrlund-Richter L.; Amit M.; Andrews P. W.; Beighton G.; Bello P. A.; Benvenisty N.; Berry L. S.; Bevan S.; Blum B.; Brooking J.; Chen K. G.; Choo A. B.; Churchill G. A.; Corbel M.; Damjanov I.; Draper J. S.; Dvorak P.; Emanuelsson K.; Fleck R. A.; Ford A.; Gertow K.; Gertsenstein M.; Gokhale P. J.; Hamilton R. S.; Hampl A.; Healy L. E.; Hovatta O.; Hyllner J.; Imreh M. P.; Itskovitz-Eldor J.; Jackson J.; Johnson J. L.; Jones M.; Kee K.; King B. L.; Knowles B. B.; Lako M.; Lebrin F.; Mallon B. S.; Manning D.; Mayshar Y.; McKay R. D.; Michalska A. E.; Mikkola M.; Mileikovsky M.; Minger S. L.; Moore H. D.; Mummery C. L.; Nagy A.; Nakatsuji N.; O'Brien M.; Oh C. S. K.; Olsson C.;

Otonkoski T.; Park K. Y.; Passier R.; Patel H.; Patel M.; Pedersen R.; Pera M. F.; Piekarczyk M. S.; Pera R. A.; Reubinoff B. E.; Robins A. J.; Rossant J.; Rugg-Gunn P.; Schulz T. C.; Semb H.; Sherrer E. S.; Siemen H.; Stacey G. N.; Stojkovic M.; Suemori H.; Szatkiewicz J.; Turetsky T.; Tuuri T.; van den Brink S.; Vintersten K.; Vuoristo S.; Ward D.; Weaver T. A.; Young L. A.; Zhang W. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat. Biotechnol.* 25: 803–816; 2007.

Amit M.; Carpenter M. K.; Inokuma M. S.; Chiu C. P.; Harris C. P.; Waknitz M. A.; Itskovitz-Eldor J.; Thomson J. A. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* 227(2): 271–278; 2000.

Barnes D.; Sato G. Serum-free cell culture: a unifying approach. *Cell* 22: 649–655; 1980.

Beattie G. M.; Lopez A. D.; Bucay N.; Hinton A.; Firpo M. T.; King C. C.; Hayek A. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells* 23: 489–495; 2005.

Bottenstein J. E.; Sato G. H. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* 76: 514–517; 1979.

Draper J. S.; Moore H. D.; Ruban L. N.; Gokhale P. J.; Andrews P. W. Culture and characterization of human embryonic stem cells. *Stem Cells Dev.* 13: 325–336; 2004.

Fassler, R.; Meyer, M. W. Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev.* 9: 1896–1908; 1995.

Furue M.; Okamoto T.; Ikeda M.; Tanaka Y.; Sasaki Y.; Nishihira K.; Sato J. D. Primitive neuroectodermal tumor cell lines derived from a metastatic pediatric tumor. *In Vitro Cell Dev. Biol. Anim.* 30A: 813–816; 1994.

Furue M. K.; Na J.; Jackson J. P.; Okamoto T.; Jones M.; Baker D.; Hata R.; Moore H. D.; Sato J. D.; Andrews P. W. Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc. Natl. Acad. Sci. USA* 105: 13409–13414; 2008.

Hayashi I.; Sato G. H. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature* 259: 132–134; 1976.

Hayashi Y.; Furue M. K.; Okamoto T.; Ohnuma K.; Myoishi Y.; Fukuhara Y.; Abe T.; Sato J. D.; Hata R.; Asashima M. Integrins regulate mouse embryonic stem cell self-renewal. *Stem Cells* 25: 3005–3015; 2007.

James D.; Levine A. J.; Besser D.; Hemmati-Brivanlou A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* 132: 1273–1282; 2005.

Ludwig T. E.; Levenstein M. E.; Jones J. M.; Berggren W. T.; Mitchen E. R.; Frane J. L.; Crandall L. J.; Daigh C. A.; Conard K. R.; Piekarczyk M. S.; Llanas R. A.; Thomson J. A. Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* 24: 185–187; 2006.

Nakatsuji N. Establishment and manipulation of monkey and human embryonic stem cell lines for biomedical research. *Ernst Schering Res. Found. Workshop.* 54: 15–26; 2005.

Okamoto T.; Tani R.; Yabumoto M.; Sakamoto A.; Takada K.; Sato G. H.; Sato J. D. Effects of insulin and transferrin on the generation of lymphokine-activated killer cells in serum-free medium. *J. Immunol. Methods* 195: 7–14; 1996.

Reubinoff B. E.; Pera M. F.; Fong C. Y.; Trounson A.; Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* 18: 399–404; 2000.

Sato J. D.; Barnes D.; Hayashi I.; Hayashi J.; Hoshi H.; Kawamoto T.; Matsuda R.; McKeehan W. L.; Matsuzaki K.; Okamoto T.; Serrero G.; Sussman D. J.; Kan M. et al. In: Davis J. M. (ed) Specific cells and their requirements. Basic cell culture: a practical approach. Oxford University Press, Oxford, pp 227–274; 2002.

Sato J. D.; Kawamoto T,.; Okamoto T. Cholesterol requirement of P3-X63-Ag8 and X63-Ag8.653 mouse myeloma cells for growth in vitro. *J. Exp. Med.* 165: 1761–1766; 1987.

Skottman H.; Hovatta O. Culture conditions for human embryonic stem cells. *Reproduction* 132: 691–698; 2006.

Thomson J. A.; Itskovitz-Eldor J.; Shapiro S. S.; Waknitz M. A.; Swiergiel J. J.; Marshall V. S.; Jones J. M. Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147; 1998.

Vallier L.; Alexander M.; Pedersen R. A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J. Cell Sci.* 118: 4495–4509; 2005.

Wang G.; Zhang H.; Zhao Y.; Li J.; Cai J.; Wang P.; Meng S.; Feng J.; Miao C.; Ding M.; Li D.; Deng H. Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem. Biophys. Res. Commun.* 330: 934–942; 2005.

Yang J. Z.; Ho A. L.; Ajonuma L. C.; Lam S. Y.; Tsang L. L.; Tang N.; Rowlands D. K.; Gou Y. L.; Chung Y. W.; Chan H. C. Differential effects of Matrigel and its components on functional activity of CFTR and ENaC in mouse endometrial epithelial cells. *Cell Biol. Int.* 27: 543–548; 2003.

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Growth factor-defined culture medium for human mesenchymal stem cells

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ABSTRACT Human bone marrow-derived mesenchymal stem cells (hMSCs) are potential cellular sources of therapeutic stem cells as they have the ability to proliferate and differentiate into a wide array of mesenchymal cell types such as osteoblasts, chondroblasts and adipocytes. hMSCs have been used clinically to treat patients with graft *vs.* host disease, osteogenesis imperfect, or alveolar cleft, suggesting that transplantation of hMSCs is comparatively safe as a stem cell-based therapy. However, conventional culture medium for hMSCs contains fetal bovine serum (FBS). In the present study, we developed a growth factor-defined, serum-free medium for culturing hMSCs. Under these conditions, TGF-β1 promoted proliferation of hMSCs. The expanded hMSC population expressed the human pluripotency markers SSEA-3, -4, *NANOG*, *OCT3/4* and *SOX2*. Furthermore, double positive cells for SSEA-3 and a mesenchymal cell marker, CD105, were detected in the population. The potential to differentiate into osteoblasts and adipocytes was confirmed. This work provides a useful tool to understand the basic biological properties of hMSCs in culture.

KEY WORDS: *mesenchymal stem cell, serum-free culture, TGF-β1*

Introduction

Bone marrow-derived cells can differentiate into osteoblasts *in vitro* and *in vivo* (Friedenstein *et al.*, 1966) and thus are considered a useful source of stem cells for bone regeneration. Recently, many studies have reported that human bone marrow contains a distinct cell fraction referred to as multipotent mesenchymal stem cells (hMSCs) which can give rise to a wide array of mesenchymal cell types, including bone, fat, and cartilage"(Pittenger *et al.*, 1999). However, hMSCs can differentiate along some ectodermal and endodermal cell lineages such as neuronal cells and liver cells (Pittenger *et al.*, 1999; Dezawa *et al.*, 2004; Dezawa *et al.*, 2005). Further, a recent study reported that hMSCs have the ability to generate the multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). It has been estimated that hMSCs comprise about 0.001 to 0.01% of total bone marrow mononuclear cells (Pittenger *et al.*, 1999). For use in cell-based therapies, hMSC populations require extensive *in vitro* expansion to obtain sufficient numbers. The conventional culture medium for hMSCs is composed of a basal nutrient medium supplemented with fetal bovine serum (FBS) (Haynesworth *et al.*, 1992; Lennon DP, 1996). Although these traditional culture conditions provide robust undifferentiated hMSC expansion, the ill-defined components of FBS is undesirable for clinical applications and also hampers analysis of the cell biological mechanisms that control cell behavior.

Abbreviations used in this paper: hES cells, human embryonic stem cells; hMSCs, human mesenchymal stem cells.

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We and others previously described serum-free media consisting of minimum essential components suitable to propagate and accurately analyze the characteristics of differentiated cells (Hayashi and Sato, 1976; Furue and Saito, 1998; Sato *et al.*, 2002; Furue *et al.*, 2005; Furue *et al.*, 2008; Hayashi *et al.*, 2010). One of these media, hESF9, supports the serial cultivation of undifferentiated human embryonic stem (hES) cells in the absence of feeder cells and thus provides an experimental system for elucidating cellular responses to specific environmental stimuli (Furue *et al.*, 2008; Na *et al.*, 2010). For example, either FGF-2 or heparin promotes proliferation of hES cells in a concentration-dependent manner although these effects were not detected under conventional culture conditions. Thus, a defined serum-free medium consisting of minimum essential components should be useful in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection (Furue *et al.*, 2010).

Because hMSCs have multipotent properties similar to hES cells, we speculated that hMSCs should be able to grow in similar culture conditions as hES cells. In the present study, we demonstrated that addition of TGF-β1 to the defined serum-free medium for hES cells supports the robust proliferation of hMSCs. The hMSC population expanded in the absence of serum expressed the mesenchymal cell markers CD44, CD73, CD90, and CD105. Further, they expressed human pluripotency surface markers, SSEA-3, -4, TRA-2-54, and also the transcription factors of *NANOG*, *OCT3/4*, and *SOX2*. We show that the serum-free expanded hMSCs can differentiate into osteoblasts and adipocytes. This work sets the stage for serum-free hMSC cell culture and thereby provides a useful tool to understand the basic biological characteristics of hMSCs.

Results

In this study we used a human bone marrow-derived hMSC line designated UE7T-13 (JCRB 1154). The life span of these cells was prolonged by infecting them with a retrovirus containing human papillomavirus E7 and telomerase reverse transcriptase (hTERT) cDNAs (Mori *et al.*, 2005; Shimomura *et al.*, 2007; Ishii *et al.*, 2008; Takeuchi *et al.*, 2007). We first tested the ability of hESF9 medium, which we had developed for use with hES cells, to support the growth of UE7T-13 cells. The cells were harvested using trypsin/EDTA, from cultures in conventional medium containing 10% FBS (POWERDBY10) and transferred to 0.1% gelatin-coated dishes in hESF9 medium. However, UE7T-13 cell growth was quite slow. We then investigated the effects of various growth factors on proliferation of the cells. UE7T-13 cells were seeded on 0.1% gelatin in hESF9 in the absence of FGF-2 and heparin (hESF9(-/-)), containing increasing concentrations of FGF-1, FGF-2, TGF-β1, activin A, or leukemia inhibitory factor (LIF) (Fig. 1). Both FGF-1 and FGF-2 promoted UE7T-13 proliferation in a dose-dependent manner, and the greatest effect was seen at 10 ng/ml FGF-2. Neither LIF nor activin A affected on UE7T-13 cell proliferation, but TGF-β1 slightly stimulated UE7T-13 proliferation. Next all five factors (FGF-1, FGF-2, TGF-β1, activin A, and LIF) or four factors with increasing concentrations of heparin were added to UE7T-13 cultures (Fig. 2). When either FGF-2 or TGF-β1 was withdrawn from the cultures, the cell numbers decreased significantly. Heparin promoted cell proliferation in a dose-dependent manner. This result suggested that addition of FGF-2 and TGF-β1 to hESF9(-/-) medium, is critical for UE7T-13 proliferation, and heparin also enhanced cell growth. hESF9 medium supplemented with TGF-β1 was designated hESF10.

L-ascorbic acid-2-phosphate (Asc 2-P) in hESF9 medium supported hES cells. However, it is known to promote hMSC cell differentiation into osteoblasts. Therefore, we examined whether the presence of Asc 2-P in hESF10 medium promoted osteoblastic differentiation of UE7T-13 cells. We analyzed the expression of *bone sialoprotein* (*IBSP*), *osteocalcin* (*BGP*), *osteonectin* (*SPOCK2*), and *osteopontin* (*SPP1*) in UE7T-13 cell cultured in hESF10 with or without Asc 2-P and in conventional medium (Fig. 2). These osteoblast genes were expressed at significantly lower levels in cells cultured in the serum-free media than in those cultured in the conventional medium. These results suggest that the serum-free medium is suitable for hMSC maintenance. *IBSP* gene expression was higher in the cells cultured in the

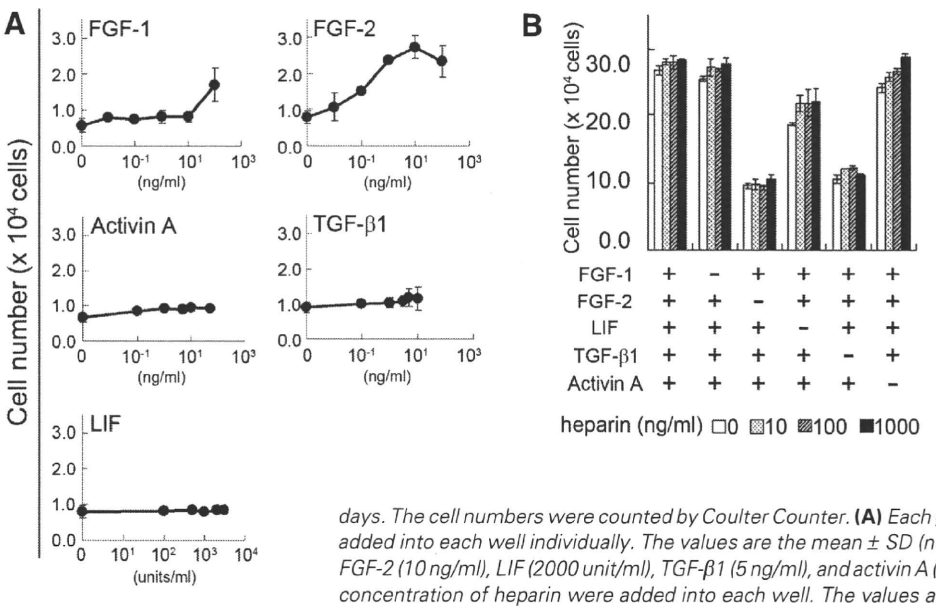

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. *After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) at 1 x10⁴ cells per well and cultured for 6 days. The cell numbers were counted by Coulter Counter.* **(A)** *Each growth factor at indicated concentration was added into each well individually. The values are the mean ± SD (n=3).* **(B)** *All five factors of FGF-1 (100 ng/ml), FGF-2 (10 ng/ml), LIF (2000 unit/ml), TGF-β1 (5 ng/ml), and activin A (10 ng/ml) or four factors of them with varying concentration of heparin were added into each well. The values are the mean ± SD (n=3).*

presence of Asc 2-P. These results suggested that Asc 2-P promoted differentiation of UE7T-13 cells into osteoblasts. We removed Asc 2-P from hESF10 medium for hMSCs, and designated the new formulation D-hESF10.

To confirm the characteristics of UE7T-13 cells expanded in the absence of serum, we performed flow cytometry with antibodies to markers for hMSCs and pluripotent cells (Fig. 3A). Cells grown in D-hESF10 medium were positive for CD44, CD73, CD90, CD105, and TRA-2-54 (tissue non-specific alkaline phosphatase antibody), but negative for CD45 (a marker of all hematopoietic cells) and CD56 (a neural cell adhesion molecule). We further stained the cells with antibodies to CD105 and SSEA-3 (Fig. 3B). The immunocytochemical analysis showed that SSEA-3$^+$/CD105$^+$ double positive cells were present in the UE7T-13 population grown in D-hESF10 although cells positive for either CD105 or SSEA-3 were also detected in the population. The cell growth rate in D-hESF10 was comparable to that in conventional culture conditions (Fig. 4).

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrin β1* (*ITGB1*), and the hES cell pluripotency markers, *OCT3/4* (*POU5F1*) and *NANOG* were similar in the cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. *SOX2* expression was significantly higher in cells cultured in D-hESF10 compared with cells cultured in conventional culture conditions. On the other hand, the expression levels of *IBSP*, *BGP*, *SPOCK2*, and *SPP1* were significantly lower in cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. These results suggest that serum-free expanded UE7T-13 cells retain an undifferentiated phenotype.

We determined the differentiation capacity of the serum-free expanded UE7T-13 cells. After the UE7T-13 cells were cultured in D-hESF10 for 7 passages, the cells were cultured in medium designed to induce differentiation into osteoblasts or adipocytes (Fig. 6). Culturing in osteoblastic differentiation medium induced the formation of nodules that stained positive with Alizarin red, suggesting that the cells had the potential to differentiate into osteoblasts. When the cells were cultured in

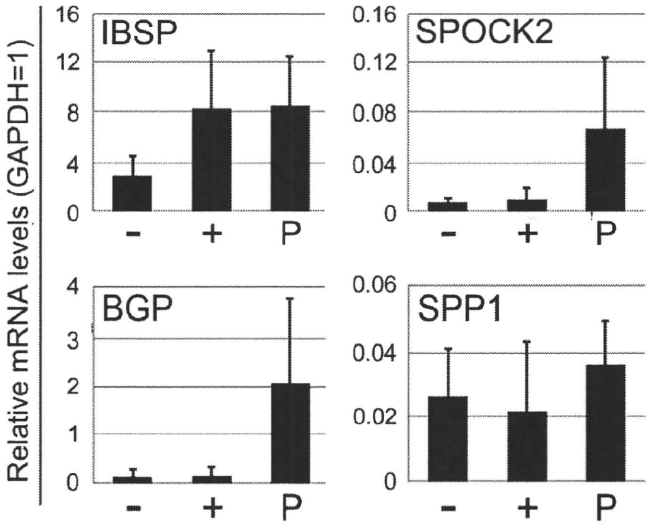

Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression was normalized by the amount of* GAPDH. *The values are the mean ± SD (n=3).*

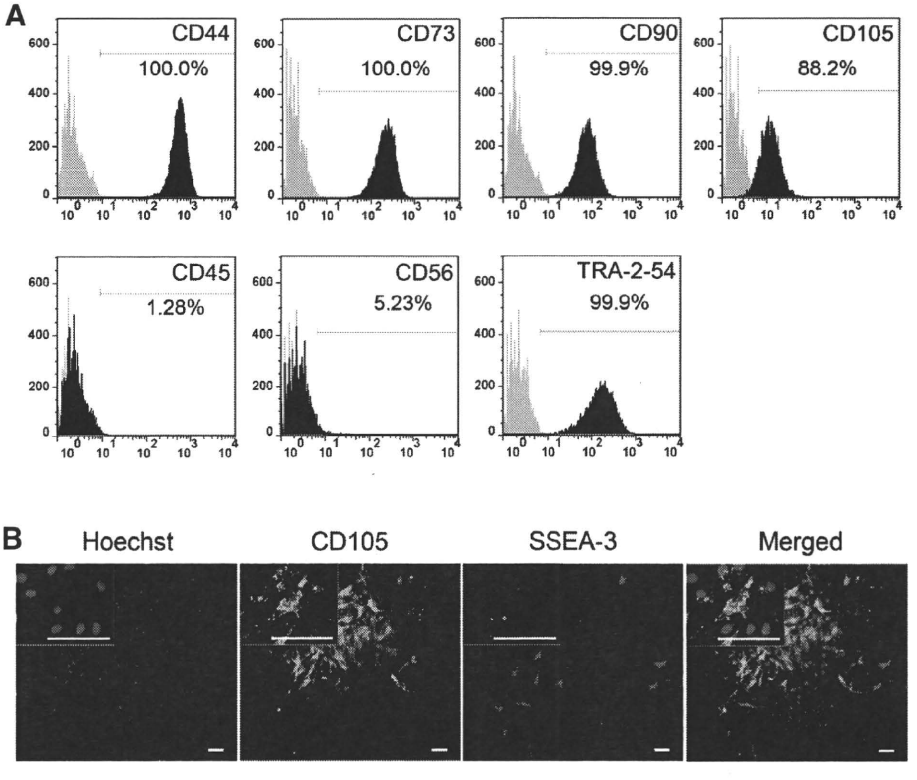

Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells.* **(B)** *Immunocytochemical analysis of SSEA-3 and CD105 expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days. Scale bars, 100 μm.*

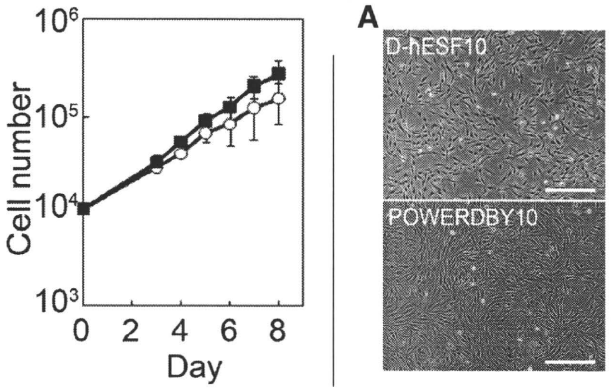

Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of 1x10^4 cells per well. Cell numbers were counted every day. The values are the mean ± SD (n=3).*

adipocytic differentiation medium, Oil red O-positive cells appeared. Taken together these results suggest that the serum-free expanded UE7T-13 cells have maintained the capacity to differentiate into osteoblasts or adipocytes.

Discussion

Developing clinical serum-free media for maintaining and expanding human stem cells is a major research topic in regenerative medicine. Our current results indicate that it is possible to culture hMSCs on gelatin in a defined medium, designated D-hESF10, in which human recombinant insulin, human transferrin, a low concentration of fatty acid-free bovine albumin conjugated with oleic acid, FGF-2, and TGF-β1 are the protein components. The basal medium ESF was developed for mouse ES cells (Furue *et al.*, 2005). For hES cell culturing, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) was removed from ESF but Asc 2-P was added (Furue *et al.*, 2008). For propagating hMSCs, Asc 2-P was removed from the hES cell culture medium because we found that Asc 2-P increased osteoblastic marker expression in hMSCs. These findings indicated that signaling by Asc 2-P in hMSCs is different from that in hES cells.

FGF-2 is a heparin-binding growth factor which stimulates the proliferation of a wide variety of cells. The biological activity of FGF-2 is efficient in the concentration range of 0.1 to 10.0 ng/ml. Addition of FGF-2 has been shown to increase the growth rate and life span of hMSCs from different species (Tsutsumi *et al.*, 2001; Benavente *et al.*, 2003), suggesting that FGF-2 play an important role in self-renewal of hMSCs. In hES cells, FGF-2 is a crucial to maintain the undifferentiated state (Amit *et al.*, 2004; Hoffman and Carpenter, 2005). We previously reported that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hES cells in serum-free without feeders (Furue *et al.*, 2008). In this study, we found that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hMSCs in a serum-free medium. These findings suggest that they share the same signal pathway to support self-renewal. Heparin at 1 mg/ml promoted hMSC cell proliferation, and we previously reported that heparin at 1 mg/ml inhibited hES cell proliferation. Thus the sensitivity to heparin is different between hMSCs and hES cells.

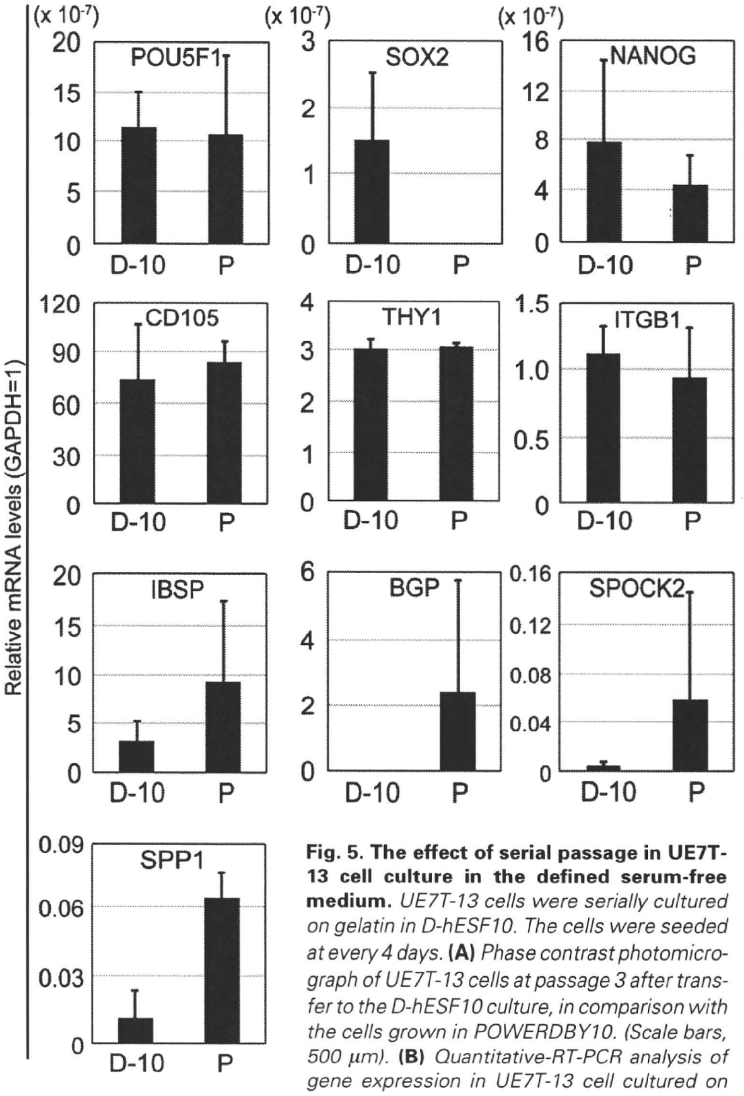

Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, in comparison with the cells grown in POWERDBY10. (Scale bars, 500 μm).* **(B)** *Quantitative-RT-PCR analysis of gene expression in UE7T-13 cell cultured on gelatin in D-hESF10 at passage 4 (D-10), in comparison with the cells grown in POWERDBY10 (P). The name of each gene is noted in each bar graph. Gene expression was normalized with respect to* GAPDH. *The values are the mean ± SD (n=3).*

The TGF-β1 pathway has been reported to be important in hMSC differentiation into the osteogenic and chondrogenic lineages (Li and Xu, 2005; Kulterer *et al.*, 2007). While we have shown that TGF-β1 alone did not promote cell proliferation of hMSCs, the combination with FGF-2 and heparin enhanced cell proliferation of hMSCs. Chase *et al.* reported the combination of TGF-β1, FGF-2, and PDGF-BB in a commercial serum-free medium for the expansion of hMSCs although the optimal concentrations of these factors were not disclosed. The cell growth rate in D-hESF10 medium was similar with that in the conven-

tional culture conditions suggesting that addition of TGF-β1 and FGF-2 is sufficient to replace serum in supporting hMSC cell growth. A culture medium consisting of the minimum components necessary to support survival and proliferation would be beneficial to understand the characteristics of naïve hMSCs. Therefore, we think that addition of PDGF-BB is not crucial for an hMSC culture medium.

Several studies reported that two distinct cell morphologies are seen in early-passage hMSC cultures: small, spindle-shaped cells that are rapidly self-renewing and large, flat cells that replicate slowly and appear more mature (Mets and Verdonk, 1981; Colter *et al.*, 2001; Sekiya *et al.*, 2002). The morphology of serum-free expanded UE7T-13 cell population contained comparably small, spindle-shaped cells. However, specific undifferentiated markers of hMSCs have not been identified yet (Pochampally *et al.*, 2004). Further, although the cells are cloned, cells within an individual colony are heterogeneous in morphology, growth rates, and efficiency with which they differentiate (Mets and Verdonk, 1981; Bruder *et al.*, 1997; Colter *et al.*, 2001). The International Society for Cellular Therapy (ISCT) has proposed three criteria to define hMSCs (Dominici *et al.*, 2006). hMSC population must be positive at least for several antigens such as CD105, CD73, and CD90, and negative for CD45. CD105 is usually used to identify an hMSC population. Many studies reported that hMSCs also expressed hES cell pluripotency markers, SSEA-3, -4, NANOG, OCT3/4, and alkaline phosphatase (Pochampally *et al.*, 2004; Roubelakis *et al.*, 2007; Battula *et al.*, 2008; Conrad *et al.*, 2008; Pang *et al.*, 2010). We also detected the expression of NANOG, OCT3/4, and SOX2. These findings suggested that hES cell pluripotency markers may be universal stem cell markers in humans. Dezawa's group recently reported that double positive CD105 and SSEA-3 cells have the ability to generate multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). We also confirmed the existence of CD105 and SSEA-3 double positive cells in the hMSC population expanded in D-hESF10. In this study, we confirmed the differentiation potential of hMSCs to generate osteoblasts or adipocytes, but in the future we will examine the ability of hMSCs to generate cells from all three germ layers.

To facilitate the transition of human stem cell biology from basic research to clinical application all the components of maintenance and differentiation media should be publicly disclosed so they can be evaluated by many researchers. A commercial xeno-free serum-free medium for hMSCs was reported recently (Chase *et al.*, 2010). However, the non-disclosure of components is problematic as the medium formulation cannot be usefully modified or improved. Because all the components of D-hESF10 medium are disclosed here, the medium can be modified to study signaling pathways involved in maintaining multipotency and to develop differentiation protocols.

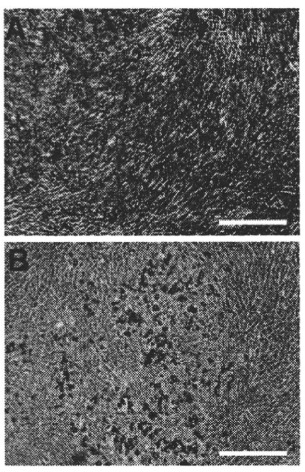

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were stained with Alizarin Red S (red).* **(B)** *Adipocytic differentiation was induced in adipocytic medium for 24 days. The cells were stained by Oil red O staining (red). Scale bars: 500 μm.*

Materials and Methods

Cell Cultures

An immortalized hMSC line UE7T-13 (Mori *et al.*, 2005) (JCRB 1154, JCRB Cell Bank, Osaka, Japan) was used in this study. Cells were maintained on 100 mm dish (BD Falcon, Oxnard, CA) in POWERDBY10 (MED-SHIROTORI, Tokyo, Japan) that was also used in the experiments as a control medium. The cells were harvested with 0.25% trypsin in 1 mM EDTA-4Na.

Serum-free Cell Culture Media

hESF9 comprises ESF basal medium (Furue *et al.*, 2005) without HEPES supplemented with nine defined factors: Asc 2-P, 6-factors (human recombinant insulin, human transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA)), bovine heparan sulfate sodium salt, and human recombinant FGF-2 (Sigma, St. Louis, MO), as described previously (Furue *et al.*, 2008) (Supplementary Table 1). ESF basal medium without HEPES supplemented with Asc 2-P (hESF-GRO), and ESF basal medium without HEPES and Asc 2-P (hESF-DIF) were purchased by the Cell Science & Technology Institute (CSTI, Sendai, Japan). All other reagents were from Invitrogen (Carlsbad, CA) and Sigma. D-hESF10 medium consists of hESF-DIF medium supplemented with 6-factors, FGF-2, heparin, and TGF-β1 (R&D Systems, Minneapolis, MN). To harvest cells, 0.25% trypsin in 1 mM EDTA-4Na was used and the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). For differentiation into osteoblasts or adipocytes, the cells were cultured according to the instruction by the suppliers (Lonza, Basel, Switzerland). The differentiated cells were stained by Alizarin Red S (Wako Pure Chemical Industries, Osaka, Japan) or Oil Red O (Wako).

Cell proliferation

Before the serum-free experiments, cells grown in POWERDBY10 were incubated by in hESF9 medium without heparin and FGF-2 (hESF9(-/-)) overnight to starve the effect of serum. Cells were replaced at the cell density of $1x10^4$ cells /well on 24-well plate (BD Falcon) coated with 0.1% porcine gelatin solution (Millipore, Billerica, MA) and cultured in hESF9(-/-) medium in the presence of varying growth factors. The cell numbers were counted by Coulter Counter (Beckman Coulter, Hialeah, FL).

Gene expression

A detailed reverse transcription-polymerase chain reaction (RT-PCR) protocol was described previously (Furue, *et al.*, 2005). Total RNA was extracted from hMSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the provider's instructions. Q-RT-PCR was carried out using the TaqMan gene expression Master Mix on in ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the supplier's instructions (ABI). Specific primers-probe set were listed in Supplementary Table 2. Expression levels were all normalized by the expression level of *GAPDH*. The relative level of each gene in cDNA of undifferentiated hES cells was defined as "1." The KhES-3 cell line was used as a control; the cells were obtained from the Institute for Frontier Medical Science, Kyoto University, and the Review Board of the National Institute of Biomedical Innovation approved this research.

Antigen expression

For *in situ* immunocytochemisty, the cells were immunostained with antibodies, as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, fluorescence images were acquired using by IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, England). Flow cytometry was performed with BD FACS Canto flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, the labeled primary antibodies were used, but the biding of anti-SSEA-3, anti-CD56, and Tra-2-54 antibodies was visualized with RPE-conjugated goat anti-mouse Ig (Dako, Carpinteria, CA) or Alexa Fluor 647 goat anti-rat IgM (Invitrogen). The primary antibodies used are listed in Supplementary Table 3.

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References

AMIT, M., SHARIKI, C., MARGULETS, V. and ITSKOVITZ-ELDOR, J. (2004). Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* 70: 837-845.

BATTULA, V.L., TREML, S., ABELE, H. and BUHRING, H.J. (2008). Prospective isolation and characterization of mesenchymal stem cells from human placenta using a frizzled-9-specific monoclonal antibody. *Differentiation* 76: 326-336.

BENAVENTE, C.A., SIERRALTA, W.D., CONGET, P.A. and MINGUELL, J.J. (2003). Subcellular distribution and mitogenic effect of basic fibroblast growth factor in mesenchymal uncommitted stem cells. *Growth Factors* 21: 87-94.

BRUDER, S.P., JAISWAL, N. and HAYNESWORTH, S.E. (1997). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* 64: 278-294.

CHASE, L.G., LAKSHMIPATHY, U., SOLCHAGA, L.A., RAO, M.S. and VEMURI, M.C. (2010). A novel serum-free medium for the expansion of human mesenchymal stem cells. *Stem Cell Res Ther* 1: 8.

COLTER, D.C., SEKIYA, I. and PROCKOP, D.J. (2001). Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. *Proc Natl Acad Sci USA* 98: 7841-7845.

CONRAD, C., ZEINDL-EBERHART, E., MOOSMANN, S., NELSON, P.J., BRUNS, C.J. and HUSS, R. (2008). Alkaline phosphatase, glutathione-S-transferase-P, and cofilin-1 distinguish multipotent mesenchymal stromal cell lines derived from the bone marrow versus peripheral blood. *Stem Cells Dev* 17: 23-27.

DEZAWA, M., ISHIKAWA, H., ITOKAZU, Y., YOSHIHARA, T., HOSHINO, M., TAKEDA, S., IDE, C. and NABESHIMA, Y. (2005). Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* 309: 314-317.

DEZAWA, M., KANNO, H., HOSHINO, M., CHO, H., MATSUMOTO, N., ITOKAZU, Y., TAJIMA, N., YAMADA, H., SAWADA, H., ISHIKAWA, H. *et al.* (2004). Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 113: 1701-1710.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. and HORWITZ, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8: 315-317.

DRAPER, J.S., PIGOTT, C., THOMSON, J.A. and ANDREWS, P.W. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat* 200: 249-258.

FRIEDENSTEIN, A.J., PIATETZKY, S., II and PETRAKOVA, K.V. (1966). Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 16: 381-390.

FURUE, M., OKAMOTO, T., HAYASHI, Y., OKOCHI, H., FUJIMOTO, M., MYOISHI, Y., ABE, T., OHNUMA, K., SATO, G.H., ASASHIMA, M. *et al.* (2005). Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In vitro Cell Dev Biol Anim* 41: 19-28.

FURUE, M.K., NA, J., JACKSON, J.P., OKAMOTO, T., JONES, M., BAKER, D., HATA, R., MOORE, H.D., SATO, J.D. and ANDREWS, P.W. (2008). Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc Natl Acad Sci USA* 105: 13409-13414.

FURUE, M.K., TATEYAMA, D., KINEHARA, M., J. NA, OKAMOTO, T. and SATO, J.D. (2010). Advantages and difficulties in culturing human pluripotent stem cells in growth factor-defined serum-free medium. *In vitro Cell.Dev.Biol. Animal* 46: 573-576.

HAYNESWORTH, S.E., BABER, M.A. and CAPLAN, A.I. (1992). Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* 13: 69-80.

HOFFMAN, L.M. and CARPENTER, M.K. (2005). Characterization and culture of human embryonic stem cells. *Nat Biotechnol* 23: 699-708.

ISHII, K., YOSHIDA, Y., AKECHI, Y., SAKABE, T., NISHIO, R., IKEDA, R., TERABAYASHI, K., MATSUMI, Y., GONDA, K., OKAMOTO, H. *et al.* (2008). Hepatic differentiation of human bone marrow-derived mesenchymal stem cells by tetracycline-regulated hepatocyte nuclear factor 3beta. *Hepatology* 48: 597-606.

KULTERER, B., FRIEDL, G., JANDROSITZ, A., SANCHEZ-CABO, F., PROKESCH, A., PAAR, C., SCHEIDELER, M., WINDHAGER, R., PREISEGGER, K.H. and TRAJANOSKI, Z. (2007). Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics* 8: 70.

KURODA, Y., KITADA, M., WAKAO, S., NISHIKAWA, K., TANIMURA, Y., MAKINOSHIMA, H., GODA, M., AKASHI, H., INUTSUKA, A., NIWA, A. *et al.* (2010). Unique multipotent cells in adult human mesenchymal cell populations. *Proc Natl Acad Sci USA* 107: 8639-8643.

LENNON DP, H.S., BRUDER SP, JAISWAL N, CAPLAN AI. (1996). Human and animal mesenchymal progenitor cells from bone marrow: identfication of serum for optimal selection and proliferation. *In vitro Cell Dev Biol Anim* 32: 602-611.

LI, W.G. and XU, X.X. (2005). The expression of N-cadherin, fibronectin during chondrogenic differentiation of MSC induced by TGF-beta(1). *Chin J Traumatol* 8: 349-351.

METS, T. and VERDONK, G. (1981). *In vitro* aging of human bone marrow derived stromal cells. *Mech Ageing Dev* 16: 81-89.

MORI, T., KIYONO, T., IMABAYASHI, H., TAKEDA, Y., TSUCHIYA, K., MIYOSHI, S., MAKINO, H., MATSUMOTO, K., SAITO, H., OGAWA, S. *et al.* (2005). Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. *Mol Cell Biol* 25: 5183-5195.

NA, J., FURUE, M.K. and ANDREWS, P.W. (2010). Inhibition of ERK1/2 prevents neural and mesendodermal differentiation and promotes human embryonic stem cell self-renewal. *Stem Cell Res* 5: 157-169.

PANG, R., ZHANG, Y., PAN, X., GU, R., HOU, X., XIANG, P., LIU, Z., ZHU, X., HU, J., ZHAO, J. *et al.* (2010). Embryonic-like stem cell derived from adult bone marrow: immature morphology, cell surface markers, ultramicrostructure and differentiation into multinucleated fibers in vitro. *Cell Mol Biol (Noisy-le-grand)* 56 Suppl: OL1276-1285.

PITTENGER, M.F., MACKAY, A.M., BECK, S.C., JAISWAL, R.K., DOUGLAS, R., MOSCA, J.D., MOORMAN, M.A., SIMONETTI, D.W., CRAIG, S. and MARSHAK, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284: 143-147.

POCHAMPALLY, R.R., SMITH, J.R., YLOSTALO, J. and PROCKOP, D.J. (2004). Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood* 103: 1647-1652.

ROUBELAKIS, M.G., PAPPA, K.I., BITSIKA, V., ZAGOURA, D., VLAHOU, A., PAPADAKI, H.A., ANTSAKLIS, A. and ANAGNOU, N.P. (2007). Molecular and

proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. *Stem Cells Dev* 16: 931-952.

SEKIYA, I., LARSON, B.L., SMITH, J.R., POCHAMPALLY, R., CUI, J.G. and PROCKOP, D.J. (2002). Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 20: 530-541.

SHEVINSKY, L.H., KNOWLES, B.B., DAMJANOV, I. and SOLTER, D. (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell* 30: 697-705.

SHIMOMURA, T., YOSHIDA, Y., SAKABE, T., ISHII, K., GONDA, K., MURAI, R., TAKUBO, K., TSUCHIYA, H., HOSHIKAWA, Y., KURIMASA, A. *et al.* (2007). Hepatic differentiation of human bone marrow-derived UE7T-13 cells: Effects of cytokines and CCN family gene expression. *Hepatol Res* 37: 1068-1079.

TAKEUCHI, M., TAKEUCHI, K., KOHARA, A., SATOH, M., SHIODA, S., OZAWA, Y., OHTANI, A., MORITA, K., HIRANO, T., TERAI, M. *et al.* (2007). Chromosomal instability in human mesenchymal stem cells immortalized with human papilloma virus E6, E7, and hTERT genes. *In vitro'Cell Dev Biol Anim* 43: 129-138.

TSUTSUMI, S., SHIMAZU, A., MIYAZAKI, K., PAN, H., KOIKE, C., YOSHIDA, E,, TAKAGISHI, K. and KATO, Y. (2001). Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun* 288: 413-419.

of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). *Journal of Clinical Oncology*, **23,** 1984–1992.

Orciuolo, E., Buda, G., Cecconi, N., Galimberti, S., Versari, D., Cervetti, G., Salvetti, A. & Petrini, M. (2007) Unexpected cardiotoxicity in haematological bortezomib treated patients. *British Journal of Haematology*, **138,** 396–397.

Orlowski, R.Z., Nagler, A., Sonneveld, P., Blade, J., Hajek, R., Spencer, A., San Miguel, J., Robak, T., Dmoszynska, A., Horvath, N., Spicka, I., Sutherland, H.J., Suvorov, A.N., Zhuang, S.H., Parekh, T., Xiu, L., Yuan, Z., Rackoff, W. & Harousseau, J.L. (2007) Randomized phase III study of pegylated liposomal doxorubicin plus bortezomib compared with bortezomib alone in relapsed or refractory multiple myeloma: combination therapy improves time to progression. *Journal of Clinical Oncology*, **25,** 3892–3901.

Sonneveld, P., Hajek, R., Nagler, A., Spencer, A., Blade, J., Robak, T., Zhuang, S.H., Harousseau, J.L. & Orlowski, R.Z. (2008) Combined pegylated liposomal doxorubicin and bortezomib is highly effective in patients with recurrent or refractory multiple myeloma who received prior thalidomide/lenalidomide therapy. *Cancer*, **112,** 1529–1537.

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Serum granulysin as a possible biomarker of natural killer cell neoplasms

Granulysin is a cytolytic and proinflammatory molecule that is excreted from cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. It is synthesized as a 15-kDa molecule and then cleaved at the amino and carboxy termini to produce an active 9-kDa form (Pena *et al*, 1997). Equivalent amounts of these two forms of granulysin are found in CTL and NK cells. However, the 9-kDa form is sequestered in cytolytic granules, while the 15-kDa form is constitutively secreted and more stable than the 9-kDa form when excreted *in vivo*. Therefore, the 15-kDa form constitutes a major portion of serum granulysin (Ogawa *et al*, 2003) and is considered to be a biomarker.

We have previously reported that serum granulysin reflects on cellular immune capacity (Ogawa *et al*, 2003), anti-tumour activity (Nagasawa *et al*, 2005) and graft-*versus*-host reaction in allogeneic transplantation (Nagasawa *et al*, 2006). Recently, it has been reported that granulysin is an important mediator of keratinocyte death in Stevens–Johnson syndrome and toxic epidermal necrolysis (Chung *et al*, 2008). Considering that granulysin is usually expressed in activated CTL, but not in resting or naive CTL, and constitutively expressed in NK cells, it was speculated that serum granulysin could be a biomarker for NK cell-related disease.

In this context, serum granulysin was retrospectively investigated in a patient with long-term NK type chronic active Epstein–Barr virus (EBV) infection (CAEBV). Serum granulysin was measured using our previously reported enzyme-linked immunosorbent assay method (Ogawa *et al*, 2003).

The patient presented with hydroa vacciniforme at the age of 8 years, and was diagnosed as NK type CAEBV when aged 9 years. In addition to the aggravation of facial skin lesion, general malaise progressed gradually. She was referred to our hospital at 16 years of age, and infusion of autologous activated T cells was started as a cell therapy. As no improvement was achieved, cytotoxic chemotherapy was started to eradicate EBV-infected cells. Although EBV load was markedly reduced after chemotherapy, the skin lesion did not improve and biopsy revealed the presence of EBV-infected cells (Fig 1B).

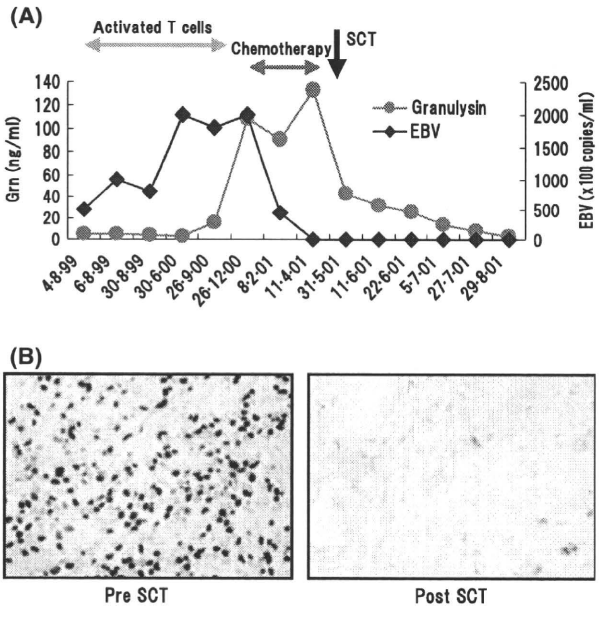

Fig 1. (A) Serum granulysin levels (Grn) and EBV genome load in the peripheral blood in a patient with NK type CAEBV. (B) EBV-infected cells in facial skin disappeared after SCT. EBER positive cells are stained dark brown. Original magnification ×400.

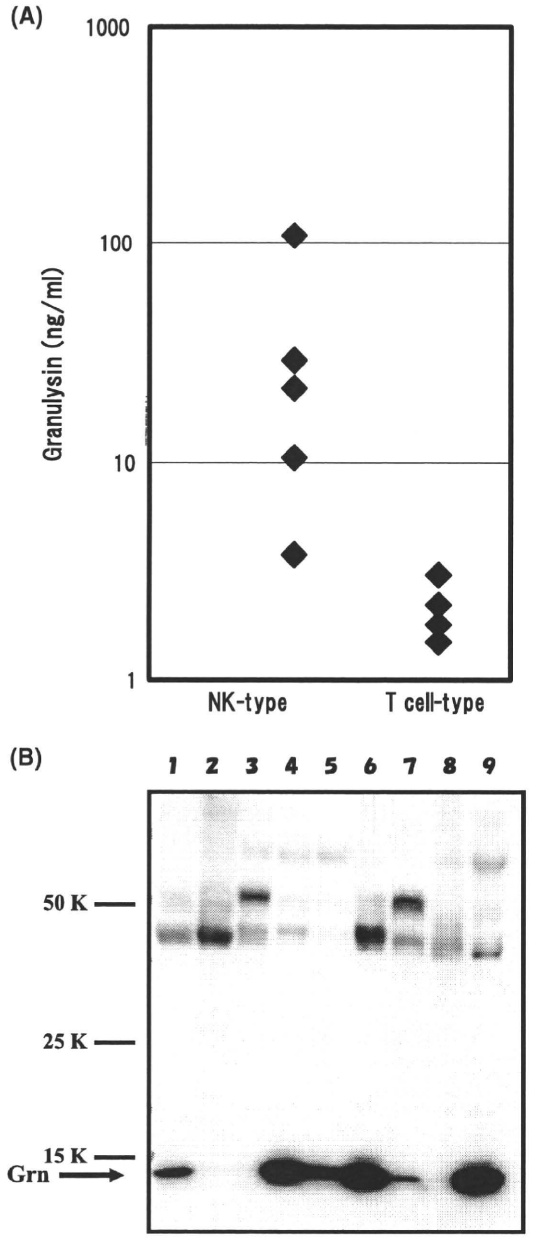

Fig 2. (A) Serum granulysin levels in five NK-type and four T cell-type (αβT) patients with CAEBV. (B) Expression of granulysin in EBV infected NK and γδ T cell lines. The monoclonal antibody, RF10 (Ogawa *et al*, 2003), which reacts with 15-kDa but not 9-kDa granulysin, was used for Western blotting. Lane number and cell line; 1:SNK1(NK) 2:SNK6(NK) 3:SNK8(γδT) 4:SNK10(NK) 5:SNK11(NK) 6:SNK5(NK) 7:SNK15(γδT) 8:SNK16(γδT) 9:SNK20(γδT).

In order to totally cure this condition, the patient received a bone marrow stem cell transplantation (SCT) from a human leucocyte antigen-identical unrelated donor at the age of 18 years. A skin biopsy performed after SCT showed complete disappearance of EBVinfected cells, and serum granulysin was reduced to levels within the normal range (1·5 ± 3·0 ng/ml; Fig 1A) (Ogawa *et al*, 2003).

We also investigated serum granulysin levels in patients with NK type and T cell-type (αβT cell) CAEBV (Kimura *et al*, 2005). Serum granulysin was elevated in NK type but not in T cell-type CAEBV (Fig 2A). Interestingly, serum granulysin was significantly elevated in one patient whose γδ T cells were infected with EBV (data not shown). Next, we investigated the expression of granulysin in several EBV-infected cell lines that were established from CAEBV patients. As expected, most of the NK and γδ T cell lines expressed granulysin (Fig 2B). (SNK11 clone was established from the patient described above and it was clonal in terms of EBV infection). Interestingly, tumour necrosis factor α (TNF-α) was exclusively excreted from the granulysin-expressing cell lines, although interferon-γ was produced in all cell lines (data not shown). From these observations, serum granulysin seems to be a useful biomarker of NK cell neoplasms and could be a marker of its malignant transformation, although the NK proliferative diseases examined here were all EBV-related. Comparison between EBV- and non-EBV- related NK or γδT cell disorders is also an interesting issue regarding not only their pathophysiology but also the mechanism of granulysin regulation, which is not precisely known yet. Further investigation is required to determine its clinical use and significance.

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References

Chung, W.H., Hung, S.I., Yang, J.Y., Su, S.C., Huang, S.P., Wei, C.Y., Chin, S.W., Chiou, C.C., Chu, S.C., Ho, H.C., Yang, C.H., Lu, C.F., Wu, J.Y., Liao, Y.D. & Chen, Y.T. (2008) Granulysin is a key mediator for disseminated keratinocyte death in Stevens–Johnson syndrome and toxic epidermal necrolysis. *Nature Medicine*, **12,** 1343–1350.

Kimura, H., Hoshino, Y., Hara, S., Sugaya, N., Kawada, J., Shibata, Y., Kojima, S., Nagasaka, T., Kuzushima, K. & Morishima, T. (2005) Differences between T cell-type and natural killer cell-type chronic active Epstein–Barr virus infection. *The Journal of Infectious Disease*, **191,** 531–539.

Nagasawa, M., Kawamoto, H., Tsuji, Y. & Mizutani, S. (2005) Transient increase of serum granulysin in stage IVs neuroblastoma patient during spontaneous regression: case report. *International Journal of Hematology*, **82,** 456–457.

Nagasawa, M., Isoda, T., Itoh, S., Kajiwara, M., Morio, T., Shimizu, N., Ogawa, K., Nagata, K., Nakamura, M. & Mizutani, S. (2006) Analysis of serum granulysin in patients with hematopoietic stem cell transplantation: its usefulness as a marker of graft-versus-host reaction. *American Journal of Hematology*, **81,** 340–348.

Ogawa, K., Takamori, Y., Suzuki, K., Nagasawa, M., Takano, S., Kasahara, Y., Nakamura, Y., Kondo, S., Sugamura, K., Nakamura, M. & Nagata, K. (2003) Granulysin in human serum as a marker of cell-mediated immunity. *European Journal of Immunology*, **33,** 1925–1933.

Pena, S.V., Hanson, D.A., Carr, B.A., Goralski, T.J. & Krensky, A.M. (1997) Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. *Journal of Immunology*, **158,** 2680–2688.

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Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR

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ABSTRACT

Aim To measure the bacterial genome in ocular fluids and to analyse the clinical relevance of infectious endophthalmitis.

Methods Nineteen ocular fluid samples (eight aqueous humour and 11 vitreous fluid samples) were collected from 19 patients with suspected bacterial endophthalmitis. Fifty ocular samples from uveitis patients were also collected along with 40 samples from patients without ocular inflammation and used as controls. Bacterial ribosomal DNA (16S rDNA) was measured by a quantitative PCR assay.

Results Bacterial 16S rDNA was detected in patients with clinically suspected bacterial endophthalmitis (18/19, 95%). With the exception of one case, high copy numbers of bacterial DNA were detected (1.7×10^3–1.7×10^9 copies/ml) in these patients. There were 10 samples (53%) with positive bacterial cultures while there were nine samples (47%) with positive Gram-staining. Real-time PCR detected bacterial 16S rDNA in three (6%) of the 50 samples from the control uveitis patients. In addition, none of the samples from the control patients without intraocular inflammation were positive.

Conclusions Quantitative broad-range PCR of bacterial 16S rDNA is a useful tool for diagnosing bacterial endophthalmitis.

INTRODUCTION

Bacterial infectious endophthalmitis occurs due to exogenous infections, such as those arising from trauma and intraocular surgery, or from endogenous infections, such as systemic infectious disorders. Previous studies have used PCR to demonstrate the presence of bacterial DNA in the ocular fluids in patients with infectious endophthalmitis.[1–10] PCR has often been used to provide evidence of bacterial involvement in the eyes with suspected intraocular infections.[8] These suspected infections include idiopathic endophthalmitis and uveitis. Recent advances in molecular biology along with the use of real-time PCR have made it possible to determine quantitative measurements of the viral load associated with viral diseases in the eye.[11–13] Several studies have recently reported finding the bacterial ribosomal RNA gene (16S rDNA) in the ocular fluids of patients with infectious endophthalmitis.[4 8 10] With primers of the bacterial 16S rRNA gene, broad-range PCR can be used to detect the presence of bacteria within the samples. In endophthalmitis patients with previous intravitreal administration of antibiotics, PCR methodology has been shown to be more effective than bacterial cultures in detecting bacterial DNA in the ocular fluids.[10] However, even broad-range PCR has not been able to determine quantitative information for the bacterial genome in the ocular sample.

In the present study, after collecting ocular samples from patients with suspected intraocular infections, which included bacterial infectious endophthalmitis, we attempted to detect and then measure the bacterial genome using real-time quantitative PCR with primers for 16S rDNA amplifications.

MATERIAL AND METHODS

Subjects

Based upon medical history and clinical observations, 69 patients with endophthalmitis and uveitis were consecutively enrolled in a prospective study that was conducted from 2008 to 2009 at the Tokyo Medical and Dental University Hospital. Samples of aqueous humour and vitreous fluids were collected from all patients. Nineteen patients (19 eyes: eight aqueous humour and 11 vitreous fluids) had bacterial infectious endophthalmitis. Of these 19 patients, six had acute postoperative endophthalmitis, four had late postoperative endophthalmitis, one had post-traumatic endophthalmitis, five had endogenous endophthalmitis, two had keratitis-associated endophthalmitis, and one had endophthalmitis after intravitreal injections of bevacizumab.

The second patient group was also a prospective study, and 50 ocular samples were collected from various patients with uveitis. The underlying pathology included idiopathic uveitis (n=21), herpetic keratouveitis (n=3), herpetic anterior iridocyclitis (n=3), acute retinal necrosis (n=5), cytomegalovirus retinitis (n=2), toxoplasmosis (n=3), toxocariasis (n=2), sarcoidosis (n=2), HTLV-1-associated uveitis (n=1), toxic lens syndrome (n=1), *Candida* endophthalmitis (n=2) and intraocular lymphoma (n=5). In this study, fungal endophthalmitis cases such as *Candida* endophthalmitis were classified as being part of this patient group. All the patients displayed active intraocular inflammation at the time of sampling.

In addition to the patient groups, we also analysed samples from a control group. These patients were enrolled in this prospective study in 2009. Forty samples (20 aqueous humour and 20 vitreous fluids) were collected from patients who did not have any type of ocular inflammation (age-related cataract, macular oedema secondary to branch retinal vein occlusion, retinal detachment, idiopathic macular hole or idiopathic epiretinal membrane).

For the ocular sampling (asepsis), the following procedures were performed in all subjects. In all of the eyes that were sampled, the ocular surfaces, including the conjunctival sacs, were rinsed once with an aqueous povidone iodine solution. Subsequently, all of these eyes were then rinsed once with a balanced-salt solution. A 0.1 ml aliquot of aqueous humour was collected aseptically in a syringe with a 30 G needle. Half of the sample was then transferred into a pre-sterilised microfuge tube and used for PCR.

In patients with endophthalmitis/uveitis who were undergoing vitreous surgery, uncontaminated non-diluted vitreous fluid samples (0.5–1.0 ml) were collected during diagnostic pars plana vitrectomy (PPV). Immediately after collection, 100 µl of the sample was transferred into a pre-sterilised microfuge tube and used for PCR. None of the asepsis samples used for analysis came from patients being given systemic antibiotics or from patients who were receiving intraocular antibiotic injections.

Conventional microbiological investigations

The Bacteria Work Station of the Tokyo Medical and Dental University Hospital processed all specimens (aqueous humour and vitreous fluids) within 1 h after the sample collection, with standard methods followed for the isolation and identification of the aerobic and anaerobic bacterial cultures. The culture methods followed conventional techniques that have been previously published.[14 15] Cultures were incubated for up to 7 days, with those lacking growth designated as culture-negative. Cytospin smears of the specimens were stained using Gram's method for detection of bacteria.

Quantitative PCR

DNA was extracted from samples using a DNA minikit (Qiagen, Valencia, California, USA) installed on a Robotic workstation for automated purification of nucleic acids (BioRobot E21, Qiagen). The real-time PCR was performed using Amplitaq Gold and the Real-Time PCR 7300 system (Applied Biosystems, Foster City, California, USA). Primers and probes of bacterial 16S rDNA and the PCR conditions are described elsewhere.[16] The sense primer (Bac349F) was 5′-AGGCAGCAGTDRGGAAT-3′ and the antisense primer (Bac806R) was 5′-GGACTACYVGGGTATCT-AAT-3′. The TaqMan probe (Bac516F) was 5′-FAM-TGCCAGC-AGCCGCGGTAATACRDAG-TAMRA-3′. Products were subjected to 50 cycles of PCR amplification, with cycling conditions set at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Bacterial copy number values of more than 100 copies/ml in the sample were considered to be significant.

Sensitivity of TaqMan real-time PCR

To confirm the real-time PCR assay sensitivity, the 458 bp fragments were amplified from the DNA of *Staphylococcus aureus* (NBRC 12732) with Bac349F and Bac806R. The PCR fragments were inserted into the pGEM cloning plasmid with the pGEM T-Easy Vector Cloning System I kit (Promega, Tokyo, Japan). The plasmid was digested with restriction enzyme ScaI. Linearised plasmid was controlled by gel electrophoresis and quantified by using the Smart Ladder DNA size and mass marker (Wako, Tokyo, Japan) and the OD260 measurement. Standard curves were constructed from serial 10-fold dilutions of linearised plasmid DNA with 10 ng/µl MS2 RNA (Basel, Roche, Switzerland). The detection limit and standard range of the TaqMan real-time PCR were determined by using serial 10-fold dilutions of linearised plasmid. The standard range of DNA was linearly quantified from one to nine log DNA copies, with a detection limit of 10 copies. The negative control (nuclease-free water) was not detected.

PCR FOR 16S rRNA GENE AND SEQUENCE ANALYSIS

PCR mix (50 µl volumes) was prepared from Low-DNA AmpliTaq Gold DNA polymerase LD (Applied Biosystems). The mix comprised dATP, dGTP, dCTP, dTTP, 2 mM $MgCl_2$ and 1×Gold buffer, along with each of the primers (500 nM) (forward primer fD1-AGAGTTTGATCCTGGCTCAG; reverse primer rp2-ACGGCTACCTTGTTACGACTT).[17]

Template DNA, 1.25U of AmpliTaq Gold DNA polymerase LD (Applied Biosystems), and nuclease-free water were added to the sample. The PCR assay was performed using the Takara Thermal Cycler TP-400 (Takara Bio Inc., Shiga, Japan). The cycling conditions used were: 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 42°C for 30 s, and 72°C for 4 min. Gel electrophoresis was performed using a 0.8% agarose gel (Takara Bio Inc.) in 40 mmol/l Tris, 1 mmol/l EDTA for 30 min at 100 V, followed by ethidium bromide staining. Before cycle sequencing, amplicons were purified using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's protocol. Cycle sequencing was performed by forward and reverse priming using the Big Dye v3.1 Terminator Reaction kit (Applied Biosystems). The PCR assay was performed using a Perkin Elmer 9700 with cycling conditions set at: 95°C for 30 s, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Electrophoresis was conducted in a 3130xl genetic analyser (Applied Biosystems).

We used the DNA sequence analysis to examine patients suspected of having bacterial endophthalmitis (patient samples that only had high amounts of total DNA and detected high copy numbers of bacterial 16S rDNA). Basic local alignment search tool (BLAST) analysis was used to examine the DNA sequences. The 16S rDNA sequences obtained were compared with those available in the GenBank BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Using a previously published method,[18] positive identification of the species level was defined as identification of a 16S rDNA sequence that had 99% similarity or greater with that of the GenBank BLAST strain sequence.

Prevention of bacterial contamination

To ensure that no contamination of the PCR preparation occurred, the DNA amplification and the analysis of the amplified products were done in separate laboratories. The preparation was performed on a laminar flow workbench and employed single-use aliquots of reagent and dedicated pipettes. Microfuge tubes and mineral oil aliquots were carefully sterilised prior to use.

RESULTS

Our initial PCR results indicated that bacterial 16S rDNA was positive in 18 ocular fluids of the clinically suspected bacterial endophthalmitis patients (18/19, 95%, table 1). These positive patients had high copy numbers of 16S rDNA ranging from 1.7×10^3 to 1.7×10^9 copies/ml, which indicated the presence of bacterial infection. In the one PCR-negative case (case 16 in table 1), PCR did not detect any bacterial genome in the vitreous fluid (<100 copies), although *Klebsiella pneumoniae* was detected in the biopsy sample of the liver abscess.

In the conventional bacterial cultures, 10 (53%) out of the 19 samples were positive (table 1). In addition, positive Gram staining was found in nine (47%) out of these samples. There were only two patients (cases 2 and 4 in table 1) that received

Table 1 Detection of bacterial 16S rDNA in suspected bacterial endophthalmitis and uveitis

Case	Diagnosis	Sample	Bacterial 16S rDNA	Culture	Smear	BLAST analysis	Treatment
1	Postoperative (acute)	AH	2.8×10^8 copies/ml	*Staphylococcus* spp.	Negative	nt	PPV, IAI, SA
2	Postoperative (acute)	VF	1.5×10^8 copies/ml	Negative	Negative	nt	PPV, IAI, SA
3	Postoperative (acute)	AH	1.5×10^6 copies/ml	*Staphylococcus epidermidis*	G (+)	*Staphylococcus epidermidis*	PPV, IAI, SA
4	Postoperative (acute)	VF	7.5×10^6 copies/ml	Negative	Negative	nt	PPV, IAI, SA
5	Postoperative (acute)	VF	9.0×10^7 copies/ml	Negative	G (+)	nt	PPV, IAI, SA
6	Postoperative (acute)	VF	1.9×10^7 copies/ml	*Streptococcus sanguinis*	G (+)	*Streptococcus sanguinis*	PPV, IAI, SA
7	Postoperative (late)	VF	8.1×10^7 copies/ml	Negative	Negative	*Bradyrhizobium elkanii*	PPV, IAI, SA
8	Postoperative (late)	AH	1.7×10^3 copies/ml	Negative	Negative	nt	SA
9	Postoperative (late)	AH	3.9×10^4 copies/ml	Negative	Negative	nt	SA
10	Postoperative (late)	AH	8.6×10^4 copies/ml	*Pseudomonas aeruginosa*	G (−)	nt	PPV, IAI, SA
11	Post-traumatic	VF	1.4×10^6 copies/ml	*Enterococcus faecalis*	G (+)	*Enterococcus faecalis*	PPV, SA
12	Endogenous	VF	1.3×10^7 copies/ml	*Pseudomonas* sp.	G (−)	*Pseudomonas* sp. PR	PPV, IAI, SA
13	Endogenous	VF	1.7×10^9 copies/ml	*α-Streptococcus*	G (+)	*Streptococcus mitis*	PPV, IAI, SA
14	Endogenous	AH	1.1×10^4 copies/ml	Negative	Negative	nt	IAI, SA
15	Endogenous	VF	5.5×10^6 copies/ml	*Staphylococcus aureus*	Negative	*Staphylococcus aureus*	PPV, IAI, SA
16	Endogenous	AH	<100 copies/ml	Negative	Negative	nt	PPV, IAI, SA
17	Keratitis	AH	3.1×10^6 copies/ml	*Streptococcus pneumoniae*	G (+)	*Streptococcus pneumoniae*	IAI, SA
18	Keratitis	VF	6.8×10^4 copies/ml	Negative	Negative	nt	IAI, SA
19	Intravitreal injection*	VF	1.8×10^6 copies/ml	*Streptococcus oralis*	G (+)	*Streptococcus* sp.	PPV, IAI, SA
20	Idiopathic uveitis	AH	1.4×10^3 copies/ml	Negative	nt	nt	IAI
21	Idiopathic uveitis	VF	6.1×10^4 copies/ml	Negative	Negative	nt	SA
22	CMV retinitis	AH	4.2×10^3 copies/ml	Negative	nt	nt	IAI, SA

AH, aqueous humour; BLAST, basic local alignment search tool; CMV, cytomegalovirus; IAI, intravitreal antibiotic injection; nt, not tested; PPV, pars plana vitrectomy; SA, systemic antibiotics; VF, vitreous fluids.
Using broad-range quantitative PCR, bacterial 16S rDNA could be detected in the ocular samples of the suspected bacterial endophthalmitis cases (18/19, 95%). Broad-range quantitative PCR was also used to measure the bacterial genome in the ocular samples collected from the uveitis patients (n=50) and from the three patients (6%) that were positive.
*Bacterial endophthalmitis after intravitreal injections of bevacizumab.

intravitreal injections of antibiotics prior to the PCR analysis. As shown in table 1, after examinations that included PCR, all patients received antibiotics (systemic and/or local medications).

With the exception of three out of the 50 uveitis patients, real-time PCR indicated the patients were negative for the bacterial 16S rDNA. Details for the three exceptions are shown in table 1.

The 16S rDNA was detected in two patients with idiopathic uveitis and one with cytomegalovirus (CMV) retinitis. Clinically, all of these patients were diagnosed with unilateral uveitis. Bacterial cultures were negative in all of the tested samples. In addition, bacterial 16S rDNA was not detected in any of the 40 control samples collected from the patients without ocular inflammation.

To identify the specific bacterial species, we used BLAST analysis to examine some of the bacterial infectious endophthalmitis patients. Analysis was only possible when the patient's samples had high amounts of total DNA and there was a detected high copy number of the bacterial 16S rDNA. As summarised in table 1, BLAST analysis identified *Staphylococcus epidermidis* (case 3), *Streptococcus sanguinis* (case 6), *Bradyrhizobium elkanii* (case 7), *Enterococcus faecalis* (case 11), *Pseudomonas* sp. *PR* (case 12), *Streptococcus mitis* (case 13), *Staphylococcus aureus* (case 15), *Streptococcus pneumoniae* (case 17) and *Staphylococcus* sp. (case 19). The results of the BLAST analysis were identical to the results of the bacterial culture with the exception of case 7, who was found to have a negative culture. However, even though the bacterial examinations such as bacterial cultures and smears were negative in this patient with late postoperative endophthalmitis, broad-range real-time PCR analysis of the vitreous sample yielded positive results (8.1×10^7 copies/ml). In the present study, once we were able to determine the bacterial species via the BLAST analysis and conclusively diagnose bacterial endophthalmitis, we were then able to begin treatment with antibiotics.

Case report

As seen in table 1, case 7 was a 75-year-old man who was referred to the uveitis clinic at our hospital during July 2007 due to keratic precipitates, cells and fibrin in the anterior chamber along with hypopyon and anterior vitreous opacity in his right eye (figure 1). The patient had undergone cataract surgery in his right eye 1 year prior to being seen in our clinic. Although visual acuity of his right eye at the time of his initial presentation to our clinic was 0.8, 2 months later, his visual acuity was less than 0.1. A vitreous sample was collected during the pars plana

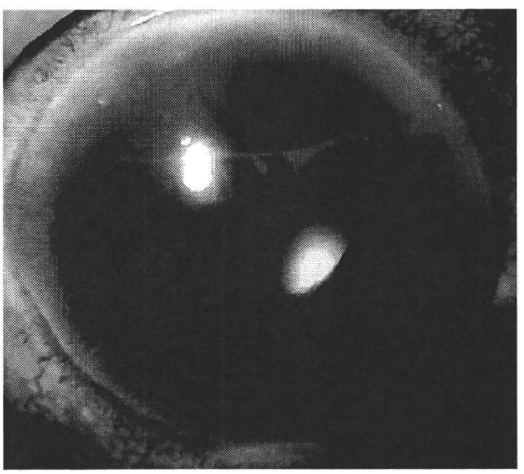

Figure 1 Case 7 (late postoperative endophthalmitis). Slit-lamp photograph in suspected bacterial endophthalmitis. In the right eye, cyclitic membrane, height of the hypopion, and severity of vitritis were seen. In this patient, broad-range quantitative PCR revealed a high copy number of the bacterial genome (8.1×10^7 copies/ml). Basic local alignment search tool (BLAST) analysis detected *Bradyrhizobium elkanii*.

vitrectomy. While bacterial culture and the Gram-staining of the vitreous sample were negative, broad-range and real-time PCR detected 8.1×10^7 copies/ml of bacterial 16S rDNA (table 1). In addition, the BLAST analysis detected *Bradyrhizobium elkanii*. After the patient was given an intravitreal antibiotic injection (vancomycin and ceftazidime) and systemic antibiotics (levofloxacin), inflammation in his right eye completely disappeared. After receiving treatment, visual acuity in his right eye recovered to 0.9 and there was no severe intraocular tissue damage noted.

DISCUSSION

In the present study, with the exception of one patient, we detected bacterial 16S rDNA in all of the cases that were clinically suspected to have bacterial endophthalmitis. In these patients, high copy numbers of the bacterial DNA were detected, which indicated the presence of a bacterial infection. In the single patient who was suspected of having infectious endophthalmitis but had no bacteria in the ocular sample, *K. pneumoniae* was detected by biopsy culture for liver infection. Thus, we were ultimately able to diagnose the patient as having endogenous endophthalmitis.

On the other hand, conventional microbiological investigations of the ocular fluid samples, such as bacterial cultures and smears, were negative in about one-half of these patients. Only three of the 50 samples collected from the patients with other clinical entities of uveitis were positive for the broad-range real-time PCR analyses of the bacterial 16S rDNA. In addition, no bacterial 16S rDNA was detected in any of the samples from the control patients without ocular inflammation.

The potential advantage of using PCR is that minute numbers of bacteria can be detected from the very small specimens that are required for the analysis. Chen *et al*[19] developed this PCR detection method for the eubacterial genome based on the conserved regions of the 16S rRNA sequence (16S rDNA) of *Escherichia coli*. As the universal primers chosen from 16S rDNA have a large amount of sequence information and highly conserved regions of the gene, primers can be synthesised for a wide variety of bacteria. In addition, the eubacterial primers used had both a high specificity and sensitivity, which was comparable to previous studies.[1 3] Hykin *et al*[1] examined 29 control vitreous samples and found four that were positive for the eubacterial genome using PCR. In a further study by Therese *et al*,[3] only a single control sample (5%) was found using the eubacterial-based PCR. In the present study, we did not detect any bacterial 16S rDNA (<100 copies/ml) in any of the samples from the control non-infectious patients when using our broad-range real-time PCR. Thus, another potential advantage of our PCR system is that it provides quantitative information for the bacterial infection. In the present study, we found false positive results (1–100 copies/ml) in only two control samples that we tested, a result that could be due to contamination caused by the conjunctival ocular flora present during the collection of the samples. Other possible causes of the contamination might be related to technical errors that occurred during the PCR preparation or perhaps due to bacterial exposure when collecting the ocular sample.

In cases of bacterial infectious endophthalmitis, it is often difficult to differentiate between inflammation caused by non-infectious and infectious agents. For example, to determine the cause of postoperative inflammation in the eye, we must consider many different possibilities, such as surgical manipulation, toxic lens syndrome, recurrent uveitis (especially if the patient has a previous history) or bacterial endophthalmitis. In the past, microbiological investigations of the ocular fluids have often failed to detect the infectious agent in bacterial endophthalmitis, resulting in a clinical dilemma regarding therapy. Deciding to use antibiotics and steroids necessitates determining whether an inflammation is infectious or sterile. Therefore, an aetiological diagnosis is essential in such cases. The use of PCR with universal eubacterial primers, which possesses broad specificities for all Gram-positive and -negative bacteria, has been recently found to be much more useful for detecting the eubacterial genome in ocular samples of postoperative endophthalmitis cases compared to the routine microbiological investigations.[2 3 5 6 8–10] In the present study, our broad-range real-time PCR for the eubacterial genome showed high correlation with the bacteriologically positive samples. This suggests that bacteriologically negative samples may include the bacterial genome. In a recent report by the French Institutional Endophthalmitis Study Groups, eubacterial PCR was found to be much more effective than bacterial cultures in detecting bacteria in vitreous samples from patients with previous intravitreal administration of antibiotics.[10] Although the previous administration of antibiotics in the PPV vitreous fluids may inhibit bacterial growth, it is assumed that PCR may still be able to detect bacterial DNA of either living or killed bacteria.

As revealed in this study, real-time PCR found only three (6%) of the 50 ocular samples from patients with unilateral uveitis to be positive. However, high copy numbers of bacterial DNA were detected in these uveitis patients, which included idiopathic uveitis (n=2) and cytomegalovirus retinitis (n=1). Endophthalmitis and uveitis positive cases with low quantification of DNA (eg, 1×10^3–1×10^4 copies/ml) cannot be differentiated according to the number of copies. Although topical or systemic steroids were administered for long periods in the idiopathic uveitis patients, the inflammation remained uncontrolled. It has also been reported that viral PCR has found cytomegalovirus DNA in the eyes of cytomegalovirus retinitis cases.[13] When these patients were given intravitreal administration of an antiviral injection (Ganciclovir), an anterior vitreous opacity was subsequently observed. There were three cases that received antibiotics (intravitreal injection and/or systemic) in our study and the intraocular inflammation, such as vitreous opacity, was well controlled by this antibiotic therapy. Although bacterial DNA amplification in such cases usually suggests contamination, antibiotic administration proved to be effective in our study. Thus, the bacterial PCR-based evidence suggests bacterial involvement in eyes that have a suspected intraocular infection. While PCR for eubacterial detection is necessary for rapid and accurate diagnosis in patients suffering from an unknown intraocular inflammatory disorder, it can also be used to accurately determine samples that are not infected. In our study we found 47 samples (94%) that had negative PCR results. Overall, our results suggest that a sensitive and rapid diagnostic test not only allows for confident verification of the diagnosis (non-infectious inflammation vs infection), but also allows for early commencement of specific and appropriate treatment. In addition, PCR analysis is able to exclude bacterial infections as the potential cause of an ocular disorder.

In conclusion, this new PCR system is an excellent diagnostic system for intraocular specimens and can be used as an alternative to further examine specimens determined to be bacteriologically negative by conventional methods. Our study also clearly demonstrated that a new diagnostic PCR system using eubacterial detection with broad-range PCR along with quantitative evaluation with real-time PCR could be extremely useful for detecting bacterial DNA within ocular samples. Recently, Goldschmidt *et al* reported that a new diagnostic test for

Propionibacteriaceae was designed using TaqMan real-time PCR.[20] Therefore, the ability to be able to collect quantitative information on bacterial infections in the eye should be useful in helping to determine clinical diagnoses and therapeutic follow-ups. Moreover, using a combination of the quantitative PCR method and the BLAST analysis to detect bacterial species is a very valuable tool for diagnosing suspected bacterial endophthalmitis. However, the DNA in 10 of 19 samples could not be sequenced using this technique and thus could not identified, which could potentially limit the clinical usefulness of this technique at the present time. In order for clinicians to be able to obtain bacterial identifications, we may need to consider additional options for the sequence analysis. In addition, in the future we will need to further verify whether this broad-range PCR can detect candidate bacterial DNA including *K. pneumoniae* in bacterial endophthalmitis.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Ethics Committee of Tokyo Medical and Dental University. The research followed the tenets of the Declaration of Helsinki.

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REFERENCES

1. **Hykin PG,** Tobal K, McIntyre G, *et al*. The diagnosis of delayed post-operative endophthalmitis by polymerase chain reaction of bacterial DNA in vitreous samples. *J Med Microbiol* 1994;**40**:408–15.
2. **Lohmann CP,** Heeb M, Linde HJ, *et al*. Diagnosis of infectious endophthalmitis after cataract surgery by polymerase chain reaction. *J Cataract Refract Surg* 1998;**24**:821–6.
3. **Therese KL,** Anand AR, Madhavan HN. Polymerase chain reaction in the diagnosis of bacterial endophthalmitis. *Br J Ophthalmol* 1998;**82**:1078–82.
4. **Knox CM,** Cevallos V, Margolis TP, *et al*. Identification of bacterial pathogens in patients with endophthalmitis by 16S ribosomal DNA typing. *Am J Ophthalmol* 1999;**128**:511–12.
5. **Lohmann CP,** Linde HJ, Reischl U. Improved detection of microorganisms by polymerase chain reaction in delayed endophthalmitis after cataract surgery. *Ophthalmology* 2000;**107**:1047–51.
6. **Anand AR,** Madhavan HN, Therese KL. Use of polymerase chain reaction (PCR) and DNA probe hybridization to determine the Gram reaction of the infecting bacterium in the intraocular fluids of patients with endophthalmitis. *J Infect* 2000;**41**:221–6.
7. **Okhravi N,** Adamson P, Lightman S. Use of PCR in endophthalmitis. *Ocul Immunol Inflamm* 2000;**8**:189–200.
8. **Okhravi N,** Adamson P, Carroll N, *et al*. PCR-based evidence of bacterial involvement in eyes with suspected intraocular infection. *Invest Ophthalmol Vis Sci* 2000;**41**:3474–9.
9. **Chiquet C,** Lina G, Benito Y, *et al*. Polymerase chain reaction identification in aqueous humour of patients with postoperative endophthalmitis. *J Cataract Refract Surg* 2007;**33**:635–41.
10. **Chiquet C,** Cornut PL, Benito Y, *et al*. Eubacterial PCR for bacterial detection and identification in 100 acute postcataract surgery endophthalmitis. *Invest Ophthalmol Vis Sci* 2008;**49**:1971–8.
11. **Sugita S,** Shimizu N, Kawaguchi T, *et al*. Identification of human herpes virus 6 in a patient with severe unilateral panuveitis. *Arch Ophthalmol* 2007;**125**:1426–71.
12. **Kido S,** Sugita S, Horie S, *et al*. Association of varicella zoster virus load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. *Br J Ophthalmol* 2008;**92**:505–8.
13. **Sugita S,** Shimizu N, Watanabe K, *et al*. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. *Br J Ophthalmol* 2008;**92**:928–32.
14. **Allen SD.** Anaerobic bacteria. In: Lennete Edwin H, ed. *Manual of clinical microbiology*. 4th edn. Washington DC: American Society for Microbiology, 1985:413–72.
15. **Baron EJ,** Peterson LR, Finegold SM. *Bailey and Scott's diagnostic microbiology*. 9th edn. St Louis: Mosby, 1994:79–136.
16. **Takai K,** Horikoshi K. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl Environ Microbiol* 2000;**66**:5066–72.
17. **Weisburg WG,** Barns SM, Pelletier DA, *et al*. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;**173**:697–703.
18. **Goldenberger D,** Kunzle A. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J Clin Microbiol* 1997;**35**:2733–9.
19. **Chen K,** Neimark H, Rumore P, *et al*. Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. *FEMS Microbiol Lett* 1989;**48**:19–24.
20. **Goldschmidt P,** Ferreira CC, Degorge S, *et al*. Rapid detection and quantification of Propionibacteriaceae. *Br J Ophthalmol* 2009;**93**:258–62.

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Ex vivo expanded cord blood CD4 T lymphocytes exhibit a distinct expression profile of cytokine-related genes from those of peripheral blood origin

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Summary

With an increase in the importance of umbilical cord blood (CB) as an alternative source of haematopoietic progenitors for allogenic transplantation, donor lymphocyte infusion (DLI) with donor CB-derived activated $CD4^+$ T cells in the unrelated CB transplantation setting is expected to be of increased usefulness as a direct approach for improving post-transplant immune function. To clarify the characteristics of activated $CD4^+$ T cells derived from CB, we investigated their mRNA expression profiles and compared them with those of peripheral blood (PB)-derived activated $CD4^+$ T cells. Based on the results of a DNA microarray analysis and quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR), a relatively high level of forkhead box protein 3 (Foxp3) gene expression and a relatively low level of interleukin (IL)-17 gene expression were revealed to be significant features of the gene expression profile of CB-derived activated $CD4^+$ T cells. Flow cytometric analysis further revealed protein expression of Foxp3 in a portion of CB-derived activated $CD4^+$ T cells. The low level of retinoic acid receptor-related orphan receptor γ isoform t (RORγt) gene expression in CB-derived activated $CD4^+$ T cells was speculated to be responsible for the low level of IL-17 gene expression. Our data indicate a difference in gene expression between $CD4^+$ T cells from CB and those from PB. The findings of Foxp3 expression, a characteristic of regulatory T cells, and a low level of IL-17 gene expression suggest that CB-derived $CD4^+$ T cells may be a more appropriate source for DLI.

Keywords: CD4; cord blood; donor lymphocyte infusion; forkhead box protein 3; interleukin 17; T cell

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Abbreviations: BIM, BCL2-like 11; CB, cord blood; CTLA-4, cytotoxic T-lymphocyte antigen-4; CDKN, cyclin-dependent kinase inhibitor; DLI, donor lymphocyte infusion; Foxp3, forkhead box protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte–macrophage colony-stimulating factor; GVHD, graft-versus-host disease; GVL, graft-versus-leukaemia; HSCT, haematopoietic stem cell transplantation; ICOS, inducible T-cell co-stimulator; IFNG, interferon γ; IL, interleukin; PB, peripheral blood; RORγt, retinoic acid receptor-related orphan receptor γ isoform t; RT, reverse transcriptase; TCR, T-cell receptor; Th, T helper cell; Treg, regulatory T cell.

Introduction

Donor lymphocyte infusion (DLI) is a direct and useful approach for improving post-transplant immune function. DLI has been shown to exert a graft-versus-leukaemia (GVL) effect and has emerged as an effective strategy for the treatment of patients with leukaemia, especially chronic myelogenous leukaemia, who have relapsed after unrelated haematopoietic stem cell transplantation (HSCT).[1] In addition, DLI has been successfully used for some life-threatening viral infections, including Epstein–Barr virus and cytomegalovirus infections after HSCT.[2]

Although DLI frequently results in significant acute and/or chronic graft-versus-host disease (GVHD), several groups have demonstrated that depletion of CD8 T cells from DLIs efficiently reduces the incidence and severity of GVHD while maintaining GVL activity.[3,4] Therefore, selective CD4 DLI is expected to provide an effective and low-toxicity therapeutic strategy for improving post-transplant immune function. Actually, selective CD4 DLI based on a recently established method for *ex vivo* T-cell expansion using anti-CD3 monoclonal antibody and interleukin (IL)-2 is now becoming established as a routine therapeutic means of resolving post-transplant immunological problems in Japan.[5]

The importance of umbilical cord blood (CB) as an alternative source of haematopoietic progenitors for allogenic transplantation, mainly in patients lacking a human leucocyte antigen (HLA)-matched marrow donor, has increased in recent years. Because of the naïve nature of CB lymphocytes, the incidence and severity of GVHD are reduced in comparison with the allogenic transplant setting. In addition, CB is rich in primitive $CD16^-$ $CD56^+$ natural killer (NK) cells, which possess significant proliferative and cytotoxic capacities, and so have a substantial GVL effect.[6]

In contrast, a major disadvantage of CB transplantation is the low yield of stem cells, resulting in higher rates of engraftment failure and slower engraftment compared with bone marrow transplantation. In addition, it was generally thought to be difficult to perform DLI after CB transplantation using donor peripheral blood (PB), with the exception of transplantations from siblings. However, the above-described method for the *ex vivo* expansion of activated T cells can produce a sufficient amount of cells for therapy using the CB cell residues in an infused bag, which has solved this problem and made it possible to perform DLI with donor CB-derived activated $CD4^+$ T cells in the unrelated CB transplantation setting.[5] It has also been reported that CB-derived T cells can be expanded *ex vivo* while retaining the naïve and/or central memory phenotype and polyclonal T-cell receptor (TCR) diversity,[7] and thus potential utilization for adoptive cellular immunotherapy post-CB transplantation has been suggested.[8]

There are functional differences between CB and PB lymphocytes, although the details remain unclear. In an attempt to clarify the differences in characteristics between activated $CD4^+$ T cells derived from CB and those derived from PB, we investigated gene expression profiles. In this paper we present evidence that CB-derived $CD4^+$ T cells are distinct from PB-derived $CD4^+$ T cells in terms of gene expression.

Materials and methods

Cell culture and preparation

CB was distributed by the Tokyo Cord Blood Bank (Tokyo, Japan). The CB was originally collected and stored for stem cell transplantation. Stocks that were inappropriate for transplantation because they contained too few cells were distributed for research use with informed consent, with the permission of the ethics committee of the bank. In addition, all of the experiments in this study using distributed CB were performed with the approval of the local ethics committee. The mononuclear cells were isolated by Ficoll-Paque centrifugation and cultured in the presence of an anti-CD3 monoclonal antibody and interleukin (IL)-2 using TLY Culture Kit 25 (Lymphotec Inc., Tokyo, Japan) as described previously.[5] Although several different methods for T-cell stimulation have been reported, this method is currently being used clinically in Japan. Thus we selected this method in this study. After 14 days of culture, $CD4^+$ cells were isolated using a magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. As a control, mononuclear cells isolated from the peripheral blood of healthy volunteers were similar examined.

Polymerase chain reaction (PCR)

Total RNA was extracted from cells using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed using a First-Strand cDNA synthesis kit (GE Healthcare Bio-Science Corp., Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Using cDNA synthesized from 150 ng of total RNA as a template for one amplification, real-time reverse transcriptase (RT)-PCR was performed using SYBR® Green PCR master mix, TaqMan® Universal PCR master mix and TaqMan® gene expression assays (Applied Biosystems, Foster City, CA), and an inventoried assay carried out on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems) according to the instructions provided. Either the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene or the β-actin gene was used as an internal control for normalization. The sequences of gene-specific primers for real-time RT-PCR are listed in Table 1.

DNA microarray analysis

The microarray analysis was performed as previously described.[9] Total RNA isolated from cells was reverse-

