Expansion of mouse primitive hematopoietic cells in three-dimensional cultures on chemically fixed stromal cell layers

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Abstract

To establish a practical and convenient method to expand hematopoietic cells (HCs), we applied chemically-fixed stromal cell layers formed within three-dimensional (3D) scaffolds to feeder of HC cultures. The HCs were expanded using two successive cultures. First, stromal cells were cultured within porous polymer scaffolds and formed tissue-engineered constructs (TECs); the scaffolds containing stromal cells, were fixed using aldehyde (formaldehyde or glutaraldehyde) or organic solvents (acetone, methanol or ethanol). Second, mouse fetal liver cells (FLCs), as a source of HCs, were cultured on the TECs for 2 weeks, and the effects of fixative solutions on expansion of primitive HCs (c-kit$^+$ and CD34$^+$ cells) were examined. In the cultures on aldehyde-fixed TECs, primitive HCs were expanded 2.5- to 5.1-fold in the cultures on TECs fixed with glutaraldehyde, whereas no expansions were detected in those fixed with formaldehyde. However, we achieved expansion of primitive HCs > 5-fold in the cultures using TECs fixed with organic solvents. Among these solvents, the highest expansions—of roughly 10-fold—were obtained using acetone fixation. Ethanol-fixed TECs also supported the expansion of the primitive HCs well (6.6- to 8.0-fold). In addition to these sufficient expansions, the procedure and storage of fixed TECs is fairly easy. Thus, HC expansion on chemically-fixed TECs may be a practical method for expanding primitive HCs.

Keywords: Hematopoietic stem progenitor cell, ex vivo expansion, fixation, stromal cell, cryopreservation, 3D culture

Introduction

Hematopoietic stem cell (HSC) transplantation is widely applied in the treatment of patients with severe hematologic diseases such as acute leukemia and malignant lymphoma. The HSCs are harvested from bone marrow, peripheral blood, or umbilical cord blood (UCB). Of these cell sources, UCB has several advantages, namely, of ease of collection, high proportion of primitive hematopoietic cells (HCs), and high tolerance to human leukocyte antigen (HLA) mismatch (Ajami et al. 2019; de Lima et al. 2012; Islami et al. 2018; Skoric et al. 2007). However, the amount of HSCs in a single UCB unit is insufficient for transplantation into adult patients, and this prevents the wider application of UCB transplantation (Ajami et al. 2019; de Lima et al. 2012; Hofmeister et al. 2007; Islami et al. 2018).

To solve this problem, ex vivo expansion of HSCs has been investigated and the effectiveness of adding and combining stimulating factors—such as stem cell factor (SCF), thrombopoietin (TPO), and Flt3 ligand—on HSC expansion has been demonstrated (Ajami et al. 2019; Chou et al. 2013; Chou and Lodish 2010; da Silva et al. 2005; Hofmeister et al. 2007; Li et al. 2019; Verfaillie 1993). Coculturing HCs with stromal cells has also been proven as an effective approach (Ajami et al. 2019; Chou et al. 2013; Darvish et al. 2019; da Silva et al. 2005; Dexter et al. 1977; Li et al. 2019). In these cocultures, stromal cell growth is generally suppressed using γ-irradiation or anticancer agents prior to the cocultures, to prevent overgrowth of the cells (Li et al. 2019; Ponchio et al. 2000; Roy et al. 2001). These treatments are known to enhance HSC expansion, but they have the disadvantages of requiring specialized equipment for γ-irradiation and there is a risk that any residual agent would inhibit HC growth. In addition, treated stromal cells are required to be maintained in cultures and are not suitable for long storage periods (Ito et al. 2006; Miyoshi et al. 2015, 2019).

The major mechanisms of stromal cells' support of HC expansion in the cocultures are thought to result from secretion of stimulating factors and cell-cell contact with HCs. The importance of direct contact between HCs and stromal cells has been revealed in coculture experiments where these cell types were separated by a membrane of cell culture insert (Kawada et al. 1999). To distinguish the effects of stimulating factors and direct contact, application of chemically-fixed stromal layers for HC expansion was considered to be potential approach (Li et al. 2017). In this method, the structures of stromal cell surface molecules involved in cell-cell contact are preserved whereas no cytokines and growth factors are secreted from dead stromal cells. Thus, this method is expected to be useful for directly evaluating the effects of cell-cell contact (Ito et al. 2006).

In previous studies, we have investigated the expansion of HCs in the three-dimensional (3D) cocultures (Miyoshi et al. 2015, 2019). In these cocultures, tissue-engineered constructs (TECs) of stromal cells that were formed by 3D culturing the cells within porous scaffolds was cryopreserved to suppress their growth and to allow for long-term storage. We found that expansion of primitive HCs when cocultured with the freeze-thawed TECs was sufficient and greater than that without cryopreservation, although the viability of freeze-thawed cells within the TECs was lower than 50% (Miyoshi et al. 2015). These results strongly suggested the primary importance of cell-cell contact between HCs and stromal cells in the expansion of primitive HCs, rather than secreted stimulating factors.

In the present study, chemical fixation was applied for the treatment of stromal cell TECs with the aim of establishing a practical and convenient expansion method for primitive HCs. Aldehyde and organic solvents were used as fixative solutions, and their effects on the HC expansion were examined.

Materials and Methods

Cells and culture medium

Fetal liver cells (FLCs) harvested from C57BL/6NCrSlc mice (Japan SLC, Hamamatsu, Japan) on embryonic day 14 were used as HCs and DAS 104-8 cell line (kindly provided by Dr. Osamu Ohneda, University of Tsukuba) were used as the stromal cells, similar to our previous studies (Miyoshi et al. 2015, 2019; Ohneda et al. 1998). This study was approved by the University of Tsukuba Animal Experimental Committee, number 19-399. All mice were treated according to guidelines developed by the committee.

In all culture experiments, cells were cultured in Hava medium composed of high-glucose Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA), 10% fetal bovine serum (FBS; MP Biomedicals, Santa Ana, CA), 10^{-4} mmol/L minimum essential medium (MEM) nonessential amino acids (Gibco), 2 x 10^{-3} mmol/L L-glutamine (Gibco), 10^{-4} mmol/L 2-mercaptoethanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and 0.5% penicillin-streptomycin (Gibco) (Miyoshi et al. 2015, 2019; Ohneda et al. 1998).

Three-dimensional scaffolds, cell seeding, and harvesting cultured cells

For the 3D scaffolds, porous polyvinyl formal resin cubes (2 x 2 x 2 mm^3; Aion, Osaka, Japan) with a mean pore size of 130 μm were used after autoclave sterilization and type I collagen coating (Koken, Tokyo, Japan) (Miyoshi et al. 2015, 2019).

At the initiation of the cultures and cocultures, stromal cells or FLCs were seeded into the scaffolds using the centrifugal cell immobilization (CCI) method, as previously reported (Miyoshi et al. 2015, 2019; Yang et al. 2001). Briefly, 100 resin cubes and cell

aliquots suspended in culture medium were put into a centrifuge bottle and centrifuged six times (300 x g) to entrap the cells within the cubes.

After the 3D culture experiments, 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid)/0.2% collagenase solution was added to the scaffold cubes containing cells (1 mL per 10 cubes), and the cubes were cut into small pieces using a surgical knife. The cells were then detached from the scaffolds by vigorous pipetting and then 2 mL of phosphate buffered saline (PBS) containing 2% FBS was added. Prior to fluorescence-activated cell sorting (FACS) analysis, the obtained cell suspension was filtered through a 70 μm nylon mesh to remove scaffold pieces and cell aggregates.

Fixation of tissue-engineered constructs

To determine suitable fixative solutions for stromal cells 3D cultured within the resin cubes (TECs) for HC expansion, aldehyde fixation (formaldehyde (FA) and glutaraldehyde (GA)) and organic solvent fixation (acetone (Ac), methanol (Me), and ethanol (Et)) were examined.

In the aldehyde fixation, the TECs were washed with PBS three times, and put into a glass centrifuge tube. Then, 10% formalin neutral buffer solution (FA fixative; FUJIFILM Wako Pure Chemical Corporation) or GA fixative (phosphate buffer containing 0.2% GA) was poured into the tube (1 mL per 10 cubes) and stored at 4^{o}C for 1-7 days. After the fixation, 60 cubes were put into a 60 mm culture dish, washed with PBS three times, stored in PBS at 4^{o}C for over 24 h, and used for the HC cultures.

In the case of organic solvent fixation, the washed TECs were placed in a glass centrifuge tube and fixed by adding an organic solvent (Ac, Me or Et) of equal measurement to the aldehyde solution used for aldehyde fixation, and then stored at -20^{o}C for 5-7 days.

Prior to culture experiments, organic solvents were removed using a vacuum or by washing. The Ac- or Me-fixed TECs were placed in a vacuum desiccator, and the solvents were removed by decompression for 1 h. The TECs were then immersed in PBS, degassed for 1 h to remove air bubbles within the TECs, and stored in PBS for over 24 h. Concerning Et-fixed TECs, they were washed similarly to the procedure of aldehyde fixation, as described above.

As a control experiment, TECs were cryopreserved and thawed just before coculturing, similar to our previous study (Miyoshi et al. 2010, 2015, 2019).

Procedure of culture experiments

The HCs contained within the FLCs were expanded using two successive cultures (Miyoshi et al. 2015, 2019). To form the TECs, stromal cells were first seeded into cubic scaffolds at a density of 1 x 10^7 cells/cm^3, and 30 cubes containing cells were cultured in a 60 mm culture dish with 7.5 mL of medium for 2 days. After the fixation of these TECs, FLCs at a density of 1 x 10^8 cells/cm^3 were secondly seeded into the TECs. Ten TEC cubes obtained were placed in a 35 mm dish and cells within these TECs were cultured with 2.5 mL of medium for 2 weeks to expand the HCs. The culture medium was replaced every other day.

In the controls, FLCs were cocultured within untreated or freeze-thawed TECs, similar to the procedures described in our previous study (Miyoshi et al. 2010, 2015, 2019).

Cell counting, fluorescence-activated cell sorting analysis, and microscopic observation

The numbers of 3D cultured cells within fixed TECs were measured using an MTT assay of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Mosmann 1983), as previously reported (Miyoshi et al. 2011, 2013, 2015).

Percentages of the HCs among the 3D cultured cells were measured via FACS analysis using a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Anti-Ter119 (erythroid cells), anti-B220 (B cells), anti-c-kit (hematopoietic progenitor cells; HPCs), and anti-CD34 (hematopoietic stem progenitor cells; HSPCs) monoclonal antibodies were used as markers of for the HCs (all from PharMingen, San Diego, CA) (Miyoshi et al. 2015). The numbers of these cells were calculated from total cell numbers and the percentage of marker-positive cells in the FACS analysis.

The morphology of the 3D cultured cells within the porous scaffolds was observed using scanning electron microscopy (JEOL, Tokyo Japan) (Yanagi et al. 1992).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analyses were carried out using *t* tests and significance was set at $p < 0.05$.

Results

Hematopoietic cell cultures on stromal layers fixed by aldehyde

Figure 1 shows the density changes of total cells and each HC type over time in the FLC 3D cultures within aldehyde-fixed TECs (FA-fix and GA-fix). The densities in control cocultures using untreated (UT) and cryopreserved TECs (CP) are also shown in Fig. 1. In UT, CP, and GA-fix, the total cell densities increased with elapse of culture period, but these changes were unremarkable (Fig. 1a). Densities of erythroid cells significantly decreased under the conditions excepting GA-fix (Fig. 1b), whereas no remarkable decreases in B cell densities were detected under any condition (Fig. 1c).

With respect to primitive HCs (HPCs and HSPCs), densities of these cells showed significant increases in the controls (UT, CP) (Fig. 1d, e). In the case of FA-fix and GA-fix, changes in these cell densities exhibited differing tendencies; with significant increases in GA-fix, but considerable decreases in FA-fix. Therefore, GA fixation of the stromal cells was far better than FA for expanding primitive HCs.

Hematopoietic cell cultures on stromal layers fixed by organic solvent

Changes in cell densities over time when FLCs were cultured within TECs fixed by organic solvent (Ac-fix, Me-fix and Et-fix) are summarized in Fig. 2. Total cell densities in Ac-fix and Me-fix significantly increased on Days 7 and 14 (Fig. 2a). Although erythroid cell densities were rather constant under all conditions (Fig. 2b), increases in B cell densities were remarkable (Fig. 2c). With respect to primitive HCs, these cells were well expanded irrespective of the organic solvent used (Fig. 2d, e). Among the three solvents, cultures in Ac-fix and Et-fix showed similar expansion of these HCs, and the expansions were slightly better than those in Me-fix.

Comparison of hematopoietic cell expansion

The expansions of HCs calculated from the densities on Day 7 or 14 and Day 1 are summarized in Fig. 3. Expansions of erythroid cells were higher in GA-fix and cultures with organic solvent-fixed TECs (Ac-fix, Me-fix and Et-fix) compared with other conditions (Fig. 3a), however, these expansions were 2-fold at most. B cells, on the other hand, were expanded over 20-fold on Day 14 in Ac- and Et-fix (Fig. 3b), indicating that these fixations efficiently facilitated B cell expansion.

In the case of HPCs, expansions over 5-fold were achieved on Day 14 under GA-fix and organic solvent fixation conditions. The expansions in GA-fix, Me-fix, and Ac-fix were significantly higher than those of controls (UT, CP) (Fig. 3c). Concerning HSPCs, high expansion of HSPCs on Day 14 were achieved in the Ac-fix and Et-fix, and these expansions were comparable to those in CP (Fig. 3d).

Among different fixation conditions, expansion of B cells, HPCs, and HSPCs showed a similar tendency. Highest expansions of these cells were obtained in Ac-fix, and Et-fix provided the second highest values.

Scanning electron microscopy observation

Figure 4 shows representative scanning electron microscopy images of FLCs cultured on GA- and Ac-fixed stromal layers. In GA-fix (Fig. 4a, b), surface of porous scaffolds that are observable in the lower left of Fig. 4c were covered by numerous cells and extracellular matrix (ECM), and the smooth surface formed by these components were observed (Fig. 4a). Spherical cells considered to be HCs were also observable within the inner pores of the scaffold (Fig. 4b). In the cultures on Ac-fixed stromal layers, cells and ECM formed cell-ECM composites on the surface of scaffolds (Fig. 4c, d). Around these composites, abundant amounts of ECM were present. Compared with the cells in the GA-fix, no marked differences in cell morphology were recognized.

Discussion

Chemical fixation is widely applied in the field of molecular biology, including immunohistochemical experiments, sample preservation, and electron microscopy sample

resin embedding (Li et al. 2017). Two major methods of chemical fixation of cells are aldehyde and organic solvent fixation, and preservation of cell morphology after the fixation depends on the fixation methods (Brock et al. 1999; Huebinger et al. 2018). Cells immersed in organic solvents such as acetone, methanol, and ethanol are fixed by dehydration. Although these solvents preserve antigenicity of proteins, changes in cell shape as well as denature, coagulation, and/or extraction of proteins are induced. Compared with these structural and morphological changes in organic solvent fixation, aldehyde fixation preserves cell structure in a more stable manner (Brock et al. 1999; Li et al. 2017).

There have been few reports of the application of fixed cells to feeder layers of cultures. In cultures of human cord blood-derived HSPCs on GA-fixed stromal cell monolayers, the fixed stromal cells revealed the ability to support HPC expansion (Ito et al. 2006). However, to our knowledge, there are no reports of the application of fixed cells to 3D cultures. In the present study, therefore, we performed HC cultures on TECs fixed with either aldehyde or organic solvents and the expansions of HCs were compared with those in CP; a method by which sufficient expansion of primitive HCs was achieved in our previous study (Miyoshi et al. 2015). We found that expansions of primitive HCs in Ac-fix and Et-fix were comparable with those in CP (Fig. 3c, d), indicating sufficient potential of chemically-fixed TECs for supporting primitive HC expansion.

Compared with cultures on aldehyde-fixed TECs, expansions of the primitive HCs were higher in those on TECs fixed with organic solvents (Fig. 3c, d), although preservation of stromal cell structures in the TECs with aldehyde fixation might be better than those with organic solvent fixation. Thus, it was suggested that some proteins or small molecules on the surface of stromal cells contributed to the HC expansion. In addition, since conditions of

aldehyde fixation such as aldehyde concentration and fixation period were not optimized in this study, improvement of these conditions might enhance the HC expansion.

For clinical application, cells expanded on acetone-treated cells or materials are likely to be problematic due to toxicity issues, although the highest expansions of primitive HCs were achieved this way in this study. Among the organic solvents, therefore, ethanol is regarded as possible reagent for stromal cell fixation because of its low toxicity and the relatively high expansions that we observed in Et-fix (Fig. 3). Glutaraldehyde is widely used for tissue fixation and as material for transplantation, as with its application in bioprosthetic heart valves (Manji et al. 2014). It was also reported that GA-fixed cell monolayers preserved their morphology after 4-week storage in a refrigerator, indicating the easy storage of fixed stromal cells (Ito et al. 2006). Compared with these fixed monolayers, storage of the fixed TECs is fairly easy. Thus, glutaraldehyde is considered to be another candidate as a fixation solution, if HC expansion in GA-fix is improved by modifying the fixation conditions.

In the cocultures of HCs with stromal cells, stimulating factors are generally added to the culture media, and this method has been reported to achieve high expansion of primitive HCs as large as dozens of times (da Silva et al. 2005; Kawada et al. 1999; Li et al. 2019). At the same time, however, the addition of these factors may increase the risk of unwanted differentiation of HSPCs; thus, establishment of culture methods that are able to expand HSPCs efficiently with no exogenous cytokine is desired (Mortera-Blanco et al. 2011). In this study, the highest expansions of primitive HCs were roughly 10-fold and, therefore, lower than those in the previous reports mentioned above. However, the primitive HCs in this study were successfully expanded without the occurrence of exogenous cytokines; furthermore, the highest expansion value is arguably sufficient for UCB transplantation into

adult patients. Thus, the HC cultures on chemically-fixed TECs is potentially a novel and practical expansion method of primitive HCs.

Compared with control cocultures, the total cell densities in cultures within fixed TECs on Day 1 were lower (Figs. 1a, 2a). These different densities might be caused by viable stromal cells in control cocultures as well as reduced FLC attachment at cell seeding due to the stromal cell fixation. A possible approach to increasing cell densities within the chemically fixed TECs at the initiation of cultures, could be via collagen coating of the TECs prior to HC seeding. However, this may reduce the beneficial effects on HC expansion induced by interaction between HCs and stromal layers. Thus, the effects of collagen coating of chemically fixed TECs on cell densities may be worth investigating in future experiments.

There is a possibility that the differences in cell density on Day 1 affect expansion of HCs in the cultures on the fixed TECs. In our previous study, effects of seeding densities ($0.1\text{-}1.5 \times 10^7$ cells/cm^3) of mouse bone marrow-derived mononuclear cells (MNCs) on HPC expansion were examined in 3D cultures and no obvious differences in the expansion were observed (Miyoshi et al. 2011). In this study, however, culture conditions, including the cells cultured and the use of stromal cells, were different from those of the previous study. Thus, the effects of cell densities on expansion using this culture method should be examined in future.

In conclusion, we cultured HCs on 3D-cultured stromal cells that were fixed using aldehyde or organic solvents prior to the culturing. In our cultures using Ac-, Et- and GA-fixed stromal cells, high expansions of primitive HCs (roughly 10-fold) were achieved without the occurrence of exogenous cytokines. These expansions were comparable to those of control cocultures using 3D freeze-thawed stromal cells. Thus, this culture method is

considered to be an effective expansion method of primitive HCs.

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Conflict of interest

The authors declare no conflicts of interest.

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Fig. 1 Changes over time in numbers of total cells and HCs in 3D culture experiment of FLCs on stromal cell layer fixed with aldehyde. Mean ± SD. Open circles, cocultures with untreated stromal cell layers (UT) (n = 3); closed circles, cocultures with 3D freeze-thawed stromal cell layers (CP) (n = 8); open squares, cultures on tissue-engineered constructs (TECs) of stromal cells fixed with formaldehyde (FA-fix) (n = 3); closed squares, cultures on TECs of stromal cells fixed with glutaraldehyde (GA-fix) (n = 5). Numbers of **a** total cells, **b** $Ter119^+$ erythroid cells, **c** $B220^+$ B cells, **d** $c\text{-}kit^+$ HPCs, and **e** $CD34^+$ HSPCs. *$p < 0.05$ vs. Day 1; $^{\dagger}p < 0.05$ vs. UT; $^{\ddagger}p < 0.05$ vs. CP; $^{\#}p < 0.05$ vs. FA-fix.

Fig. 2 Changes over time in numbers of total cells and HCs in 3D culture experiment of FLCs on stromal cell layer fixed with organic solvent. Mean ± SD. Closed triangles, cultures on TECs of stromal cells fixed with acetone (Ac-fix) (n = 5); Open diamonds, cultures on TECs of stromal cells fixed with methanol (Me-fix) (n = 5); closed diamonds, cultures on TECs of stromal cells fixed with ethanol (Et-fix) (n = 4). Numbers of **a** total cells, **b** $Ter119^+$ erythroid cells, **c** $B220^+$ B cells, **d** $c\text{-}kit^+$ HPCs, and **e** $CD34^+$ HSPCs. *$p < 0.05$ vs. Day 1; $^{\dagger}p < 0.05$ vs. UT; $^{\ddagger}p < 0.05$ vs. CP; $^{\S}p < 0.05$ vs. Me-fix; $^{\P}p < 0.05$ vs. Et-fix.

Fig. 3 Expansions of HCs in 3D culture experiments. These values demonstrate density ratios on Day 7 or 14 and Day1. Mean ± SD. Gray bars, Day 7; closed bars Day 14. **a** $Ter119^+$ erythroid cells, **b** $B220^+$ B cells, **c** $c\text{-}kit^+$ HPCs, and **d** $CD34^+$ HSPCs. $^{\dagger}p < 0.05$ vs. UT; $^{\ddagger}p < 0.05$ vs. CP; $^{\#}p < 0.05$ vs. FA-fix; $^{\P}p < 0.05$ vs. GA-fix; $^{\S}p < 0.05$ vs. Me-fix; $^{*}p < 0.05$ vs. Et-fix.

Fig. 4 Scanning electron micrographs of FLCs cultured on stromal cell layers fixed with either glutaraldehyde or acetone. **a, b** FLCs cultured on TECs of stromal cells fixed with glutaraldehyde (GA-fix, Day 14). **c, d** FLCs cultured on TECs of stromal cells fixed with acetone (Ac-fix, Day 14). Bars = 100 μm (**a**, **c**); bars = 10 μm (**b**, **d**).

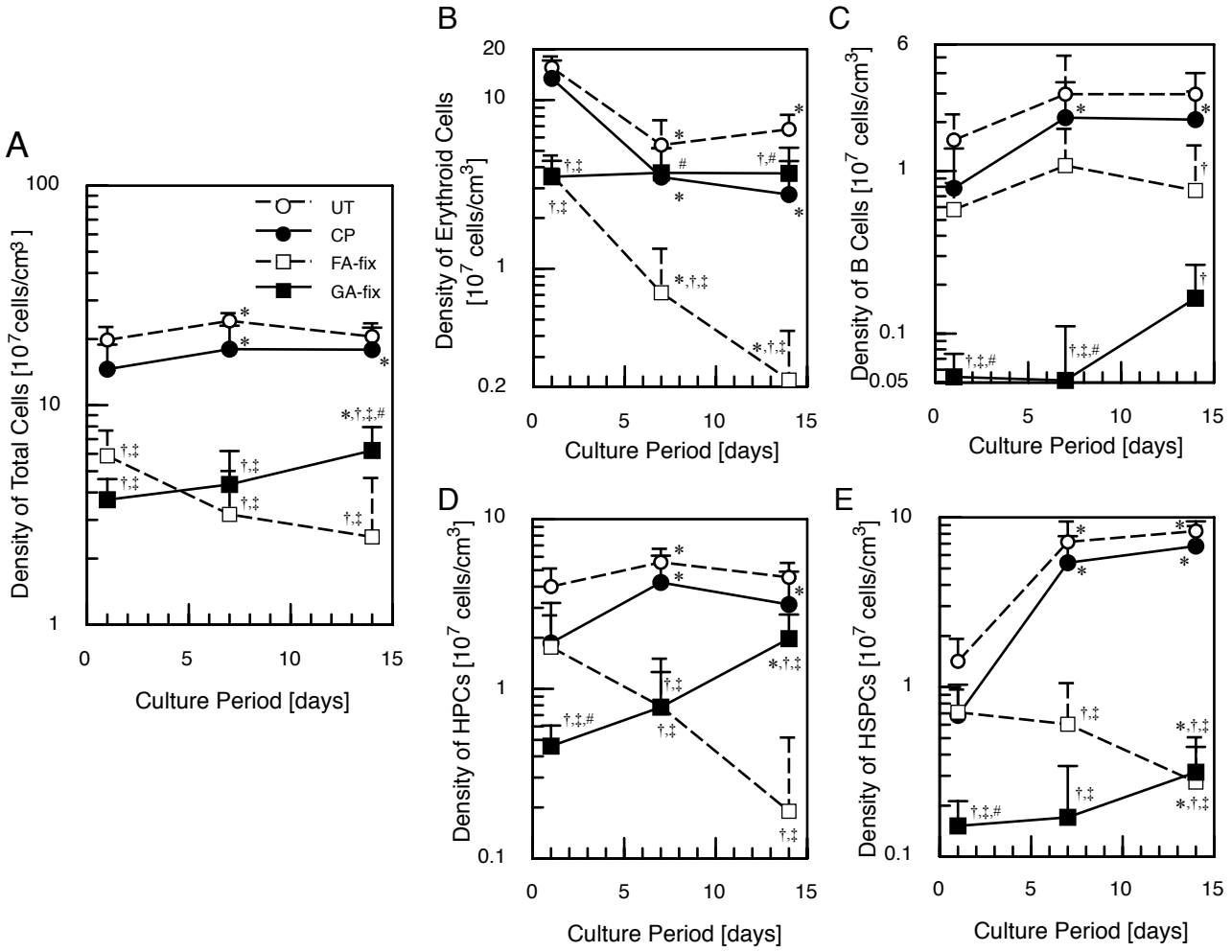
A
Density of Total Cells [10^7cells/cm^3]
100
10
1
UT
CP
FA-fix
GA-fix
0
5
10
15
Culture Period [days]
B
Density of Erythroid Cells [10^7 cells/cm^3]
20
10
1
0.2
Culture Period [days]
C
Density of B Cells [10^7 cells/cm^3]
6
1
0.1
0.05
Culture Period [days]
D
Density of HPCs [10^7 cells/cm^3]
10
1
0.1
Culture Period [days]
E
Density of HSPCs [10^7 cells/cm^3]
10
1
0.1
Culture Period [days]

Figure 1

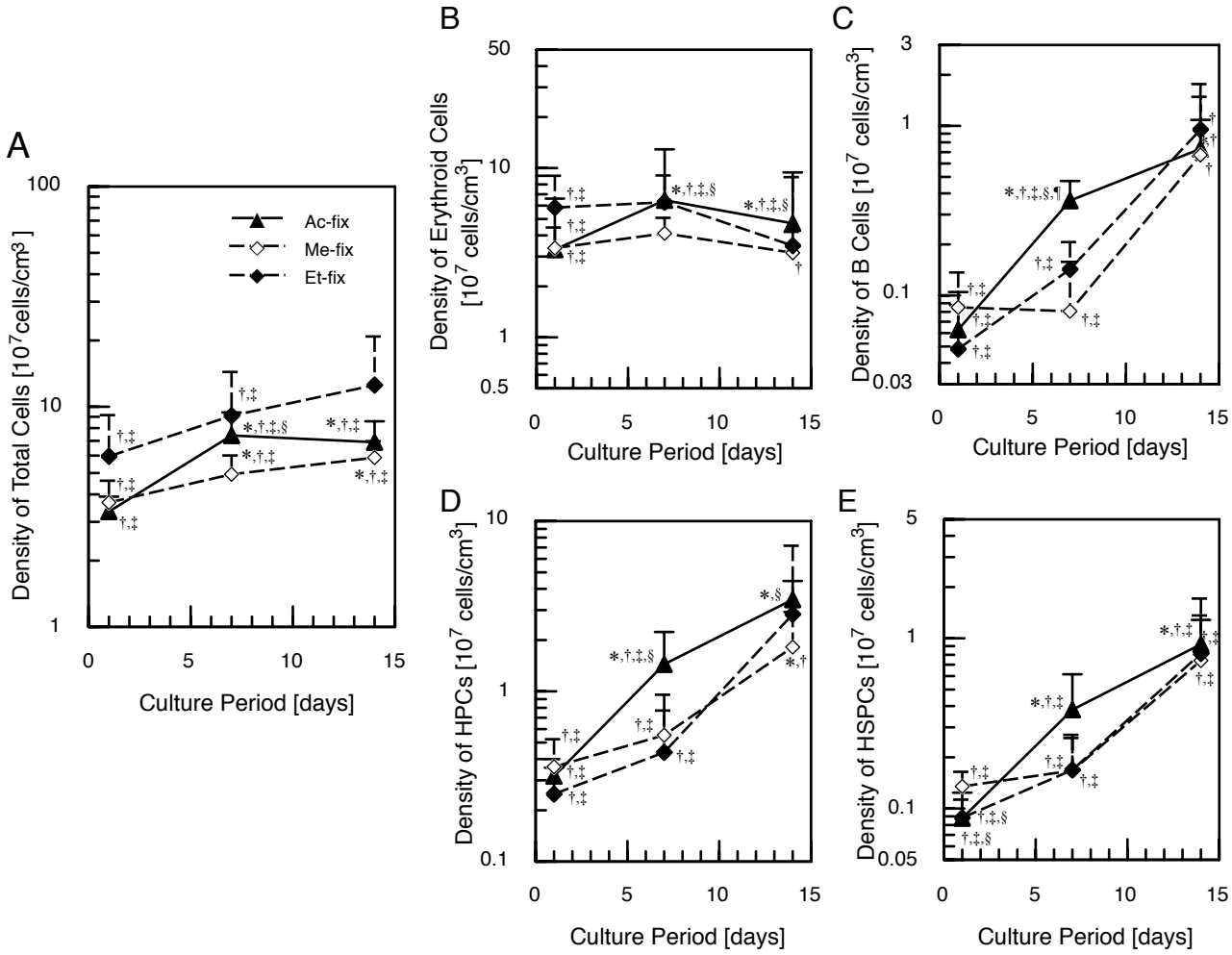
A
Density of Total Cells [10^7cells/cm^3]
Ac-fix
Me-fix
Et-fix
Culture Period [days]
B
Density of Erythroid Cells [10^7 cells/cm^3]
Culture Period [days]
C
Density of B Cells [10^7 cells/cm^3]
Culture Period [days]
D
Density of HPCs [10^7 cells/cm^3]
Culture Period [days]
E
Density of HSPCs [10^7 cells/cm^3]
Culture Period [days]

Figure 2

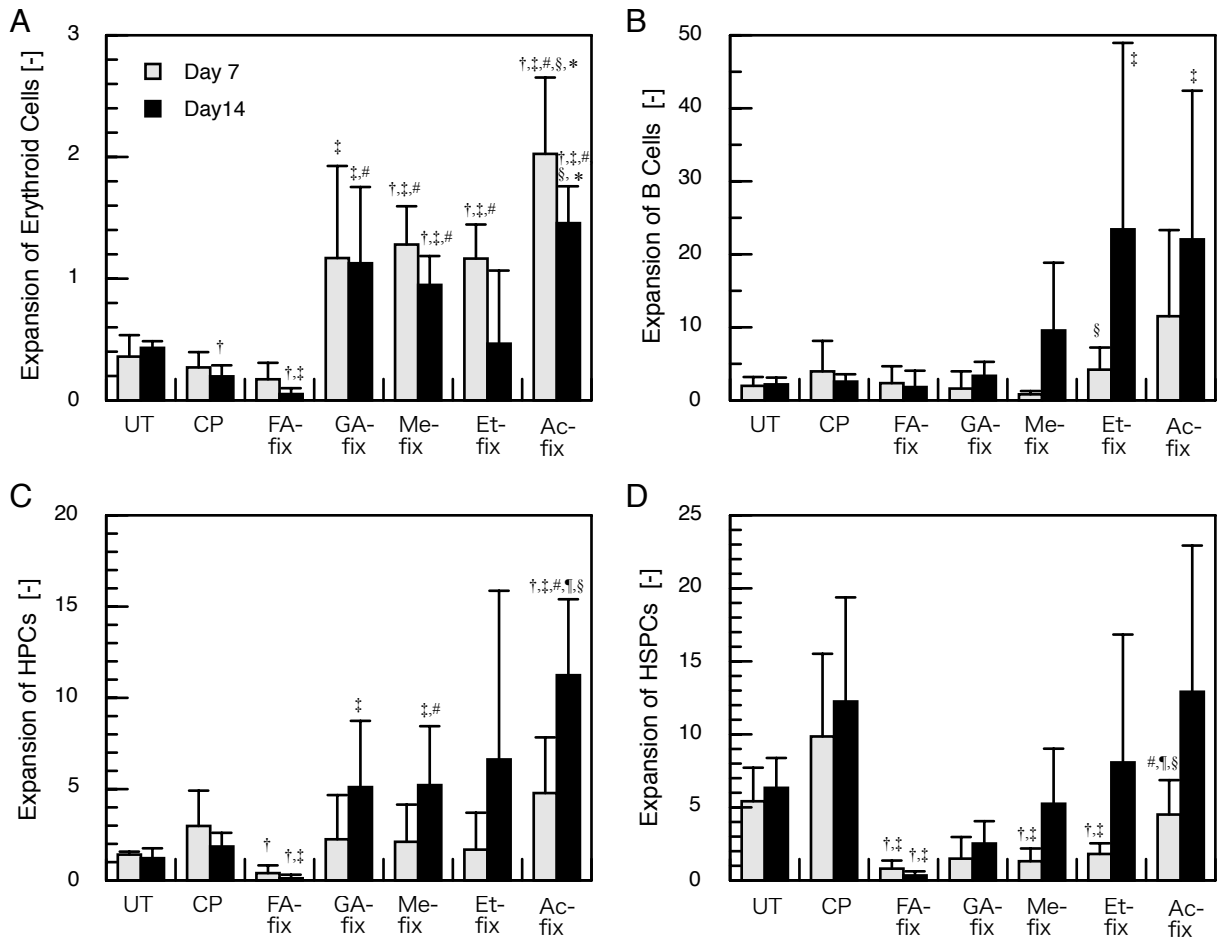
A
Expansion of Erythroid Cells [-]
Day 7
Day14
UT
CP
FA-fix
GA-fix
Me-fix
Et-fix
Ac-fix
B
Expansion of B Cells [-]
C
Expansion of HPCs [-]
D
Expansion of HSPCs [-]

Figure 3

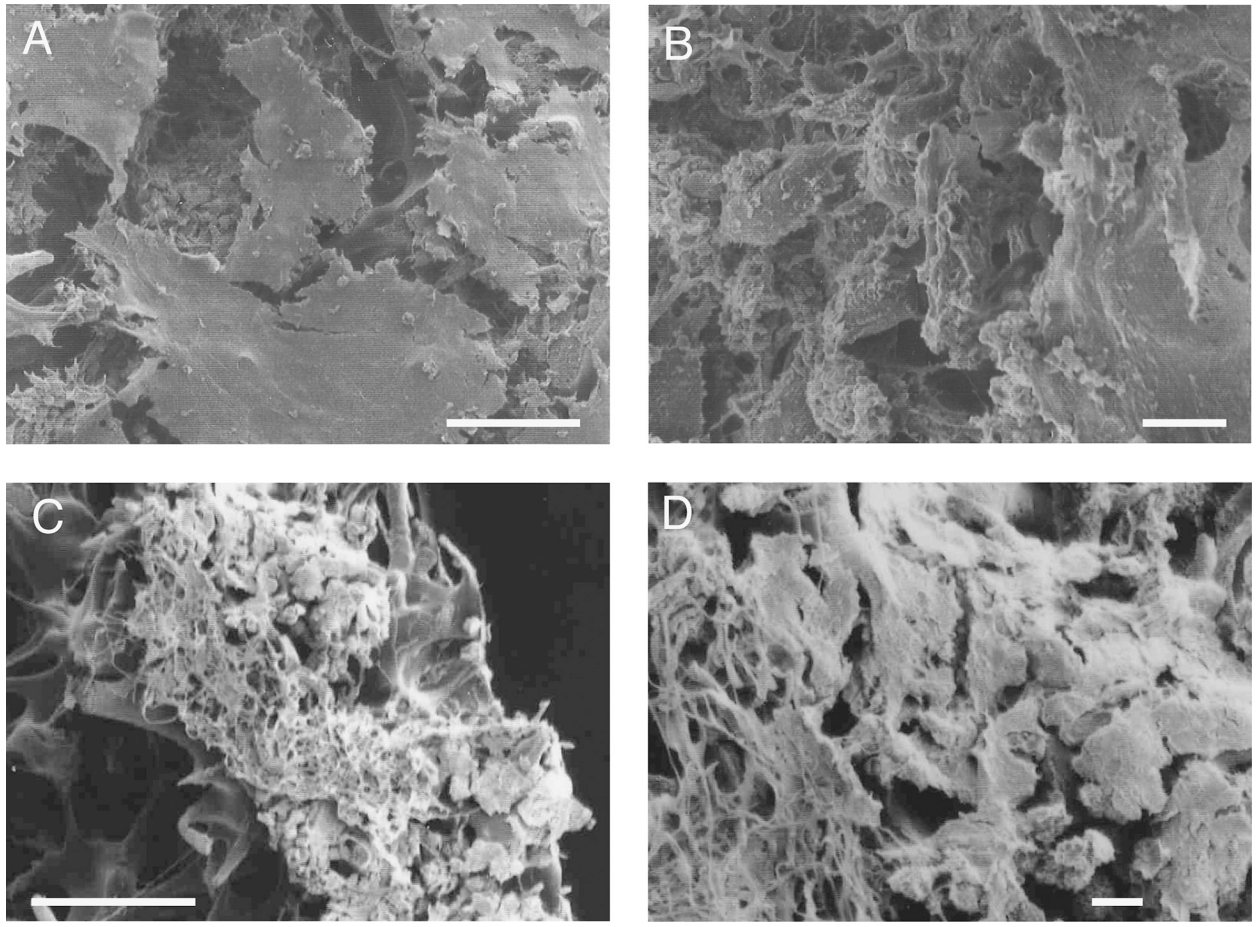

Figure 4