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In vitro chemosensitivity study of human leukemic cells in a three-dimensional bone marrow culture system

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Abstract

In the bone marrow, hematopoietic stem cell proliferation and differentiation are regulated by the hematopoietic microenvironment. This is reflected by the ability of the bone marrow microenvironment to alter the growth of leukemic cells, and protect leukemic cells from anti-cancer agents. Previously, we established a three-dimensional (3D) bone marrow culture system that maintained normal hematopoiesis, including prolongation of hematopoietic stem cell proliferation and differentiation. In the present study, we analyzed the effects of the anti-cancer drug, cytarabine, on the human leukemic cell line (K562) co-cultured with stromal cells in the 3D system. Comparisons were made with K562 cells treated with cytarabine in suspension or grown on a two-dimensional stromal cell monolayer (2D). We demonstrated that K562 cells cultured in the 3D system were more resistant to cytarabine treatment compared with cells grown in 2D or in suspension. Furthermore, there was a significant increase in the number of K562 cells in G_0/G_1 phase in 3D culture compared with cells grown in 2D or suspension cultures. These findings suggest that the differential response to cytarabine treatment in 3D culture may be related to the cell cycle period, which was modulated by stromal cells in the 3D microenvironment. Thus, the 3D culture system may be a valuable new tool for investigating leukemic cell-stromal cell interactions and the leukemic cell response to anti-cancer agents *in vitro*.

Keywords: 3D culture; bone marrow stromal cell; cell cycle; leukemia; cytarabine

Introduction

Hematopoietic stem cells (HSCs) can self-renew and produce progenitors that are committed to differentiate in to a wide range of blood cell types, including erythrocytes, leukocytes, lymphocytes, and platelets. Earlier studies demonstrated that the proliferation and differentiation of HSCs are regulated by the bone marrow microenvironment [1-3]. Stromal cells, which are distinct from hematopoietic cells, are an essential component of this microenvironment, and are necessary for the long-term maintenance of HSCs *in vitro* [4, 5]. Several reports have shown that stromal cells regulate the proliferation and differentiation of HSCs through the production of diffusible hematopoietic regulatory factors and extracellular matrix, as well as through physical cell-cell interactions involving adhesion molecules and gap junction-mediated cell communication [1, 6-12]. Furthermore, the physiological function of stromal cells requires an anatomically appropriate three-dimensional (3D) orientation to determine HSC fate, which is linked to a normal and/or dysfunctional hematopoietic system [10, 13-16].

Stromal cells within the bone marrow microenvironment influence the proliferation of leukemic cells, as well as the normal hematopoietic cells from which they were derived. In addition, stromal cells likely protect leukemic cells from the effects of cytotoxic anti-cancer agents [17, 18]. In fact, current *in vitro* preclinical anti-cancer drug testing does not always predict *in vivo* responses [19]. Furthermore, even among patients who achieve complete remission with initial chemotherapy, the vast majority of patients with leukemia relapse because of residual disease in the bone marrow [20, 21]. However, the ability of stromal cells to support the proliferation (and differentiation) of HSCs or leukemic cells and to maintain their self-renewal potential has generally been investigated in long-term, two-dimensional (2D) bone marrow culture systems, which are structurally fundamentally different from the

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native hematopoietic environment. Hematopoiesis *in vivo* takes place in the bone marrow, where hematopoietic or leukemic cell proliferation is intimately linked with the 3D hematopoietic microenvironment, the so-called hematopoietic niche, which is composed of stromal cells. HSCs or leukemic cells are found in spaces within the bone marrow, and they rely on cell-cell interactions and the local milieu to determine their immediate fate [22].

We recently developed novel polymer particles with grafted epoxy polymer chains to support cell immobilization in a new 3D cell cultivation system [23-25]. The base polymer particles were synthesized by suspension polymerization of acrylic monomer and 2,2′-azobis[N-(2-propenyl)-2-methylpropionamide] (APMPA), and the epoxy polymer chain was extended from the particle surface by graft polymerization. The advantage of these particles is that the particle size, length and number of chains and the composition of the base polymer can be easily manipulated. The unique fibroblast cell line, MS-5, which was established from irradiated mouse bone marrow stromal cells and supports both murine and human HSC proliferation and differentiation *in vitro* [26-29], was used as a source of stromal cells in our 3D culture. The 3D cell bridges formed by MS-5 cells in the 3D cell cultivation system likely overcome the peeling problem associated with previous 3D and 2D systems, allowing long-term maintenance of HSCs *in vitro* [23]. Most HSCs in the 3D culture adhered (homed) to stromal cells, remained in the resting state, established an equilibrium between proliferation and differentiation, and similar to what is seen in hematopoietic tissue *in vivo*.

In this study, we designed our experiments to address the regulation of leukemic cell proliferation in the hematopoietic niche in 3D culture. As part of our approach, we examined the kinetics of leukemic cell growth in the presence of the anti-cancer drug, cytarabine (Ara-C), in our 3D culture system compared with 2D culture models. The combination therapy of cytarabine and anthracycline has been the standard and first line anti-leukemic regimen for more than 30 years [30]. Cytarabine is a nucleoside analog that is activated by phosphorylation to form cytosine arabinoside triphosphate (Ara-CTP), which competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA. Thus, the anti-cancer effect of cytarabine depends on the cell cycle of target cells. The K562 human leukemic cell line was co-cultivated with MS-5 stromal cells, and the influence of cytarabine on the growth and cell cycle of K562 cells were assessed.

Materials and methods

Materials

2,2′-azobis (isobutyronitrile), APMPA, dipotassium hydrogen phosphate, glutaraldehyde aqueous solution (25% w/v), glycidyl methacrylate (GMA), hydrochloric acid, methacrylic acid (MA), methanol, methyl methacrylate, paraformaldehyde aqueous solution (36% w/v), saccharose, sodium dihydrogen phosphate, and trypan blue were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cresol red, poly (vinylpyrolidone) K-90, sodium hydroxide, and toluene were purchased from Nacalai Tesque (Kyoto, Japan). Pentaerythritol triacrylate was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cytarabine was purchased from Sigma-Aldrich Co., dissolved in pyrogen-free saline to produce a concentration of 1 mg/mL, then diluted with Iscove's modified Dulbecco's medium (IMDM) for use. A previous study showed that the half-maximal effective concentration (EC50) of cytarabine for K562 cells was 0.064 μM (15 ng/mL) [31]. In the present study, cytarabine was used at concentrations ranging from 1 ng/mL (0.004 μM) to 10 μg/mL (40.1 μM).

Preparation of cell support with grafted polymer chains

Polymer particles with grafted epoxy polymer chains were prepared as described previously [24]. For graft polymerization, GMA and MA were used at a ratio of 4:1 (w/w), respectively, and the resulting particles were washed in a funnel with a 40 × volume of distilled water and methanol. These polymer particles are subsequently referred to as G-02 polymer particles.

The distribution of particle diameters was measured using the laser particle diameter analyzer Microtrac FRA (Microtrac Inc., Montgomery, PA, USA), and 150-200 μm particles were used in this study. The amount of epoxy group in the polymer particles was measured using the hydrochloric acid-dioxane method, and particles with more than 1.03×10^{-3} μmol/g-particle of epoxy group were selected [23].

Cultivation of MS-5 stromal cells and K562 cells

The murine stromal cell line, MS-5, was maintained in 7 mL IMDM supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), penicillin (50 U/mL; Gibco BRL, Grand Island, NY, USA), and streptomycin (100 μg/mL; Gibco BRL) in 25 cm^2 flasks (Falcon 3013; Corning, One Riverfront Plaza, NY, USA). The cells were cultured in a humidified incubator at 37°C with 5% CO_2, sub-cultured at a split ratio of 1:4 every 7 days using 0.25% trypsin plus 0.02% EDTA in phosphate-buffered saline (PBS), and maintained as described previously [23].

Human leukemic K562 cells [32] were maintained in 7 mL IMDM supplemented with 10% FCS, penicillin (50 U/mL), and streptomycin (100 μg/mL) in 25 cm^2 flasks. The cells were cultured in a humidified incubator at 37°C and 5% CO_2, and sub-cultured at a split ratio of 1:10 every 5 days. For all experiments, the cells were used in their logarithmic growth phase.

3D co-culture system

MS-5 cells ($5\text{-}10 \times 10^5$) were added to 5 mL IMDM supplemented with 10% FCS in the presence of $1–5 \times 10^4$ G-02 polymer particles in a 14 mL round-bottomed polypropylene tube (Falcon 2006; Corning). The mixture was incubated in a humidified incubator at 37°C and 5% CO_2 for 24 h, then transferred into 35 mm plastic dishes (Falcon 3046; Corning), and the incubation was continued. Once cells were immobilized on the surface of the particles, they remained attached, proliferated and formed bridges

between the particles. The supernatant was replaced with fresh growth medium every 7 days. After 2-3 weeks, when the MS-5 cells had grown and formed a 3D layer in the culture dish, K562 cells (5×10^4/mL) suspended in 5 mL growth medium were inoculated onto the MS-5 cells. Control cultures included MS-5 cells grown in dishes without G-02 particles, and K562 cells inoculated onto MS-5 cells in traditional 2D cultures.

Treatment with cytarabine

Three different experiments were designed. In the first, various concentrations of cytarabine were added to the culture at the same time as K562 cell inoculation, and the cells were left to grow undisturbed for the duration of the experiment. In the second, cytarabine was added to the culture at the same time as K562 inoculation and the cells were harvested (including some K562 cells adhered to stroma) 5 days later, washed once with IMDM to remove cytarabine, and then re-inoculated into the cytarabine free culture. Residual K562 cells in the supernatant or adherent layer after 5 days cytarabine treatment were able to grow in cytarabine free culture conditions. In the third, K562 cells were inoculated into the culture 2 days before cytarabine treatment. Pre-inoculated K562 cells adhered to the MS-5 cells, and the effect of cytarabine on the adhered cells were investigated.

Cultured K562 cells were harvested at various intervals for analysis. The supernatant (including floating cells) and cells in the adherent layer were collected separately. Adherent K562 cells were easily dislodged from the MS-5 cells in both 2D and 3D culture without trypsin treatment, and were harvested by repeated pipetting. The harvested cells from the supernatant and adherent layers were counted using a hemocytometer, and viable cells were distinguished by trypan blue dye exclusion. An aliquot of cells was assayed for CD45 and APO2.7 surface antigen expression, and the cell cycle was analyzed by flow cytometry.

Cell surface antigen analysis

Harvested cells were washed with PBS containing 2% FCS and passed through a 35 μm filter (Cell Strainer; Falcon 352235) to remove the polymer particles and aggregated cells (most of which were stromal MS-5 cells). Cells (2×10^5) were suspended in 0.5 mL of PBS containing 2% FCS and 0.02% NaN_3 and incubated with one or both of the following monoclonal anti bodies (mAbs) for 30 min at 4°C: Phycoerythrin (PE)-conjugated mAb APO2.7 (clone 2.7 A6A3) for detection of the APO2.7 antigen expressed by apoptotic cells (Becton Dickinson, San Jose, CA, USA) [33, 34], and fluorescein isothiocynate (FITC)-conjugated anti-human CD45 (clone H130, Becton Dickinson) for the detection of hematopoietic cells. The cells were washed three times with PBS and assessed by flow cytometry on a Cytomix FC500 (Beckman Coulter, Brea, CA, USA).

Cell cycle analysis

Harvested cells were washed with PBS and passed through a 35 μm filter to remove the polymer particles and aggregated cells, and cell cycle analysis was performed using with Coulter DNA PREP Reagents Kit (Beckman Coulter) in accordance with the manufacturer's instructions [35]. Briefly, 2×10^5 cells suspended in 0.1 mL of PBS were treated with 0.1 mL of DNA Prep LPR reagent, vortexed, and 2 mL of DNA PrepStain was immediately added. After further vortexing, the cells were incubated for 25 min at room temperature in a dark box, and the cell cycle was assessed by flow cytometry (Cytomix FC500, Beckman Coulter).

Statistical analysis

The results are expressed as the mean ± SD of triplicate experiments. Differences between the means were determined using two-way analysis of variance (ANOVA), and $p \leq 0.05$ was considered significant.

Results

Sensitivity of K562 and MS-5 cells to cytarabine treatment

K562 cells in suspension culture (stromal cell free culture) grow logarithmically in the absence of cytarabine, and reach a growth plateau after 5 days of culture. Introduction of 10 ng/mL of cytarabine suppressed the growth of K562 cells significantly in suspension (stromal cell free) culture, consistent with the known EC50 of cytarabine for K562 cells (0.064 μM, 15 ng/mL) (Figure 1a) [31]. At concentrations of 100 ng/ml or greater, growth was effectively completely abrogated (Figure 1a). Thus, cytarabine at the concentrations of 1 μg/mL and 10 ng/mL were used for the following co-culture experiments.

Stromal MS-5 cells were seeded into 35 mm plastic dishes with or without G-02 particles, and high dose cytarabine (10 or 100 μg/mL) was introduced when MS-5 cells formed a 2D or 3D stromal layer on the bottom of the culture dish. After 5 days of cytarabine treatment, cells were collected using 0.25% trypsin/ 0.02% EDTA in PBS, and viable cell number was determined by trypan blue dye exclusion (Figure 1b). More than 90% of MS-5 cells collected from 2D or 3D culture were viable after treatment with 10 or 100 μg/mL of cytarabine. Thus, MS-5 cells were resistant to cytarabine treatment once the cells had formed a stromal layer.

Influence of cytarabine on the growth of K562 cells in culture with stromal cells

K562 cells (5×10^4/mL) were inoculated into the 2D or 3D cultures. Figures 2a-c shows phase contrast images of K562 cells in stromal cell free, 2D, and 3D cultures, respectively. Inoculated K562 cells adhered to the surface of the flat MS-5 layer (2D culture, Figure 2b), and infiltrated the 3D structural MS-5 layer (3D culture, Figure 2c). After 5 days of cytarabine treatment (1 μg/mL) in stromal cell free or 2D culture, the shape of K562 cells become irregular with a slightly reduced cell-size (Figure 2d, e). In 3D culture, K562 cells remained adherent to the 3D structural MS-5 cells and maintained a rounded shape (Figure 2f).

The number of viable K562 cells was counted after cytarabine treatment. Firstly, 1 μg/mL or 10 ng/mL of cytarabine was added to the culture at the same time as K562 cell inoculation, and the viable cell number was counted after five days of cultivation (Figure 3a). In control cultures without cytarabine, the number of K562 cells in the 3D co-culture system was significantly lower than

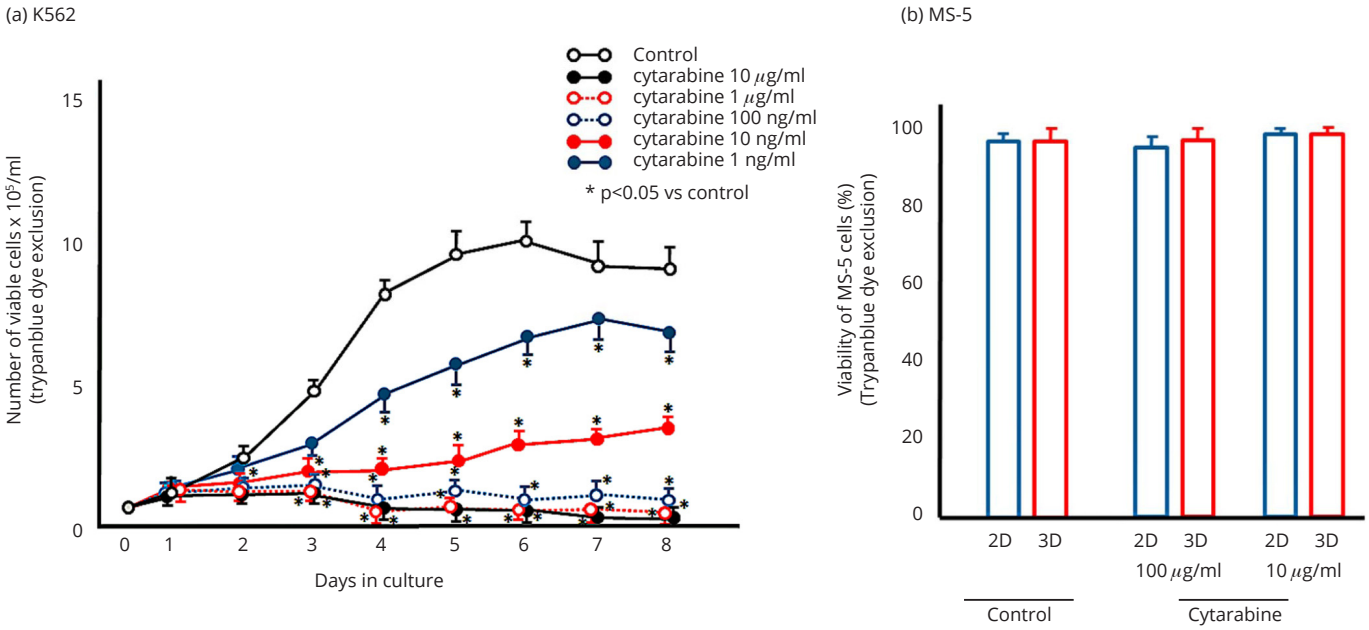

Figure 1 Effects of cytarabine on the growth of K562 and MS-5 cells: (a) K562 cells (5×10^4/mL) were cultured without stromal cells and with various concentrations of cytarabine. Cells were harvested at day 1 to 8 days of culture, and the number of viable cells was counted by trypan blue dye exclusion. (b) MS-5 cells (5×10^5) were cultured in plastic dishes with (3D) or without (2D) G-02 particles, and when cells formed a stromal layer on the bottom of dish, 10 or 100 μg/mL of cytarabine was added to the culture. After 5 days, MS-5 cells were collected by trypsin treatment and the number of viable cells was counted. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD.

*Significant difference ($p < 0.05$) with respect to the cytarabine free control culture.

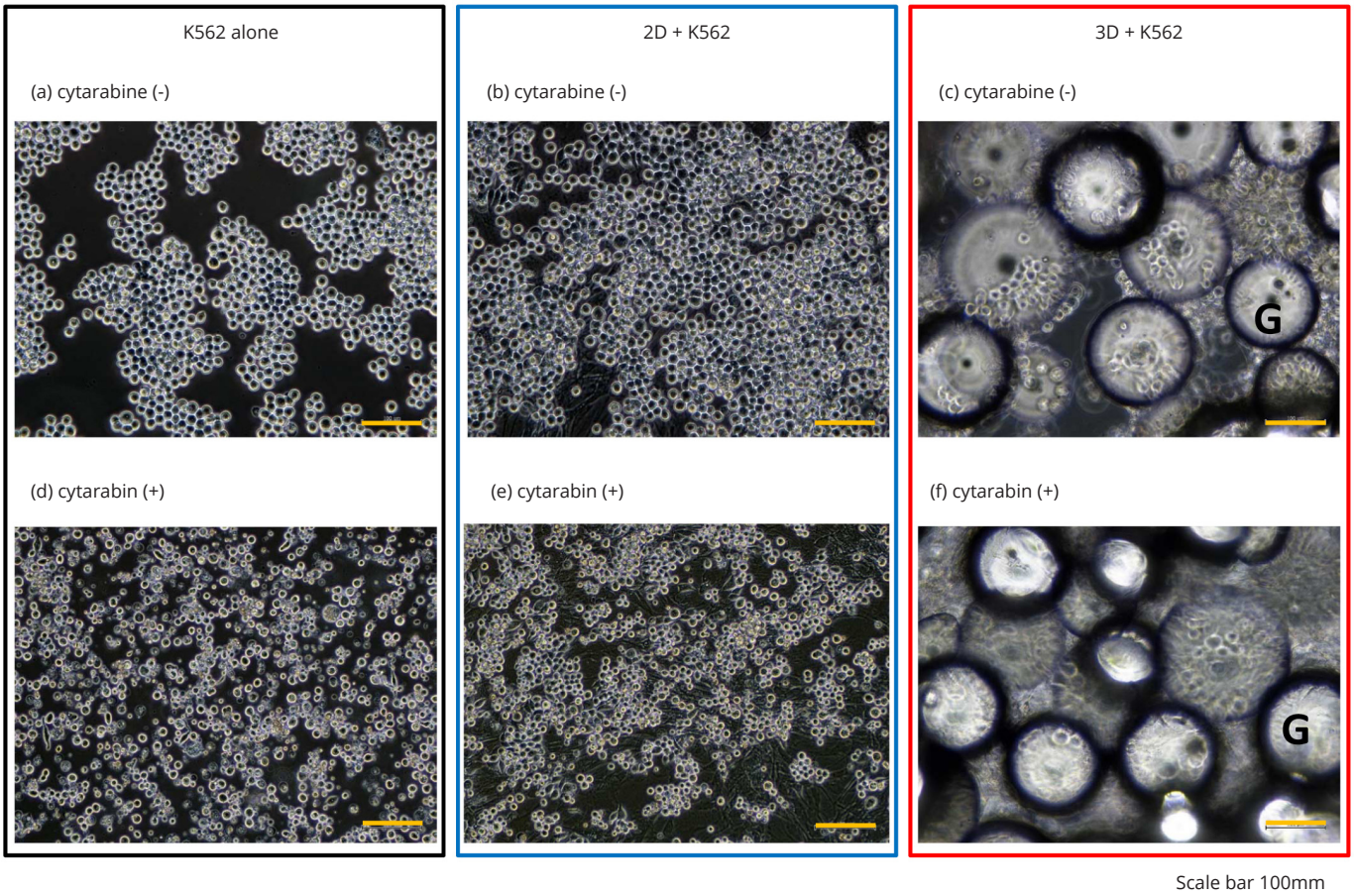

Figure 2 Phase contrast images of K562 cells in stromal cell free, 2D and 3D cultures. Images of cultures after 5 days in the absence (a-c) or presence of 1 μg/mL of cytarabine (d-f). Scale bar shows 100 μm. G: G-02 particles, diameter between 150 to 200 μm.

that without stromal cells, or in the 2D co-culture system. Although cytarabine reduced the number of K562 cells in all groups, the number of K562 cells in 3D co-culture was significantly higher than that in culture without stromal cells.

Next, 1 μg/mL of cytarabine was introduced into the culture at the same time as inoculation of K562 cells, and cells were collected after 5 days, washed, and re-cultured without cytarabine (Figure 3b). The surviving residual K562 cells began to proliferate in cytarabine free culture conditions. K562 cells in 3D culture were much more proliferative compared with the cells in stromal cell free or 2D culture, indicating that the K562 cells in 3D culture were more resistant to cytarabine treatment.

To examine the effect of cytarabine on adhered K562 cells, the drug was introduced 2 days after inoculation of K562 cells (Figure 3c), which allowed enough time for K562 cells to adhere to stromal MS-5 cells. The number of cells in 3D culture was significantly greater than that in stromal cell free or 2D culture. Furthermore, the number of K562 cells in 3D culture was higher than the number obtained when K562 cells and cytarabine were added simultaneously, indicating that once K562 cells adhered to the stroma the cytoreductive effect of cytarabine was diminished.

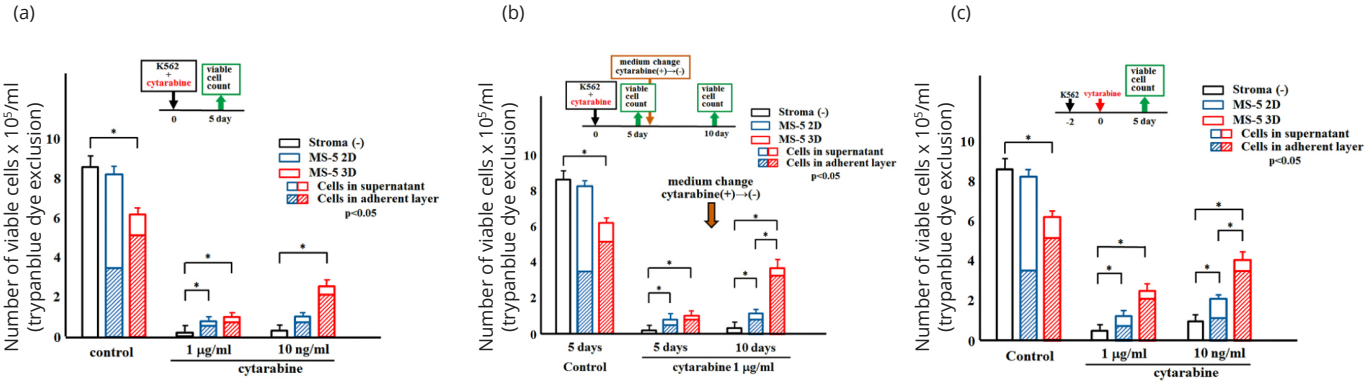

Figure 3 Cytocidal activity of cytarabine on K562 cells in 3D culture: (a) 1 μg/mL or 10 ng/mL of cytarabine was added to the culture at the same time as K562 inoculation into stroma free, 2D and 3D culture. After 5 days, K562 cells in the supernatant and adherent layer were collected separately, and the number of viable cells was counted by trypan blue dye exclusion. (b) Cytarabine was introduced at the same time as K562 inoculation. After 5 days, cytarabine was removed by washing the culture, and the culture was continued. After five days of cytarabine free culture, K562 cells were collected and the number of viable cells was counted. (c) K562 cells were pre-co-cultured with stroma for two days. After K562 cells were well adhered to the stroma, cytarabine was added, and K562 cells were collected at 5 days. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD.

* Significant difference ($p < 0.05$)

Expression of the APO2.7 antigen on K562 cells after treatment with cytarabine

To address the mechanisms underlying the reductions in K562 cell numbers in response to cytarabine treatment, the expression of the APO2.7 apoptosis antigen was examined. An aliquot of cells was collected from each culture 5 days after treatment (using the same protocol as for Figure 3a) and double stained with anti-CD45 and APO2.7 antibodies (Figure 4). Human leukemic K562 cells were positive for CD45, and murine MS-5 cells were negative for CD45. Figure 4a shows the typical dot plot histogram of two-color cytometry determined by CD45 (FL-1) and Apo2.7 (FL-2). Figure 4b represents the typical flow cytometry histograms of K562 cells treated with cytarabine and stained for APO2.7, and the results are summarized in Figure 4c. Significantly higher numbers of APO2.7 positive cells were observed in culture without stromal cells, suggesting that apoptosis was induced under those conditions (Figure 4c). The lowest APO2.7 expression was observed on MS-5 adherent cells in 3D culture compared with floating cells and adherent cells in 2D culture (Figure 4d). Overall, these data demonstrate that in 3D culture MS-5 cells protect K562 cells from apoptosis induced by cytarabine treatment.

Cell cycle analysis in cultured K562 cells

K562 cells were collected from stromal cell free, 2D, and 3D cultures without cytarabine treatment, and the cell cycle was analyzed by flow cytometry. Figure 5a shows the typical cell cycle pattern at day 1 of culture. The percentage of resting K562 cells (G_0/G_1) was 40.1 ± 2.8% in stromal cell free culture, 42.2 ± 3.2% in 2D culture and 61.4 ± 3.5% in 3D culture. The percentage of resting K562 cells in stroma free and 2D culture increased daily as K562 cell numbers increased, and no significant differences between cells collected from stroma free and 2D culture were observed at day 3 and 4 of culture (Figure 5b). On the other hand, the percentage of resting cells in 3D culture did not change over 4 days (Figure 5b), even though the cell number in culture increased (Figure 3). In addition, a higher percentage of adherent K562 cells were in the resting state compared with floating cells in 3D culture (Figure 6). K562 cells in 3D culture appeared to maintain a stable cell cycle, and more than 50% of cells were in resting phase at any one time.

Discussion

In vivo, hematopoiesis takes place in the bone marrow, where hematopoietic cells are in intimate association with distinctive stromal cell elements. The results of several previous studies have suggested that the stromal cell microenvironment plays an important role in hematopoiesis [1-3], and Schofield proposed the concept of a "niche" as a specialized microenvironment housing HSCs [36]. A variety of *in vitro* co-culture experiments with stromal and hematopoietic cells demonstrated that both stromal cell function and niche anatomy regulate stem cell quiescence, self-renewal, and differentiation [6-9, 11, 12, 37, 38]. Abnormal clones originating from hematopoietic cells, including leukemic clones, also arise within the niche. Stromal cells in the niche may supervise and suppress the growth of abnormal clones to maintain normal hematopoiesis [34] by producing soluble factors and mediatory cell-to-cell interactions via adhesion molecules

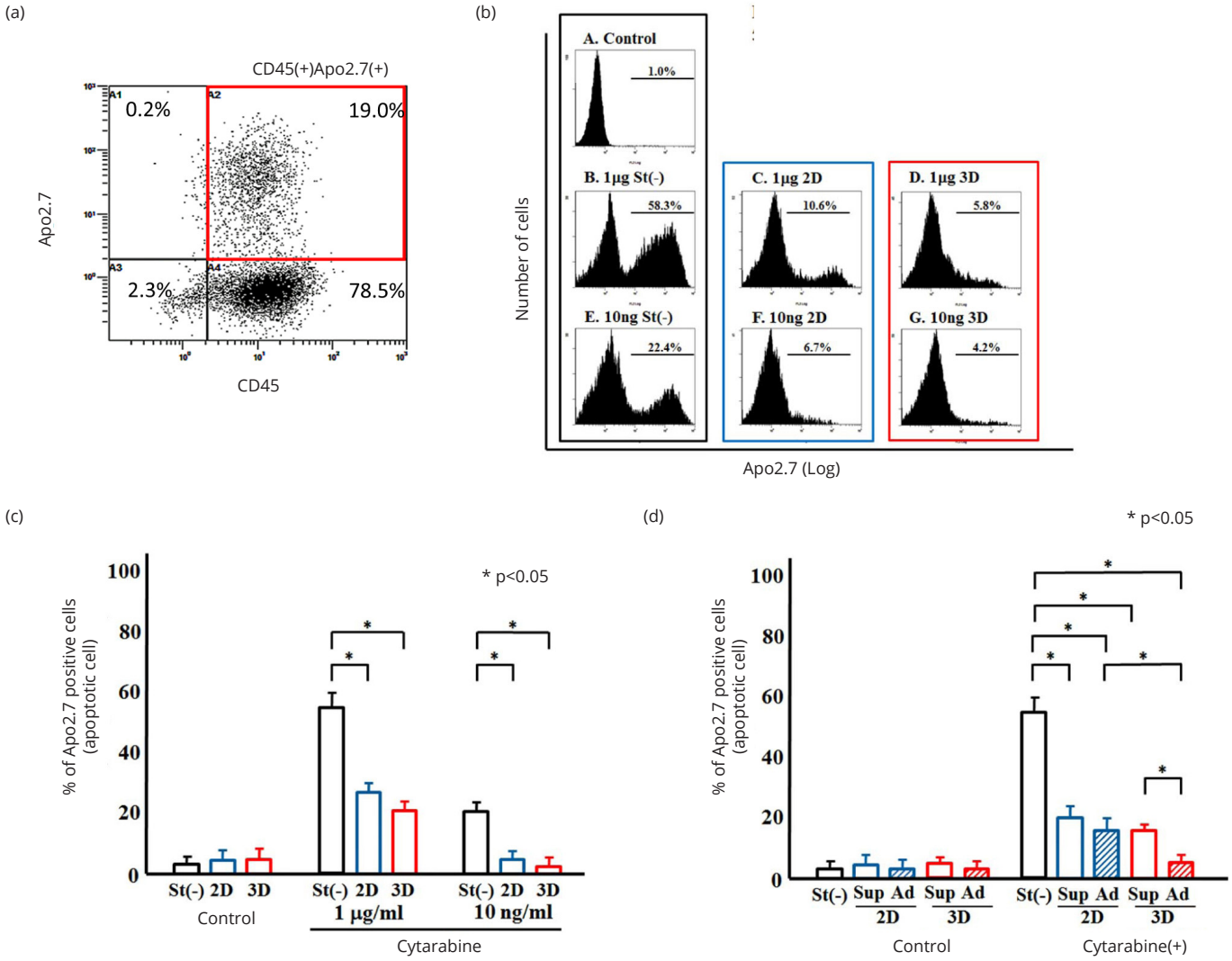

Figure 4 The expression of APO2.7 antigen on the surface of K562 cells after cytarabine treatment. Cytarabine was added to the culture at the time of K562 cell inoculation (as in Figure 3a), and cells were collected 5 days after treatment. (a) A typical dot plot histogram of two-color flow cytometry determined by CD45 (FL-1) and Apo2.7 (FL-2) was shown. (b) Histograms of APO2.7 positive K562 cells among CD45 positive cells after treatment with cytarabine were shown. (c) Bar graph summary of the percentages of APO2.7 positive cells. (d) The percentage of APO2.7 positive cells collected from the supernatant and adherent layer after 5 days of 1 μg/mL cytarabine treatment. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD. St(-) – stromal cell negative; Sup – supernatant cells; Ad – adherent cells.

∗Significant difference ($p < 0.05$)

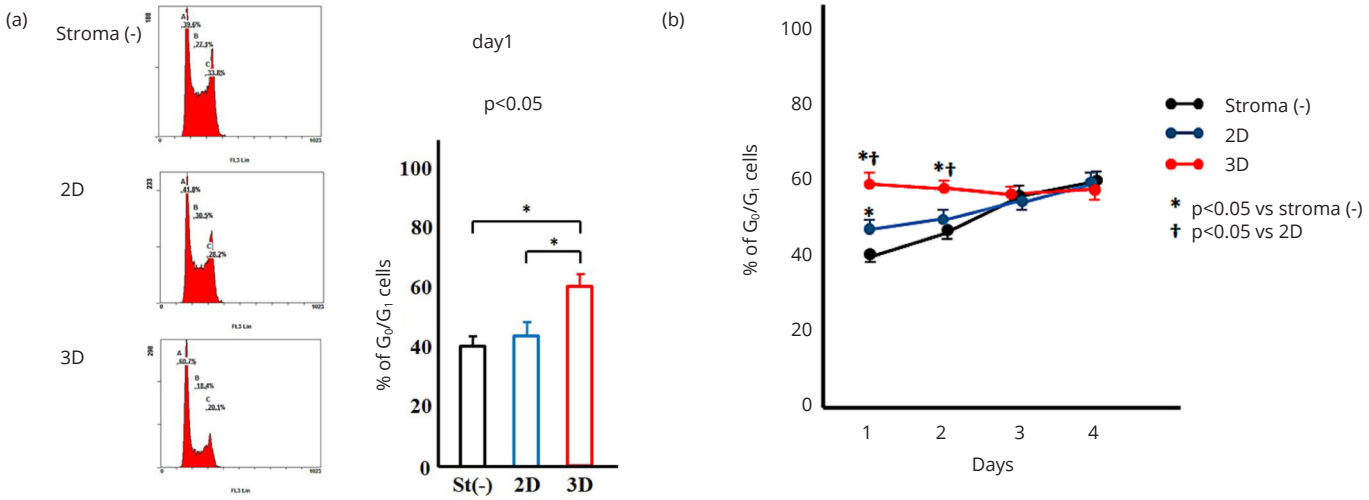

Figure 5 Cell cycle analysis of K562 cells. K562 cells were collected at day 1, 2, 3 and 4 after inoculation into stromal cell free, 2D or 3D culture without cytarabine: (a) Typical histograms of the cell cycle analyzed by flow cytometry and a bar graph summary from three experiments at day 1. (b) Summary of the fluctuations in the percentages of G_0/G_1 cells in each culture. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD. Stroma (-) – stroma free culture.

∗Significant difference ($p < 0.05$)

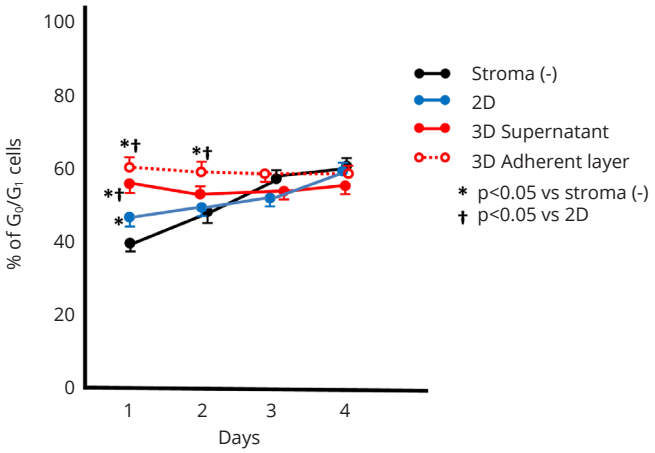

Figure 6 Cell cycle analysis of K562 cells in the supernatant and adherent layer. K562 cells in the supernatant and adherent layer in 3D culture was separately collected and subjected to cell cycle analysis. Two different wells were prepared for each point, and experiments were performed in triplicate. The results are expressed as the mean ± SD.

∗Significant difference ($p < 0.05$)

[39-41]. It is possible that if stromal cell regulatory mechanisms are compromised, hyper-proliferation of abnormal clones may occur. Thus, stromal cells regulate the proliferation of not only normal hematopoietic cells but also abnormal clones. However, the mechanisms underlying stromal cells regulatory functions remain unclear.

Previously, we established a novel 3D culture system using polymer particles with grafted epoxy polymer chains for cell immobilization [23, 24]. In our 3D culture system, MS-5 cells are used as stromal cells [23], and HSC proliferation and differentiation are maintained for significantly longer, recapitulating more closely the natural hematopoietic niche compared with traditional 2D culture systems. Interestingly, most HSCs were in the S phase in both the supernatant and adherent layer in 2D cultures at 4 weeks of co-culture, although more than 50% of those cells were outside S phase in the 3D cultures [25], indicating that the stromal cells in 3D culture control the proliferation and differentiation of hematopoietic cells by regulating the cell cycle. Similarly, the results from this study indicate that stromal cells also regulate the proliferation of leukemic cells in 3D culture, consistent with the importance of the hematopoietic niche in leukemogenesis.

Although our study did not address the mechanisms by which stromal cells regulate the K562 cell cycle and apoptosis, the data highlight the fact that the investigation of anti-cancer drugs *in vitro* is not straightforward. An unique 3D *in vitro* culture model was shown by Torisawa et al. [42]. They use a poly(dimethylsiloxane) (PDMS) device and histological examination showed the mimic cultured specimen to *in vivo*. This model showed the organ-level marrow protective effects to toxic agents, however, PDMS devise was necessary to implant to subcutaneously on the back of the mouse for 4-8 weeks prior to the culture, then cultured in special microfluidic system. In this regard, the practical advantages of our 3D culture system, including easy visual microscopic observation, and identification of human K562 leukemic cells and murine stromal cells by differential surface maker expression, make our system a particularly attractive new approach to address the complexities associated with the analysis of cancer cell chemosensitivity *in vitro*. In addition, our 3D co-culture system with stromal cells does not require the addition of any biological agents, such as hematopoietic growth factors to support hematopoietic and leukemic cell proliferation.

In relation to cytarabine sensitivity, our data accord with several previous studies that demonstrated that apoptotic changes induced by cytarabine in leukemic cell lines or primary cells were reduced by co-culture with stromal cells in 2D *in vitro* models. Lee et al. [43] and Macanas-Pirard et al. [44] demonstrated that soluble factor(s) produced by stromal cells protected leukemic cells from apoptosis induced by cytarabine treatment. In addition, Shishido et al. [45] and Kogoshi et al. [46] showed that stromal cells-mediated resistance to cytarabine treatment by leukemic cells through cell-to-cell interactions via adherent molecules. Recent 3D existing models showed that cell adhesion-mediated drug resistance protects the leukemic cells from apoptosis induce by cytotoxic agents. Damiano et al. [47] and Kuzelova et al. [48] reported that leukemic cells (e.g. K562 cells) adhered to fibronectin were found to be resistant to apoptosis induced by DNA damaging agents. Aljitawi et al. [49] reported that leukemic cells in 3D culture using Scaffold fibers, were resistant to drug-induced apoptosis compared to cells cultured in 2D, and chemo-resistant response of leukemic cells was related to the expression of N-cadherin in 3D culture system. In facts, Xishan et al. [50] also demonstrated that the influences of N-cadherin on K562 cell proliferation *in vitro*. All these models provide an opportunity to study leukemic cell responses to anti-cancer agents in 3D culture system. Importantly, our results revealed that although stromal MS-5 cells in 2D culture partially protected K562 cells from cytarabine induced apoptosis, greater protection was observed in our 3D system. This is the first report demonstrating that the capacity of stromal cells to mediate the resistance of leukemic cells to cytarabine is dependent on the culture system (2D and 3D). Why this is the case requires further investigation. However, we previously demonstrated that the expression of various genes involved in the regulation of hematopoietic cell proliferation and differentiation was "stabilized" in stromal cells in 3D culture compared to those in 2D culture, suggesting that 3D culture promotes the establishment of a "resting" niche in which most hematopoietic cells are outside S phase [25].

In the future, it will be necessary to clarify the mechanisms regulating hematopoietic or leukemic cell homing to the stromal niche, and the maintenance of the resting condition. Comparative studies of 2D and 3D cultures to investigate stromal cell production of various soluble factors, extracellular matrix [51] and adherent molecules may help to resolve these questions.

Conclusion

Stromal cells in our 3D culture system regulated leukemic (K562) cell proliferation by controlling the cell cycle. In 3D culture, most K562 cells were maintained in a stable resting state and the cytocidal activity of cytarabine was reduced compared with cells grown in 2D culture. Although

further studies are required to analyze the mechanisms responsible for the cell cycle regulatory activity of MS-5 cells in 3D culture, the results indicate that our 3D culture system may be a valuable new tool for investigating the cell-to-cell interactions between stroma and leukemic cells *in vitro*.

Author contributions

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. NF, TH, IT, SF, MY and SA conducted the experiments, SF and MY made polymer particles, and NF, TH and SA wrote the manuscript.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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