Original Article

Establishment of an ATLL cell line (YG-PLL) dependent on IL-2 and IL-4, which are replaced by OX40-ligand+ HK with poly-L-histidine and dermatan sulfate

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We established an IL-2 and IL-4 (IL2/4) - dependent adult T-cell leukemia/lymphoma (ATLL) cell line (YG-PLL) by adding poly-L-lysine (PLL) to the culture medium. YG-PLL originates from lymphoma cells and contains a defective HTLV-I proviral genome. Although YG-PLL cannot survive without IL-2/4, the follicular dendritic cell (FDC)-like cell line HK expressing OX40-ligand gene (OX40L+HK) inhibited their death in the presence of soluble neutral polymers. After the prevention of cell death, YG-PLL proliferated on OX40L+HK without IL2/4 in the presence of two kinds of positively or negatively charged polymers. In particular, dermatan sulfate and poly-L-histidine supported growth for more than 4 months. Therefore, the original lymphoma cells proliferated transiently in the presence of IL2/4, and their growth arrest was inhibited by the addition of PLL. Furthermore, YG-PLL lost IL2/4 dependency by the following 3-step procedure: preculture with IL2/4 and neutral polymers, 3-day culture with neutral polymer on OX40L+HK to inhibit cell death, and co-culture with OX40L+HK in the presence of the positively and negatively charged polymers. The extracellular environment made by soluble polymers plays a role in the growth of ATLL *in vitro*.

Keywords: Adult T-cell leukemia/lymphoma, Interleukin, OX-40 ligand, Follicular dendritic cell, Soluble high-molecular-weight polymer

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a clonal disorder caused by human T-cell leukemia virus I (HTLV-I) infection.[1,2] Although HTLV-I induces the immortalization of lymphocytes *in vitro*, there is no evidence that HTLV-I is involved in the tumorigenesis of ATLL. Currently, ATLL is thought to be caused by additional gene abnormalities that accumulate from HTLV-I infection.[3]

ATLL cells exhibit the phenotype of activated T-cells,[4] which express the IL-2 receptor. However, ATLL cells proliferate *in vitro* for a short period and die through apoptosis, which is inhibited by IL-2.[5] Stimulation by IL-2 or IL-4 rarely induces the proliferation of tumor cells, and reports of a growth system *in vitro* are rare.[6,7]

To proliferate ATLL cells *in vitro*, we established a cell line (Hu-ATTACK) in the presence of IL-2 by coculturing with human umbilical vein endothelial cells (HUVECs), which express OX40 ligand (OX40L).[8] In normal CD4 and CD8 T-cells, T-cell receptor stimulation with the help of OX40L expands activated cells.[9] Stimulation of ATL cells with OX40L inhibited Fas-induced apoptosis.[10] Growth of Hu-ATTACK required IL2 and OX40L, which suggested that OX40L blocked apoptosis induced during cell growth by IL-2.

Furthermore, among ATLL cases unresponsive to IL2/IL4 and OX40L, two cell lines (HKOX1 and HKOX2) were established by the co-culture of follicular dendritic cells (FDCs), such as the cell line HK expressing OX40L (OX40L+HK) with IL2/IL4.[11] Therefore, IL2/IL4, OX40L, and feeder effects of HK are necessary for the long-term growth of some ATLL cells. However, additional factors along with IL2/IL4 are necessary for ATLL cells that are unresponsive to IL2/4 and OX40L+HK.

Compared with the extracellular environment *in vitro*, the extracellular space *in vivo* is narrow and cells are packed

with negatively charged adjacent cells. If negatively charged, soluble high-molecular-weight molecules can be added to the *in vitro* culture system to make the extracellular environment similar to the intercellular space *in vivo*. An ATLL case in which leukemic cells that were unresponsive to IL2/4 and OX40L⁺HK became responsive by the addition of negatively charged, soluble polymers was previously reported. These cells developed into a cell line dependent on negatively charged polymers (HKOX3).[12] This suggested that the interaction with specific negatively charged polymers is related to the growth of ATLL with growth factors.

We report that in the presence of a positively charged soluble polymer, an IL2/IL4-dependent cell line was able to be established from lymph node cells that demonstrated IL2/IL4-dependent transient growth with or without negatively charged polymers, and this cell line proliferated on OX40L⁺HK without IL2/IL4 by adding other soluble polymers.

MATERIALS AND METHODS

Cell culture

The FDC-like cell line HK[13] was kindly supplied by Dr. Choi (Laboratory of Cellular Immunology, Alton Ochsner Medical Foundation, New Orleans, LA. USA). OX40L⁺HK cells were established by introducing human OX-40 ligand cDNA to HK, which was described in a previous study[14] and Iscove's modified Dulbecco's medium (IMDM) + 20% FCS was used for the maintenance of OX40L⁺HK. Frozen primary ATLL cells and their cell lines were cultured in IMDM containing 10 U/ml of heparin, 20% human plasma, 10 ng/ml of human IL-2 (Peprotech), and 10 ng/ml of human IL-4 (Peprotech). The detection of mycoplasma infection was not examined throughout the cell culture.

The original lymphoma cells and YG-PLL were cultured in 24-well or 96-well cluster dishes. While subculturing the growing cells, the volume of cultured cell suspension was adjusted to 1000 μl or 200 μl in 24-well or 96-well plates, respectively, and the precisely fractioned cell suspension was transferred to the adjacent well. Except for the primary culture, viable cell numbers were counted using 20 μl of the cell suspension, which was mixed with 20 μl of 0.4 w/v% Trypan Blue Solution [Wako Pure Chemical industry].

The culture of YG-PLL on OX40L⁺HK was performed in 24-well culture plates. Upon transfer to a new dish containing OX40L⁺HK, the mononuclear cells in the cell suspension were separated from dead cells using Ficoll® Paque Plus. The cell growth rate was measured by counting viable cells using the same method described above.

High-molecular-weight polymers and their concentration in the culture medium

High-molecular-weight (M.W.) soluble polymers and their final concentration in culture medium (CiCM) were as follows: Poly-ethylene glycol (PEG) [M.W. 15000-25000, nacalai tesque]: CiCM 0.5-1 mg/ml; polyvinyl alcohol (PVA)

[M.W. 146000-186000, Sigma-Aldrich]: CiCM 0.5-1 mg/ml; polyvinylpyrrolidone (PVP) [M.W. 360000, Sigma-Aldrich]: CiCM 1 mg/ml; dextran (Dex) [M.W. 190000-230000, nacalai tesque]: CiCM 1 mg/ml; poly-γ-glutamic acid (PGA) [M.W. 200000-500000, Wako Pure Chemical industry]: CiCM 1 mg/ml; chondroitin sulfate C sodium salt (CSC) [M.W. 40000-80000,Wako Pure Chemical industry]: CiCM 0.5 mg/ml; hyaluronic acid sodium salt (HRL) [M.W. not determined, Wako Pure Chemical industry]: CiCM 0.5 mg/ml; chondroitin sulfate A sodium salt (CSA) [M.W. 40000-80000, Sigma-Aldrich]: CiCM 0.5 mg/ml; dermatan sulfate sodium salt (DS) [M.W. 85000~100000, Tokyo Chemical Industry]: CiCM 0.5 mg/ml; poly-L-lysine hydrobromide (PLL) [M.W. 30000-70000, Sigma-Aldrich] CiCM 5-50 μg/ml; poly-L-ornithine hydrobromide (PLO) [M.W. 30000-70000, Sigma-Aldrich]: CiCM 12.5 μg/ml; poly-D-lysine hydrobromide (PDL) [M.W. 30000-70000, Sigma-Aldrich]: CiCM 12.5 μg/ml; poly-L-histidine (PLH) [M.W. 5000-25000, Sigma-Aldrich]: CiCM 12.5 μg/ml.

Phenotype and genotype analyses of cell lines

Flow cytometry was performed using the following panel of lymphoid-associated monoclonal antibodies: CD4 (RPA-T4), (DAKO Denmark), CD2 (RPA-2.10), CD3 (UCHT1), CD5 (L17F12), CD8 (SK1), CD25 (M-A251), and CD7 (8H8.1) (Beckman Coulter, Inc. CA, USA).

Human T-cell leukemia virus-I (HTLV-I) proviral integration into cell lines was performed by an inspection agency (Special Reference Laboratory, Japan).

The origin of cell lines was determined by the fragment length comparison of the V-N-J rearrangement portion of the T-cell receptor γ-chain gene.[15] The high-molecular-weight DNA was extracted and amplified by PCR using three sets of primer mixtures labeled with fluorescent dyes. The primers used in this analysis were as follows: Vγ (1−8) II 5'ACCAGGAGGGGAAGGCCCCACAG, Vγ9 5'GGAAAGGAATCTGGCATTCCG, Vγ10 5'AATCCGCAGCTCGACGCAGCA, Vγ 11 5'GCTCAAGATTGCTCAGGTGGG, Vγ12 5'CCTCTTGGGCACTGCTCTAAA, Jγ1/2 5'ACCTGTGACAACAAGTGTTGTTC(NED), Jγ P1/2 5'AGTTACTATGAGCT(T/C)TAGTCCC(6-FAM), Jγ P 5'TGTAATGATAAGCTTTGTTCC(HEX). The three mixtures were Mixture I Vγ (1−8) II: Jγ1/2, JγP1/2, JγP, Mixture II Vγ9: Jγ1/2, JγP1/2, JγP, and Mixture III Vγ10−12: Jγ1/2, JγP1/2, JγP. The PCR products of each mixture were analyzed using an ABI PRISM 310 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA).

RESULTS

Establishment of cell lines

A 66-year-old, HTLV-I-positive male developed generalized lymph-adenopathy after chemotherapy for erythroderma due to ATLL. He did not present with leukemia and the left inguinal lymph-node biopsy revealed ATLL.

When frozen lymphoma cells were cultured in the presence of IL2/4, they proliferated vigorously until approximately two months, and stopped abruptly on repeated trials. When lymphoma cells were cultured in the same IL2/4 containing medium with or without negatively charged CSC or HRL, they grew for 54 days with HRL and 72 days with CSC, compared with 54 days in control culture.

In the next step, to examine the effects of a positively charged polymer, culture in the presence of 5 μg/ml or 50 μg/ml of PLL with or without CSC was conducted. The two cultures with 5 μg/ml or 50 μg/ml of PLL alone continued to proliferate for more than 3 months; however, those growing in the two other culture conditions i.e. PLL+ CSC, stopped growing at day 66 (Figure 1). Therefore, the lymphoma cells proliferated for long periods with IL2/4 and 5 μg/ml or 50 μg/ml of PLL, and these cell lines were named YG-PLL.

YG-PLL exhibited defective and monoclonal integration of HTLV-I on Southern blot analysis (Figure 2A). Flow cytometry of YG-PLL revealed CD2, CD3, CD4, CD5, and CD25 to be positive, and CD7, CD8, CD10, CD19, and CD20 to be negative, typical of ATLL cells (Figure 2B). The rearrangement pattern of the T-cell receptor γ-chain gene was identical to that of the original lymphoma cells (Figure 2C). This suggested that YG-PLL originated from the lymphoma cells of ATLL.

Induction of IL2/4-free culture

Three months after the start of culture, YG-PLL continued to proliferate with a doubling time of approximately 35 hours (Figure 3A). Then, comparing the IL2/4 dependent growth rate with or without PLL, YG-PLL proliferated at the same rate as PLL for 100 days (Figure 3A). Therefore, the phenotype of PLL dependency changed after long-term exposure to PLL.

When YG-PLL was cultured without IL2/4, most of the cells died and the remaining cells survived without proliferation.
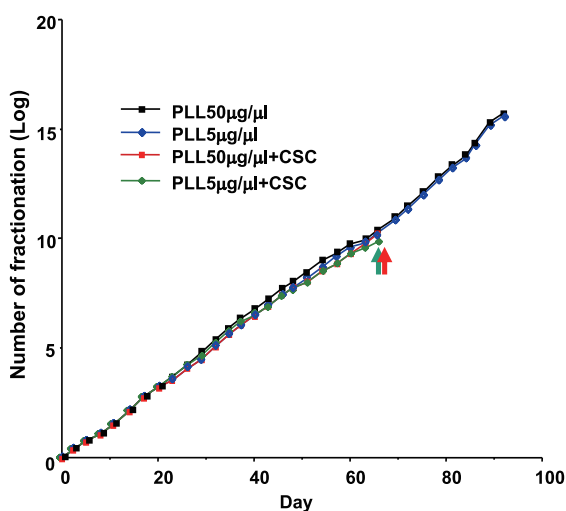

Fig. 1. Cell growth rate of primary culture.
The numbers of exact fractionation of subculture were graphed. Stable proliferation from the start of the primary cultures in PLL+CSC stopped at day 66 (arrows).

Growth promotion by different types of polymers was examined by adding them to the culture medium; however, 11 polymers (PLL, PDL, PLO, PLH, PGA, CSC, HRL, PVA, PEG, DEX, and DS) had no effects on proliferation, irrespective of transient growth in CSC (Figure 3B).

Inhibition of cell death by neutral polymers on OX40L⁺HK

Previously, ATLL-derived cell lines were established by co-culture with HUVECs or OX40L⁺HK, which was the transfectant of human OX40 ligand cDNA. First, YG-PLL was cocultured with HUVECs or HK with or without polymers in the absence of IL2/4. However, the inhibition of death or the promotion of growth of YG-PLL was not observed (data not shown).

Then, the activity of OX40L⁺HK was examined by its coculture in the presence of different soluble polymers without IL2/4. In the presence of the four negatively charged polymers, YG-PLL died within 7 days (Figure 4A). Thus, the negatively charged polymers had no activity regarding the inhibition of cell death. In the case of positively charged polymers, YG-PLL died within 7 days with PLL, PDL, or PLO, demonstrating no activity regarding the inhibition of cell death, and only PLH supported the survival of some cells for approximately two weeks (Figure 4B).

In the case of neutral polymers, all four polymers partially inhibited cell death at day 20, and PVA and PEG maintained survival for more than a month (Figure 4C). Therefore, OX40L⁺HK supported the survival of YG-PLL in the presence of PVA or PEG.

OX40L⁺HK with DS and PLH maintained proliferation after the inhibition of cell death by PVA and PEG

Although OX40L⁺HK with PVA or PEG inhibited cell death by the deprivation of IL2/4, no growth of YG-PLL was observed. There is also the possibility that charged polymers instead of neutral polymers can sustain the growth after the inhibition of death by OX40L⁺HK with PVA or PEG.

Next, after culturing with IL2/4 and 0.5 mg/ml of PVA and PEG (PVA/PEG) for two weeks, and a subsequent three-day co-culture with OX40L⁺HK with PVA/PEG in the absence of IL2/4, YG-PLL were transferred to OX40L⁺HK with a single charged polymer.

In the presence of the eight kinds of single polymers, YG-PLL exhibited no growth except for transient proliferation in CSC (Figure 5A). Then, the synergistic effects of positively and negatively charged polymers was examined. A few combinations induced proliferation for more than two months, but it stopped eventually (Table 1). However, DS and PLH (DS/PLH) promoted cell growth for over four months (Figure 5B). Thus, in YG-PLL, OX40L⁺HK with PVA/PGA inhibits cell death caused by cytokine depletion, and OX40L⁺HK with DS/PLH promotes cell growth for a long period.
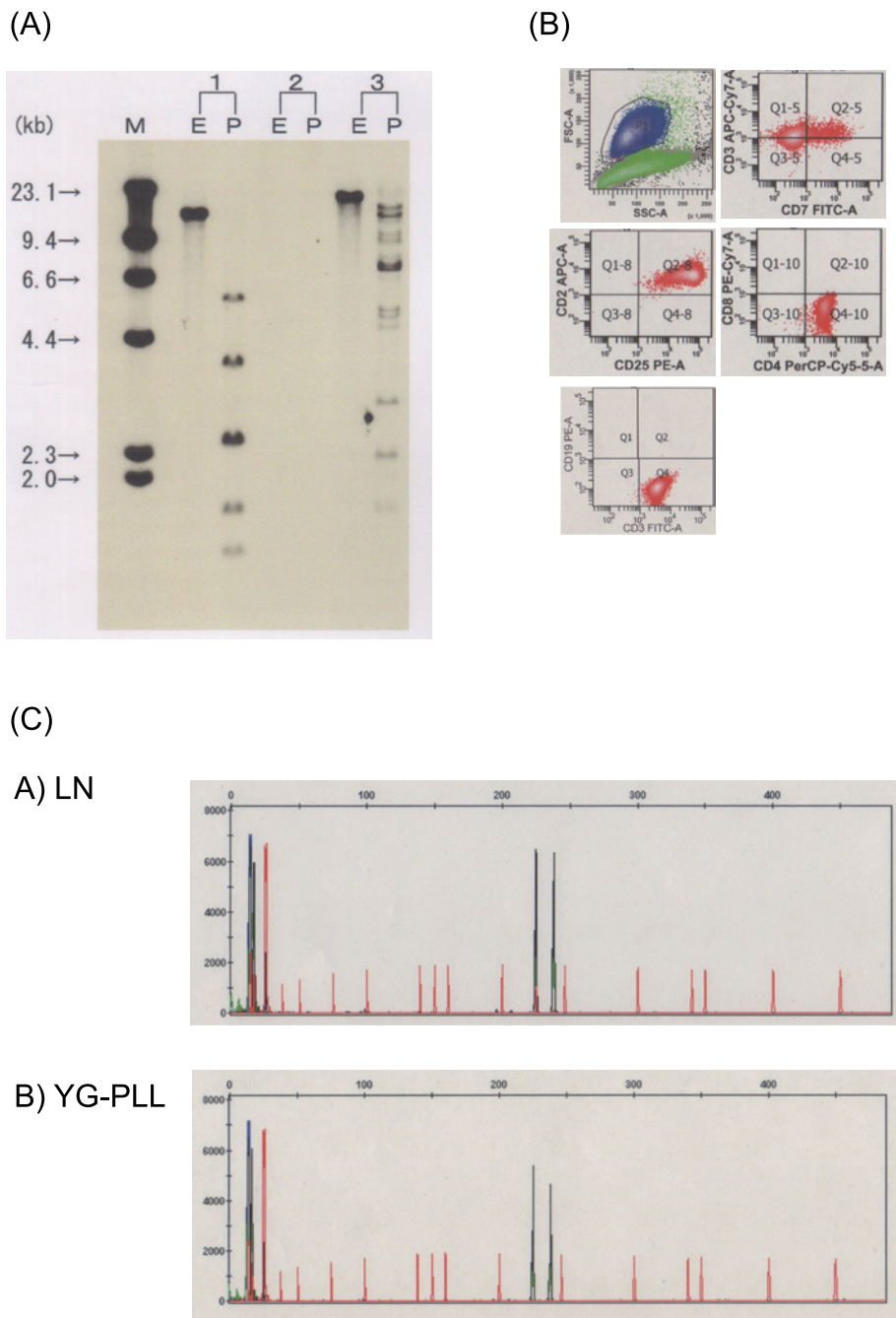
(A)

(B)

(C)

A) LN

B) YG-PLL

Fig. 2. (*A*) HTLV-I proviral integration in YG-PLL. Lane M: size markers; E: *Eco*RI diges-
tion; P: *Pst*I digestion; Lane 1: positive control DNA; Lane 2: negative control DNA; Lane 3:
YG-PLL (*B*) Flow cytometry analysis of YG-PLL (*C*) T-cell receptor γ chain rearrangement in
lymph nodes and YG-PLL. *A*) Original lymphoma cells and *B*) YG-PLL.

DISCUSSION

An ATLL-derived IL2/4-dependent cell line, YG-PLL,
was established by the addition of PLL to the culture
medium.   From the start of the culture of lymphoma cells,
the growth rate of lymphoma cells by IL2/4 was steady with
or without CSC or PLL.   This suggested that the steady pro-
liferation by IL2/4 progressively induced growth inhibition,
and PLL may counteract the inhibitory mechanism.   PLL is a
high–molecular-weight, positively charged polymer, and this

molecule may interact with the outer cell membranes of cells
that are negatively charged.

Three months after the start of culture, YG-PLL grew
without PLL for more than three months.   This phenomenon
suggested that PLL was not directly involved in cell prolifer-
ation, but introduced resistance to growth arrest.   These
mechanisms remain to be clarified.

In general, IL2-dependent ATLL cell lines undergo
growth arrest and apoptosis upon IL2 deprivation.   Cell
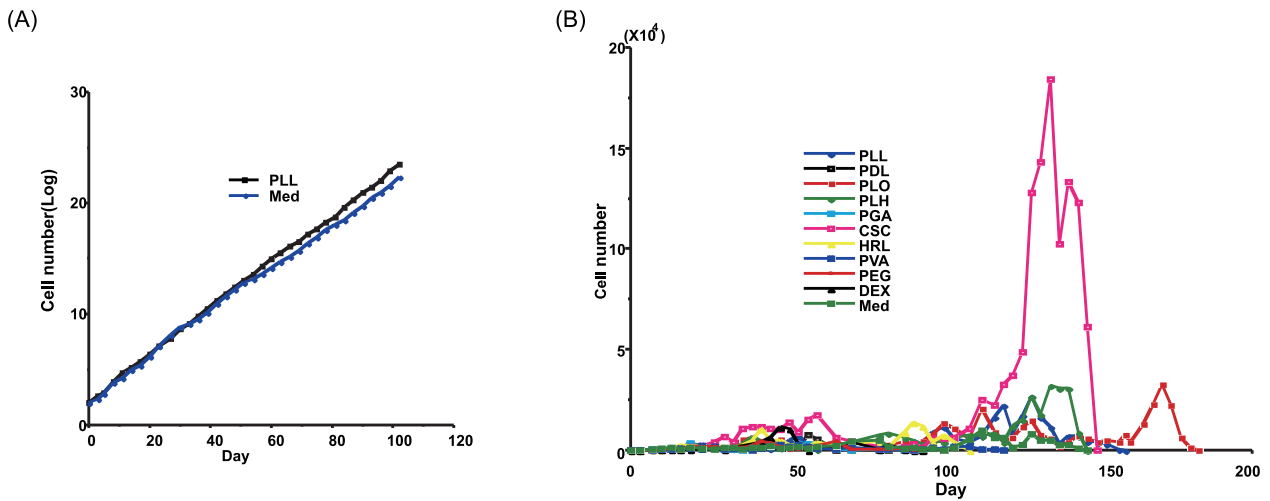death of YG-PLL was also induced by the deprivation of

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(A)

(B)

Fig. 3. (*A*) Growth curve of YG-PLL with or without poly-L lysine with IL2/4, (*B*) Growth of YG-PLL with one polymer each without IL2/4.
Only CSC showed transient growth. DS was not used in this experiment. PLL: poly-L-lysine hydrobromide, PDL: poly-D-lysine, PLO: poly-L-ornithine, PLH: poly-L-histidine, PGA: poly -γ-glutamic acid, CSC: chondroitin sulfate C, HRL: hyaluronic acid, PVA: polyvinyl alcohol, PEG: poly-ethylene glycol, DEX: dextran, Med: no polymer
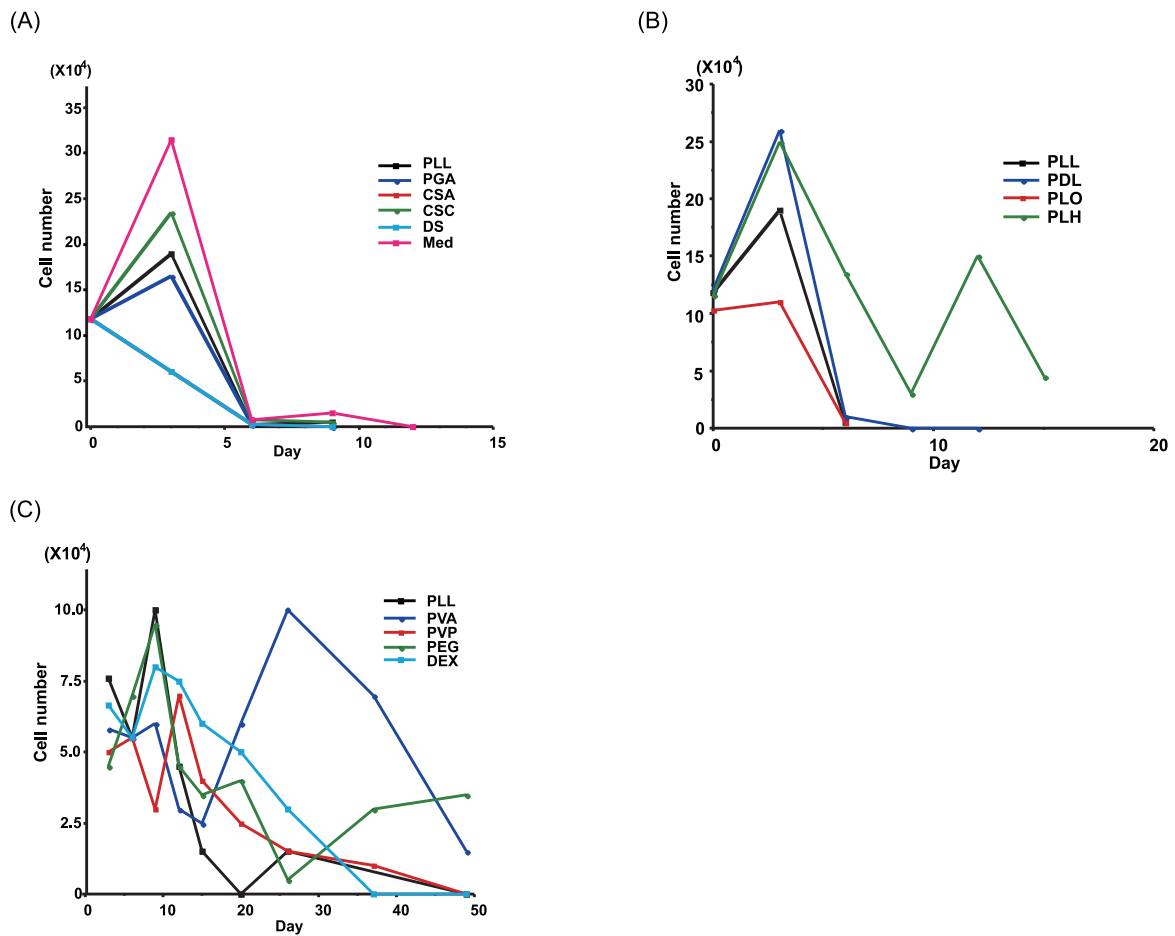
(A)

(B)

(C)

Fig. 4. Inhibition of cell death by OX40L⁺HK with polymers.
YG-PLL was cultured on OX40L⁺HK with negatively charged polymers (*A*), with positively charged polymers (*B*), or neutral polymers and PLL (negative control) (*C*). Negatively charged polymers and positively charged polymers except PLH had no activity to inhibit cell death, and neutral polymers supported the survival of YG-PLL.
PLL: poly L-lysine hydrobromide, PGA: poly -γ-glutamic acid, CSC: chondroitin sulfate C, CSA: chondroitin sulfate A, DS: dermatan sulfate, Med: no polymer, PDL: poly-D-lysine, PLO: poly-L-ornithine, PLH: poly-L-histidine, PVA: poly-vinyl alcohol, PVP: polyvinylpyrrolidone, PEG: poly-ethylene glycol, DEX: dextran
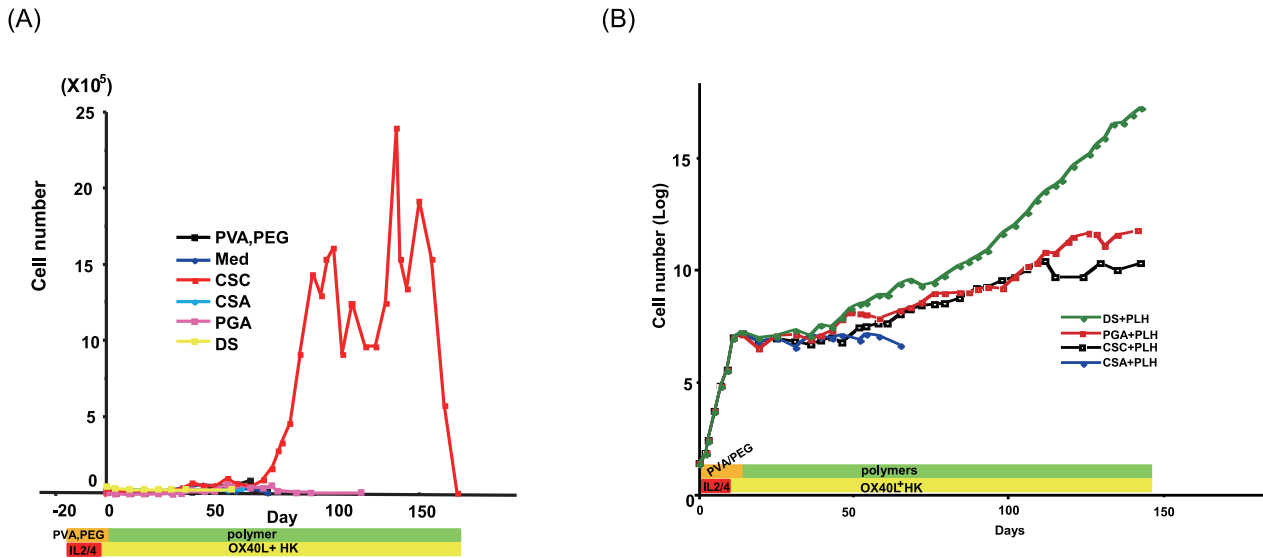
Fig. 5. Growth of YG-PLL on OX40L⁺HK with polymers after the inhibition of cell death.
After the pre-culture containing IL2/4 and PVA + PEG, 3 days of culture with PVA + PEG on OX40L⁺HK to inhibit cell death was followed by co-culture with OX40L⁺HK in the presence of a single polymer (***A***) or both positively and negatively charged polymers (***B***).
(***A***) Growth of YG-PLL on OX40L⁺HK with a single negatively charged polymer. Transient growth was detected in CSC. (***B***) Growth of YG-PLL on OX40L⁺HK with two kinds of polymers. DS + PLH induced constant growth. PVA: polyvinyl alcohol, PEG: poly-ethylene glycol, CSC: chondroitin sulfate C, CSA: chondroitin sulfate A, PGA: poly-γ-glutamic acid, DS: dermatan sulfate, PLH: poly-L-histidine, Med: no polymer

Table 1. Proliferation period with two kinds of charged polymers

	CSC	CSA	PGA	DS
PLL	26	7	7	9
PDL	110	7	7	9
PLO	45	7	7	9
PLH	95	48	110	125+

The proliferation period was defined as the length of time from the start date of culture with the combination of polymers to the day when the absolute cell number reached the maximum value. The result of DS+PLH was confirmed in another independent experiment. PLL: poly-L-lysine hydrobromide, PDL: poly-D-lysine, PLO: poly-L-ornithine, PLH: poly-L-histidine, CSC: chondroitin sulfate C, CSA: chondroitin sulfate A, PGA: poly-γ-glutamic acid, DS: dermatan sulfate.

IL2/4 unless neutral polymers and OX40L⁺HK were present. OX40L⁺HK alone has no ability to inhibit the death of YG-PLL, as previously reported, because the OX40L⁺HK and IL2/4-dependent cell lines HKOX1, HKOX2, and HXOX3 were unable to survive on OX40L⁺HK without IL2/4.[11,12] By unknown mechanisms, neutral polymers altered the properties of YG-PLL, which facilitated the inhibition of cell death by OX40L⁺HK.

After pre-treatment with neutral polymers and OX40L⁺HK, YG-PLL continued dependent proliferation on OX40L⁺HK and DS/PLH without IL2/4, and relative long-term proliferation was observed in PGA + PLH, CSC + PDL, and CSC + PLH. This suggests that OX40L⁺HK-dependent growth requires two supportive actions: growth facilitation and blocking of growth inhibition. DS/PLH possesses sufficient power for both of these actions, and the other three combinations of polymers are less effective.

In a previous report, the HK cell line supported the growth of a follicular lymphoma cell line[16] or Burkitt cell lines.[17] In addition, OX40L⁺HK directly induced the growth of ATLL, which suggested that ATLL cells can proliferate *in vivo* near FDC in the presence of OX40L and molecules similar to DS/PLH. OX40L may be induced on many cell types, such as dendritic cells, endothelial cells, and blood cells,[9] which may exist near ATLL cells.

The action of DS/PLH on YG-PLL remain to be elucidated. Both DS and PLH are high-molecular-weight molecules, and their interaction is limited to the outer cell membrane. *In vivo*, outer membranes are exposed to adjacent cells through the extracellular space, which may be substituted for specific soluble polymers *in vitro*. All polymers used in this study were high-molecular-weight polysaccharides, polypeptides, and polymers of hydrophilic small molecules, whose molecular structures vary. Therefore, the mechanism of action may be related to physical interactions. If similar growth mechanisms exist *in vivo*, their analysis is difficult because of the lack of technology. Further examination of the proliferation mechanism of YG-PLL may clarify the growth mechanism *in vivo*.

CONFLICT OF INTEREST

None

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