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Original Article

Establishment of cell lines from adult T-cell leukemia cells dependent on negatively charged polymers

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Growing adult T-cell leukemia/lymphoma (ATLL) cells *in vitro* is difficult. Here, we examined the effects of static electricity in the culture medium on the proliferation of ATLL cells. Six out of 10 ATLL cells did not proliferate *in vitro* and thus had to be cultured in a medium containing negatively charged polymers. In the presence of poly-γ-glutamic acid (PGA) or chondroitin sulfate (CDR), cell lines (HKOX3-PGA, HKOX3-CDR) were established from the same single ATLL case using interleukin (IL)-2, IL-4, and feeder cells expressing OX40L (OX40L$^+$HK). Dextran sulfate inhibited growth in both HKOX3 cell lines. Both PGA and OX40L$^+$HK were indispensable for HKOX3-PGA growth, but HKOX3-CDR could proliferate in the presence of CDR or OX40L$^+$HK alone. Thus, the specific action of each negatively charged polymer promoted the growth of specific ATLL cells *in vitro*.

Keywords: Adult T-cell leukemia/lymphoma, Cell line, Follicular dendritic cell, Interleukin, Charged polymer

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a highly aggressive lymphoid neoplasm[1,2] that occurs in T-cells infected with human T-cell leukemia virus I (HTLV-I)[3]. Its tumorigenesis is related to the accumulation of genomic abnormalities[4,5]; next-generation sequencing clarified the complicated genomic abnormalities and revealed the activation of the signaling pathway involving nuclear factor κ-B (NFκB)[6]. However, the genomic abnormalities are so divergent that their common pathway still remains to be explored.

We have established ATLL-derived cell lines to help the understanding of pathogenesis. Generally, ATLL cells die ex vivo due to apoptosis, which is inhibited by the addition of interleukin (IL)-2[7], although IL-2 does not support acute-type ATLL cell growth[8]. With regard to the microenvironment of different organs where ATLL was involved, interaction with the surrounding cells was suspected.

First, we cultured 10 ATLL cases in the presence of IL-2 and/or IL-4, and only one cell line was established. Then, from the remaining nine ATLL cases, one cell line (HU-ATTAK) was established by co-cultivation with human umbilical vein endothelial cells (HUVEC)[9]. HU-ATTAK was dependent on the OX40 ligand (OX40L), which exists on the surface of HUVEC[10,11].

The follicular dendritic cell (FDC)-like cell line HK[12] could support the growth of a follicular lymphoma derived cell line[13]. When we used the HK transformant that was introduced with the human OX40L gene (OX40L$^+$HK)[14], two cell lines out of the remaining eight ATLL cases were established. The established cell lines HKOX1 and HKOX2 proliferated in the presence of IL-2, IL-4 (IL-2/IL-4), and OX40L$^+$HK on 24-well culture plates. However, HKOX2 growth was occasionally retarded when it was cultured on 96-well plates.

Subsequent preliminary experiments suggested that the growth was suppressed by static electricity on the walls of the 96-well plates, which are made of polystyrene. The static electricity more markedly influenced the cells in 96-well plate than those in the 24-well plate due to their proximity to the walls of the 96-well plate. Thus, we hypothesized that the growth of some ATLL cells may be influenced by the electric fields produced by negative charge, which may cause growth promotion or inhibition in some ATLL cases. However, the remaining six ATLL cases did not proliferate in the 96-well plates.

Generally, viable cells are negatively charged and exist densely *in vivo*, which produces electric fields outside the cells. In order to expose ATLL cells to more potent electric fields, we thought that negatively charged, soluble high-molecular weight polymers would construct an efficient electric field if in the culture medium. Thus, we prepared three

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kinds of negatively charged polymers and cultured the six ATLL cells with one polymer each in the presence of IL2/IL4 with or without OX40L$^+$HK. We succeeded in establishing two cell lines from one ATLL case. These results indicated that negatively charged polymers support the growth of some ATLL cells.

MATERIALS AND METHODS

Cell culture

The FDC-like cell line HK[12] 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 the human OX-40L gene to HK, as described in a previous article[15,14]. Iscove's modified Dulbecco's medium (IMDM) + 10% FCS was used for maintaining OX40L$^+$HK. Frozen primary ATLL cells were maintained in IMDM with 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). They were co-cultured with OX40L$^+$HK on 24-well culture plates (Coster, Cambridge, MA, USA). To measure cell growth, the number of viable cells was counted using the trypan blue dye exclusion test.

Preparation of negatively charged polymers and measurement of ζ potentials of their solutions

Poly-γ-glutamic acid (PGA) [molecular weight (M.W.) 200–500 kDa] (Wako Pure Chemical Industry) was dissolved with 1 N NaOH solution, its pH adjusted to 7.5 by adding 1 N HCl, and then sterilized. Chondroitin sulfate C sodium salt [M.W. 40–80 kDa] (Wako Pure Chemical industry) and sodium dextran sulfate [M.W. 500 kDa] (Wako Pure Chemical Industry) were dissolved in distilled water and sterilized. The ζ potential of each solution was determined at 0.1, 0.5, 1.0, and 5.0 mg/ml polymer concentrations using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) at 25°C. The ζ potentials were calculated from the electrophoretic mobility of the polymers by laser Doppler electrophoresis under an applied potential.

Phenotype and genotype analyses of cell lines

Flow cytometry was performed using the following panel of lymphoid-associated monoclonal antibodies: HLA-DR (CR3/43), CD4 (RPA-T4), (DAKO, Denmark), CD2 (RPA-2.10), CD3 (UCHT1), CD5 (L17F12), CD8 (SK1), CD25 (M-A251), TCR-αβ (WT31), TCR-γδ (11F2, BD Pharmingen, Japan BD, Tokyo, Japan), CD7 (8H8.1, Beckman Coulter, Inc. CA, USA), CD134 (W4-3), and CD252 (TAG-34) (Medical & Biological Laboratories, Nagoya, Japan).

HTLV-I proviral integration in cell lines was performed by an inspection agency (Special Reference Laboratory, Japan).

The origin of cell lines was determined by examining the fragment length of the V-N-J rearranged segment of the T-cell receptor's γ-chain gene[16]. The high molecular weight DNA was extracted and amplified by PCR using three mixture sets of primers labeled with fluorescent dyes. The primers used in this analysis were as follows: Vγ(1–8) II 5′-ACCAGGAGGGGAAGGCCCCACAG-3′, Vγ9 5′-GGAAAGGAATCTGGCATTCCG-3′, Vγ10 5′-AATCCGCAGCTCGACGCAGCA-3′, Vγ11 5′-GCTCAAGATTGCTCAGGTGGG-3′, Vγ12 5′-CCTCTTGGGCACTGCTCTAAA-3′, Jγ1/2 5′-ACCTGTGACAACAAGTGTTGTTC-3′ (NED), JγP1/2 5′-AGTTACTATGAGCT(T/C)TAGTCCC-3′ (6-FAM), and JγP 5′-TGTAATGATAAGCTTTGTTCC-3′ (HEX). The three mixtures were Mixture I Vγ(1–8) II: Jγ1/2, JγP1/2, and JγP; Mixture II Vγ9: Jγ1/2, JγP1/2, and JγP; and Mixture III Vγ10–12: Jγ1/2, JγP1/2, and JγP. The PCR products of each mixture were analyzed with an ABI PRISM 310 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA).

RESULT

PGA supports the growth of an ATLL case

Three cell lines from nine ATLL cases were established in the presence of IL-2 + HUVEC or IL-2/IL-4, and andanOX40L$^+$HK, but the remaining six ATLL cases did not grow under these conditions. First, we examined the growth-promoting effects of PGA. The cell culturing was initiated with 1 mg/ml PGA in the presence of IL-2/IL-4 and with or without OX40L$^+$HK. Consequently, one of six ATLL cells proliferated vigorously for over 3 months in the presence of OX40L$^+$HK. This ATLL case exhibited aggressive leukocytosis consisting of flower-like cells, but revealed no skin rash or lymphadenopathy. Flow cytometry of the cell line gave a positive signal for CD2, CD3, CD4, and CD25, which is the typical phenotype of ATLL. The HTLV-I proviral integration pattern obtained by Southern blot analysis demonstrated defective clonal bands (Fig. 1A). T-cell receptor γ chain (TCR-γ) rearrangement analysis revealed the pattern to be identical to that of the original peripheral leukemic cells (Figs. 2A, B). These data indicate that one out of six ATLL cases proliferated with the aid of IL-2/IL-4, OX40L$^+$HK, and 1 mg/ml of PGA. We named this cell line HKOX3-PGA.

Other negatively charged polymers affect ATLL cell growth.

To assess growth promotion by other negatively charged polymers, we attempted to establish cell lines from the six ATLL cells using chondroitin sulfate (CDR) and dextran sulfate (DEX). To compare the negative electric potential of the medium containing CDR or DEX with that containing 1-mg/ml PGA, the ζ potential of the culture medium containing each molecule was measured (Table 1). The ζ potential of the culture medium containing 0.5-mg/ml CDR or 0.5-mg/ml DEX was comparable to that containing 1-mg/ml PGA. The six ATLL cells were cultured in the presence of IL-2/IL-4 and 0.5-mg/ml CDR, or 0.5-mg/ml DEX, with or without OX40L$^+$HK.

Consequently, only the same ATLL case that gave rise to HKOX3-PGA showed constant growth for over 3 months in the presence of IL-2/IL-4 and 0.5-mg/ml CDR with OX40L$^+$HK. The HTLV-I proviral integration pattern was identical with that of HKOX3-PGA (Fig. 1B), and TCR-γ rearrangement was the same as that of the original leukemic cells and HKOX3-PGA (Fig. 2). We named this cell line HKOX3-CDR. These data indicated that one of the six ATLL cases responded to PGA or CDR in the presence of IL-2/IL-4 and OX40L$^+$HK.

Dependency on negatively charged molecules was different in HKOX3 cell lines.

Several factors on which growth was dependent were compared between HKOX3-PGA and HKOX3-CDR cell lines. Both cell lines were critically dependent on IL-2/IL-4 (data not shown). HKOX3-PGA was critically dependent on OX40L$^+$HK, and the growth stopped after 1 week without OX40L$^+$HK (Fig. 3A). In cultures with or without PGA on OX40L$^+$HK, the growth rate was identical for a month, which then slowly decreased in the culture without PGA (Fig. 3B). When the growth rate of the culture without PGA became low, adding PGA promptly restored the growth (Fig. 3C). Then, the proliferative effects of 3 negatively charged polymers on HKOX3-PGA were examined. In the presence of PGA or CDR, the growth rate was identical to the culture without polymers for 2 months. However, the growth stopped within 10 days in the presence of DEX (Fig. 3D). These data indicate that HKOX3-PGA is dependent on both OX40L$^+$HK and PGA, whereas DEX inhibited its growth in the presence of OX40L$^+$HK.

In HKOX3-CDR, the CDR-dependent growth pattern was different from that in HKOX3-PGA. In the culture with OX40L$^+$HK, the growth rate with or without CDR was identical for over 3 months (Fig. 3E). In the absence of OX40L$^+$HK, the growth rate with or without CDR was identical for a month, then the culture without CDR stopped growing. HKOX3-CDR without OX40L$^+$HK continued to grow in the presence of CDR, although the original leukemic cells of HKOX3-CDR did not proliferate in the presence of CDR without OX40L$^+$HK in the primary culture. In HKOX3-CDR, replacing CDR with DEX rapidly suppressed

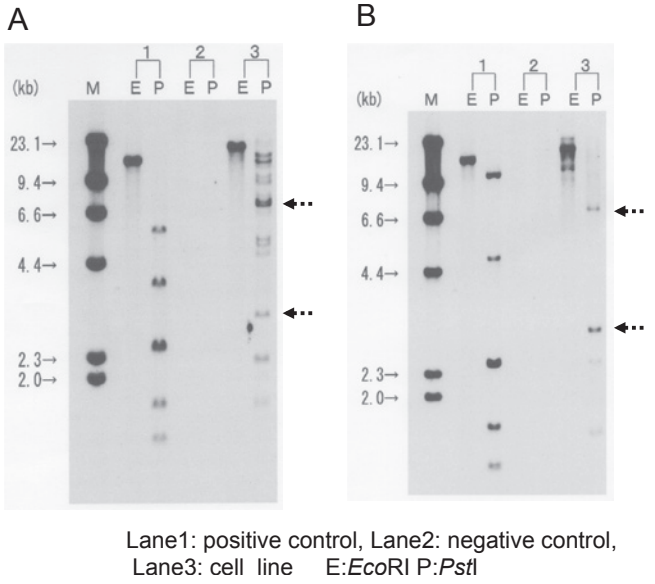

Fig. 1. HTLV-I proviral integration in HKOX3 cell lines. ***A***) HKOX3-PGA; ***B***) HKOX3-CDR. The dotted arrows show the flanking sequence.

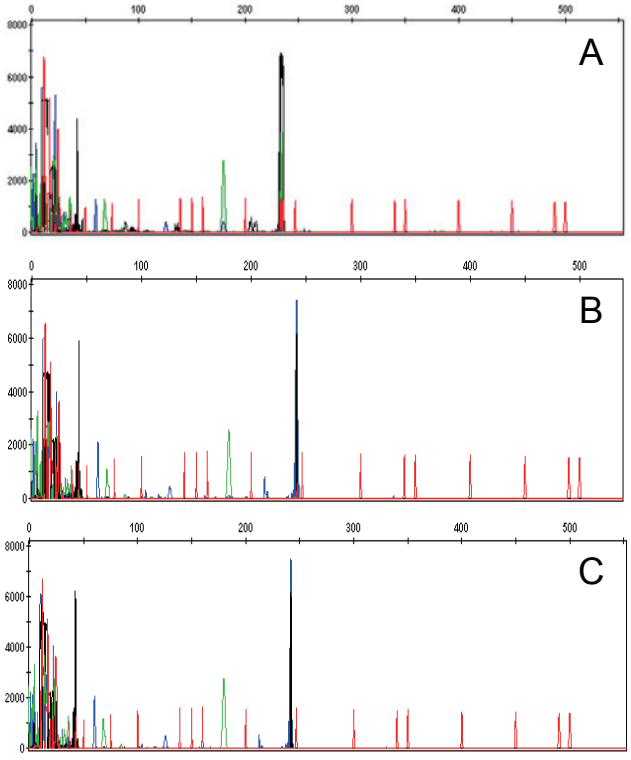

Fig. 2. TCR γ rearrangement pattern. ***A***) Original leukemic cells; ***B***) HKOX3-PGA; and ***C***) HKOX3-CDR. The distance of the major peak was identical in the original leukemia and cell lines.

Table 1. ζ potential of the culture medium containing negatively charged polymers.

(mg/ml)	Poly-γ-glutamic Acid (mV)	Chondroitin Sulfate (mV)	Dextran Sulfate (mV)
0.1	-6.4±1.2	-7.4±0.1	-6.7±0.9
0.5	-10.0±0.9	-7.2±1.0	-9.2±1.1
1	-11.1±0.4	-9.2±0.6	-9.2±1.4
5	-23.0±0.5	-9.7±0.6	-18.8±0.5

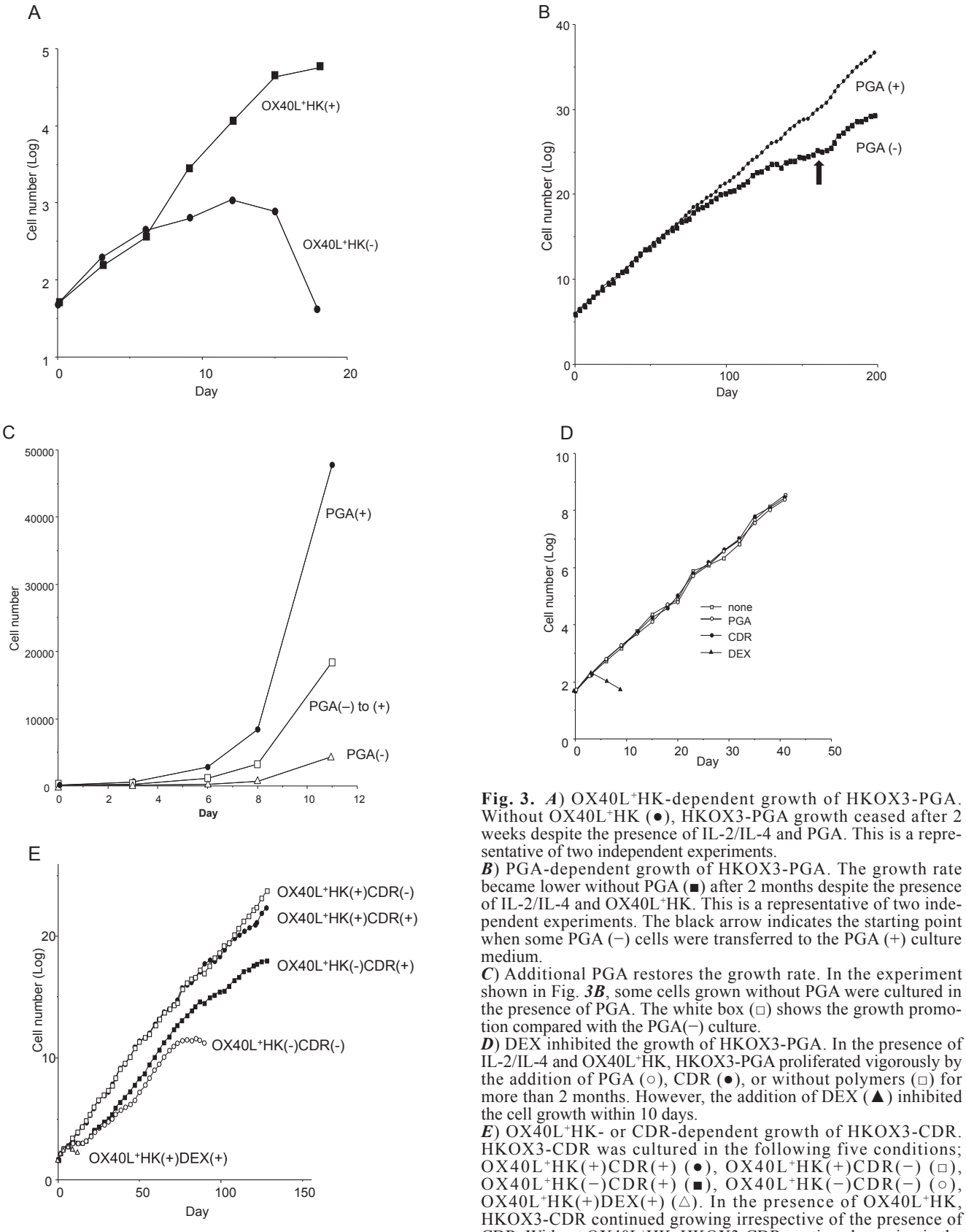

Fig. 3. ***A***) OX40L$^+$HK-dependent growth of HKOX3-PGA. Without OX40L$^+$HK (●), HKOX3-PGA growth ceased after 2 weeks despite the presence of IL-2/IL-4 and PGA. This is a representative of two independent experiments.
B) PGA-dependent growth of HKOX3-PGA. The growth rate became lower without PGA (■) after 2 months despite the presence of IL-2/IL-4 and OX40L$^+$HK. This is a representative of two independent experiments. The black arrow indicates the starting point when some PGA (−) cells were transferred to the PGA (+) culture medium.
C) Additional PGA restores the growth rate. In the experiment shown in Fig. ***3B***, some cells grown without PGA were cultured in the presence of PGA. The white box (□) shows the growth promotion compared with the PGA(−) culture.
D) DEX inhibited the growth of HKOX3-PGA. In the presence of IL-2/IL-4 and OX40L$^+$HK, HKOX3-PGA proliferated vigorously by the addition of PGA (○), CDR (●), or without polymers (□) for more than 2 months. However, the addition of DEX (▲) inhibited the cell growth within 10 days.
E) OX40L$^+$HK- or CDR-dependent growth of HKOX3-CDR. HKOX3-CDR was cultured in the following five conditions; OX40L$^+$HK(+)CDR(+) (●), OX40L$^+$HK(+)CDR(−) (□), OX40L$^+$HK(−)CDR(+) (■), OX40L$^+$HK(−)CDR(−) (○), OX40L$^+$HK(+)DEX(+) (△). In the presence of OX40L$^+$HK, HKOX3-CDR continued growing irrespective of the presence of CDR. Without OX40L$^+$HK, HKOX3-CDR continued growing in the presence of CDR. In the presence of DEX, cells stopped growing within 10 days. The concentration of CDR was generally 0.5 mg/ml, but it accidentally became 5 mg/ml during days 52–119.

the growth (Fig. 3E). These data indicate that HKOX3-CDR could proliferate without either CDR or OX40L$^+$HK. The inhibitory effects of DEX on both HKOX3-PGA and HKOX3-CDR indicate that a DEX-specific inhibitory mechanism exists.

DISCUSSION

We have demonstrated that ATLL cell growth is promoted by the cooperation of IL-2/IL-4 and OX40L$^+$HK *in vitro*; however, non-responsive ATLL cells continue to exist. Based on the observation that static electricity influences ATLL cell growth, we examined whether negatively charged molecules in the culture medium aid ATLL cell proliferation.

Using negatively charged polymers that have a relatively simple molecular structure, PGA- or CDR-responsive cell lines could be established from one of six ATLL cases. However, while its negative electric potential is similar, DEX, the other molecule, exhibited no proliferative effects on six ATLL cells, but inhibited the growth of both HKOX3-PGA and HKOX3-CDR. As these polymers have a completely different structure, interactions with ATLL cells, the mechanisms of which remain unknown but include a negative electric charge, may play a role in ATLL cell proliferation.

The mechanism of action of PGA and CDR was deemed to be different. HKOX3-PGA proliferation was dependent on PGA and OX40L$^+$HK. In a culture without PGA, HKOX3-PGA lost its growth capacity after more than two months, and its growth recovery upon adding PGA was prompt. Therefore, the action of PGA was rapid and long lasting. HKOX3-CDR had acquired independence from OX40L$^+$HK and CDR. ATLL cells grown in a primary culture with IL-2/IL-4 and CDR without OX40L$^+$HK did not proliferate much and died. This suggests that the nature of HKOX3-CDR is changed during primary culture in the presence of OX40L$^+$HK and CDR. Further analysis comparing the genotypes of HKOX3-PGA and HKOX3-CDR is necessary to clarify the mechanisms of action of negatively charged polymers.

The specific actions of PGA, CDR, and DEX on ATLL cells suggest that the interactions are divergent. Our data indicate that the growth of some ATLL cells is enhanced not by a negative electric field, but by negatively charged polymers, and that the mechanism of growth promotion is different among polymers. Thus, ATLL cells that did not grow upon the addition of PGA or CDR may react to other kinds of polymers. Recently, we have succeeded in establishing cell lines from some ATLL cells using positively charged polymers. However, preliminary experiments have shown no growth in B-cell lymphomas upon the addition of negatively charged polymers.

In any tissue in the body, viable cells are negatively charged and packed in a limited space. The biochemical reactions of cell surface molecules may be influenced by charged molecules, which are not reproduced in a liquid culture system. The addition of soluble polymers into culture media may resemble the tissue microenvironment. Further analysis of growth promotion by negatively charged polymers may clarify the growth of ATLL cells *in vivo*.

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CONFLICT OF INTEREST

None of the authors declare conflicts of interest.

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