J. Life Science. (2002), 12(1), 22~26

JOURNAL OF
Life Science

Proliferation Effect of Conditioned Medium Produced by Lymph Node Stromal Cells

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Received: March 13, 2002

Abstract CS21 lymphoma cells that preferentially metastasize to lymph nodes after s.c. inoculation into BALB/c mice were grown *in vitro* in the presence of CA12 stromal cells isolated from lymph nodes. To obtain fundamental data on identification and characterization of the soluble factor(s) produced by CA12 stromal cells, we investigated the biological profile of the conditioned medium produced by CA12 stromal cells. CA12 conditioned medium has no affinity with Con A. CA12 conditioned medium is associated with the proliferation of splenic T- and thymic T-cells without adding mitogen, although the conditioned medium cannot induce the differentiation of thymocytes. Additionally, we showed that H-7, not HA-1004 inhibits CS21 cell proliferation. These results suggest that CA12 conditioned medium has a specific soluble factor(s) produced by lymph node stromal cells.

Key words: T-lymphoma, lymph node stromal cells, conditioned medium

Introduction

Many studies on the proliferation and differentiation of thymic lymphocytes have been investigated, but little consideration has been given to the possibility that lymph node stromal cells control the proliferation of lymphocytes. It is reported that Tsuruo *et al.* have previously prepared and characterized mouse malignant T-lymphoma CS21 cells that are highly metastatic to auxillary lymph nodes when inoculated s.c. into the right flank of BALB/c mice [1]. CS21 cells grew *in vitro* when cells were coculture with CA12 lymph node stromal cells, but eventually underwent apoptosis after separation from the stromal cells [2]. CA12 stromal cells exerted bi-directional regulations on the growth of CS21 lymphoma cells. They promoted the growth of CS21 cells by direct contact and/or by a soluble factor(s). We successfully raised monoclonal antibodies that inhibited CS21 cell adhesion to CA12 stromal cells and found that monoclonal antibodies MCS-5 and -19, which recognize a M_r 168,000 and a M_r 23,000 protein, respectively, suppressed apoptotic cell death of CS21 cells even after the cells were separated from CA12 cells [3]. Therefore, we proposed that the cell adhesion molecules, such as CD45 [4] and Thy-1 (CD 90) [5], played a crucial role in CS21 cell survival. Besides these adhesion molecules, the effects of soluble factor(s) on apoptosis of these cells were not yet investigated.

In this study, we examined the characterization of conditioned medium of CA12 stromal cells that induce proliferation of CS21 lymphoma cells. Using inhibitor, we showed that a soluble factor(s) is associated with the signaling of CS21 cell proliferation.

Materials and Methods

Cell lines and reagents

CS21 cells and CA12 stromal cells were cloned from Lymph A cells. Lym A cells were originally established from spontaneously developed tumor mass in the auxillary lymph node of BALB/c mice. Although CA12 stromal cells could not form a tumor mass following s.c. inoculation in BALB/c mice, CS21 cells form a tumor mass and selectively metastasized to lymph node. CA12 stromal cells have properties similar to those of stromal cells in lymph nodes.

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We have already reported that CS21 cells underwent apoptotic cell death with DNA fragmentation when they were cultured alone (data not shown). CS21 cells, however, express Thy-1 and CD4 antigen but not CD8, like as mature helper-T-lymphocytes (data not shown). Wortmannin, herbimycin A, genistein, staurosporine, H-7, HA-1004, bovine serum albumin, ethylene glycol were commercially available.

Separation of CS21 cell-enriched population

CA12 stromal cells (1×10^5 cells/plate) were plated in a culture dish (10-cm diameter) containing 10 ml of RPMI growth medium and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 16 h of incubation, CS21 lymphoma cells (5×10^5 cells/ml) were added to the CA12 cell culture and allowed to grow further. After 5 or 6 days, CS21 cells were harvested with CA12 stromal cells from the dishes by pipetting. The mixed cell suspension was first centrifuged at 50 *g* for 5 min to precipitate large CA12 stromal cells. The suppressant was transferred to a new 50 ml-tube and centrifuged further at 800 *g* for 5 min to collect small CS21 cells. The precipitated cells were resuspended in RPMI growth medium and incubated for 1 h in a culture dish to allow to attach and to remove the remaining adhesive CA12 stromal cells to the dishes. The unattached cells were carefully transferred to a new 50 ml-tube and centrifuged at 50 *g* for 5 min. The supernatant was harvested and centrifuged again at 800 *g* for 5 min to collect CS21 cells [4].

Measurement of DNA synthesis

CS21 cells (5×10^4 cells/ml), harvested as described above, were incubated in 1 ml of the RPMI growth medium containing various concentrations of the conditioned medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Fifty-four hours later the cells were pulse-labeled with 1 mCi of [*methyl*-^{3}H] thymidine for 18 h and harvested. The radioactivity incorporated into cellular DNA was measured using an LKB liquid scintillation counter (Beckman, Irvine, CA).

Preparation of thymocytes, splenic B- and T-cells

BALB/c mouse (3- to 4-week-old) was killed and thymus and spleen was cut into pieces. The cell clumps were centrifuged at 800 rpm for 5 min and then added 20 ml of 0.83% NH_4Cl in 20 mM HEPES (pH 7.2) on ice for 5 min. To remove NH_4Cl, additional 20 ml of RPMI medium was added and recentrifuged. Finally, the thymocytes that suspended in RPMI medium and counted viable cells, were used for differentiation of $CD4^+CD8^+$ thymocytes into a $CD4^-CD8^+$ or $CD4^+CD8^-$ thymocytes. The other part, the recentrifuged splenic cells were passed through by a nylon wool (Biotest Diagnostics, Dreieich, Germany) column to separate splenic T- and B-cells.

Inhibitor assay

CS21 cells were freshly separated from coculture plates and added to 24 cell wells with a concentration of 1×10^5 cells/ml plus conditioned medium by CA12 stromal cells every 6 h. Various inhibitors were added to the culture plates at the beginning of culture. After further incubation for 12 h, the cells were counted by trypan blue dye exclusion assay.

RESULTS

Comparison of culture conditions and stability of CA12 conditioned medium

Because CA12 conditioned medium induced CS21 cell proliferation, we investigated the effects of stabilizing agents and cell density, and culture time of CA12 stromal cells. Ethylene glycol is widely used stabilizing agents for purification of enzymes (data not shown). Bovine serum albumin (BSA) is also used as a stabilizer of proteins such as growth factors. Fig. 1 shows that addition of BSA (0.1%) in CA12 conditioned medium lengthens the activity of CS21 cell growth (column 5), whereas ethylene glycol has no cell growth activity or has cytotoxicity against CS21 cells (column 6). In addition, we examined the CS21 cell growth by CA12 conditioned medium. When CA12 stromal cells were seeded at various concentrations and conditioned medium cultured for 6, 12, or 24 h was collected, then used for the relative growth of CS21. As a result, the growth of CS21 was most optimal at a concentration of 1×10^4 cells/ml (Fig. 2, black columns)

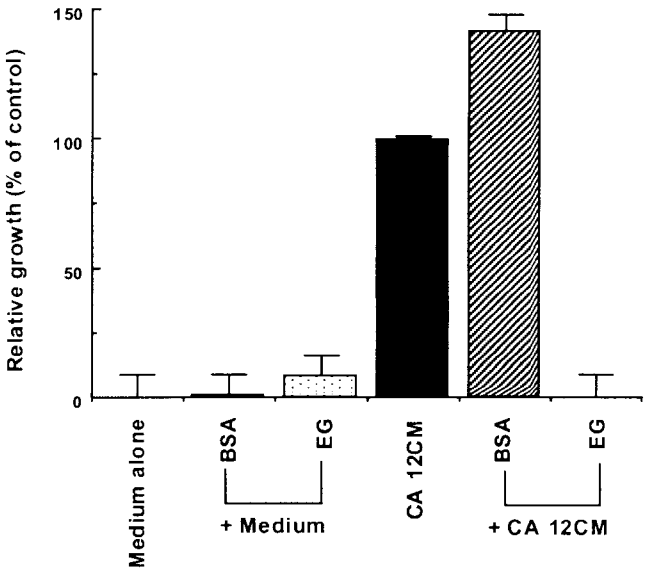

Fig. 1. Effect of stabilizing agents on stability of CA12 conditioned medium. CA-21 cells (5×10^4 cells/ml) were freshly isolated and added to cell walls with medium alone, BSA (0.1%) and ethylene glycol (0.1%) as controls or with BSA (0.1%), ethylene glycol (0.1%) in CA12 conditioned medium. One hundred % relative growth denotes [^{3}H]thymidine uptake of CA12 conditioned medium (22,050 cpm±250).

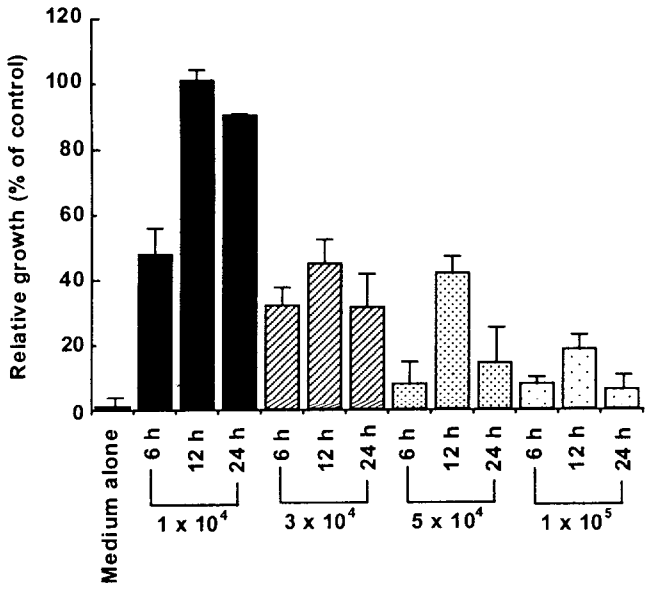

Fig. 2. Effect of cell density and culture time. CA12 stromal cells were seeded at a concentration of 1×10^4, 3×10^4, 5×10^4, or 1×10^5 cells/ml and incubated for 36 h to 48 h. After discarding the medium, prewarmed RPMI medium was added and incubated for a further 6, 12, 24 h as indicated above. The CS21 cell activity was assayed as [^{3}H] thymidine uptake.

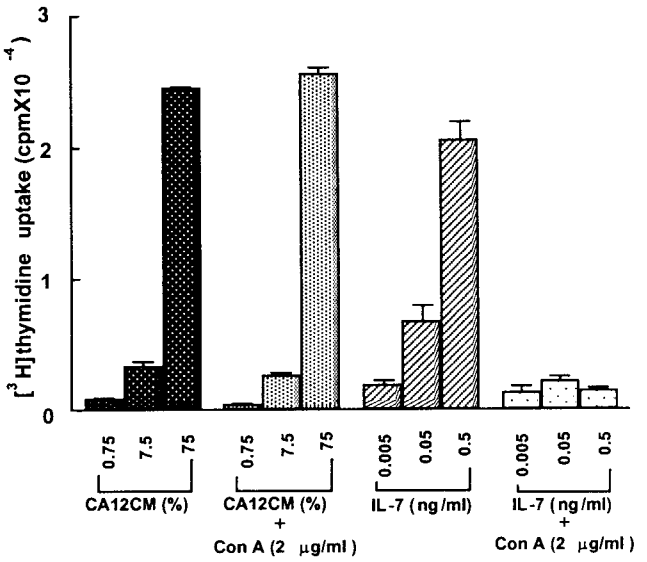

Fig. 3. Response of CS21 lymphoma cells to Con A plus CA12 conditioned medium or interleukin-7. CS21 cells (1×10^5 cells/ml) were cultured for 48 h with CA12 conditioned medium, CA12 conditioned medium plus Con A, IL-7, or IL-7 plus Con A. The cpm of Con A alone and medium alone was 369, and 113, respectively.

Effects of mitogen on CS21 cell growth

To identify the soluble factor(s) on CS21 cell growth, we examined the properties of the soluble factor(s) that are very important for further characterization. Among the molecular media for chromatographical analysis, we examined that whether CA12 conditioned medium has an affinity with heparin. It is known that IL-7 is produced by bone marrow stromal cells and proliferates the pre-B, T-cells, and mature T-cells. In this case, we used IL-7 as a control to examine whether the soluble factor(s) on CS21 cell growth is IL-7 (data not shown). It is known that Con A has its binding affinity with some glycosylated proteins. CS21 cells were added to the cell wells and incubated for 48 h with CA12 conditioned medium, CA12 conditioned medium plus Con A, IL-7, or IL-7 plus Con A (Fig. 3). We obtained a result that a soluble factor(s) in CA12 conditioned medium did not bind to Con A, but IL-7 binded to Con A. These results suggest that CA12 conditioned medium has not an affinity with Con A.

Response of splenic T and thymic T cells to CA12CM

It has become clear that some growth factors including IL-7 have an activity to proliferate T and/or B lymphocytes [6]. To investigate the effects of CA12 conditioned medium on growth of splenic T and thymic T cells, we examined whether CA12 conditioned medium promote the growth of splenic T and thymic T cells (Fig. 4). We compared IL-7 to CA12CM stimulated with or without Con A. In splenic T cells, IL-7

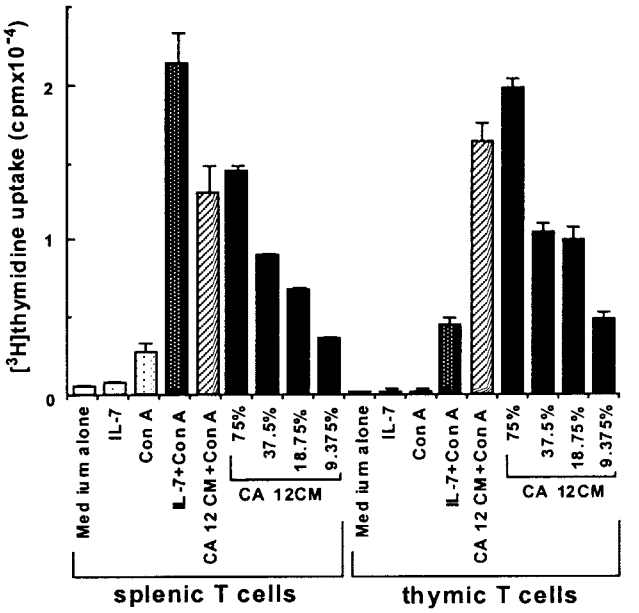

Fig. 4. Response of splenic T and thymic T cells to CA12 conditioned medium. Thymic T and splenic T cells were cultured for 48 h at the concentrations. The concentrations of IL-7 and Con A were 1 ng/ml and 5 mg/ml, respectively. The CA12 conditioned medium (75%) was prepared and added as described in Materials and methods. Bars mean SD values of triplicate determinations.

stimulated with Con A promotes cell growth, CA12CM did not change on cell growth by Con A. In thymic T cells, we got a similar result (Fig. 4., columns 10~18). Con-

sequently, these results show that CA12CM do not require any mitogen for stimulation of splenic T and thymic T cell-growth.

Activation of PKC by CA12 conditioned medium

By using metabolic inhibitors, we can know about how inhibitors block a signal transduction pathway induced by a growth factor(s). There are some reports that some cytokines including IL-7 induce tyrosine phosphorylation in T and B cell precursors and in mature T-cells [7], but the signal transduction pathway of T-lymphoma cells remains to be studied. It is well known that the activation by phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC)-activating agent, is blocked by PKC inhibitors. In order to investigate the mechanism of the CS21 cell growth induced by CA12 conditioned medium, we tried to use H-7, a PKC inhibitor, compared to a control (HA-1004) while compared to PMA as a positive control. We found that the soluble factor(s) produced by CA12 stromal cells is blocked by H-7 as well as did PMA and IL-7 (Fig. 5 and data not shown). These results suggest that CA12 conditioned medium may contain a soluble factor(s) that makes active PKC of CS21 cells.

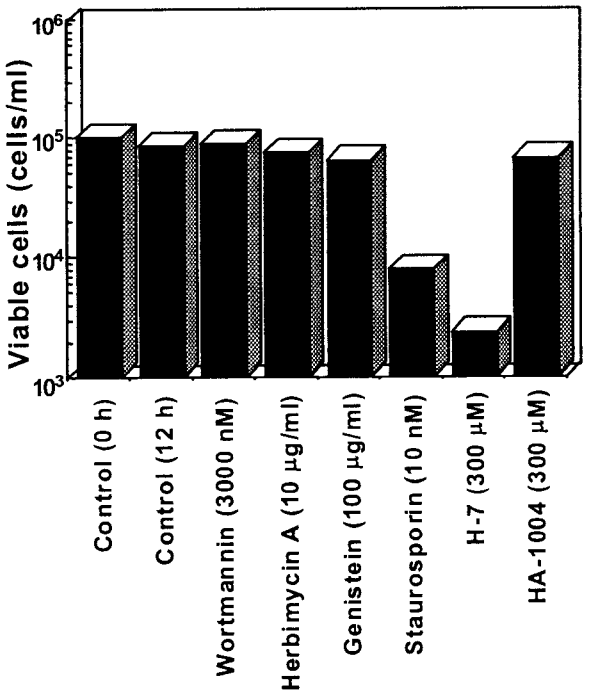

Fig. 5. Effects of CS21 cell growth by inhibitors. CS21 cells were freshly separated from coculture plates and added to 24 wells with a concentration of 1×10^5 cells/ml plus IL-7 (1 ng/ml). After further incubation for 12 h, the cells were counted by trypan blue dye exclusion assay. The data represents mean of duplicate determinations. Two independent experiments showed similar results.

DISCUSSIONS

Proliferation and differentiation of hematopoietic stem cells depend on the presence of positive and negative signals [8]. These signals are mediated by growth factors, which are produced by different cells of the blood compartment and by cell contact. Despite the abundant documentation for the role of growth factors and the adhesion molecules in metastatic process, no report is known about the mechanisms by which growth factor(s) and/or adhesion molecule(s) excreted from stromal cells influence metastatic T-lymphoma cells in lymph node. Because the growth and apoptotic cell death of T-lymphoma CS21 cells are largely affected by cytokines and adhesion molecules, the identification of the factors provides information of CS21 cell-growth supported by CA12 stromal cells.

It is proposed that CA12 stromal cells exert bi-directional regulations on the growth of CS21 lymphoma cells. They promoted the growth of CS21 cells by direct cell-cell contact or by a soluble factor(s). In the course of investigating roles of cell adhesion molecules, we successfully developed some clones of the mAbs raised against CS21 cell surface molecules that partially inhibited adhesion of lymphoma cells to lymph node stromal cells [3]. In this study, we demonstrated that CS21 cells proliferate in the presence of conditioned medium produced from CA12 stromal cells. To elucidate the cellular interaction between CA12 lymph node stromal cells and CS21 lymphoma cells that affect the growth of the lymphoma cells and, to the ultimate, to identify the soluble factors and to examine the possibility of prevention of CS21 lymphoma cell metastasis in lymph node that preferentially metastasized in the organ *in vitro*. The effect of conditioned medium from various fibroblasts indicated that CA12 conditioned medium is the most effective on CS21 cell growth (data not shown). Because CS21 cells selectively metastasized to lymph node (8-fold higher metastatic activity than control), it was no doubt that CA12 stromal cells were very useful tool for investigating lymphatic metastasis. To identify and characterize the soluble factor(s) produced by CA12 stromal cells, comparison of physicochemical properties of the CA12CM is required. It is reported that the addition of cysteine to the culture medium greatly enhanced the survival of rat chondrocytes [9]. They showed that conditioned medium from rat chondrocytes cultures was fractionated by ultrafiltration using a membrane with molecular mass cut-off at 10 kDa, the survival-promoting activity was recovered in the concentrate and not in the filtrate. Our cell system showed that the CS21 cell promoting activity contained in the concentrated fraction (molecular mass cut-off of 100 kD) and in the pass-through fraction (molecular mass cut-off of 10 kD) (data not shown).

There are increasing numbers of examples where several extracellular signaling molecules have been shown to collaborate to promote the cell growth in culture [10], pre-

sumably reflecting the advantages of combinatorial control. Not one, we suggest, but more soluble factors from CA12 conditioned medium enable CS21 cells to proliferate and finally to avoid cell death.

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