J. Life Science. (2001), 11(2), 99~102

JOURNAL OF
Life Science

Nursing Effects of Thiols Including Cysteine in Lymph Node Stromal Cells and P388 Cells

Jin Yeul Ma*, Kap Joo Park, Hyunmin Kang***, Taekyu Park****, Doo-Sang Park and Sang-Han Lee†**

Korea Research Institute of Bioscience & Biotechnology (KRIBB), Taejon 305-333, Korea
**Korea Institute of Oriental Medicine, Seoul 135-100, Korea*
***Institute of Genetic Engineering, Konkuk University, Seoul 143-701, Korea*
****Department of Life Science, Yongin University, Yongin 449-714, Korea*
*****Division of Life Science, Konkuk University, Chungju 380-701, Korea*

Received: October 2, 2001

Abstract Mouse malignant T-lymphoma CS21 cells can grow when cocultured with CA12 lymph node stromal cells, but they undergo apoptotic cell death with DNA fragmentation when separated from CA12 stromal cells. In the course of examining the effects of the soluble factor (s) secreted by CA12 stromal cells on CS21 cell growth, we found that thiols including cysteine promoted CS21 cell growth. P388 cell growth was also promoted by various thiols. These results suggest that thiols including cysteine participate in CA12 and P388 cell growth.

Key words: T-lymphoma, thiols, cysteine, stromal cells, mouse leukemia

Introduction

Mouse malignant T-lymphoma CS21 (CMS) cells metastasize to the lymph node when inoculated s.c. in the right flank of BALB/c mice [1]. CS21 cells grew *in vitro* when they were cocultured with CA12 lymph node stromal cells, but CS21 cells underwent apoptosis with DNA fragmentation after separation from CA12 stromal cells. The results of our studies on the growth properties of CS21 cells revealed that the growth of CS21 cells depended upon two types of molecules provided by CA12 stromal cells: soluble factor (s) and cell adhesion molecules [2]. In order to elucidate the adhesion molecules, we raised monoclonal antibodies that inhibited CS21 cell binding to CA12 stromal cells [3]. We finally identified that CD45 protein tyrosine phosphatase and Thy-1 glycoprotein were involved in CS21 cell adhesion to CA12 stromal cells [4,5].

* Corresponding author
Phone: 82-42-860-4667, Fax: 82-42-860-4595
E-mail: sang@mail.kribb.re.kr

On the other hand, the mechanism of T-lymphoma cell growth by the soluble factor (s) secreted by CA12 stromal cells, however, has not yet been investigated except IL-7 [6]. In this study, we examined the biological activities of other soluble factor (s) from CA12 stromal cells that promoted CS21 cell growth. The major finding is that thiols including cysteine, not cystine, induce cell proliferation in CS21 cells. This nursing effect was also examined in P388 mouse leukemia cells.

MATERIALS AND METHODS

Cell lines

CS21 lymphoma cells and CA12 lymph node stromal cells were established and cultured as described previously [1,2]. P388 mouse leukemia cells were cultured in RPMI Medium supplemented with 5% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

Reagents

Thiol-bearing and dithiol-cleaving compounds used in this study were L-cysteine (Nacalai Tesque, Kyoto, Japan), N-acetylcysteine (NAC) (Wako Pure Chemical Co., Tokyo, Japan), 2-mercaptoethanol (2-ME) (Nacalai), dithiothreitol (DTT) (Serva, Heidelberg, Germany), reduced glutathione (GSH) (Wako), oxidized glutathione (GSSG) (Wako), and DL-homocysteine (Aldrich Chemicals Co., Milwakee, WI).

Preparation of the conditioned medium of CA12 stromal cells

CA12 stromal cells were seeded at a concentration of 5×10^4 cells/ml and then cultured for 48 h until reaching to the subconfluent condition. The medium was changed with prewarmed RPMI growth medium containing 5% FBS. Because the preincubation of the medium at 37°C in the CO_2 incu-

bator resulted in the conversion of reduced thiols in the medium to oxidized thiols by autooxidation (detected by DTNB assay) (data not shown), we used prewarmed RPMI growth medium to exclude the effects of reduced thiols contained in the medium. After a 24-h incubation, the conditioned medium (CA12CM) was harvested and immediately used for the further study.

DTNB assay

To measure the total thiol content of the culture medium, CA12 cells were incubated in 1 ml of the medium in 24 cell wells at 37°C in a 5% CO_2 atmosphere. After incubation for a given time, 1.2 ml of the medium that separated from the cells was mixed rapidly with 1.2 ml of 0.2 M potassium phosphate-10 mM EDTA, pH 8.0. The absorbance at 412 nm of the mixture was measured. And then 0.1 ml of 10 mM 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma) was added. After 5 min of incubation, the increase in absorbance at 412 nm was measured. The thiol content was calculated from the value for cysteine as a standard. In case of determination of TCA-insoluble thiol, equal volume of 10% TCA was added to the CA12 conditioned medium, which vortexed vigorously for 1 min. After 15 min of incubation at 15,000 rpm, the supernatants were used for the determination of thiol content.

Proliferation assay

The proliferation of CS21 cells was measured by [*methyl*-^{3}H]thymidine incorporation. CS21 cells (1×10^5 cells/well) were incubated with various concentrations of conditioned medium or factors in 24-well tissue culture plates for 48 h and then labeled with 37 kBq/ml (1 mCi/ml) of [*methyl*-^{3}H]thymidine (^{3}H-TdR) (NEN, Wilmington, DE) for the last hour. The cells were harvested on glass filters, and the radioactivity associated with cellular DNA was measured using a LS-6000LL liquid scintillation counter (Beckman, Irvine, CA), as described previously [3].

RESULTS

The mouse malignant T-lymphoma CS21 cells [1] could not grow *in vitro* when cultured alone; however, they could grow in the presence of CA12 lymph node stromal cells [2]. Although we previously identified that the cell adhesion molecules took part in the transmission of apoptosis-inhibitory signals from CA12 stromal cells [3-5], the soluble factor(s) secreted by CA12 stromal cells had not been identified. Among the known cytokines, interleukin-1, -2, -3, -4, -5, -6, -9, -10, and -12 did not stimulate the growth of CS-21 cells (data not shown). In the course of investigating these soluble factor(s), we found that the CA12 conditioned medium (CM) contained low-molecular-weight factors that induced CS21 cell growth. Because thiols have low molecular weights and have reported beneficial effects in some types of mammalian cells [6-9], we examined the possibility that the soluble factors might be thiols.

We then investigated the concentration of total thiols in CA12CM (Fig. 1) and found that the incubation of CA12 stromal cells increased the content of total thiols in a time-dependent fashion (data not shown). The [^{3}H]thymidine incorporation of CS21 cells also increased in proportion to the concentration of cysteine in the CA12CM (data not shown). When the CA12CM (collected after a 24-h incubation with CA12 stromal cells) was incubated at 37°C for 2-6 h, the concentration of cysteine decreased rapidly (Fig. 2, closed circles). The growth-promoting effects of CA12CM also decreased in proportion to the cysteine concentrations (Fig. 2, open triangles). This decreased concentration might occur through the autooxidation of reduced thiols [7-9].

Because total thiols enhanced the growth of CS21 cells (Fig. 1 and 2), we examined the effect of other thiol-bearing and dithiol-cleaving compounds on CS21 cell proliferation. Cysteine, DL-homocysteine, NAC, GSH, 2-ME, and DTT promoted the CS21 cell proliferation (Fig. 3 and data not shown). Cystine, the oxidative product of cysteine, had no effect on cell growth (Fig. 3, dotted columns). The growth of CS21 cells was, therefore, thiol dependent *in vitro* (Fig.3 and data not shown). Additionally, we also examined the effect of CA12CM on P388 leukemia cell growth (Fig. 4). P388 leukemia cells can grow in the presence of 2-ME in

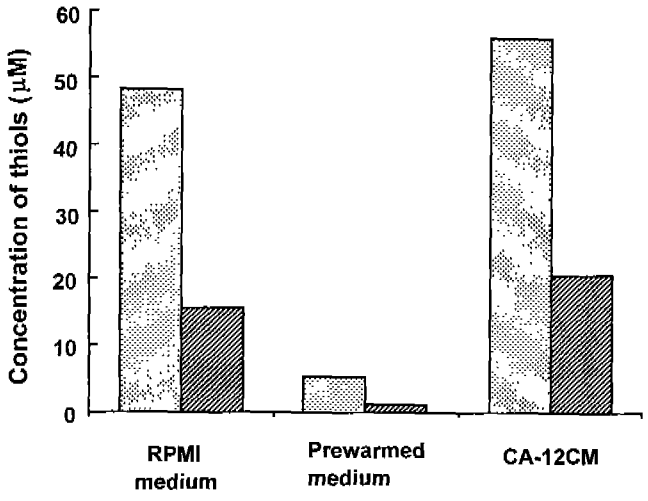

Fig. 1. Determination of thiol(s) from CA12CM.
CA-12 stromal cells were seeded at a concentration of 5×10^5 cells/ml and cultured for 48 h in 60~70% cell confluence. Prewarmed medium (5 ml), containing a low concentration of total thiol (<1 mM) by DTNB assay, was poured into the dish and then reincubated for 12 h. After filtration of the CA12CM, an equal volume of 10% TCA solution was added to precipitate the protein thiols in the medium. Vigorous vortexing was required prior to centrifugation. The supernatants were examined using DTNB assay, using L-cysteine as a standard. Prewarmed medium showed that the concentration of thiol in the medium is rapidly reduced by autooxidation. Dotted bar; total thiol, slashed bar; TCA-insoluble thiol. The data denote mean value of duplicate determinations.

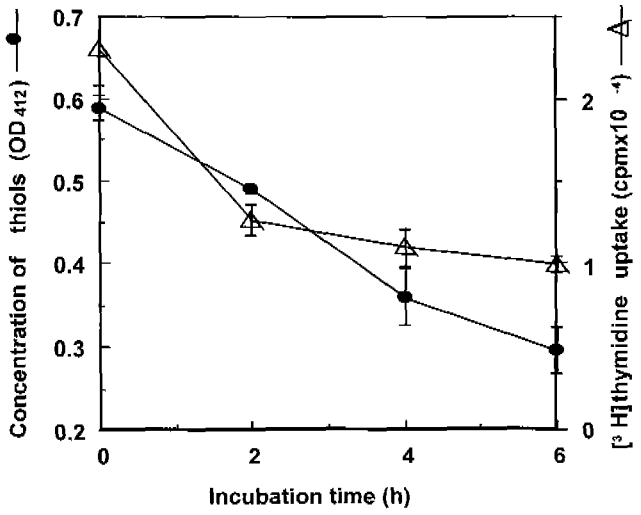

Fig. 2. Instability of cysteine as a soluble factor contained in CA12CM.
CA12 stromal cells were incubated for 24 h and then the CA12CM was collected. CA12CM was added to 24-well plates and incubated for 2, 4, or 6 h in a CO_2 incubator at 37°C. The incubated CA-12CM was used as a sample for cysteine content by DTNB assay (closed circles) and for growth proliferating activity by [^{3}H]thymidine incorporation (open triangles).

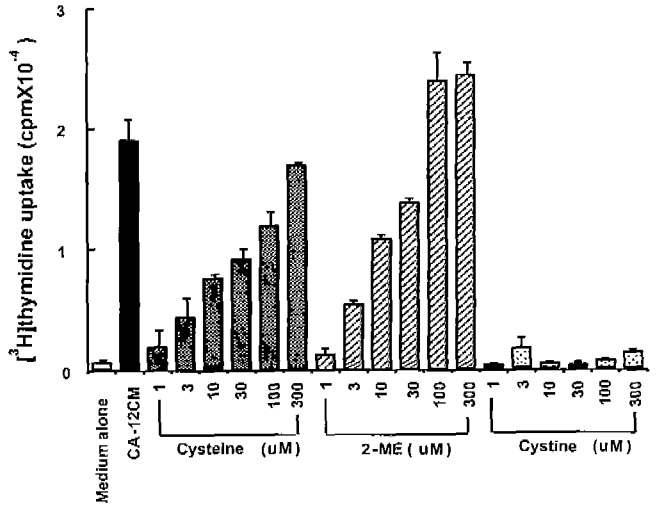

Fig. 3. Concentration-dependency of thiols on CS21 cell proliferation.
CS21 cells were freshly isolated from CA12 stromal cells and incubated for 48 h with various concentrations of cysteine, 2-ME, or cystine. CS21 cells were then labelled with 37 kbq/ml of [^{3}H]thymidine for the last hour and harvested, as described in Materials and Methods. Bars mean SD values of triplicate determinations.

RPMI medium (data not shown). In this case, 2-ME converts cystine to cysteine. Because addition of CA12CM (7.5 or 75%), and cysteine (10,30,100, or 300 mM) proliferates P388 leukemia cells, we concluded that thiols such as cysteine promote cell growth of P388 leukemia as well as of

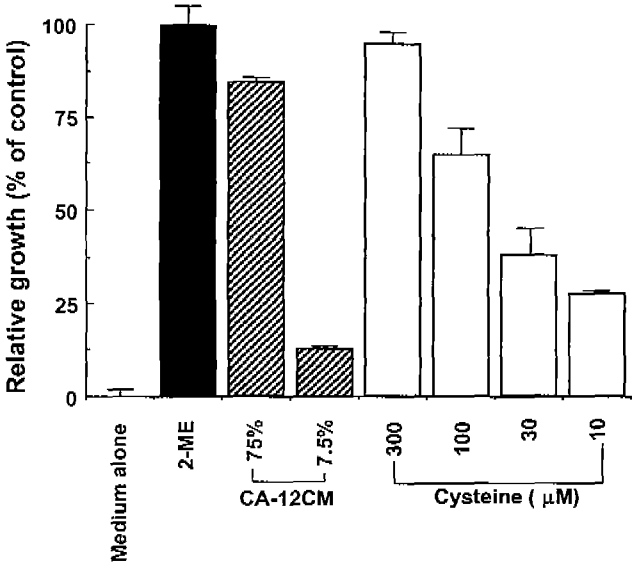

Fig. 4. Effect of CA12CM and cysteine on p388 cell proliferation.
P388 leukemia cells were cultured and maintained in RPMI medium. CA12CM was harvested from CA12 stromal cell plates that seeded at 1×10^5 cells/ml of an initial concentration and incubated for 36 h. In 24 cell wells, 450 ml of the above sample were added and P388 cells were added to 5×10^4 cells/ml. After a 48 h-incubation, the cells were harvested and counted for [^{3}H] thymidine incorporation. Dotted columns; cysteine added, slashed columns; CA12CM added, black column; 2-ME added.

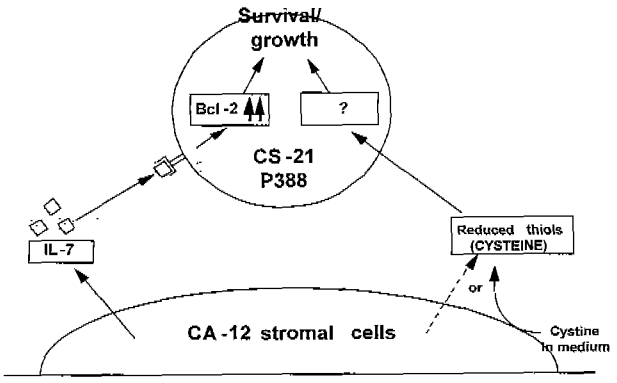

Fig. 5. A possible mechanism of CS21 or P388 cell proliferation on CA-12 lymph node stromal cells.

CS21 lymphoma cells.

DISCUSSIONS

The mechanism of CS21 cell growth induced by the soluble factor(s) produced from CA12 stromal cells has not yet been well documented. In this study, we confirmed the soluble factor(s) secreted by CA12 stromal cells that promoted CS21 cell growth. It has been reported that some thiol compounds, including cysteine, are produced by fibroblasts, but it is not clear whether one or many thiols are produced by these

fibroblasts [7,10]. We here examined that thiol(s) compound was produced by CA12 stromal cells. Furthermore, we found that thiols induced CS21 cell growth. Therefore, the continuous production of thiols including cysteine during CA12 stromal cell culture may play an important role in CS21 cell growth promotion; although some other factor (s) secreted by CA12 stromal cells could also partially be involved in CS21 cell growth, as we described previously.

It has been well known that cysteine is not only an essential amino acid but also a thiol compound that plays a critical role in cell growth. It converts to glutathione and exerts a variety of biological processes in lymphocytes or lymphoma cells [10]. Thiols including cysteine would include protection of the cells from oxidative damage, participation in the g-glutamyl cycle, and/or disulfide exchange between glutathione and proteins may be a control mechanism in lymphocyte activation [10]. Lymph nodes are the important arena for immune reactions, and for such activity, various interactions or functional networks occur among component cells. The role of cysteine in lymph nodes is also not investigated yet but there is a possibility of a pivotal factor for lymphocyte education. The educated lymphocytes play various roles in target cells. It is worthy to examine a possibility that cysteine produced by lymph node stromal cells may educate lymphocytes not to die in apoptosis (until arrive at target cells). Further studies on roles of lymph node stromal cells will give us more information about cell-to-cell interactions as well as the involvement of lymphatic metastasis.

In summary, we have demonstrated, herein, that thiols including cysteine play a key role in growth stimulation of CS21 cells. The further study will be focused on a signal transduction pathway (s) triggered by thiols.

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