J. Life Science. (1999), **9**(1), 9~13

JOURNAL OF
Life Science

Cysteine Participates in Cell Proliferation by Inhibiting Caspase3-like Death Protease

Sang-Han Lee* and Soon-Duck Hong†

Department of Microbiology, Kyung-pook National University, Taegu 702-701. Korea
**Samsung Biomedical Research Institute, Sungkyunkwan University, College of Medicine, Suwon 440-746, Korea*

Received: April 12, 1999

Abstract Reduced thiols were important compounds for the maintenance of leukemia and lymphoma cell survival (and growth). In the course of examining the microenvironmental effects on lymphoma and leukemia cell growth, we found that cysteine suppressed apoptosis in these cells. In a present study, in order to investigate the role of cysteine on the suppression of apoptotic cell death, we used CS21, P388, and L1210 cell lines. The addition of BSO, an inhibitor of glutathione synthase, induced apoptosis of these cells by blocking the cellular uptake of cysteine in CS21 cells. Although L1210 cells underwent apoptosis without thiol compounds, the addition of these compounds suppressed the apoptosis and promoted the growth of L1210 cells. When specific inhibitors of caspase3-like proteases were added into the cell culture, L1210 cells evaded undergoing apoptosis even in the absence of thiol compounds. The caspase3-like proteases, but not caspase1-like proteases, were activated during the L1210 cell apoptosis but the addition of thiol compounds suppressed the activation of caspase3-like proteases. These results suggest that reduced thiols including cysteine play an important role in the suppression of cell apoptosis by inhibiting the activation of caspase3-like proteases.

Key words: Cysteine, apoptosis, caspase3-like death protease, thiols, protease inhibitor

Introduction

It has previously characterized that mouse malignant T-lymphoma CS21 cells grew *in vitro* when cocultured with CA12 lymph node stromal cells [14]. As a result of the growth properties of CS21 cells, it is found that CS21 cells require at least two types of molecules for cell growth provided by CA12 stromal cells: cell adhesion molecules and soluble factors [2,4]. We recently identified cysteine as a soluble factor produced by CA12 stromal cells [8,9]. Cysteine suppressed CS21 cell apoptosis by inhibiting the activation of caspase3-like proteases via a Bcl-2-independent mechanism. As thiol-bearing and dithiol-cleaving compounds also suppressed CS21 cell apoptosis, reduced thiols were essential for the maintenance of CS21 cell survival [9]. Indeed, thiol compounds are well studied in order to provide beneficial effects for lymphocytes cultured *in vitro*. One of the most effective and frequently used thiol compound is 2-mercaptoethanol (2-ME). In most cases, 2-mercaptoethanol is effective at concentrations from 10 to 100 mM, and its action resembles that of macrophages or feeder layer cells. L1210 is one of the most extensively studied thiol-dependent cell lines *in vitro*. L1210 cells require cysteine for cell survival because they have a low capability to synthesize cysteine. Cysteine can be utilized for L1210 cell culture, but it is easily oxidized to cystine in the culture medium. It has been shown that L1210 cells are deficient in their capacity to take up cystine and that the cellular cysteine and glutathione contents decrease considerably in normal culture media containing cystine. There is a possibility that the feeder layer cells promote growth of L1210 cells by supplying cysteine continuously.

To elucidate the mechanism of thiol-mediated apoptosis suppression, we examined the effect of some thiol-bearing and dithiol-cleaving compounds on the growth and the apoptosis of thiol-dependent cell lines. Furthermore, we found that the apoptotic cell death is involved with a caspase3-like protease in these cell lines.

Materials and Methods

Cell lines and reagents

L1210, P388, and CS21 cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2-95% air in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 mM of 2-mercaptoethanol (2-ME), and kanamy-

†Corresponding author
Phone: 82-331-299-6142, Fax: 82-331-299-6149
E-mail: shlee@medical.skku.ac.kr

cin at 100 ug/ml. The fluorogenic substrates of proteases, DEVD-AMC (Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-amino-4-methylcoumarin) and YVAD-AMC (acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspart-1-amino-4-methylcoumarin) were obtained from Bachem Co., and used for the substrates of proteases. Diamidino-2-phenylindole (DAPI) and L-buthionine-[S, R]-sulfoximine (BSO) were obtained from Sigma. The other reagents are all commercially available.

Preparation of the conditioned medium

CA12 stromal cells were seeded at a concentration of 5×10^4 cells/ml and then cultured for 48 h until reaching the subconfluent condition. The medium was changed with pre-warmed RPMI growth medium containing 5% FBS. The preincubation of the medium at 37℃ in a CO_2 incubator resulted in the conversion of reduced thiols in the medium to oxidized thiols by autooxidation (detected by DTNB assay) (data not shown). Thus, 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 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℃ in a 5% CO_2 atmosphere. After incubation for a given time, 1.2 ml of the medium, which 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 vortexes vigorously for 1 min. After centrifuging, the supernatants were used for the determination of thiol content.

Cell proliferation assay

Cells (5×10^4 cells/ml) were cultured in 24-well culture plates with or without 2-ME. Viable cells were counted every day over a four-day period by trypan blue dye exclusion assay. The cells with or without various thiols for 48 h were harvested on glass filters, and the radioactivity associated with cellular DNA was measured as previously described [2].

DNA fragmentation and morphological evaluation

The DNA of cells incubated with or without various thiols for 24 h was separated and electrophoresed, as previously described [8].

Estimation of prorease activity

The cells (1×10^5 cells/ml) were incubated with medium alone or medium with cysteine (100 μM). After a 24 h-incubation, the cells were harvested and lysed with the lysis buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiothreitol]. The cell lysate was then incubated with 20 μM of the fluorogenic substrate of proteases, DEVD-AMC or YVAD-AMC in ICE buffer [20 mM HEPES (pH 7.5), 10% glycerol, 2 mM dithiothreitol] at 37℃. The AMC released from the fluorogenic substrates was measured with the excitation at 380 nm and the emission at 460 nm using a fluorescence spectrophotometer (Hitachi, Tokyo, Japan), model F-2000 [9].

Western blotting analysis

Western blot analysis was performed as previously described [8]. In brief, cells were solubilized with lysis buffer containing 2% NP-40 and 0.2% SDS and then the cell lysates (20 μg/lane) were applied to a 10-20% gradient polyacrylamide gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking with PBS containing 5% skim milk for 1 h, the membrane was incubated with anti-mouse Bcl-2 mAb for 1 h and then incubated with peroxidase-conjugated anti-hamster IgG. The membrane was developed with the ECL detection system.

FACS analysis

The propidium iodide (PI)-stained cells were analyzed using a Beckton Dickinson FACS flow cytometer as described by a manufacture's manual.

Results

Thiol compounds enhance the cell growth of thiol-dependent cell lines. The mouse malignant T-lymphoma CS21 cells could not grow *in vitro* when cultured alone. However, they could grow in the presence of CA12 lymph node stromal cells [2,3,4]. It has been identified that the cell adhesion molecules took part in the transmission of apoptosis-inhibitory signals from CA12 stromal cells[8,9,17]. In the course of investigating the soluble factor(s) that can enhance CS21 cell growth, it has been found that the CA12 conditioned medium contained low-molecular-weight factors that induce CS21 cell growth (data not shown). When CA12 stromal cells were cultured for preparation of CA12CM, the concentration of thiols produced by CA12 stromal cells increased up to 4-fold within 6 h (data not shown). Recently, cysteine was identified as a low-molecular-weight factor [10]. As shown in Fig. 1A, CS21 cells were grown in a concentration dependent manner. Additionally, when the CA-12CM was added to the culture of P388 cells (thiol-dependent mouse leukemia cells), the cell growth increased as shown in Fig. 1B. Furthermore, 2-ME addition induced the growth of L1210 cells, which is known to be another thiol-dependent cell line (Fig.

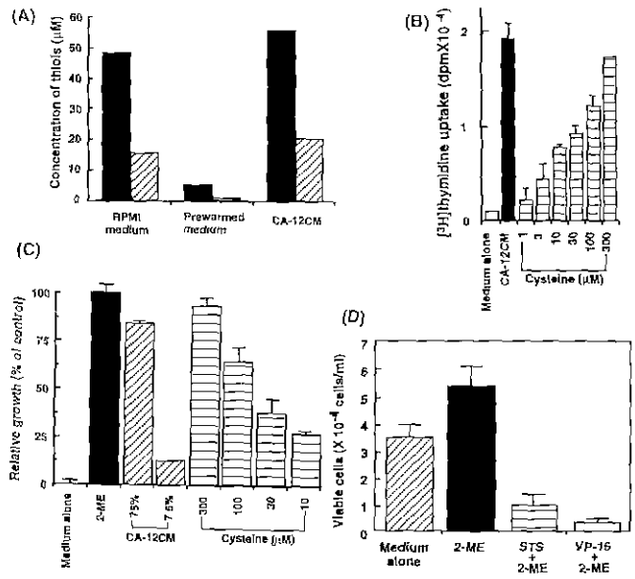

Fig. 1. Thiols induced thiol-dependent cell proliferation. (A), Concentration dependency of cysteine on CS21 cell growth. CS21 cells (1×10^4 cells/ml) were cultured for 48 h with various concentrations of cysteine. The average cpm of the medium alone was 855. (B), Cysteine induced P388 cell growth. P388 leukemia cells were cultured and maintained in RPMI medium. CA12CM was harvested from CA12 stromal cell plates that were seeded at 1×10^4 cells/ml of an initial concentration and incubated for 36 h. In 24 cell wells, 450 μl of the above sample or of fresh medium with various concentration of cysteine, were added and 150 ml of P388 cells (5×10^4 cells/ml) were also added. After a 48 h-incubation, the cells were harvested and counted for thymidine incorporation. The results represent relative growth. The average cpm of medium alone and the addition of 2-mercaptoethanol (2-ME; 100 μM) was 120, 23518, respectively. (C), Effect of 2-ME on L1210 cell growth. L1210 cells (5×10^4 cells/ml) were incubated for 24 h with (black column) or without (dotted column) 2-ME (100 μM), with staurosporine (STS; 15 ng/ml) plus 2-ME (shaded column), or with VP-16 (20 μg/ml; as a positive control) plus 2-ME (white column). The vertical bars represent standard deviation values of triplicate determinations. Repeated experiments gave similar results.

1C and data not shown). Treatment of the broad specificity protein kinase inhibitor staurosporine (15 ng/ml) induced cells to die within less than 24 h with the morphological feature of apoptosis (Fig. 1C and not shown). These above results suggest that thiols such as cysteine may be involved in a thiol-dependent signaling pathway for cell growth (or survival).

Inhibition of cell growth by BSO

Because cysteine enhanced the growth of CS21 cells (Fig. 1A), P388 cells (Fig. 1B), and L1210 cells (Fig. 1C), we examined the effect of BSO, which is known to inhibit glutathione synthase, in CS21 cells. The addition of BSO inhibited CS21 cell growth (Fig. 2A), and induced apoptosis in the cells by blocking the cellular uptake of cysteine (confirmed by trypan blue exclusion assay and DAPI staining; data not shown). The addition of cysteine saved the cells from the apoptotic execution pathway (Fig. 2B). We had similar results in P388 and L1210 cells (unpublished results). This data suggests that cysteine (produced by CA12 stromal cells) plays an important role in suppressing CS21 cell apoptosis.

Induction of apoptosis and activation of caspase3-like protease

As the growth of L1210 cells was known to be dependent on reduced thiols contained in culture medium, we examined what kinds of compounds supported the growth of L1210 cells. When 2-ME was added to the L1210 cell culture, 2-ME could convert cystine, which was contained in the culture medium, to cysteine. In this condition, the viable cell numbers of L1210 cells gradually increased. However, the viable cell numbers decreased when L1210 cells were cultured without 2-ME. Other reduced thiols and dithiol-cleaving compounds, but not oxidized thiols triggered the proliferation of L1210 cells. Therefore, reduced thiols, but not oxidized thiols, were indispensable for L1210 cell growth.

We then studied whether L1210 cells underwent apoptosis when cultured without thiol-bearing or dithiol-cleaving compounds. As shown in Fig. 3B, apoptotic bodies occurred in L1210 cells cultured without these compounds. Moreover, thiol-deprivation induced the nuclear condensation in L1210

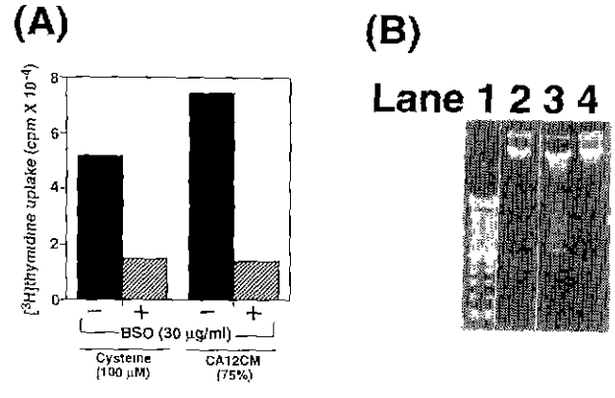

Fig. 2. Effect of BSO on CS21 cell growth. (A), Inhibition effect of BSO on CS21 cell growth by cysteine. CS21 cells were harvested from coculture plates and added to 24-cell wells in a concentration of 1×10^5 cells/ml. CA12CM was harvested from CA12 stromal cell plates of 80-90% cell plates and filtered with a membrane filter (Millipore). After 24-h incubation, the radioactivity was counted as described in Materials and Methods. (B), DNA fragmentation pattern of CS21 cells by BSO. The harvested cells were incubated with proteinase K and RNAse, respectively, for 2 h at 50℃, and analyzed with a UV transilluminator, as described in Materials and Methods. The molecular size marker was the 100-base pair DNA ladder (Lane 1).

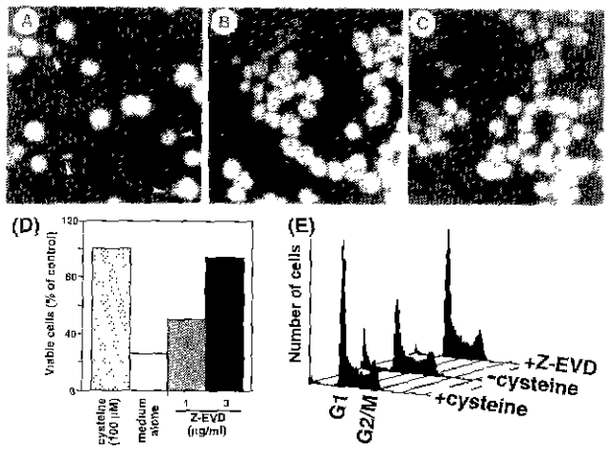

Fig. 3. Caspase3-like pretease is inhibited by cysteine. (A-C), DAPI staining of L1210 cells. The cells treated with (A) or without (B) cysteine (100 μM), and Z-EVD (100 μM; C) for 24 h were stained with DAPI and photographed. (D), FACS analysis of cysteine-treated L1210 cells. The cells were treated with (forward) or without (middle) cysteine, and Z-EVD (backward) for 24 h. The PI-stained cells were analyzed using a FACScan. (E), Effect of cysteine treatment on caspase3-like protease activation. The L1210 cells, treated with cysteine, was incubated for 24 h. The cell lysates were prepared as described in Materials and Methods and then measured for the AMC liberated when cultured with DEVD-AMC or YVAD-AMC. (F), Western blotting of Bcl-2-family protein expression. The cell lysates as treated above were analyzed the expression of Bcl-2-related proteins with the antibodies.

cells (confirmed by DAPI staining: see Fig. 3B). However, the apoptosis was inhibited by the addition of 2-ME. Therefore, L1210 cells evaded apoptosis by the addition of reduced thiols.

To clarify the molecules, which associated with the apoptosis induced by thiol withdrawal, we examined whether ICE-like proteases were involved in the apoptosis using Z-EVD, an inhibitor of CPP32 protease. When L1210 cells were cultured without thiol-bearing or dithiol-cleaving compounds, L1210 cells underwent apoptosis. The addition of Z-EVD suppresses the apoptosis even in the absence of thiol-bearing or dithiol-cleaving compounds. This result suggested that interleukin-1beta converting enzyme (ICE)-like or caspase3-like proteases were involved in the promotion of L1210 cell apoptosis. As Z-EVD could inhibit the activity of both ICE-like proteases and caspase3-like proteases, we then investigated the activity of ICE-like proteases and caspase3-like proteases using the fluorogenic substrates of ICE-like proteases and caspase3-like proteases. When the cell lysates from L1210 cells cultured without 2-ME were incubated with these fluorogenic substrates, AMC was liberated from DEVD-AMC but not from YVAD-AMC (Fig. 3E). However, the cell lysates from L1210 cells cultured with 2-ME could not cleave AMC from both YVAD-AMC and DEVD-AMC. These results indicate those caspase3-like proteases, but not caspase1-like proteases were associated with the induction of L1210 cell apoptosis and those reduced thiols could suppress the activation of caspase3-like proteases.

Discussion

As we mentioned in the previous study [7], CA12 stromal cells produce cysteine and support the proliferation of CS21 lymphoma cells. 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 some types of mammalian cells [17]. These would include protection of the cells from oxidative damage, participation in the g-glutamyl cycle, and/or disulfide exchange between glutathione and proteins that may be a control mechanism in lymphocyte activation [5]. It is probable that there is a control system of cysteine in lymphoid as well as blood tissues, suggesting that some cells (for example, lymph node stromal cells) produce (or convert) to support the constant level of cysteine. The cysteine may participate in the inhibition of apoptosis, cell proliferation and moreover, the regulation of metastasis [6,10,15].

Apoptotic cell death is a cellular suicide functionally different from mitosis [11,16]. It may play a pivotal role in the growth control of damaged and premalignant cells [11]. Pathologically, failure in an apoptotic execution program can break the balance in a viable cell number, and finally some of these abnormalities might lead to oncogenesis

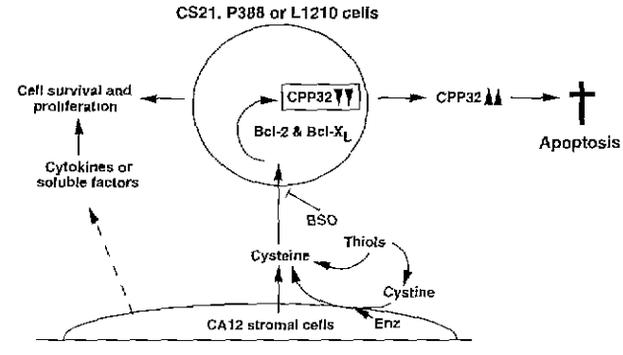

Fig. 4. A possible mechanism for the apoptosis suppression of lymphoma or leukemia cells by cysteine. Cysteine produced by CA12 lymph node stromal cells is an antiapoptotic factor in CS21 lymphoma cells including P388 and L1210 cells. Although it was not proved whether CA12 stromal cells excrete cysteine into medium or they continuously produce an enzyme that can convert cystine into cysteine in order to protect the cells from apoptosis. Some cytokines and/or soluble factors can survive and proliferate these cells. But basically, in the initial stage, cysteine is the only factor that inhibits the apoptosis execution machinery, as suggesting that cysteine excreted into medium within 1 h when cultured the CA12 cells alone.

[1,12,13]. So, control of apoptotic cell death has emerged as a novel approach for cancer chemotherapy [1]. As Z-EVD, which is an ICE-family inhibitor, suppresses apoptosis of L1210 cells (Fig. 3B and D), we may modulate apoptosis by using inhibitors or inducers, if they developed, for the possibility of therapeutic treatment of cancer cells. Further studies on the roles of thiol compounds during apoptosis as well as on the usefulness of ICE-family modulators (to modulate the cells which are derived to induce or inhibit the enzyme) will give us more information about cell-to-cell interactions (or communications) as well as the involvement of metastasis.

In summary, we demonstrated that the 2-ME−depleted L1210 cells might undergo apoptosis via a mechanism of caspase3-like protease activation. Further investigation on the mechanism and physiological significance of thiol compound-depleted apoptosis will contribute to the understanding of microenvirnmental oncogenesis in leukemia as well as lymphoma.

References

1. Fisher, D. E. 1994. Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**, 539.
2. Fujita, N., Naito, M., Lee, S.-H., Hanaoka, K., and Tsuruo, T. 1995. Apoptosis inhibition by anti-M(r) 23,000 (Thy-1) monoclonal antibodies without inducing bcl-2 expression. *Cell Growth Differ.*, **6**, 355.
3. Fujita, N., Kataoka, S., Naito, M., Heike, Y., Boku, N., Nakajima, M., and Tsuruo, T. 1993. Suppression of T-lymphoma cell apoptosis by monoclonal antibodies raised against cell surface adhesion molecules. *Cancer Res.*, **53**, 5022.
4. Hanaoka, K., Fujita, N., Lee, S.-H., Seimiya, H., Naito, M., and Tsuruo, T. 1995. Involvement of CD45 in adhesion and suppression of apoptosis of mouse malignant T-lymphoma cells. *Cancer Res.*, **55**, 2186.
5. Ishii, T., Bannai, S., and Sugita, Y. 1981. Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol in vitro. Role of the mixed disulfide of 2-mercaptoethanol and cysteine. *J. Biol. Chem.*, **256**, 12387.
6. Ishizaki, Y., Burne, J. F., and Raff, M. C. 1994. Autocrine signals enable chondrocytes to survive in culture. *J. Cell. Biol.*, **126**, 1069.
7. Lee, S.-H., Fine, R. L., and Tsuruo, T. 1997. Biological role of lymph node stromal cells in mouse T-lymphoma cells. *In Proceedings of 8th KSEA Conference*, Rutgers University, Piscataway, New Jersey, pp120-124.
8. Lee, S.-H., Fujita, N., Imai, K., and Tsuruo, T. 1995. Cysteine produced from lymph node stromal cells suppresses apoptosis of mouse malignant T-lymphoma cells. *Biochem. Biophys. Res. Commun.*, **213**, 837.
9. Lee, S.-H., Fujita, N., Mashima, T., and Tsuruo, T. 1996. Interleukin-7 inhibits apoptosis of mouse malignant T-lymphoma cells by both suppressing the CPP32-like protease activation and inducing the Bcl-2 expression. *Oncogene*, **13**, 2131.
10. Lin, K.-I., Lee, S. H., Narayanan, R., Baraban, J. M., Hardwick, J. M., Ratan, R. R. 1995. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. *J. Cell. Biol.*, **131**, 1149.
11. Raff, M. C. 1992. Social controls on cell survival and cell death. *Nature*, **356**, 397.
12. Steller H. 1995. Mechanisms and genes of cellular suicide. *Science*, **267**, 1445.
13. Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456.
14. Tsuruo, T., Oh-Hara, T., Yamori, T., Tsukagoshi, S., Ishikawa, T., and Sugano, H. 1988. Development of lymphosarcoma lines with high metastatic ability to lymph nodes and visceral organs in BALB/c mice. *Clin. Exp. Metastasis*, **6**, 141.
15. van den Dobbelsteen, D. J., Nobel, C. S. I., Schlegel, J., Cotgreave, I. A., Orrenius, S., and Slater, A. F.: Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *J. Biol. Chem.*, **271**, 15420 (1996).
16. Wyllie, A. H., Kerr, J. F., and Currie, A. R. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68**, 251.
17. Zmuda, J., and Friedenson, B. 1983. Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. *J. Immunol.*, **130**, 362.