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Pyruvate Secreted by Human Lymphoid Cell Lines Protects Cells from Hydrogen Peroxide Mediated Cell Death

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Reactive oxygen species (ROS) released from polymorphonuclear leukocytes and macrophages could cause DNA damage, but also induce cell death. Therefore inhibition of cell death must be an important issue for accumulation of genetic changes in lymphoid cells in inflammatory foci. Scavengers in the post culture medium of four lymphoid cell lines, lymphoblastoid cell lines (LCL), Raji, BJAB and Jurkat cells, were examined. Over 80% of cultured cells showed cell death 24 h after xanthine (X)/xanthine oxidase (XOD) treatment, which was suppressed by addition of post culture medium from four cell lines in a dose-dependent manner. H_2O_2 but not $O_2^{\bullet-}$ produced by the X/XOD reaction was responsible for the cytotoxity, thus we used H_2O_2 as ROS stress thereafter. The H_2O_2-scavenging activity of post culture media from four cell lines increased rapidly at the first day and continued to increase in the following 2–3 days for LCL, Raji and BJAB cells. The scavenging substance was shown to be pyruvate, with various concentrations in the cultured medium among cell lines. Over 99% of total pyruvate was present in the extracellular media and less than 1% in cells. α-Cyano-4-hydroxycinnamate, a specific inhibitor of the H^+-monocarbohydrate transporter, increased the H_2O_2-scavenging activity in the media from all four cell lines via inhibition of pyruvate re-uptake by cultured cells from the media. These findings suggest that lymphoid cells in inflammatory foci could survive even under ROS by producing pyruvate, so that accumulation of lymphoid cells with DNA damage is possible.

Keywords: Hydrogen peroxide, scavenger, pyruvate, α-cyano-4-hydroxycinnamate, cell death, malignant lymphoma

INTRODUCTION

From the pathoepidemiological studies on the two types of B cell malignant lymphomas, lymphoma of thyroid developing in Hashimoto's thyroiditis[1] and lymphoma in chronic pyothorax patients,[2,3] we proposed the concept of malignant lymphoma developing in chronic inflammation.[1] Malignant lymphoma of mucosa-associated lymphoid tissue (MALT) is also included in this category,[4] in that

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Helicobacter pylori-induced follicular gastritis predisposes to gastric lymphoma.[5] In the inflammatory lesions, reactive oxygen species (ROS) released from polymorphonuclear leukocytes and macrophages might exert a genotoxic effect on cells,[6] thus could be carcinogenic.[7,8] Indeed previous studies have shown that ROS can cause DNA strand breaks[9] and chromosomal alterations[10] in resting peripheral blood lymphocytes or phytohemagglutinin-stimulated lymphocytes. Cytokines secreted by inflammatory cells also have growth promotional effects.[11,12]

Alternatively ROS are cytotoxic agents and can induce apoptotic cell death, which is inhibited by the *bcl-2* family of apoptosis regulatory genes.[13] Using xanthine(X)/xanthine oxidase (XOD) system for generation of ROS,[14] we recently showed that pretreatment with IL-6 protected LCL cells from cell death (submitted for publication). IL-6 is also supposed to be secreted in inflammatory foci.[12,15,16] In the course of that study, we noticed that extracellular medium of LCL cells protected lymphoid cells from cell death which was independent of IL-6 treatment. This medium also reduced H_2O_2 in the cell free system. These findings might suggest the presence of some agents which protect LCL cells from apoptosis via reducing H_2O_2. Several anti-oxidant enzymes such as catalase and glutathione peroxidase and low molecular weight compounds such as glutathione, uric acid,[17] pyruvate,[18,19] and bilirubin[20] are known to scavenge H_2O_2. But little is known about anti-oxidant enzymes and scavengers in human lymphoid cells. In this study, we demonstrated the emergence of the H_2O_2-scavenging activity of extracellular medium from all four lymphoid cell lines examined and identified it as pyruvate.

MATERIALS AND METHODS

Cell Culture

LCLs were established from peripheral blood lymphocytes of healthy adults by infection with Epstein–Barr virus (EBV). Other human lymphoid cell lines, Raji, BJAB, and Jurkat cells were obtained from the Japanese Cancer Research Resources Bank. Cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS, BioWhittaker, Walkersville, MD), 100 U/mL penicillin, and 100 μg/mL streptomycin, and 1 mM glutamine, in a humidified atmosphere with 95% air, and 5% CO_2 at 37°C. Cells, washed twice with RPMI 1640 medium and collected by centrifugation at 400*g* for 5 min, were suspended in fresh medium, counted in a hemocytometer after treatment with trypan blue, and seeded at a density of, usually, 2.0×10^5 cells/mL. At various elapsed time after cultivation, extracellular medium was obtained by centrifugation at 400*g* for 5 min and kept at −20°C until use. Cells were collected by centrifugation at 3000*g* for 5 min, washed twice with phosphate buffered saline (PBS), and kept at −80°C until use.

Detection of DNA Ladder

The DNA ladder in X/XOD-treated cells was determined by the method described by Sellins and Cohen[21] with some modification. Briefly, the cell pellet was lysed with lysing buffer (10 mM Tris, 10 mM EDTA, pH 7.5) containing 0.5% Triton X-100, and the lysates were centrifuged. The supernatant, containing fragmented DNA, was incubated with RNase A, and further incubated with proteinase K. DNA was quantified by measuring OD at 260 nm, and electrophoresed in 1.5% agarose gel and visualized on an ultraviolet transilluminator after stained with ethidium bromide.

Preparation and Treatment of Each Reagent

X (ICN biochemical Inc., Aurora, OH) dissolved in PBS and XOD (Sigma Chemical Co., St. Louis, MO) in RPMI 1640 medium were added to culture at 200 μM and indicated units, respectively. H_2O_2 (Mitsubishi Gasu Kagaku Inc., Tokyo, Japan)

and sodium pyruvate (Sigma) dissolved in PBS and sterilized deionized water, respectively, were used for experiments. α-Cyano-4-hydroxycinnamate (Sigma) was dissolved in dimethylsulfoxide, superoxide dismutase from bovine erythrocytes (4100 U/mg protein) (SOD, Sigma) in PBS, and catalase from bovine liver (11,000 U/mg protein) (Sigma) and lipopolysaccharide (Sigma) in sterilized deionized water. Human recombinant IL-6 and human IFN γ were kindly provided by Ajinomoto Co. (Tokyo, Japan) and Ohtsuka Pharmaceutical Co. (Tokyo, Japan), respectively. IL-6 and IFN γ were dissolved in RPMI 1640 medium and PBS, respectively. The prepared solutions containing each reagent with a volume of less than 10% of total culture medium were added to culture. As controls, the same volume of solvent without reagent was used. After incubation for the indicated time, extracellular medium and cells were prepared as described above. Cell viability was estimated with trypan blue assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[11,22]

Determination of H_2O_2

The concentration of H_2O_2 was estimated with a colorimetric assay using ferrous ammonium sulphate.[23] Briefly, 200 µL of 4% trichloroacetic acid (TCA) was added to 200 µL sample. The supernatant was obtained after centrifugation at 3000*g*, and 40 µL of 10 mM $FeSO_4(NH_4)_2SO_4$ solution was added to 200 µL of supernatant and mixed. Then 16 µL of 3.0 M NaSCN solution was added and mixed, kept for 10 min at room temperature, and absorbance at 480 nm was read.

Assay of the H_2O_2-Scavenging Activity

For assay of the H_2O_2-scavenging activity, 100 µL of 280 µM H_2O_2 solution was added to 100 µL of sample and the mixture was incubated at 37°C for 2 h. Then 200 µL of ice-cold TCA was added to stop the reaction and cooled on ice for 20 min. After centrifugation at 3000*g* at 4°C, the supernatant was assayed for H_2O_2. The initial amount of H_2O_2 was determined by measuring the reaction mixture containing the medium and TCA prior to incubation. The background level of H_2O_2 was obtained by assay of the reaction mixture prepared with PBS without addition of H_2O_2 solution.

Assay of Pyruvate

The concentration of pyruvate in extracellular medium and cell lysate was estimated with a colorimetric assay[18] using LDH (Boehringer Mannheim, GmbH, Germany) and NADH (Oriental Yeast Co., LTD, Tokyo, Japan). Briefly, 5 U of LDH, 100 µM of NADH, and 100 µL of sample in 1 mL potassium phosphate buffer (100 mM, pH 7.4) were incubated at 37°C. The decrease of fluorescence of NADH (excitation, 340 nm; emission, 460 nm) was followed by a Hitachi F-2000 fluoresence spectrophotometer.

Assay of Uric Acid, Bilirubin, and LDH

The concentrations of uric acid, bilirubin, and LDH in extracellular medium were determined using commercial kits, COBAS INTEGRA Uric Acid (Roche Diagnostics K.K. Tokyo, Japan), IATRO D-BIL (Iatron Laboratories, Inc. Tokyo, Japan), and L-Type-Wako-LDH (Wako Pure Chemical Indusries, Ltd. Osaka, Japan), respectively. For determination of uric acid, the media were lyophilized and dissolved in a small volume of sMQ before assay.

Statistical Analysis

Statistical significances of differences in LD_{50} of H_2O_2 for cell lines cultured in the presence or absence of post culture medium, and differences in the H_2O_2-scavenging activity of the medium from cell lines cultured between in the presence and absence of 1 mM α-cyano-4-hydroxycinnamate were evaluated using Two group *t*-test: Paired.

RESULTS

The H_2O_2-Scavenging Activity in the Medium of LCL Cells

Twenty-four hours after X/XOD treatment, cell death began in a dose-dependent manner. A parallel relationship was observed between trypan blue assay and MTT assay (Figure 1). Electrophoretic analysis of DNA at 4–8 h after X/XOD treatment revealed the occurrence of DNA laddering, which suggests that this cell death was partly due to apoptosis. Addition of allopurinol,[24] a specific inhibitor of XOD, with concentration of more than 50 μM, completely suppressed cell death, indicating that ROS was produced by X/XOD. It has been reported that two ROS, $O_2^{\bullet -}$ and H_2O_2, are produced in X/XOD system.[14] The production rate of H_2O_2 in our system was 30 ± 1 μM/h, when 200 μM of X and 4 mU/mL of XOD were used. Effects of elimination of $O_2^{\bullet -}$ by SOD and H_2O_2 by catalase were examined. SOD up to 1000 U/mL in the culture of LCL cells could not suppress X/XOD-mediated cell death. In the cell free system, catalase with concentration higher than 125 U/mL reduced H_2O_2 from 140 μM to undetectable level (less than 10 μM) after incubation at 37°C for 2 h. Alternatively addition of H_2O_2 instead of X/XOD in LCL culture induced cell death in a dose-dependent manner (Figure 2B). These findings indicated that H_2O_2 but not $O_2^{\bullet -}$ was responsible for the cytotoxicity. Thus in the following studies, we used H_2O_2 instead of X/XOD as ROS stress.

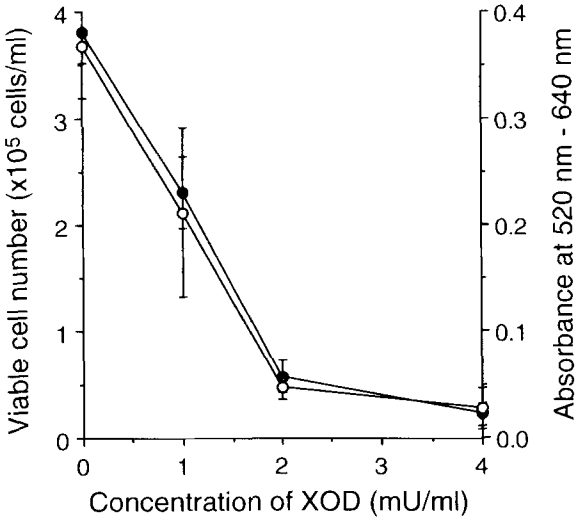


FIGURE 1 Correlation between cell viability (trypan blue assay) and MTT assay; 4.5×10^4 LCL cells were seeded in a well of 96-well tissue culture plate in a 150 μL of medium in the presence of 200 μM of X and XOD (at indicated concentration). After 24 h incubation, an aliquot of culture was subjected to trypan blue assay and the mean value of four counts from independent wells was presented with SD (○). For MTT assay, a 50 μL of MTT solution was added to each well and the cells were further incubated for 4 h. The mean absorbance value of quadruplicate wells was indicated with SD (●).

Cell death induced by either X/XOD (Figure 2A) or H_2O_2 (Figure 2B) was suppressed by adding the post culture medium in a dose-dependent manner. The post culture medium used was prepared as follows: LCL cells were started to cultivate at a density of 2.0×10^5 cells/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. At Day 4, extracellular medium was obtained by centrifugation at 400*g* for 5 min, and used for the experiment. As shown in Table I, LD_{50} of H_2O_2 for all four cell lines increased by adding an equal amount of Day 2 culture medium to fresh medium, although the increases were variable among cell lines, with the highest increase in

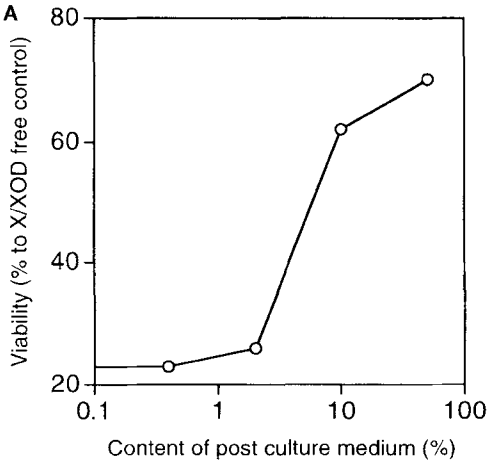
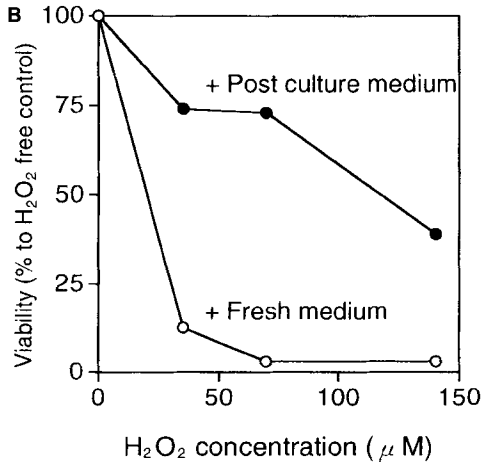


FIGURE 2A



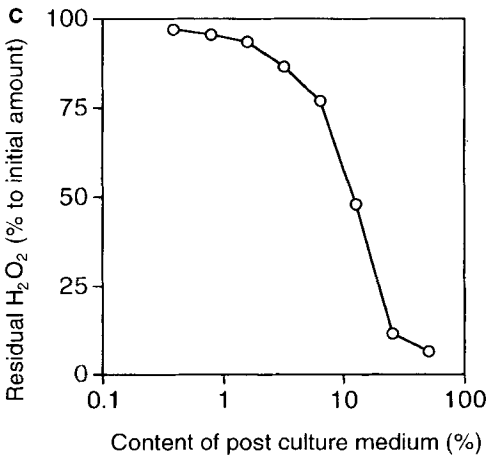


FIGURE 2B and C

FIGURE 2 Cytoprotective activity of post culture medium against X/XOD A; and H_2O_2 B; in LCL cell culture, and the H_2O_2-scavenging activity of post culture medium C. Post culture medium was prepared from the LCL cell culture on Day 4. (A) LCL cells were cultured in the medium supplemented with post culture medium at indicated extents in the presence of 200 μM xanthine and 4 mU/mL XOD, respectively. Twenty-four hours after culture starting, cell viability was measured by MTT assay. Relative cell viability was expressed as percentage to a X/XOD free control. Means of duplicate assay. (B) LCL cells were cultured in the medium supplemented at 50% with fresh medium (○) or post culture medium (●) in the presence of H_2O_2 at indicated concentrations. Twenty-four hours after culture starting, cell viability was measured by MTT assay. Means of duplicate assay.(C) Post culture medium was diluted with PBS at indicated extents and incubated with 140 μM H_2O_2 at 37°C for 2 h. Residual H_2O_2 was determined with a colorimetric assay using ferrous ammonium sulphate. Means of duplicate assay.

TABLE I Effect of supplement of post culture medium on LD_{50} of H_2O_2 (μM)* for four lymphoid cell lines

Cell line	Supplement of post culture medium		Ratio	*P*-value
	No	Yes		
LCL	17 ± 5	65 ± 13	3.9	0.024
Raji	16 ± 4	57 ± 10	3.6	0.008
BJAB	13 ± 2	22 ± 5	1.7	0.052
Jurkat	31 ± 8	37 ± 12	1.2	0.097

*Means of triplicate assay for LCL, Raji and BJAB cells, and quadruplicate assay for Jurkat cells with SD; LCL: lymphoblastoid cell line.

LCL cells. Day 4 culture medium of LCL cells removed H_2O_2 in a dose-dependent manner (Figure 2C). This H_2O_2-scavenging activity was dependent on the reaction temperature: 0.22 nmol/min at 0°C and 1.6 nmol/min at 37°C. In fresh medium, over 90% of the initial H_2O_2 amount was maintained even 24 h after incubation at 37°C. The increased LD_{50} of H_2O_2 must result from the H_2O_2-scavenging activity of post culture medium. We tentatively defined the activity as unit: one unit being the activity of eliminating 14 nmol H_2O_2 within 2 h of incubation at 37°C.

Time Course of the H_2O_2-Scavenging Activity of the Medium in Culture

The extracellular media of four cell lines were daily prepared for 7 days and their H_2O_2-scavenging activities were assayed in the cell free system. All cell lines grew exponentially by Day 4, and then cell density decreased (Figure 3). The emergence of the H_2O_2-scavenging activity was observed in all cell lines, though the degrees of activity were variable among cell lines and with time elapsed after culture. The activity increased rapidly at the first day in all cell lines and continued to increase in the following 2–3 days for LCL, Raji and BJAB cells. In Jurkat cells, the activity remained at a low level by Day 7. The rapid decrease of the activity was observed in Raji and BJAB cells after reaching the peak, while the activity in LCL cells remained high

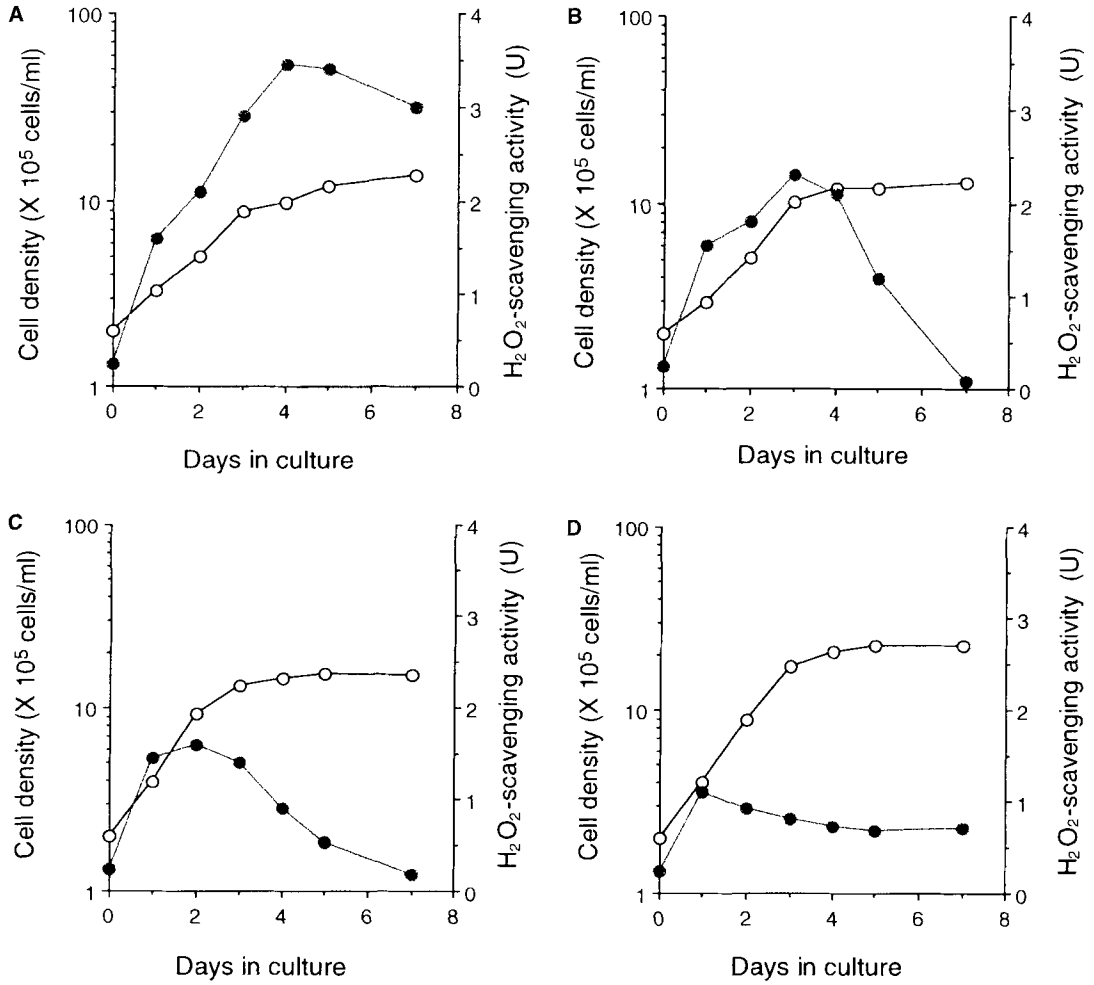


FIGURE 3 Changes of cell density and the H_2O_2-scavenging activity in the media from four lymphoid cell lines during culture for seven days. Culture of four lymphoid cell lines, LCL (A), Raji (B), BJAB (C), and Jurkat cells (D), were started at 2.0×10^5 cells/mL. An aliquot of cultures was obtained daily and prepared for measurements of cell density (○) and the H_2O_2-scavenging activity in the medium (●). Means of duplicate assay.

by Day 7. A positive relationship was observed between the initial cell density in culture and the H_2O_2-scavenging activity of extracellular medium in LCL cells.

Identification of the H_2O_2-Scavenging Substance

The H_2O_2-scavenging activity of the cultured medium was preserved after boiling, treatment with 0.05% trifluoroacetic acid (pH 1), and treatment with proteinase K. Over 90% of total activity passed through a dialysis membrane with a pore size of 3500 Da. These results indicated that the H_2O_2-scavenging substance was stable to heat and acid, thus not an enzyme, a low molecular-mass scavenger.

The concentrations of glutathione, uric acid,[18] pyruvate,[19,20] and bilirubin[21] in the medium were measured before and after cell culture (Table II). Changes of concentration were observed in pyruvate and uric acid. The H_2O_2-scavenging activity of these substances was determined as follows: (1) uricase treatment did

TABLE II Scavenger concentrations in fresh medium and Day 4 culture medium from LCL cell culture

Scavenger	Concentration of scavenger (μM)*	
	Fresh medium	Day 4 culture medium
Pyruvate	64	820
Uric acid	< 0.7	1.5
Bilirubin	< 0.5	< 0.5

*Means of duplicate assay.

TABLE III Pyruvate concentrations in the media and cells at 48 h in cultures of four lymphoid cell lines

Cell line	Pyruvate concentration*	
	Medium (μM)	Cells (nmol/10^7 cells)
LCL	520	28
Raji	225	18
BJAB	215	29
Jurkat	180	13

*Means of duplicate assay.

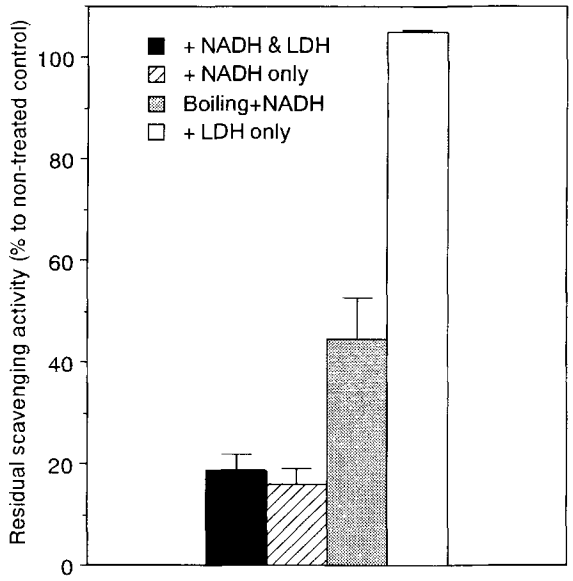


FIGURE 4 Effects of treatment of post culture medium from LCL cell culture with LDH and NADH on the H_2O_2-scavenging activity. Post culture medium was prepared from LCL cell culture at Day 2. Five mU LDH and 2 mM EDTA were added to the medium. After incubation at 37°C for 1 h, the mixture was boiled for 5 min. The residual H_2O_2-scavenging activity was measured. Means of triplicate assay with SD.

not reduce the activity, (2) addition of either glutathione up to 50 mM or uric acid up to 50 μM in fresh medium did not raise the H_2O_2-scavenging activity. These results showed that glutathione, uric acid, and bilirubin were not the scavengers in post culture medium. On the other hand, enzymatic elimination of pyruvate from post culture medium by LDH and NADH brought about over 80% loss of the activity (Figure 4). Though LDH alone did not affect the H_2O_2-scavenging activity of post culture medium, NADH alone brought about the activity loss to the same extent as obtained by LDH and NADH. When the medium was boiled prior to treatment with NADH, the activity loss was limited. These results indicated the presence of endogenous LDH in the medium. Because the amount of LDH in the medium did not change between before and after culture, i.e., 10 U, it must be derived from fetal calf serum supplemented to RPMI 1640 medium. Treatment of post culture medium from Raji, BJAB and Jurkat cells with LDH and NADH also removed the H_2O_2-scavenging activity by over 80% of 1 mM pyruvate.

The concentrations of pyruvate in the extracellular media and in cells were measured at 48 h in culture (Table III). Pyruvate concentrations in the media were variable among cell lines, but those in cells were rather similar. Over 99% of total pyruvate was present in the extracellular media and less than 1% in cells. Pyruvate lowered the H_2O_2 concentration (Figure 5A), and suppressed cell death in a dose-dependent manner in Jurkat cell culture (Figure 5B). As a summary, pyruvate secreted rapidly from cells and accumulated in the extracellular medium was responsible for the H_2O_2-scavenging activity: 250 μM pyruvate was equivalent to one unit of the H_2O_2-scavenging activity in this study.

Elevation of the Scavenging Activity in the Medium by α-Cyano-4-hydroxycinnamate

In order to elucidate the accumulation mechanism of pyruvate in the culture medium, several experiments were performed. Catalase

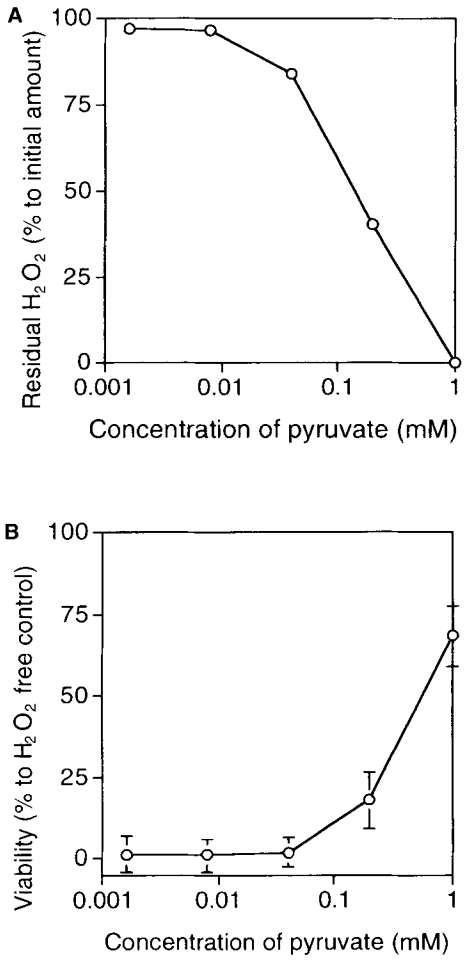


FIGURE 5A and B

FIGURE 5 The H_2O_2-scavenging activity of pyruvate (A), and its cytoprotective activity against H_2O_2 in Jurkat cell culture (B). A; Pyruvate at indicated concentrations was incubated at 37°C for 2 h with 140 μM of H_2O_2. The residual H_2O_2 was determined. Means of duplicate assay. B; Jurkat cells were cultured in the medium containing 140 μM of H_2O_2 and pyruvate at indicated concentrations. Twenty-four hours after culture, cell viability was measured by MTT assay. Means of quadruplicate assay with SD.

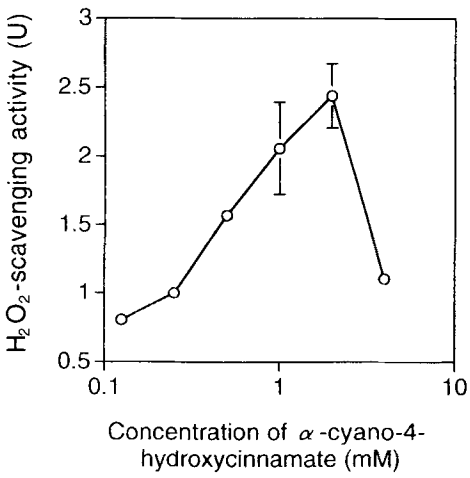


FIGURE 6 Dose effects of α-cyano-4-hydroxycinnamate on the H_2O_2-scavenging activity in post culture medium from Jurkat cell culture. Jurkat cells were cultured in the presence of α-cyano-4-hydroxycinnamate at indicated concentrations. Forty-eight hours after culture starting, post culture medium was prepared for measurement of the H_2O_2-scavenging activity. Means of triplicate assay with SD.

TABLE IV Effects of α-cyano-4-hydroxycinnamate on the H_2O_2-scavenging activity (U)* in culture medium from four lymphoid cell lines

Cell line	Treatment with α-cyano-4-hydroxycinnamate		Ratio	*P*-value
	No	Yes		
LCL	1.95 ± 0.06	2.34 ± 0.01	1.20	0.010
Raji	1.86 ± 0.04	2.50 ± 0.01	1.34	0.001
BJAB	1.87 ± 0.06	2.30 ± 0.02	1.23	0.011
Jurkat	0.91 ± 0.07	2.26 ± 0.04	2.49	0.001

*Means of triplicate assay with SD.

was reported to elevate the pyruvate concentration in the medium of several cell lines via elimination of the endogenous H_2O_2 produced by cells.[18] Addition of catalase at 1000 U/mL in culture of four lymphoid cell lines did not change the H_2O_2-scavenging activity after 48 h. Neither lipopolysaccharide at 10 μg/μL, IL-6 at 100 U/mL, nor IFN γ at 100 U/mL changed the activity. On the contrary α-cyano-4-hydroxycinnamate, a specific inhibitor of the H^+-monocarbohydrate transporter (MCT),[25,26] increased the H_2O_2 scavenging activity in the culture medium 48 h after in Jurkat cell culture in a dose-dependent manner (Figure 6). This effect of α-cyano-4-hydroxycinnamate was also observed in the media from other three cell lines, though the increasing degrees were variable among cell lines, with the highest increase in Jurkat cells (2.1 times higher than that in LCL cells) (Table IV). Cell growth and viability were not affected

by 1 mM α-cyano-4-hydroxycinnamate. In this experiment, concentration of pyruvate could not be determined because of the presence of α-cyano-4-hydroxycinnamate, which has intrinsic fluorescence and affects the colorimetric assay. However the combined findings that α-cyano-4-hydroxycinnamate-mediated scavenging activity was stable after boiling and 86% of its activity was removed by the treatment with LDH and NADH indicated that the pyruvate increased in the media. Considering that α-cyano-4-hydroxycinnamate inhibits the pyruvate transporters, MCTs, this elevation of pyruvate in the extracellular media might result from inhibition of pyruvate re-uptake by cultured cells from media. Differences in response to α-cyano-4-hydroxycinnamate among cell lines suggested differences in MCT types among cell lines.

DISCUSSION

Cell death by apoptosis and necrosis plays a principal role in tumor development. In chronic inflammatory foci, ROS produced by polymorphonuclear leukocytes and macrophages could cause DNA damage and contribute to lymphomagenesis.[9,10] ROS are also cytotoxic agents and can induce apoptotic and necrotic cell death,[19] therefore suppression of cell death of lymphoid cells with DNA damage must be essential for accumulation of genetical changes. Recently using the X/XOD system we observed that ROS cause structural abnormalities of chromosomes in LCL cells and IL-6 inhibited ROS-induced cell death of LCL cells through upregulation of *bcl-2* expression (submitted for publication). The present study showed that pyruvate produced by lymphoid cell lines protected cells from ROS.

Pyruvate, a major end-product of glycolysis, nonenzymatically reacts with H_2O_2 and forms acetate, carbon dioxide and water,[27] therefore having the H_2O_2-scavenging activity. Indeed cytoprotective activity of pyruvate against H_2O_2 in renal cells,[28] heart muscle,[29] neuron[30] and lymphocyte[19] has been reported. Secretion of pyruvate and its cytoprotective activity in several mammalian cell lines was also reported by O'Donnell *et al.*[18] However little is known about secretion of pyruvate from human lymphoid cells. This is the first report demonstrating that secretion of pyruvate is common in human lymphoid cell lines. The concentrations of pyruvate in post culture media from lymphoid cell lines (180–800 μM at plateau with 2.0×10^5 cells/mL) were much higher than those reported by O'Donnell *et al.*[18] on human cell lines derived from fibroblasts and breast adenocarcinoma or mouse cell lines derived from mastocytoma and connective tissue (60.4–149.9 μM with 2.5×10^6 cells/mL). The concentrations of pyruvate were much higher in B cell lines than in T cell lines, with the highest concentration in EBV-positive B cell lines. The intracellular concentration of pyruvate was roughly similar among each cell line. Thus concentration of pyruvate in the extracellular medium might be regulated through secretion and/or re-uptake by lymphoid cells, and EBV infection might accelerate the accumulation of pyruvate in the extracellular medium.

Recently several free radical scavengers have been reported to protect carcinoma from apoptosis by eliminating ROS. TGF-β-induced apoptosis of human hepatoma cells is suppressed by carboxyfullerene, a novel free radical scavenger.[31] N-(4-hydroxyphenyl) retinamide enhances ROS production in cervical carcinoma cells and causes apoptosis which is suppressed by pyrrolidine dithiocarbamate, an oxygen radical scavenger.[32] These findings together with the results from our study suggested that ROS scavengers might play an important role in tumor development through suppressing apoptotic cell death.

In our experiments, several chemical mediators such as lipopolysaccharide, IL-6, and IFN γ did not affect the H_2O_2-scavenging activity of post culture medium from all cell lines, indicating that these mediators might not be involved in

the accumulation mechanism of pyruvate. On the other hand, α-cyano-4-hydroxycinnamate increased the H_2O_2-scavenging activity which was removed by the treatment with LDH and NADH, suggesting that α-cyano-4-hydroxycinnamate increases pyruvate concentration in the medium via inhibition of re-uptake of pyruvate by cultured cells from media. Meanwhile a rapid decrease of pyruvate concentration in the medium of Raji and BJAB cells during the long term culture might be due to pyruvate re-uptake by cultured cells from the media. It is well-known that transport of pyruvate is mediated by MCTs. MCT1 is firstly cloned from Chinese hamster ovary cell,[33] and subsequently its homologues were cloned and sequenced from human fibroblast genomic library,[34] rat small intestinal cDNA library,[35] and mouse Ehrlich Lettre tumor cells.[36] A second MCT, termed as MCT2, was isolated from a Syrian hamster liver library,[37] and its homologues were cloned and sequenced from human liver cDNA library[38] and rat testis,[39] and its expression was reported in the several human cancer cell lines including Raji cells.[38] MCT2 has a higher affinity for pyruvate than MCT1 and shows different tissue distribution from MCT1.[38] Recently the third MCT, MCT3, was isolated from chicken retinal pigment epithelium.[40] Differences in response to cinnamate observed in four cell lines might be due to differences in MCT type used.

O'Donnell *et al.*[18] reported that addition of catalase in culture of a mastocytoma cell line enhanced pyruvate accumulation in the medium at 1.5 times, suggesting that endogenous H_2O_2 produced by cultured cells could be eliminated by catalase. In this study, however, addition of catalase did not change pyruvate concentration, suggesting that the secretion level of H_2O_2 from lymphoid cell lines might be lower than that from a mastocytoma cell line.

In conclusion, the present study showed the anti-apoptotic effect of pyruvate produced by lymphoid cell lines via scavenging H_2O_2. Therefore lymphoid cells in inflammatory foci could survive even under influence of lowered level of ROS, which makes the accumulation of lymphoid cells with DNA damage possible. This might provide a basis for lymphoma development in inflammatory foci.

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