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Mechanistic aspects of apoptosis induction by L-lysine in both HTLV-1-positive and -negative cell lines

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Abstract

The retrovirus human T-cell lymphotropic virus type-1 (HTLV-1) causes adult T-cell leukemia (ATL), which remains with no cure. This study evaluates the effects of L-lysine on proliferation and induction of apoptosis using non-cytotoxic concentrations of the test compound against HTLV-1 positive and negative malignant cell lines. The anti-proliferative effect of lysine was established and confirmed by studying the effects of the test compound on the expression of TGF mRNA expression by RT-PCR. To investigate the effect of L-lysine on the induction of apoptosis, DNA flow cytometry analyses was done and the results verified by cell death ELISA. The results indicated that a significant increase in the preG₁ phase and a decrease in the S phase of the cell cycle in all of the ATL cells tested. L-Lysine up-regulated p53, p21, and Bax protein levels and a down-regulation of Bcl-2 α in all the cell lines tested. L-Lysine was found to exert its effect through the NF- κ B pathway by inhibiting the p65 subunit specifically. Also L-lysine caused a decrease in the levels MMP-2 and MMP-9 as well as their enzymatic activity.

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1. Introduction

The human body cannot synthesize the amino acid L-lysine, making it an essential one obtained through alimentary diet (red meats, fish, milk, eggs, cheese, beans, peas and lentils). Lysine is a building block for proteins and is an essential amino acid that is needed for growth and bone development especially in children. It also helps with calcium absorption, it keeps the correct nitrogen balance throughout the body, and ensures lean body mass. Natural protein consists of 2–9% lysine and

it is the source of glutaric acid and 3-hydroxyglutaric acid [1].

Since cancer patients have a decreased food intake, they will surely suffer from a deficiency in essential nutrients such as the amino acids lysine and praline and Vitamin C (ascorbic acid), leading to the formation of a weak extracellular matrix prone to breakdown by cancer cells [2,3].

In fact, matrix metalloproteinases (MMPs), enzymes secreted by cancer cells are the agents responsible for the collagen fibers breakdown. Similar to many enzymes, most MMPs are secreted in an inactive form and get activated through a regulated and well-organized pathway. One of the steps of this pathway is regulated by TPA (tumor-promoting agent), which is responsible for

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the conversion of plasminogen to plasmin: an essential enzyme in the MMP activation cascade and lysine has the ability to interfere with TPA [2,3].

The interference of lysine is thus an obstacle for the activation pathway leading thus to a decrease in collagen fibers breakdown in the ECM, basement membrane and blood vessels and thereby to the reduction of metastasis. Therefore, it would be beneficial for cancer patients to have a proper intake of the abovementioned essential nutrients in their diet. In fact, the addition of lysine, proline and ascorbic acid at physiological concentrations (similar to those in healthy patients) inhibits metastasis by 50%, 10% and 30% in breast cancer cells (MDA-MB-231), melanoma cells (A2058) and colon cancer (HCT116), respectively [4].

Human T-cell lymphotropic virus type 1 (HTLV-1) is the first retrovirus to be discovered in humans [17]. There are around 10–20 million carriers of the virus worldwide [18]. One of the maladies the virus causes is a malignancy of activated T-lymphocytes called adult T-cell leukemia (ATL). ATL is very serious and the median survival time is around 8.5 months with therapy [19]. Treatments exist but unfortunately the results are not satisfactory.

2. Materials and methods

2.1. Cell lines and treatment

Four leukemic cell lines, C91-PL and HuT-102 (HTLV-1 positive cell lines) and CEM and Jurkat (HTLV-1 negative cell lines) were used and were a gift from A. Gessain, Institut Pasteur, Paris, France.

The HTLV-1 cell lines are ATL-derived HTLV-1 infected CD4⁺ T cell lines that constitutively express the virus upon replication. The HTLV-1 negative cell lines are uninfected human T-cell line obtained from T-cell leukemic patients. CEM cells (T-lymphoblastoid cell lines) and Jurkat were originally derived from peripheral blood of a Caucasian female infected with acute lymphoblastic leukemia (ALL) [5]. Jurkat cells are characterized by having the same markers as normal lymphocytes. Cells were grown in RPMI 1640 with 25 mM of the indicator Hepes containing 10% heat-inactivated Fetal Bovine Serum (Gibco-BRL, Paisley, Scotland), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco-BRL, Paisley, Scotland). The cells were kept in a humidified incubator at 37 °C with 95% air and 5% CO₂. The cultured cells were then treated with lysine daily.

2.2. Lysine

L-Lysine dihydrochloride was purchased in powder form from Sigma (Saint Louis, MO, USA). L-Lysine

stock solutions were prepared by dissolving it in RPMI 1640 media and adjusting the pH of the medium to 7, aliquoted and frozen until the day of the experiment. Each aliquot was used for only one experiment and discarded later.

2.3. Cytotoxicity and proliferation

The cell suspension was loaded in 96 well plate microtiter plates at a 1×10^5 density and treated daily with the test compound for 2 and 4 days depending on the experiment. Cytotoxicity and cell proliferation were determined using the Cell Titer96TM Non-radioactive cell proliferation assay and the CytoTox 96 non-radioactive cytotoxicity assay following the manufacturer's directions (Promega Corp. Madison, WI). The proliferation assay is an MTT-based method, which measures the conversion of tetrazolium salt into a formazan product by mitochondria of metabolically active cells and its absorbance is recorded at 570 nm. The CytoTox 96 assay quantitatively measures the enzyme lactate dehydrogenase (LDH), which is a stable cytosolic enzyme released from the cells upon lysis. This latter is measured upon release in culture supernatants with a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product, the absorbance of which is measured at 490 nm [6].

2.4. Flow cytometry analysis of DNA content

Cells were suspended in RPMI complete growth media and distributed in 25 cm² tissue culture flasks, and were treated daily with lysine at varying concentrations for two or four days. After treatment, cells were then spun down, washed twice with phosphate buffered saline (PBS) (Gibco-BRL, Paisley, Scotland), permeabilized with 70% ethanol, treated with 1% RNase and finally stained with propidium iodide (Molecular Probes, Eugene, Oregon) (100 µg/ml final concentration). The distribution of cell cycle phases with different DNA content was determined using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). If an increase in the amount of cells in the preG₁ (<2n) phase occurs, it shows an increase in apoptosis. Diploid G₀/G₁ cells are indicated by (2n), S-phase is (>2n but <4n), and G₂/M-phase is (4n) and the DNA content was determined using Cell Quest software.

2.5. Cell death by ELISA

To assess cell death, an ELISA kit was used to quantitatively detect cytosolic histone-associated DNA

fragments (Roche Molecular Biochemicals, Mannheim, Germany). Treated cells were seeded in 96-well culture plates at a density of 1×10^4 cells per well and then lysed and their whole nuclei were spun down by centrifugation. The antigen source used was an aliquot obtained from the supernatant and was treated in a sandwich ELISA with primary anti-histone monoclonal antibody coated to the streptavidin-coated well.

A second anti-DNA monoclonal antibody coupled to peroxidase was then added in order to quantify the number of histones by measuring the peroxidase activity which was determined photometrically using 2, 2'-azino-di (3-ethylbenzthiazolin-sulfonate) (ABTS) as a substrate, at 405 nm.

2.6. Protein extraction and western blot analysis

For protein extraction, lysis of the cells was done in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% Sodium deoxycholate, 4% protease inhibitors and 1% phosphatase inhibitors. The cells were then kept in boiling water for 5 min and after cooling down, were centrifuged at 14,000 rpm for 10 min. After collecting the supernatants, they were kept on ice and their protein concentrations were determined using the DC BioRad protein assay kit (BioRad Laboratories, Hercules, CA) with the standard being bovine serum albumin. After that, 30 μ g of cellular protein were loaded onto 12% SDS-polyacrylamide gels. The protein bands obtained were transferred to PVDF membranes (NEN Life Sciences Products, Boston, MA). For the immunoblotting technique, different primary antibodies were used: Bcl-2 α (N-19), p53 (FL-393), p21 (F-5) and Bax. Then, membranes were probed with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). To ensure equal protein loading, the actin antibody was used (Biogenesis, England).

Finally the protein bands' detection was performed using the enhanced chemiluminescence system according to the manufacturer's instructions and results were obtained on film developed using a Xomat (Amersham, Pharmacia, Biotech) [7].

2.7. Analysis of TGF mRNA by RT-PCR

Total RNA was isolated from cells using the SV Total RNA Isolation Kit (Promega, Madison, WI) following the manufacturer's directions. After precipitation of the nucleic acids, they were washed with 75% ethanol, dried and resuspended in water. 10 U of avian myeloblastosis reverse transcriptase was used (Promega) in order to perform transcription of the total RNA into cDNA. A total volume of 50 μ l containing Oligo(dT)17 primer (50 pM), 0.2 mM each of deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP; Promega), reaction buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM dithiothreitol) was added for 60 min at 42 °C. Finally, in order to amplify the cDNA, polymerase chain reaction (PCR) was performed using specific oligonucleotide primers (TGF α ; TGF β 1 and TGF β 2), specific for the detection of the target cDNAs (Table 1) [7].

The PCR reactions were performed using 100 μ l of 75 mM Tris-HCl, pH 9, 20 mM (NH₄)₂SO₄ 0.01% Tween 20, 1 mM MgCl₂, 0.2 mM of each dNTP, 0.5 U of DNA polymerase, 50 pM of each primer and 2 ml of the cDNA mixture. The following program was performed: denaturation for 45 s at 94 °C, annealing at 50 °C for 45 s, and elongation at 72 °C for 45 s, followed by a final 5 min at 72 °C. In order to determine the appropriate range of cycles consistent with an exponential increase in the amount of DNA product, a preliminary analysis was conducted for each oligonucleotide pair and for every RNA sample. Then a 3% agarose gel electrophoresis was per-

Table 1
Synthetic oligonucleotides and experimental conditions used for RT-PCR analysis

Gene	Size	Sequence	Number of cycles	Hybrid temperature (°C)
Ribosomal protein	486 bp	5'GTTACCAAGGAGGACCTCA3', 3'AGACACCTCGCCTAATGTG5'	28	50
TGF- β 1	661 bp	5'GAAGTCACCCGCGTGCTAATGG3', 3'GGATGTAAACCTCGGACCTGTGTG5'	37–40	50
TGF- β 2	192 bp	5'TTCGCAGGTATCGATGGCACCT3', 3'CGTCGTATTAACGACGGAAGCGG5'	37–40	50
TCF- α	373 bp	5'ATGTTGTTCCCTGCAAGTCC3', 3'ACTATGGAGAGGGTCCGCTT5'	30	50
MMP-2		3'GTGCTGAAGGACACACTAAAGAAGA3', 5'TTGCCATCCTTCTCAAAGTTGTAGG3'	35	55
MMP-9		3'CACTGTCCACCCCTCAGAGCS', 5'GCCACTGTCCGGCGATAAGG3'	35	55

formed followed by staining with ethidium bromide, in order to separate the 10 μ l of the PCR products stained; the intensity of the luminescent bands was evaluated using the Molecular Analyst/PC image analysis software (BioRad Laboratories, Hercules, CA). Results were expressed as relative densitometric units, normalized to the values of the phosphoribosomal protein mRNA used as an internal control. Control PCR was performed by using RNA samples not exposed to reverse transcription, and that to detect the presence of any amplified fragment(s) corresponding to genomic DNA.

2.8. Zymography

Cells were seeded for 3 days in complete growth medium and on the 4th day cells were spun down and resuspended in serum-free media. The supernatant was collected and concentrated 10-fold. The supernatant was loaded onto 10% polyacrylamide gels with gelatin and run at 90 V at 4 °C. The gels were washed twice for 30 min each with wash buffer [substrate buffer: (50 mM Tris-Cl, 5 mM CaCl₂, 150 mM NaCl) and Triton X-100]. The gels were incubated in substrate buffer for 18 h at 37 °C. The gels were then stained with 0.25% solution of Coomassie Brilliant Blue for 4 h at 37 °C and then washed with double distilled water and bands of digestion appeared as white and were photographed.

2.9. Electrophoretic mobility shift assay (EMSA)

The two HTLV-1-positive cell lines were seeded at a density of 1×10^6 cells/ml in the presence or absence of L-lysine. Nuclear proteins were extracted by suspending the cells in hypotonic ice-cold buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT)), and the cell suspension was pelleted and the nuclei were lysed in buffer C (20 mM HEPES, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 25% glycerol). Protein quantification was done using the Bio-Rad DC Protein Assay Kit (BioRad Laboratories, Hercules, CA). NF- κ B consensus oligonucleotides (Santa Cruz) (wt sense sequence: AGT TGA GGG GAC TTT CC CAG GC; antisense sequence: GCC TGG GAA AGT CCC CTC AAC T) were end-labeled with γ -³²P ATP using T4 polynucleotide kinase. The radioactively labeled probe was purified in a G-25 spin column in a microfuge at 3000 rpm for 2 min. The EMSA gel apparatus (Hoefler Pharmacia Biotech Inc., San Francisco, CA) was assembled according to the manufacturer's instructions. The gel was prepared by the addition of 70 ml

of 1 \times TBE (0.9 M Tris Base, 0.9 M boric acid, 20 mM EDTA, pH 8.0) to 10 ml 40% acrylamide/0.8% *N,N'*-bismethylene-acrylamide solution. Gels were poured immediately after the addition of 500 μ l 10% APS and 37 μ l TEMED. The combs were placed between the glass plates and the gels were allowed to polymerize overnight at 4 °C. Before loading, gels were pre-run for 30 min at 200 V to ensure that samples run at same rate. Samples were prepared by combining of 10 μ g of nuclear protein extract and ddH₂O to a final volume of 13 μ l into sterile eppendorf tubes. For the competition assay, 1 μ l of cold or mutant probes (mutant sense sequence: AGT TGA GGC GAC TTT CCC AGG C; antisense sequence: GCC TGG GAA AGT CGC CTC AAC T) were added to the appropriate tubes at a 10-fold excess of labeled probe in order to assess specificity of binding through competition assays. For each sample, 6 μ l of cocktail solution (1 μ g poly(dNG), 1 μ l DTT (1M), 1 μ l of 1% bovine serum albumin (BSA), 1 μ g poly(dIdC), and 2 μ l of 10 \times HDKE buffer (50% glycerol, 0.5 M KCl, 10 mM EDTA) were added. Finally, the hybridization reaction was allowed to proceed for 30 min following the addition of 1 μ l labeled probe (0.4 ng \geq 30,000 cpm). To perform the supershift assay, ddH₂O was added to a final volume of 12 μ l and 1 μ l of an antibody specific for either p65 or p50 was added to the nuclear extracts.

The gels were loaded and run in 1 \times TBE buffer for 1.5 h at 200 V. The gels were then dried on Whatman filter paper (80 °C, 2 h) and processed for autoradiography at -80 °C overnight on X-ray film.

2.10. ELISA for p65 NF- κ B subunit

Nuclear extracts obtained from cells treated with various non-cytotoxic concentrations of EGCG were used after diluting them in appropriate amounts of buffer. The 96-well plate supplied with the kit was coated with anti-p65 antibody according to manufacturer's instructions and incubated overnight at 4 °C. Next day, the samples were added and incubated overnight at 4 °C. Anti-p65 antibodies were added and then were probed with a secondary antibody specific to the first and conjugated to alkaline phosphatase (AKP). The reaction was allowed to take place for an hour. Later the optical density was measured at 405 nm.

2.11. Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA). The differences between the means of treated and control groups were tested for significance using Fisher's least significant differences at $p \leq 0.05$

(Fisher PLSD). An effect was considered significant when the value (+ or -) of mean difference between groups exceeded Fisher PLSD in the one-factor ANOVA test.

3. Results

3.1. Effects of lysine on cytotoxicity and proliferation

The effects of lysine on cytotoxicity were evaluated at 48 and 96 h exposure using four leukemic cell lines. Cells were grown in the presence and absence of different concentrations of the test compound. The infectious dose at which 50% (IC_{50}) of the cells died was determined. The corresponding reduction in proliferation was recorded at the IC_{50} . The results revealed a dose-dependant decrease in viable cell counts in all the cells tested. In the case of the positive cell lines, HuT-102 showed an IC_{50} of 530.7 and 219.5 $\mu\text{g/ml}$ at 48 and 96 h, respectively with corresponding inhibition in proliferation of 46.7% and 55.8% at IC_{50} . As for the C91-PL cell line, the IC_{50} at 48 h of exposure was 1033.1 $\mu\text{g/ml}$ and 403.5 $\mu\text{g/ml}$ at 96 h. The corresponding inhibition in proliferation was 31.9% after 48 h and 48.7% after 96 h. Concerning the negative HTLV-1 cell lines, CEM's IC_{50} was 1430.8 $\mu\text{g/ml}$ at 48 h and 1148.2 $\mu\text{g/ml}$ at 96 h of treatment with a corresponding inhibition in proliferation of 19.3% at 48 h and 26.8% at 96 h. In the case of Jurkat, the IC_{50} was 1240.8 $\mu\text{g/ml}$ at 48 h and 510.1 $\mu\text{g/ml}$ at 96 h. Corresponding reduction in proliferation revealed 24.3% at 48 h and 74.5% at 96 h (Fig. 1 a and b).

3.2. Effects of lysine on transforming growth factor (TGF) expression

The effects of lysine on the expression of TGF mRNA using RT-PCR were investigated. TGF is a growth factor that regulates essential functions such as proliferation, differentiation and apoptosis. Cells were treated with different non-cytotoxic concentrations of the compound.

RT-PCR was conducted on RNA from the cells and then transferred to agarose gels for electrophoresis. The effects of lysine on TGF- α , TGF- β 1 and TGF- β 2 expression were evaluated by RT-PCR. These three proteins are involved in proliferation. Further verifications on TGF mRNA were done using RT-PCR. Lysine decreased cellular proliferation of ATL by down-regulating TGF- α (an anti-apoptotic protein) and up-regulating TGF- β 2 (pro-apoptotic protein). However, there was no change in the case of TGF- β 1 expression (Fig. 2).

3.3. Effects of lysine on cell cycle progression and apoptosis

The growth inhibitory effects of Lysine on cell cycle progression and/or induction of apoptosis were assessed using flow cytometry and ELISA-based apoptosis assay.

3.3.1. DNA flow cytometry analyses

The DNA content of different cells treated with Lysine was determined using FACScan Flow Cytometer, which measures the intensity of the signal, emitted by the DNA stain propidium iodide. DNA content is indicative of the different phases of the cell cycle such that an increase in cells in the preG₁ phase indicates an increase in apoptosis.

At non-cytotoxic doses of Lysine, cell cycle distribution changes became apparent in all ATL cells treated with the test compound. The apoptotic effects of Lysine were shown by the significant increase in the preG₁ (apoptosis) phase of the cell cycle and the decrease in the S phase of the cell cycle in all tested ATL cells. A trend of increase in cell cycle distribution in the preG₁ was seen at 96 h with an increase of around 27 seen in the case of Hut-102 and around 12.55 folds in the case of Jurkat (Fig. 3).

3.3.2. Cell death by ELISA

Cell Death ELISA was used to confirm the results of flow cytometry. This method quantitatively measures cell death by detecting cytosolic histone associated DNA fragments using optical density measurements. The ELISA data indicated that Lysine induced apoptosis in all the cell lines tested. The HTLV-1 positive cell lines, C91-PL and HuT-102, showed a different response to Lysine than the negative cell lines. For example, C91-PL showed a 3.47-fold increase and HuT-102 showed a 9.8-fold increase in apoptosis at the respective concentrations of lysine used after 96 h (Fig. 4).

In the case of the HTLV-1 negative cell, a 1.20-fold increase in cell death was observed in the case of CEM and a 6.14-fold increase in the case of Jurkat at 96 h contact as compared to the control.

3.4. Effects of lysine on the expression of apoptosis related proteins

The effects of lysine on certain regulatory proteins involved in apoptosis were investigated using β -actin as a control to ensure equal loading. Four proteins were included: Bcl-2 α , an anti-apoptotic protein, Bax, a proapoptotic protein, p53, a tumor suppressor gene, and p21, a cyclin dependent kinase inhibitor. Cells were

