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Lipoprotein isolated from attenuated *Leishmania donovani* induces apoptotic cell death in human cervical carcinoma

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Abstract

Cancer is class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to spread, either by direct growth into adjacent tissue through invasion, or implantation into distant sites by metastasis. Cervical cancer is a type of cancer that starts in the cervix (the narrow opening into the uterus from the vagina). It occurs most often in women over the age of 30s. Cervical cancer is the fourth-most common cause of death from cancer in women worldwide. Although it is one of the most preventable types of cancer, it is about 8% of the total cases and total deaths from cancer. Nearly 70% of cervical cancers and 90% of deaths occur in developing countries where as in low-income countries, it is one of the most common causes of cancer death. Not all of the causes of cervical cancer are yet known, however, infection with some types of human papillomavirus and several other contributing factors like smoking, oral contraceptives and multiple pregnancies have been implicated. Surgery and radiation therapy are the most familiar treatments for invasive cervical cancer. But, chemotherapy and biological therapy are also used to treat cervical cancer. For searching of new therapeutic agents one membrane lipoprotein has been isolated from an attenuated strain of *Leishmania donovani* promastigotes (MHO/IN/1978/UR6) and tested its efficacy in inducing cell death in HeLa (the first immortal human cell line obtained from a cervical cancer patient, Henrietta Lacks during 1951) cells. In this study, it has been revealed that ALLP [membrane lipoprotein isolated from attenuated *Leishmania* strain (UR 6)] induces apoptotic cell death in HeLa cells.

Introduction

Drugs against tumor or cancer are targeted to control the growth of those cells and/or to kill those cells. Chemotherapy has been used for many years and is one of the most common treatments for cancer. Chemotherapeutic drugs could theoretically target all metastatic sites, but current treatments do not provide a significant therapeutic benefit (Soengas and Lowe, 2003). One of the main causes of failure in the treatment of cancer is the development of drug resistance by the cancer cells (Johnstone et al, 2002). The result is the re-growth of a tumor that is not sensitive to the original drug. Additionally, inactivation of apoptosis is a 'hallmark of cancer', an obligate ritual in the malignant transformation of normal cells. This is a very serious problem that may lead to recurrence of disease or even death (Banik et al., 2018). Therefore, induction of apoptosis selectively in hyper proliferating cancer cells is the

main focus of cancer treatment today (Thompson CB, 1995; Douglas et al., 2001).

There exist strong needs for searching of new therapeutic agents that can remove cancer completely without damaging the other parts of the body. In these respects, bio-molecules having apoptosis (programmed cell death) inducing activities are now achieving much attention in biomedical research fields. Today, integral membrane proteins (IMPs) or membrane lipoproteins of several microbial origins are emerging as important regulators of numerous cellular functions like immuno-stimulation, cell growth or proliferation and death. While, the major functions of the IMPs are to maintain the structural integrity of cell membrane and sub-cellular particles like mitochondria, a number of IMPs /microbial membrane lipoproteins are also reported to have apoptotic effects on a variety of immortal mammalian cell types (Into et al., 2002; Lopez et al., 2003; Majumdar et al., 2020).

So, keeping these probabilities in mind one membrane lipoprotein [attenuated leishmanial lipoprotein (ALLP)] has been isolated from an attenuated strain of *Leishmania donovani* promastigotes (MHO/IN/1978/UR6) and tested its efficacy in inducing cell death in cervical cancer cell line, HeLa cells. In this study, it has been revealed that ALLP induces apoptotic cell death in HeLa cells.

Methodology

Reagents:

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from NUNC (Roskilde, Denmark). All organic solvents used were of HPLC grade. All other chemicals used were from Sigma Chem. Co. (St. Louis, MO, USA) or mentioned otherwise.

Culture of HeLa cells

In this study, mammalian cervical carcinoma cell line, HeLa (the first immortal human cell line obtained from a cervical cancer patient, Henrietta Lacks during 1951) was taken to test the efficacy of ALLP. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic (PSN) in polystyrene tissue culture flasks obtained from NUNC (Roskilde, Denmark) at 37 ^{0}C in a humidified incubator with 5% CO_2. For experimental studies, cells were grown to 70 - 80% confluence, harvested with 0.025% trypsin and 0.52mM EDTA in phosphate buffered saline (PBS) and plated at desired density and allowed to re-equilibrate for 24 hours before any treatment. All experiments were conducted in DMEM containing 10% FBS and 1% PSN antibiotic.

Culture of *Leishmania donovani* promastigote:

Leishmania donovani promastigote (MHO/IN/1978/UR-6) were routinely maintained and grown in modified Ray's medium (J.C Roy, 1932). Cells were collected at stationary phage of growth and washed with PBS and stored at – 20 ^{0}C.

Isolation and purification of ALLP by Triton X-114 phase separation

In vitro cultured *Leishmania donovani* promastigotes, UR-6 were taken for ALLP isolation. Leishmanial cells were treated with a nonionic detergent, Triton X-114 to extract membrane lipoproteins according to the method described previously (Into et al., 2002; Bordier C, 1985; Gerlic et al., 2007) with little modifications (Majumdar et al., 2020). Briefly, leishmania promastigotes were suspended in Tris-EDTA buffer (50mM Tris-hydrochloride buffer, pH 8.0 with 0.15M NaCl and 1mM EDTA) containing protease inhibitor cocktail and combined with one-tenth volume of 25% (v/v) Triton X-114 working stock solution. The leishmanial proteins were solubilized for 2 hr at 4˚C and were then centrifuge at 10,000×g for 10 min to remove insoluble materials. The supernatant was transferred to a fresh tube. The tube was incubated at 37˚C for 10 min for phase separation, and then centrifuged at 10,000×g for 10 min. The upper aqueous phase was discarded. To the Triton X-114 phase, 9 volume of Tris-EDTA buffer was added and treated again in the same way. Lipoproteins present in the final Triton X-114 phase were taken for further purification with DEAE-cellulose chromatography. After DEAE-chromatography, purified lipoprotein was precipitated by adding 9 volumes of chilled ethanol. After a brief centrifugation at 10,000×g for 2 min at 4˚C, ethanol was discarded and the pellet was then dried out. The isolated lipoprotein was re-suspended in PBS and then used for stimulation. Protein concentrations of the isolated lipoprotein were determined by BCA protein estimation kit (Pierce,

USA). Lipoprotein samples were electrophoreticaly separated on a 10% SDS-PAGE gel and stained with Coomassie blue R250.

Estimation of protein content in ALLP

The quantity of protein content in ALLP was colorimetrically detected by the BCA™ Protein Assay kit (Pierce, USA) which is a detergent-compatible formulation based on bicinchoninic acid (BCA). The estimation was carried out according to the manufacturer's instructions.

Cell viability Assay

Cell viability was determined by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann et al., 1983). Cells were exposed to ALLP at concentrations ranging from 0 - 50 µg/ml for 48 hr. The intracellular formazon crystals formed were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm using an ELISA reader (Model: Emax, Molecular device, USA).

Assessment of cell morphology:

HeLa cells (3×10^4/well) were grown in 6-well plates in DMEM supplemented with 10% FBS for 24 hours and then were treated with or without ALLP (50µg/ml) for 48 hours. Morphological changes were observed with an inverted phase contrast microscope (Model: OLYMPUS 1X70, Olympus Optical Co. Ltd., Tokyo, Japan) and photographs were taken with the help of a digital camera (Olympus, Inc. Japan).

Fluorescence microscopy study:

To detect nuclear damage or chromatin condensation, cells were treated with or without ALLP (50µg/ml) for 48 hours, stained with 10 µg/ml of 4',6'-diamidino-2phenylindole (DAPI) and observed under fluorescence microscope. In order to distinguish the live, apoptotic and necrotic cells, the conventional Acridine orange/Ethidium bromide (AO/EB) staining procedures was followed. Cells treated with or without ALLP (50µg/ml) for 48 hr were stained with acridine orange (50µg/ml) and ethidium bromide (50µg/ml) and analyzed under fluorescence microscope with LASER beam excitation at 488 nm and 550 nm. Photographs were taken by a digital camera.

Detection of cell death by ELISA

Cell Death ELISA (Roche Molecular Biochemicals, Mannheim, Germany) was applied to detect cytosolic histone bound DNA fragments quantitatively in ALLP treated HeLa cells. Lysates of control and treated cells (with or without ALLP for 48 hours) were prepared and ELISA was carried out according to the manufacturer's instructions. Briefly, in the sandwich ELISA, cell lysates and whole nuclei (served as an antigen source) were added to wells pre-coated with primary anti-histone monoclonal antibody. This was followed by a second peroxidase coupled anti-DNA monoclonal antibody. The amount of histones was measured by the peroxidase retained in the immunocomplex. Peroxidase activity was determined spectrophotometrically at 405 nm with 2, 2'-azino-di (3-ethylbenzthiazolin-sulfonate) (ABTS) as a substrate by an ELISA reader (Model: Emax, Molecular device, USA).

Caspase-3, -8 activity assay

To further investigate the mechanism by which ALLP induced death in HeLa cells, activation of caspase-3 (an essential caspase of apoptotic process) and caspase-8 were examined. Caspase-3 and caspase-8 activities were quantified with commercially available CaspAceTM Assay system, Colorimetric (Promega, Madison, USA) and FLICE/Caspase-8 Colorimetric Assay kit (BioVision Research Products, Mountain View, CA) respectively. Assay was performed according to the manufacturer's instructions using Ac-DEVD-*p*-nitroaniline (Ac-DEVD-*p*NA) and Ac-IETD-*p*NA as substrates for caspase-3 and caspase-8 respectively. The proteolytic cleavage of the chromophore *p*-nitroaniline (*p*NA) from the labeled substrate Ac-DEVD-*p*NA and Ac-IETD-*p*NA was spectrophotometrically detected at 405 nm on ELISA reader (Model: Emax, Molecular device, USA).

Statistical analysis

All values were reported as mean ± SEM and Student's t test was used for determining statistical significance.

Results

SDS-PAGE profiles of attenuated leishmanial lipoprotein (ALLP)

The protein pattern obtained from TX-114 phase separation of stationary-phase promastigotes of *L. donovani* (UR-6) is shown in Figure 1. Lane 1: Standard protein markers; Lane-2 shows purified ALLP of approximately M_r 58,000; Lane-3 illustrates the pattern of proteins present in aqueous phase. The protein profiles of the aqueous phase clearly differ when compared with the ALLP. In lane-4, no band is shown due to the complete digestion of protein part of ALLP with Proteinase-K. Lane-5 shows the lipase digested ALLP band.

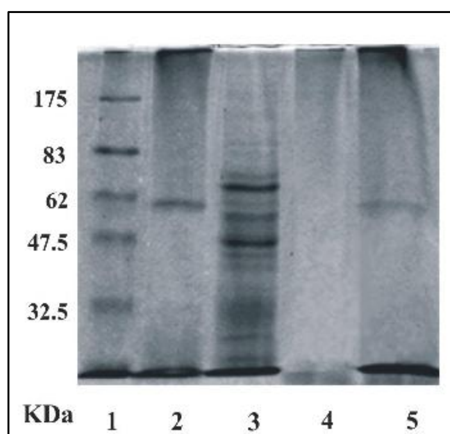

Figure 1

Figure 1. SDS-PAGE gel analysis of TX-114 phase separated proteins of L. donovani promastigotes UR6 (Majumdar et al., 2020).

ALLP reduces the viability of HeLa cells

To determine the effect of ALLP on the viability of HeLa cells was performed by MTT assay in which tetrazolium salt is cleaved and converted to a blue formazan by mitochondrial dehydrogenases of living cell. ALLP reduced the viability of HeLa cells in a dose (Figure 2A) and time-dependent manner (Figure 2B). After 48 hr treatment with 50 µg/ml of ALLP, viability of HeLa cells was found to be ~37%. Treatment with ALLP confirmed a decrease in the cell viability and an accumulation of cells floating in the culture medium, indicating ALLP-induced cell death.

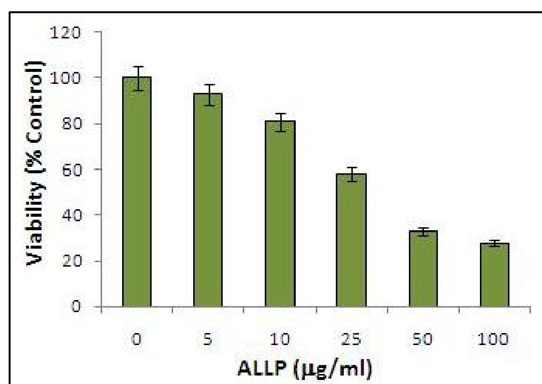

Figure 2A

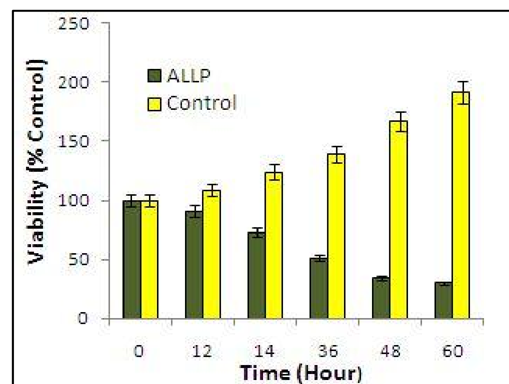

Figure 2B

Figure 2. ***Effect of ALLP on viability of HeLa cells. ALLP decreases the viability of HeLa cells in a dose (A) and time (B) dependent manner. HeLa cells (2 x10^6) were treated with indicated doses (0-100~g/ml) of ALLP for indicated time periods (0-60 h) and MTT assay was performed. The results are expressed as the mean ± SEM of three independent experiments. The viability index was compared to untreated control cells (100 %).***

ALLP induces morphological changes in HeLa cells

In order to determine whether ALLP induce apoptotic cell death, different cellular and morphological changes were studied. Phase contrast microscopy of ALLP treated HeLa cells revealed distinct morphological changes such as cell rounding, cell shrinkage, cytoplasmic blabbing and irregularities in cell counter indicating apoptotic cell death, compared to untreated control (Figure 3 Upper panel).

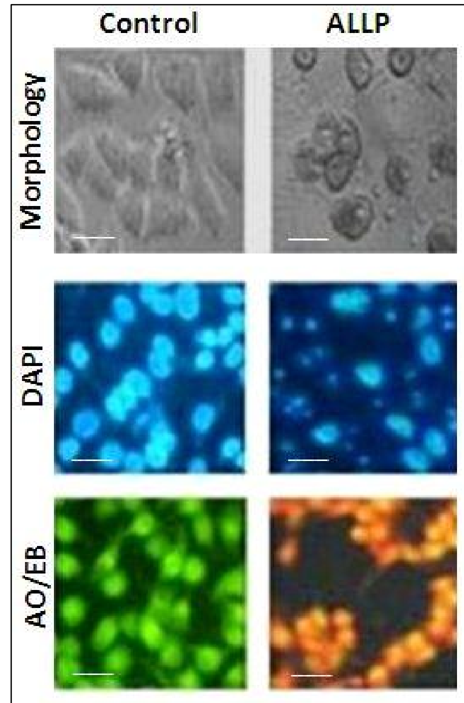

Figure 3

Figure 3. ***Morphology of HeLa cells were observed under a phase contrast microscope after treatment with or without (control) ALLP (50 µg/ml) for 48 hours (upper panel). Distinct morphological changes like cell rounding, cell shrinkage, cytoplasmic blebbing and irregularities in cell shape occur in ALLP treated cells. Middle and lower panel show distinct apoptotic nuclei in ALLP (50µg/ml) treated HeLa cells compared to untreated control cells. Cells were stained with DAPI (middle panel) or Acridine orange and Ethidium bromide (lower panel) and viewed under fluorescence microscope (bar represents 10 ~m for upper panel, 35 ~m for middle and lower panel).***

ALLP causes condensation and fragmentation of DNA in HeLa cells

The ALLP treated HeLa cells were examined for chromatin condensation and nuclear fragmentation (characteristic features of apoptotic cell death) using fluorescent DNA binding dye. Staining with DAPI revealed the appearance of chromatin condensation and nuclear fragmentation in much higher rate in ALLP treated HeLa cells than in the control (Figure 3. middle panel). When ALLP treated HeLa cells were stained with AO/EB dye mix, a major proportion of cells showed bright orange fluorescence with condensed or fragmented chromatin representing apoptosis in compare with control cells which showed green fluorescence (Figure 3. lower panel).

Cell Death ELISA was performed to detect DNA fragmentation quantitatively in ALLP treated HeLa cells. Figure 4 indicates a significant increase in the internucleosomal DNA fragmentation in ALLP treated HeLa cells after 48 hours, when analyzed by an ELISA based cell death detection kit. These ELISA based results correlates with the results of DNA fragmentation observed under fluorescence microscope.

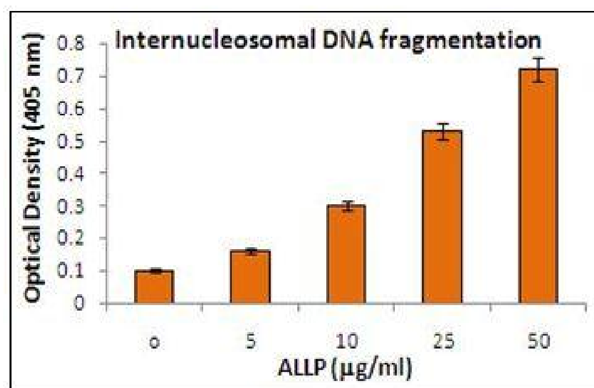

Figure 4

Figure 4. ***Effect of different concentrations of ALLP on internucleosomal DNA fragmentation in HeLa cells as measured by ELISA. The results are expressed as the mean ± SEM of three independent experiments.***

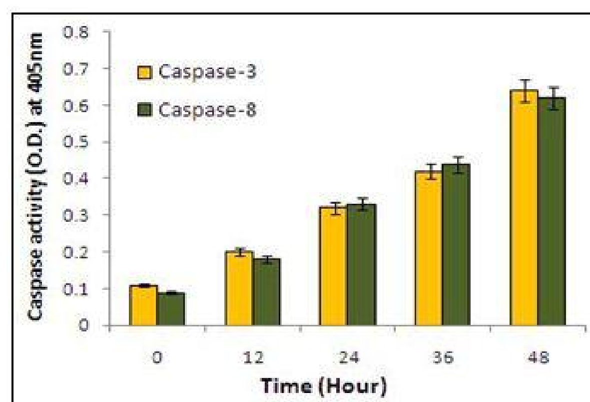

Figure 5

Figure 5. ***Spectrophotometric detection of caspase-3 and caspase-8 activities in HeLa cells treated with ALLP (50 µg/ml) for 48 hr. Cells were harvested by trypsinization, then lysed and processed for colorimetric assay as described in materials and methods. Result indicates an increasing caspase-3 and caspase-8 activity in ALLP treated cells in a time dependent manner. The results are expressed as the mean ± SEM of three independent experiments.***

As shown in Figure 5, Caspase-3 and Caspase-8 activity increased significantly in ALLP treated cells in a time dependent manner. As well as pretreatment with a specific caspase-3 inhibitor z-DEVD-fmk and caspase-8 inhibitor z-IETD-fmk prevented cell death in ALLP treated HeLa cell lines significantly. Moreover, a general caspase inhibitor z-VAD-fmk suppressed cell death considerably at a concentration of 100 µM (Figure 6).

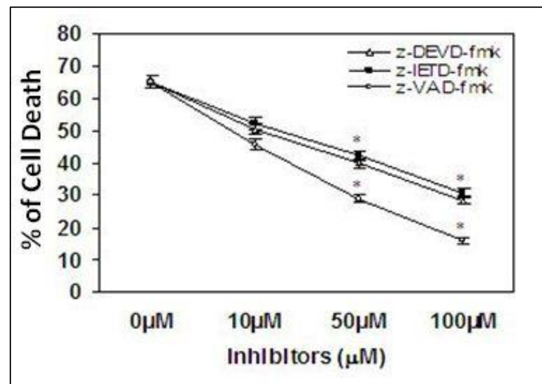

Figure 6

Figure 6. ***Effects of caspase inhibitors on the apoptotic activity of ALLP in HeLa cells. Cells were pretreated with different concentrations of each of the caspase-3 specific inhibitor z-DEVD-fmk, caspase-8 specific inhibitor z-IETD-fmk, and broad caspase inhibitor z-VAD-fmk separately for 1 hr and then exposed to 50μg/ml of ALLP for 48 hr. The results are expressed as the mean ± SEM of three independent experiments. * P≤0.001 represent significant differences between the experimental and control values.***

Discussion

The primary abnormality resulting in the development of cancer is the continual unregulated proliferation of transformed cells. Today, the treatment of cancer is highly variable and dependent on a number of factors including the type, location, and amount of disease and the health status of the patients. Chemotherapy is one of the most familiar treatments for cancer and has been applied for long days. While chemotherapy can be quite effective in treating certain cancer, chemotherapy drugs reach all parts of the body not just the cancer cells, thus results in a number of side effects during the cancer treatment. Chemotherapeutic drugs could theoretically treat all metastatic sites, but existing treatments do not provide a significant therapeutic benefit (Soengas and Lowe, 2003). One of the main reasons of disappointment in the treatment of cancer is the development of drug resistance by the cancer cells (Banik et al., 2018; Johnstone et al., 2002). The consequence is the re-growth of a tumor that is not susceptible to the original drug. Moreover, failure of apoptosis is a 'hallmark of cancer', an obligate ritual in the malignant transformation of normal cells. This is a very severe problem that may lead to recurrence of disease or even death. Therefore, induction of apoptosis selectively in hyper proliferating cancer cells is the main focus of cancer treatment today.

Microbial lipoproteins are now known to regulate a number of important cellular functions including cell growth or proliferation and death. On the other hand, *Leishmania sp.* (an eukaryotic protozoan parasite) has already been reported to have apoptotic effects on CD4+ and CD8+ cells of visceral leishmaniasis patients (Bertho et al., 2000). There is also an indication of apoptotic cell death with the reports of increased DNA damage, enhanced nitric oxide production and oxidative stress in patients with cutaneous leishmaniasis (Kocyigit et al., 2005). Furthermore, *Leishmania major* is known to induce apoptotic cell death in epidermal keratinocytes during cutaneous leishmaniasis (Eidsmo et al., 2007). Also, in a previous investigation it has been established that sphingolipid from an attenuated strain of *Leishmania donovani* promastigote (MHO/IN/1978/UR6) have apoptotic effects on melanoma cells (Ratha et al., 2006). So, there is an obvious interest to search of new components excepting the nature of apoptotic activity. It has also been found that lipoprotein isolated from attenuated *Leishmania donovani* promastigote (MHO/IN/1978/UR6) induces growth inhibition and cytotoxicity in melanoma cells (Majumdar et al., 2020). In this study, it has been investigated whether leishmanial lipoprotein has any growth inhibitory or apoptotic effects on mammalian cervical carcinoma cell line, HeLa.

Generally, all integral proteins of the cell membrane that bind asymmetrically to the phospholipids bilayer are combinations of proteins with lipids, such as fatty acids, triacylglycerol, cholesterol and phospholipids etc.

This type of proteins which have affinity both for hydrophobic groups and for water can be solubilized with detergents. Detergents are amphipathic substances that disrupt membranes by intercalating into phospholipid bilayers and solubilizing lipids and proteins. Triton X-114 (Polyethylene glycol tert-octylphenyl ether) is a nonionic detergent that lacks a charged group, enables protein solubilization with phase-partitioning of hydrophilic and amphiphilic proteins at low cloud point (23˚C). This detergent is homogeneous at 0˚C but divides in an aqueous phase and a detergent phase at above 20˚C. In this study, the non-ionic detergent Triton X-114 phase separation method had been performed to separate lipoprotein (ALLP) from stationary phase promastigotes of *L. donovani*. Triton X-114 detergent phase separation procedure, a well established method for the isolation of lipoprotein had also been successfully applied to separate integrated membrane proteins in *L. donovani* (Into et al., 2002; Gerlic et al., 2007). The protein profile of ALLP showed a single major protein of ~58 kDa.

To investigate the effect of ALLP on cell growth inhibition and apoptosis, in this study HeLa cells were taken. This study has revealed that ALLP reduced the viability of HeLa cells in a dose and time dependent manner.

Apoptosis is executed through an orchestrated series of biochemical events leading to characteristic cell morphology like cytoplasmic membrane blebbing, cell shrinkage, irregularities in cell shape, chromatin condensation, and internucleosomal DNA fragmentation etc which are distinct from necrosis. Several parameters were examined to establish whether ALLP induces apoptotic cell death rather than necrosis in the cell line. Phase contrast and fluorescence microscopy revealed distinct apoptotic morphology, chromatin condensation and fragmentation in ALLP treated cells. Inter-nucleosomal DNA fragmentation was again confirmed by cell death ELISA.

Apoptotic process is also characterized by a series of complex biochemical events carried out by a family of cysteine proteases called caspases (Cohen GM, 1997). The effects of caspases on ALLP induced cell death were examined using several caspase inhibitors. The broad caspase inhibitor z-VAD-fmk greatly suppressed the activity of ALLP at a concentration of 100µM. As well as inhibitors like z-DEVD-fmk and z-IETD-fmk also had significant suppressing effects on apoptosis inducing activity of ALLP. These findings indicate that caspase-3 and caspase-8 are involved in ALLP mediated cell death and there may be the possibilities of involvement of several other caspases, the activities of which are blocked by z-VAD-fmk.

It seems to be the first study to show that leishmanial lipoprotein is able to induce death in HeLa cells through apoptosis. This study demands further investigations to establish the detail mechanism of action of ALLP to induce apoptotic cell death in cancer cells.

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