DETERMINATION OF HUMAN T-LYMPHOCYTE APOPTOSIS MEDIATED BY BACTERIAL HEAT SHOCK PROTEIN

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ABSTRACT

DETERMINATION OF HUMAN T-LYMPHOCYTE APOPTOSIS MEDIATED BY BACTERIAL HEAT SHOCK PROTEIN

Periodontal diseases are the most common inflammatory disease worldwide which caused by the pathogenic organism living in biofilm. *Aggregatibacter Actinomycetemcomitans* (Aa) is the main player of the periodontitis disease pathology. Although some of the virulence factors of Aa has been identified up to now, its cytotoxic mechanism has not been clearly known yet. Although known virulence factors of Aa; ltx and cdt has been knocked out, the mutant Aa strains have retained the ability to induce apoptosis. Depending on the literature there must be another important virulence factor. 64kDa GroEL protein which is a molecular chaperone and a heat shock protein can be the potential candidate for being a virulence factor. AaGroEL protein has not been studied in terms of apoptosis up to now and it is not known how AaGroEL mediate immune regulation of T cells. In this study AaGroEL protein has been purified by using ATP Affinity chromatography and electroelution methods. After the purification step lps contamination has been removed by detoxi-gel endotoxin removal gel and detected by LAL Assay. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll Hypaque Density Gradient Centrifugation method. It was found that AaGroEL protein induces T cell apoptosis in dose and a time dependent manner. AaGroEL protein mediated T cell apoptosis has been detected by plasma membrane changes, activation of caspase-3 and DNA fragmentation. In conclusion, AaGroEL has antigenic properties that effect T lymphocytes by regulating immune response that would play important role in periodontal pathology.

ÖZET

BAKTERİYEL ISI ŞOKU PROTEİNİ KAYNAKLI İNSAN T-LENFOSİT APOPTOZUNUN TANIMLANMASI

Periodontal hastalıklar biofilmde yaşayan patojenik organizmaların sebep olduğu kronik inflammasyona yol açan en önemli hastalıklardır. *Aggregatibacter Actinomycetemcomitans* diş eti hastalıklarının patolojisinde rol oynayan bir bakteridir. Bazı önemli antijenik bileşenleri belirlendiği halde literatürde patolojideki rolü henüz tam olarak aydınlatılmamıştır. Aa bakterisinin önemli virulans faktörleri, cdt ve ltx genleri silindiği halde apoptotik aktivitesini kaybetmemiş olması diğer önemli bir antijenik proteinin varlığı düşündürmüştür.

Son yıllarda yapılan çalışmalar ile AaGroEL proteininin periodontal hastalıkların patolojisindeki rolü önem kazanmıştır. Bu proteininin T lenfositlerini nasıl etkilediği ve AaGroEL'e karşı oluşan T lenfosit immun yanıtının profili henüz bilinmemektedir. AaGroEL proteini ATP affinite kromatografi ve ardından elektroelution yöntemi ile izole edilmiştir. Lps kontaminasyonu detoxi-gel endotoxin removal kolonu kullanılarak elimine edilmiş ve LAL Assay ile belirlenmiştir. Periferal kandan beyaz kan hücrelerinin (PBMC) izolasyonu Ficoll Hypaque Density Gradient Centrifugation methodu ile yapılmış ve hücreler AaGroEL protein ile stimule edilmiştir. AaGroEL proteininin doza ve zamana bağlı olarak T lenfosit hücrelerinde apoptozu indüklediği ortaya konmuştur. AaGroEL proteininin T hücre apoptozu aktif kaspaz-3 enzimi ve DNA fragmentasyonu ile desteklenmiştir. Sonuç olarak AaGroEL proteini T lenfosit hücrelerinde apoptoza yol açarak bağışıklık sisteminin regulasyonunda rol oynamaktadır.

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ABBREVIATIONS

Aa	Aggregatibacter actinomycetemcomitans
AaGroEL	Aggregatibacter actinomycetemcomitans GroEL protein
cdt	Cytolethal distending toxin
ltx	Leukotoxin
Hsp	Heat shock protein
HRP	Horse radish peroxidase
EE	Electroeluted
BSA	Bovine serum albumin
LPS	Lipopolysaccharide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
Caspase	Cysteine-aspartic acid proteases
PE	Phycoerythrin
7AAD	7-Amino-Actinomycin D
FBS	Fetal bovine serum

CHAPTER 1

INTRODUCTION

Apoptosis is highly regulated programmed cell death which provides tissue homeostasis, regulation of the immune system, remodeling of tissues during development, removal of infected cells and keeps the balance of homeostasis (Opferman 2008), (Kerr, et al. 1972), (Horvitz 1999). Apoptosis is defined by condensation of cytoplasm, DNA cleavage, plasma membrane blebbing, nuclear degradation and formation of apoptotic bodies (Elmore 2007). It controls the immune response of pathogen-host relationship during infectious diseases (Hacker, et al. 2006). Defects in cell death cause a variety of pathological conditions (Yeretssian, et al. 2008), (Labbe and Saleh 2008), (Elmore 2008**).** Autoimmune diseases usually caused by disregulation of apoptosis in the immune response (Maniati, et al. 2008).

Periodontal diseases are the most common inflammatory disease world wide caused by the pathogenic organisms living in biofilm (Seymour, et al. 2007). *Porphyromonas gingivalis, Tannerella forsytenesis, Aggregatibacter actinomycetemcomitans* (Aa) are the major players of periodontitis. Periodontopathogenic bacteria live in the oral cavities and give a rise to chronic inflammation of the tissue by antigenic molecules (Oscarsson, et al. 2008). Chronic inflammation breaks down the balance of the system so it causes other systemic diseases such as cancer and asthma (Yeretssian, et al. 2008).

As a periodontal pathogen Aa is a gram negative nonmotile, anaerobic bacteria which also causes systemic diseases such as endocarditis, pericarditis, pneumonia, infectious arthritis, osteomylitis and skin infections (Slots 1982), (Van, et al. 2000), (Henderson, et al. 2002). Antigenic property of Aa has been defined up to now and it was shown that Aa induces apoptosis in macrophages, epithelial cells, T cells (Kato, et al. 1995), (Kato, et al. 2000), (Nalbant, et al. 2002). Some of the virulence factors have been identified such as; cdt which causes cell cycle inhibition , induces apoptosis, and

causes DNA fragmentation in T cells (Shenker, et al. 2001), (Shenker, et al. 2006) and Leukotoxin which has lethal effects in human T cells (Mangan, et al. 1991), (Korosstoff, et al. 1998), (Kieba, et al. 2008).

When both of the virulence factors (cdtABC, ltxA) were knocked out, the mutant strain of Aa retained the ability to induce apoptosis in T cells (Nalbant, et al. 2003). This statement shows that there must be another important virulence factor of Aa.

The antigenic activity of Aa can be related to the heat shock proteins, because it induces expression of Hsps during pathogenesis of periodontitis (Lokensgard, et al. 1994), (Goulhen, et al. 2003). Microbial Hsps are highly conserved proteins whose main role is to provide survival of microorganisms under stress conditions (Goulhen 2003). Hsps are expressed in normal conditions but up regulated under stressful condition. In spite of mediating physiology of the cells, heat shock proteins play a role in microbial pathogenesis (Gaston, et al. 1989), (Zugel and Kaufmann 1999), (Goulhen, et al. 2003), (Henderson, et al. 2006).

GroEL protein is a heat shock protein and a molecular chaperone which assists protein folding with its co-chaperone GroES, found mostly in cytoplasm, periplasm and membrane associated vesicles (Goulhen, et al. 1998), (Paju, et al. 2000). Although GroEL is a molecular chaperone, it has been suspected as being an antigen (Koga, et al. 1993), (Tabeta, et al. 2001). *Aggregatibacter Actinomycetemcomitans* GroEL (AaGroEL) causes epithelial cell proliferation in lower doses (Paju, et al. 1999), cytotoxic to epithelial cells in higher doses (Goulhen, et al. 1998). But the information about its cytotoxic effect is not clear, based on the literature it has not found any information about AaGroEL mediated cell death. It was proved that AaGroEL causes skin keratinocyte (HaCaT cell line) cell death by the activation of ERK and MAP (Zhang, et al. 2004). AaGroEL should have an important role in the bacterial-host pathogenity because induces bone resorption (Kirby, et al. 1995). Bacterial Hsps show high homology with eukaryotic Hsps. AaGroEL protein reacts with human fibronectin, (Yoshioka, et al. 2004), (Ando, et al. 1995) and has a high homology with human Hsp 60 (Ford, et al. 2005). Because of the similarities, immune response against bacterial Hsps can be destructive and end with an autoimmune disease (Wick, et al. 1999). Molecular mimicry shows that it is the major linkage between autoimmunity and microbial infection pathology.

Moreover depending on the literature there is no evidence about GroEL protein mediate T cell apoptosis. It is not known yet how AaGroEL regulate T cell immune response.

Studies about GroEL protein of other pathogenic microorganisms has support its antigenic property. GroEL protein of *Leqiolla pneumophila* has been defined as a virulence factor which plays role in bacterial adherence (Garduno, et al. 1998). *Chlamydia trachomatis* Hsp60 causes trophoblast apoptosis through Toll Like Receptor-4 (Equils, et al. 2006). *Mycobacterium tuberculosis* Hsp 65 plays a major role in bacterial virulence and induce cytokine expression (Lewthwaite, et al. 2001). *Chlamydia pneumonia* GroEL protein is related with the invasion of the bacteria to host cell (Frederick, et al. 2008).

In conclusion AaGroEL is a potential candidate for being a virulence factor. Because Nalbant and coworkers have show that Aa has another important virulence factor other that Cdt and Ltx, this study refers antigenic property of GroEL protein. There is not any evidence about antigenic property of GroEL protein and none of the studies report that it induces apoptosis. Although Goulhen and coworkers has reported that AaGroEL is cytotoxic to epithelial cells, it has not been investigated in terms of its apoptotic and antigenic property on T cells. The purpose of this study is to understand the antigenic property of AaGroEL protein in terms of apoptosis and to understand AaGroEL effect on T cell immune response.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All the materials used in this study follows as; *Aggregatibacter Actinomycetemcomitans* (Aa) (29522) type strain was obtained from American Type Cell Culture (ATCC) (Rockville, MD). Trypticase soy broth and Trypticase soy agar was purchased from Becton Dickinson. Adenosine 5'-triphosphate (ATP) agarose obtained from AppliChem. LAL Chromogenic Endpoint Assay Kit was provided by Hycult Biotechnology. Primary antibodies were obtained from StressGen Biotechnologies, HRP conjugated secondary antibodies were purchased from BioRad. Jurkat T cell line was obtained from American Type Cell Culture (ATCC), RPMI was taken from Invitrogene. Camptothecin was purchased from Sigma. Apoptosis labeling antibodies; AnnexinV, 7AAD, Caspase-3 were obtained from BD (phycoerytrin-PE). Phenotype labeling antibodies (anti-CD3) were purchased from Beckman coulter and BD. Mebstain Apoptosis detection kit was obtained from Immunotech.

2.2. Antigen Preparation

2.2.1. Bacterial Culture

Aa was first plated on selective trypticase soy agar (TSAV) and then colonies were selected and confirmed by their morphologies, gram staining, H_2O_2 test and polymerase chain reaction (PCR). Selected colonies of Aa were grown on selective trypticase soy broth (TSBV) for 48 h the media contains; 3% trypticase soy broth , 0.1% yeast extract and 10% horse serum with 75 µg/ml bacitracin (AppliChem, Darmstadt, Germany) and 5 µg/ml vancomycin (AppliChem, Darmstadt, Germany). Optimum conditions for bacterial growth were 37^{o}C and 5% CO_2 (Slots 1982). Bacterial cultures were incubated at 43^{o}C for 1 h in water bath to induce expression of heat shock proteins and then harvested. Cells were disrupted by sonification (30 cycle) and cell debris was removed by centrifugation 7500 rpm 1 h. Protein concentration of Aa sonic cell extracts (AaCE) were measured by Bradford protein assay. Cell extracts were aliquoted and stored at -80^{o}C.

2.2.2. Purification of *Aggregatibacter Actinomycetemcomitans* GroEL Protein

GroEL protein has been purified by ATP affinity chromatography method and electroeluted from SDS-PAGE (Hinode, et al. 1996). Briefly Adenosine 5'-triphosphate (ATP) agarose (5ml) (AppliChem, Darmstadt, Germany) was loaded on gravity column, washed with 5 bed volumes of dH_2O and equilibrated by 5 bed volumes equilibration buffer (20mM Hepes-NaOH, 2mM MgAc, 25mM KCl, 10mM Ammonium sulfate, 0.8mM DDT pH 7.0). After the ATP resin has been prepared, 1ml AaCE (2mg/ml) was loaded and incubated for 2 h, unbound proteins are washed with 5 bed volumes of

equilibration buffer. Proteins which have the affinity for ATP molecule bind to the agarose beads and then eluted by 5mM ATP in 5 bed volumes of elution buffer. ATP binding proteins are fractionated by elution buffer.

ATP Affinity chromatography fractions (400µg/ml) were diluted in gel loading buffer (62.5mM Tris-HCl, 1% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.05% Bromophenol blue), boiled for 5 min and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done under cooling conditions. SDS-PAGE gel rinsed with water for 5 min. After protein bands were stained with 300mM CuCl2, 64 kDa GroEL protein band was cut and destained by destaining buffer (250mM Tris-250mM EDTA buffer pH 9.0) for 10 min. Gel slices were washed with elution buffer (25mM Tris-125mM glycine-0.1% SDS). Samples were electroeluted for 3 h 50V in elution buffer, during the second hour of elution, buffer was exchanged to elution buffer without SDS. Purified AaGroEL protein was dialyzed over night against 10mM Tris-HCl buffer (pH 7.0). LPS contamination of AaGroEL protein was removed by using lps removing gel (Thermo, Fisher Scientific Inc, USA).

Furthermore AaGroEL protein was dialyzed against 1xPBS and concentrated against concentration buffer (Pierce Chemical Co, Rockford, IL) by using snakeskin dialysis tubing (Pierce Chemical Co, Rockford, IL).

2.2.3. Lps Removal

Lps concentration of purified AaGroEL was analyzed by LAL Chromogenic Endpoint Assay Kit (Hycult biotechnology, Canton, MA, USA). Detoxi-Gel Endotoxin Removing Gel (Thermo, Fisher Scientific Inc, USA) was used to remove lps contamination of purified samples (Ronneberger, 1977), (Kreeftenberg, 1977). Briefly Detoxi-Gel resin has immobilized polymyxin B to detect lps. Detoxi-Gel resin (1ml) was packed into centrifuge columns (Pierce Chemical Co, Rockford, IL), regenerated by 5 bed volumes of 1% sodium deoxycholate and washed by using endotoxin free dH_2O. Purified AaGroEL (5ml) was loaded on Detoxi-Gel resin and incubated for 1 h.

After the incubation AaGroEL was collected by using gravity column and Detoxi-Gel resin was regenerated to remove bound lps from the resin.

2.2.4. Characterization of AaGroEL Protein

Lps removed AaGroEL protein run on SDS-PAGE electrophoresis and AaGroEL protein bands were stained with coomassie brilliant blue R-250. Protein bands on SDS-PAGE gel were transferred to polyvinylidene fluoride membrane (PVDF) by using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA) and non specific binding sites on membrane were blocked in 5% non fat dried milk. Membrane was incubated with mouse monoclonal or polyclonal antibody for *E. coli* rGroEL at 1:5000 dilution (StressGen Biotechnologies, Victoria, BC, Canada) 2 h at 4^{o}C. After washing, membrane was incubated with anti-mouse HRP secondary antibody (StressGen Biotechnologies, Victoria BC, Canada) at 1:20000 dilution for 1 h. Following the washing, membrane was developed by Luminol/Enhancer-Peroxidase Buffer (Bio-Rad Laboratories, Richmond, CA) and exposed against film for 30 seconds. Samples were cut from gel slices and send to Proteome Factory (Berlin, Germany) in 5% acetic acid solution, and analyzed by LC-ESI-MSMS by tyrpsin cleavage.

2.2. Determination of Antigenic Property of AaGroEL Protein

2.3.1. Subject Selection and Isolation of Peripheral Blood Mononuclear Cells

Peripheral Blood was taken from healthy volunteers by İYTE Health Service persons. Blood donors were systemically healthy, non smoking and under the age of 50. They have signed an inform consent form which was submitted by Dokuz Eylül University Medical School, Clinic and Laboratory Research Ethical Committee Permission. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll Hypaque Density Gradient Centrifugation method (Böyum 1968).

2.3.2. Cell Culture

PBMCs and Jurkat T cells are cultured with RPMI 1640 (Invitrogene, Carlsbad, CA) with 10% FBS (Invitrogene, Carlsbad, CA) and 25mM Hepes (Invitrogene, Carlsbad, CA) as $2x10^6$ cells/ml at 5% CO_2 and 37^oC.

RPMI alone used as a negative control and camptothecin 4μM (Sigma-Aldrich, St. Louis, MO) as a positive control. Stimulants in cell culture were AaGroEL protein, recombinant AaGroEL protein, *E.coli* recombinant GroEL protein, BSA and also other bacterial heat shock proteins, Hsp 65 from *Mycobacterium bovis*, Hsp 71 from *Mycobacterium tuberculosis.* Different dose of stimulants has been used in this study.

BSA and *E.coli* recombinant GroEL protein were purified and used as a control of the purification process. Electroelution buffer was used as a control of the side effects of the purification process.

During this study all the samples were triplicate and samples were compared to negative control by using student's t test. (*$p<0.05$)

2.3.3. Detection of Early Apoptosis by Annexin V and 7AAD Labeling

PBMCs or Jurkat T cells were washed with 500µl 1xPBS and cells resuspended in 20µl Ca^{++} binding buffer, stained with AnnexinV and 7AAD (Becton Dickinson, Mountain View, CA). AnnexinV and 7AAD incubated with cells for 30 min. After the incubation, cells were acquired by Flow Cytometry and analyzed.

AnnexinV (Becton Dickinson, Mountain View, CA) is bound to phosphatidylserine to detect early apoptotic cells. 7-amino-actinomycin (7AAD) (Becton Dickinson, Mountain View, CA) is a DNA binding probe which detects late apoptotic cells. AnnexinV+ cells are early apoptotic; AnnexinV+7AAD+ cells are apoptotic, only 7AAD+ cells are late apoptotic cells.

2.3.4. Detection of Late Apoptosis by Active Caspase-3 Staining

Cells were washed two times with 500µl 1xPBS and supernant was removed. If PBMCs were used in the culture, lymphocytes were labeled by CD3 antibody. Cells fixed by 50 µl fixation solution (5.5% formaldehyde with 1xPBS) for 15 min in room temperature. Fixed cells were washed and then permeabilized by 30µl permation buffer (saponin, 0.1% NaN_3 with 1xPBS) for 5 min in room temperature. Permeabilized cells were incubated with active caspase-3 antibody (Becton Dickinson, Mountain View, CA) for 15 min in room temperature. Cells were washed and pellet was resuspended in 1xPBS with 0.5% PFA and analyzed with Flow Cytometry.

2.3.5. Inhibition of Apoptosis by Caspase Inhibitor

PBMCs were pretreated with 50µM general caspase inhibitor Z-VAD-FMK (Becton Dickinson, Mountain View, CA) for 1 h at 37^{o}C 5% CO_2**.** After the incubation cells was washed with 1xPBS and cultured with antigens for 72 h. At the end of the incubation cells were labeled by AnnexinV and 7AAD, and then acquired by Flow Cytometry.

2.3.6. Detection of DNA Fragmentation by Fluorescent Microscopy

DNA fragmentation was detected by using MEBSTAIN Apoptosis detection kit (Immunotech, France). Briefly, PBMCs were washed with 1ml 1xPBS with 0.2% BSA and fixed by 4% paraformaldehyde buffer 30 min at 4^{o}C. After fixation cells were washed and pellet was permeabilized with 100 µl 70% ethanol 30 min in -20^{o}C. Then cells were washed with 1ml 1xPBS with 0.2% BSA and ethanol was discarded. Fixed and permeabilized cells resuspended in DNA labeling solution 30µl TdT (FITC) and incubated for 1 h at 37^{o}C. After incubation cells were washed with 1xPBS with 0.2% BSA and analyzed by Fluorescent Microscopy (magnification x20) (Olymphus).

CHAPTER 3

RESULTS

3.1. Purification and Characterization of AaGroEL Protein

Aa 64kDa GroEL protein has been purified and characterized to used as an antigen. The bacterial culture samples were heat shocked (43^{o}C) to induce the expression of the heat shock proteins, but as a control group some of the samples were left at 37^{o}C (Figure 3.1.a). GroEL protein is a heat shock protein, so that its expression has been upregulated by heat before purification process. There is an apparent expression difference between heat shocked (43^{o}C) AaCE and non-heat shocked (37^{o}C). GroEL is a molecular chaperone which has an affinity for ATP molecule. In the purification process ATP affinity chromatography method is used, concentrations of the fractions were measured by Bradford protein assay. ATP fractions were analyzed by SDS PAGE electrophoresis and Western Blotting (Figure 3.1.b). AaCE proteome has more than one protein which has the affinity for ATP molecule so that ATP Affinity fractions were not pure enough (Figure 3.1.b). Other heat shock proteins such as Hsp27, Hsp45, Hsp70 can be seen in the fractions. It is not possible to purify pure GroEL protein by ATP affinity chromatography, there has to be more specific purification method**.** 64kDa GroEL protein was electroeluted from SDS gel. Protein bands were stained with 300mM $CuCl_2$, 64kDa GroEL band was cut and destained. Gel slices were washed with elution buffer and then samples were electroeluted for 3 h for 50 V in elution buffer, during the second hour of elution, buffer was exchanged to elution buffer without SDS. Purified AaGroEL protein was dialyzed over night against 10mM Tris-HCl buffer (pH 7.0). Protein concentrations are determined by Bradford protein assay. Western blotting was performed to identify the GroEL protein in the samples by using

mouse polyclonal antibody for *E. coli* rGroEL (Figure 3.1.c). AaGroEL protein has been confirmed by Mass spectrophotometry after purification step.

After the preparation of the antigens, antigenic property of AaCE, ATP fraction and AaGroEL protein examined on T cells for 48 h, RPMI alone has been used as a negative control and camptothecin has been used as a positive control. Cultures were labeled by AnnexinV and 7AAD, acquired by Flow Cytometry. At the end of the 48 h incubation 7% cells were AnnexinV+ for unstimulated cells but AaCE (100ng) and ATP (100ng) fractions induced 40% of AnnexinV+ cells. AaGroEL (100ng) induce 2 fold increased of AnnexinV+ cells when compared to negative control (Figure 3.1.d).

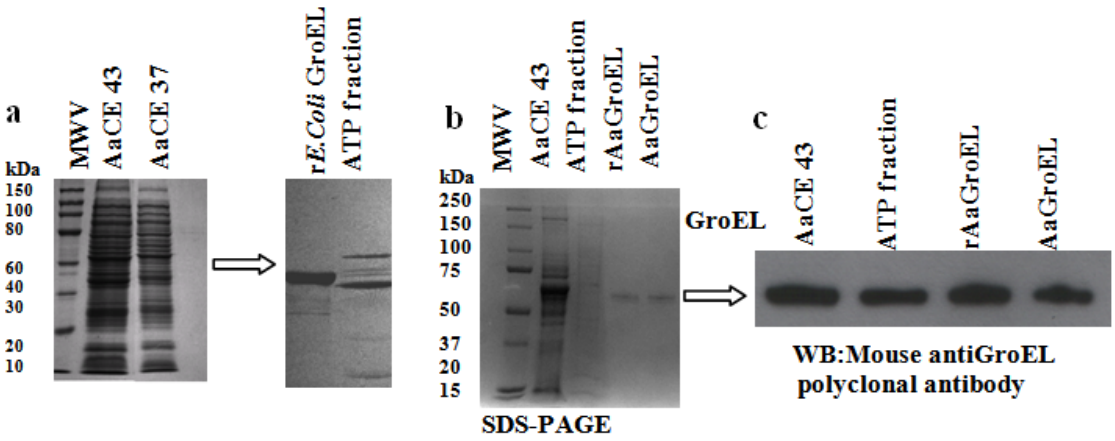

Figure 3.1.Purification and Characterization of AaGroEL Protein. a) Aggregatibacter *Actinomycetemcomitans* (29522) Cell Extract and ATP fractions in SDS-PAGE Gel. Lane 1;Molecular weight marker, lane 2;AaCE heat shocked at 43^{o}C, lane 3; AaCE 37^{o}C control sample not heat shocked. Samples were loaded on 8% SDS gel and runned at 56V 30 min and 105V 1 h, stained by coomassie brilliant blue R-250. Purification by ATP Affinity Chromatography; AaCE was loaded on Adenosine 5'-triphosphate (ATP) agarose and fractions were collected by 5mM of ATP. ATP fractions loaded on SDS gel, lane 1;*E.coli* rGroEL (500ng), lane 2;ATP fraction (5µg), run at 56V 30 min and 105V 1 h. SDS gel stained with Coomassie brilliant blue R–250. b) Purification by Electroelution from SDS PAGE Electrophoresis. AaGroEL protein was electroeluted from SDS gel. Samples were loaded on 8% SDS gel, runned 56V 30 min, and 105V 1 h. Lane1;Molecular weight marker, lane2;AaCE (500ng), lane 3;ATP (250ng), lane 4;rAaGroEL (100ng), lane 5;AaGroEL (250ng). SDS gel was stained by coomassie brilliant blue R-250. c) AaGroEL protein was identified by mouse anti-GroEL polyclonal antibody. Lane 1;AaCE (500ng), lane 2;ATP (250ng), lane 3;rAaGroEL (100ng), lane 4;AaGroEL (250ng) Samples were loaded on 8% SDS gel and run at 56V 30 min and 105V 1 h and transferred to PVDF membrane. Membrane was blocked by 5% non fat dried milk, and incubated by mouse anti-GroEL polyclonal antibody (1:5000) for 2 h, anti-mouse HRP secondary antibody (1:20000) for 1 h following the washing membrane was developed by Luminol/Enhancer-Peroxidase Buffer and exposed against film for 30 seconds.

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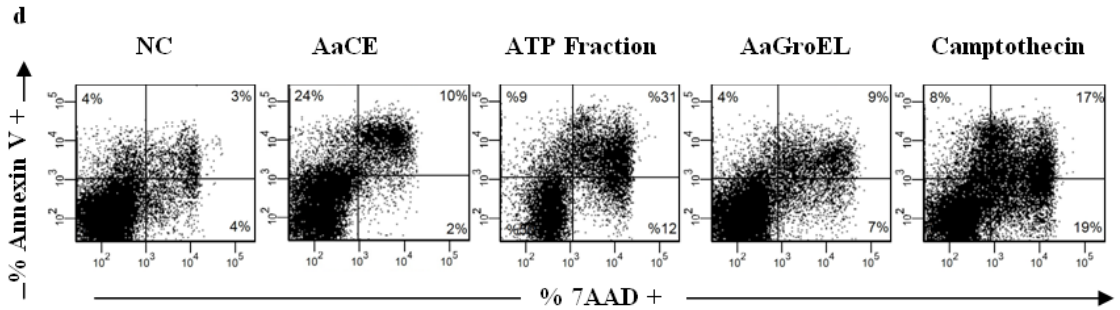

Figure 3.1. (cont.) Purification and Characterization of AaGroEL Protein. d) Lymphocyte Apoptosis Induced by Antigenic Property of AaGroEL protein. PBMCs were cultured with AaCE (100ng), ATP fraction (100ng), AaGroEL (100ng) and Camptothecin(4 μM) for 48 h. RPMI alone was used as a negative control, at the end of the incubation time cells were labeled with Annexin V and 7AAD, analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's ttest. (*$p<0.05$)

3.2. AaGroEL Mediated T Lymphocyte Apoptosis

3.2.1. Dose Response Assay

AaGroEL mediated apoptosis has been measured in terms of plasma membrane changes by AnnexinV and 7AAD. Antigenic and apoptotic effect of AaGroEL protein was determined by using PBMCs and Jurkat T cell. According to the experiments it induces apoptosis in a time and dose dependent manner. AaGroEL protein has been cultured with both PBMCs and Jurkat T cells to investigate the antigenic effect, it was reported that GroEL protein induces apoptosis both PBMCs and jurkat T cells depending on the dose.

PBMCs were isolated from peripheral blood and cultured with AaGroEL protein (1ng, 50ng, 100ng, 250ng, 500ng, 1000ng) for 0-96 h, RPMI alone was used as a negative control and camptothecin was used as a positive control. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD and acquired by Flow Cytometry. AaGroEL protein induced T cell apoptosis in an dose dependent manner, in response to 1ng AaGroEL T cells were nearly same as negative control but

500ng of AaGroEL protein cause apoptosis of 50% of T cell at 48 h, which has chosen as lethal dose (LD50) (Figure 3.2).

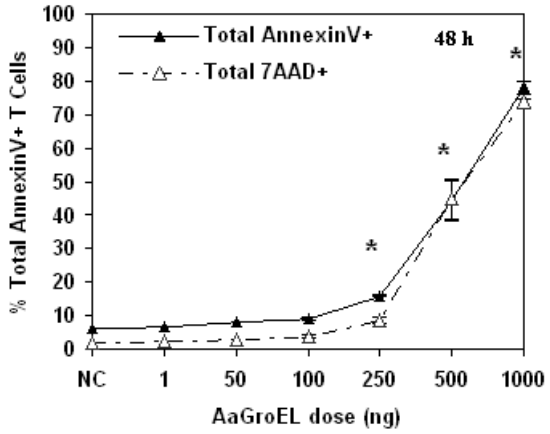

Figure 3.2. AaGroEL Mediated T Lymphocyte Apoptosis. PBMCs were isolated from peripheral blood and cultured with AaGroEL protein (1 ng, 50ng, 100ng, 250ng, 500ng, 1000ng) for 48 h, RPMI alone was used as a negative control. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD and analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's t test. (*$p<0.05$)

3.2.2. Time Course of T Lymphocyte Apoptosis Mediated by AaGroEL Protein (0-96H)

PBMCs were isolated from peripheral blood and cultured with AaGroEL protein (500ng) and rAaGroEL (20µg) for 0-96 h. RPMI alone was used as a negative control, Camptothecin used as a positive control. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD and acquired by Flow Cytometry. Apoptotic T cells were increased by time from 3% to 39% (Figure 3.3). AaGroEL induced AnnexinV+ T cells were 46% at 24 h, 57% at 48 h, but decreased after 72 h to 38%. AaGroEL mediate apoptotic process, its toxic effect was clear even at 24 h. Apoptotic activity of rAaGroEL protein was increased from 8% AnnexinV+ T cells to 36% AnnexinV+ T cells.

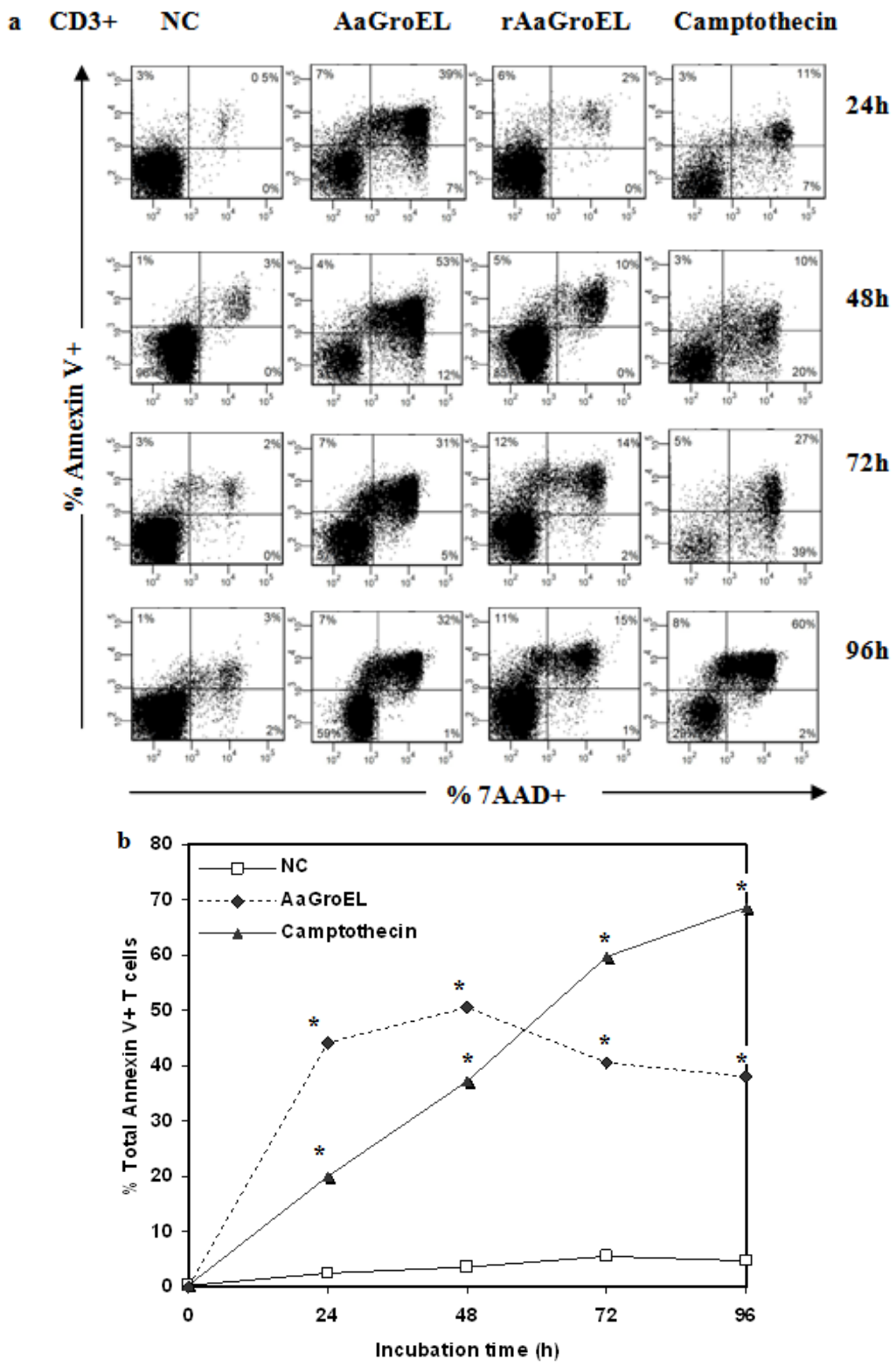

Figure 3.3. Time Kinetics of AaGroEL Mediated T Lymphocyte Apoptosis (0-96H). PBMCs were isolated from peripheral blood and cultured with AaGroEL protein (500ng) and rAaGroEL (20µg) for 0-96 h. RPMI alone was used as a negative control, Camptothecin used as a positive control. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD and analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's t test.

3.2.3.Determination of Purification Process Contributions to Apoptosis

Side effects of purification process were examined by using electroeluted *E.coli* rGroEL protein and BSA which were electroeluted at the same time with AaGroEL protein. Based on the literature *E.coli* rGroEL hasn't got an antigenic property; it has been shown that its property and functions weren't changed by purification process.

AaGroEL (100ng), *E.coli* rGroEL (100ng), Electroeluted- *E.coli* rGroEL (100ng), BSA (100ng), Electroeluted-BSA (100ng), Elution buffer, Camptothecin (4µm) were used in the culture as stimulants. At the end of the culture cells labeled with AnnexinV and 7AAD, acquired by Flow Cytometry.

BSA (bovine serum albumin) is not an antigenic protein that has no apoptotic effect; it has been proved that BSA (100ng) and EE-BSA (100ng) have the same effect on T cells; 7% AnnexinV+, 4% 7AAD+ nearly same as negative control (Figure 3.4). EE-*E.coli* GroEL protein did not show any antigenic property after purification process, 6.5% AnnexinV+, 3.5% 7AAD+. Another important control was elution buffer but also it has no side effect or any contamination. Obviously it has been shown that protein purification process has not got any side effects. When T cells cultured with AaGroEL, 100ng AaGroEL stimulated T cells were 12% apoptotic (Figure 3.4).

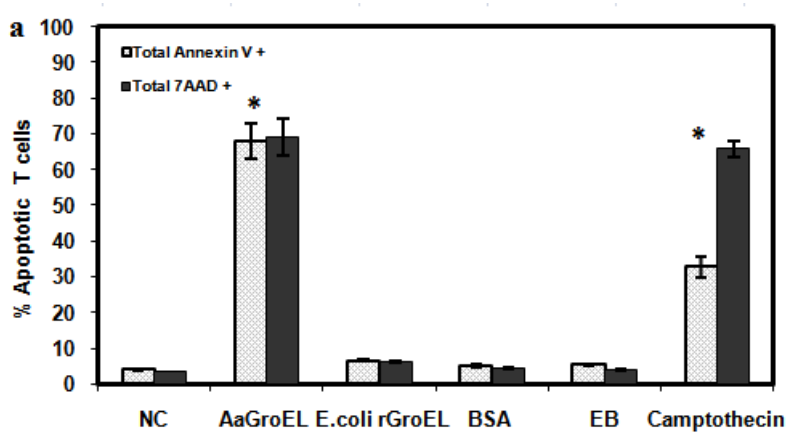

Figure 3.4. Determination of Purification Process Contributions to Apoptotic Effect of AaGroEL Protein. AaGroEL protein and other antigens were incubated with PBMCs for 48 h; RPMI alone was used as a negative control, AaGroEL (100ng), electroeluted- E.coli rGroEL (100ng), electroeluted-BSA (100ng), Camptothecin (4µm) were used in the culture as stimulants. At the end of the culture cells labeled with CD3, AnnexinV and 7AAD and analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's t test. (*$p<0.05$)

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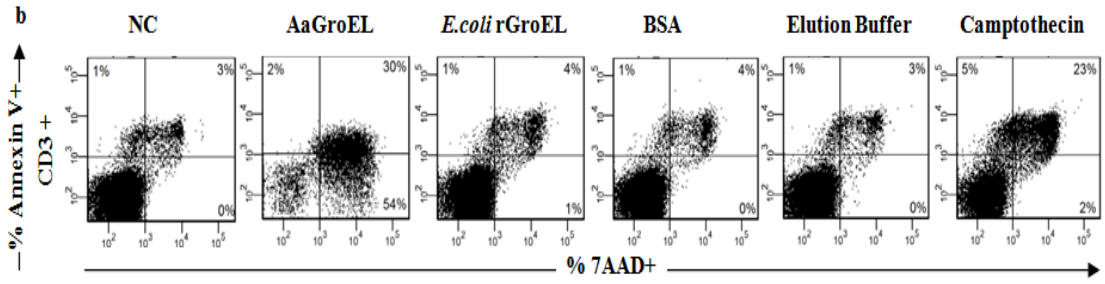

Figure 3.4. (cont.) Determination of Purification Process Contributions to Apoptotic Effect of AaGroEL Protein. AaGroEL protein and other antigens were incubated with PBMCs for 48 h; RPMI alone was used as a negative control, AaGroEL (100ng), electroeluted- E.coli rGroEL (100ng), electroeluted-BSA (100ng), Camptothecin (4µm) were used in the culture as stimulants. At the end of the culture cells labeled with CD3, AnnexinV and 7AAD and analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's t test. (*$p<0.05$)

3.2.4. LPS Effect on Apoptosis

Lps contamination of purified AaGroEL protein was detected by using LAL Assay and removed by Detoxi-Gel Endotoxin Removing Gel. All the experiments have been done by using AaGroEL after lps removal.

AaGroEL (100ng) was cultured with polymyxin B (PMN) to neutralize the effect of LPS (Lewthwaite, et al. 2001). LPS effect of T cell apoptosis was investigated by using both AaGroEL before lps removal, AaGroEL after lps removal and both of the samples were incubated with PMN. RPMI alone was used as a negative control, at the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD, analyzed by Flow Cytometry. Apoptotic effect of AaGroEL protein decreases after lps removal, but lps removed AaGroEL (100ng) induced apoptosis of T cells 13% and lps removed AaGroEL (100ng) with PMN induced apoptosis of T cells 14%. Lps effect on antigenic property of AaGroEL can be obviously seen but after lps removal PMN treatment did not cause any significant decrease in apoptosis. This results show that lps contamination of AaGroEL protein has been removed. (Figure 3.5.a)

Lps contamination can be controlled by heat inactivation because lps doesn't disrupted by heat (Lewthwaite, et al. 2001). AaGroEL protein (250ng) and lps (250ng) were boiled for 20 min, autoclaved and treated with proteinase K for 1 h and cultured

with PBMCs for 48 h. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD, analyzed by Flow Cytometry. After these steps lps is stable in high temperatures but AaGroEL has lost its. Lps activity can not inhibited by heat treatment but AaGroEL activity decreased (Figure 3.5.b).

Lps contamination has been removed from AaGroEL protein and its activity of apoptosis decreased in response to lps removal but remained as an important antigenic property (Figure 3.5.b).

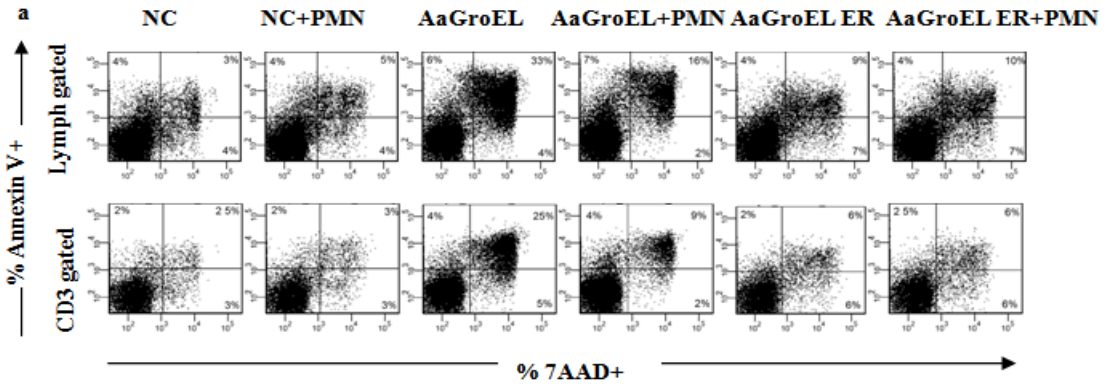

Figure 3.5. LPS Effect on AaGroEL Induced Apoptosis. PBMCs were isolated from peripheral blood and cultured with AaGroEL protein for 48 h. a) AaGroEL protein (100ng) cultured with polymyxin B to neutralize the effect of LPS. RPMI alone was used as a negative control, at the end of the incubation time cells were labeled with AnnexinV and 7AAD, analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's ttest.

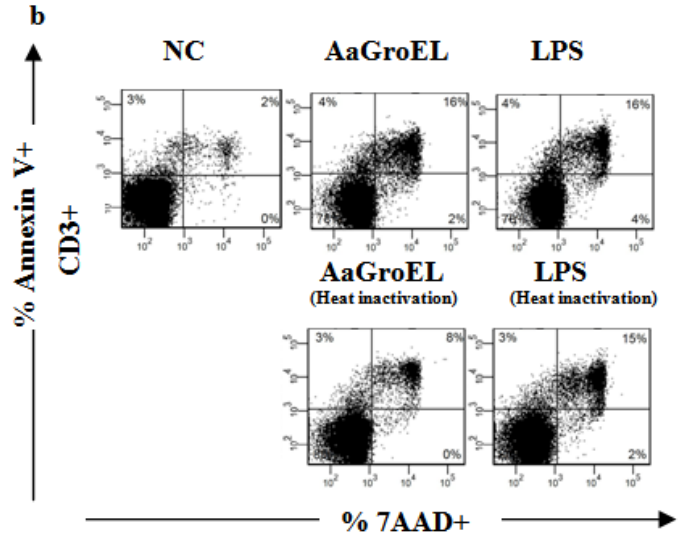

Figure 3.5. LPS Effect on AaGroEL Induced Apoptosis. b) AaGroEL protein (250ng) and LPS (250ng) were boiled for 20 min, autoclaved and treated with proteinase K for 1 h and cultured with PBMCs for 48 h. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD, analyzed by Flow Cytometry.

3.2.5. Determination of Apoptosis by Active Caspase-3

Activation of caspase-3 is an important step in the apoptotic process. Apoptotic pathways are results with caspase-3 activation which has been called as point of no return in the programmed cell death. Caspase-3 activation and DNA fragmentation is the important features of apoptosis.

PBMCs were cultured with AaGroEL. RPMI alone used as a negative control and camptothecin (4µM) used as a positive control. Following the culture cells were labeled by CD3 antibody, fixed and permeabilized cells were labeled with anti active caspase-3 and analyzed by Flow Cytometry. Significantly 8 fold increase of active caspase-3 was detected in response to AaGroEL (500ng), Camptothecin induce expression of active caspase-3 for 7 fold at 72 h (Figure 3.6.a). Our data reported that caspase-3 is important in AaGroEL mediated apoptosis.

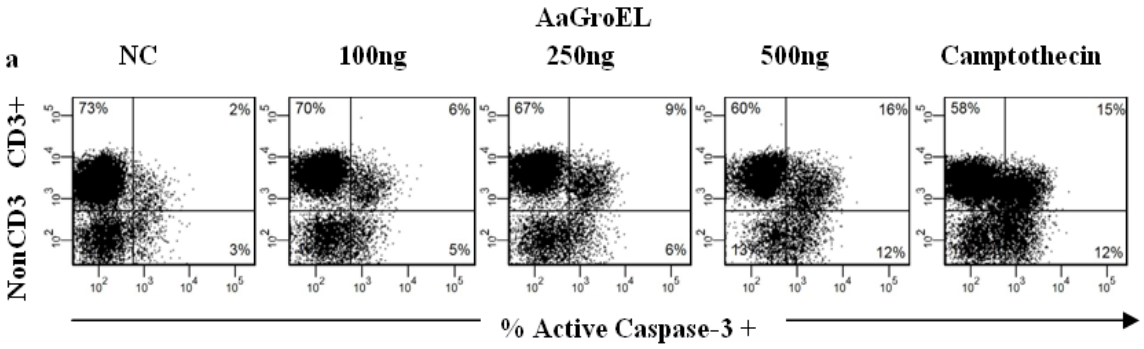

Figure 3.6. AaGroEL Mediated T cell Apoptosis Measured by Active Caspase-3 and Caspase Inhibition assay. a) PBMCs have been cultured with AaGroEL (100ng, 250ng, 500ng). RPMI alone used as a negative control and camptothecin (4µM) used as a positive control. Following the culture cells were labeled by CD3 antibody, fixed by fixation solution (5.5% formaldehyde with 1xPBS). Fixed cells were permeabilized by using permation buffer (saponin, 0.1% NaN_3 with 1xPBS). Cells were labeled with anti active caspase-3 and analyzed by Flow Cytometry. All the samples were triplicate and compared to negative control by using student's t test.

PBMCs were incubated with general caspase inhibitor (Z-VAD-FMK) for 1 h at 37^{o}C to inhibit caspases during apoptotic process. After the incubation, PBMCs were cultured with AaGroEL (100ng), rAaGroEL (20µg) and camptothecin (4µM) for 72 h. At the end of the culture cells were labeled with anti-CD3 antibody, AnnexinV and 7AAD and acquired by Flow Cytometry. Significant amount of decrease has been seen in AnnexinV+ cells inhibited by caspase inhibitor at 72 h. AaGroEL protein (100ng)

stimulated lymphocytes were 13% AnnexinV+, 9% 7AAD+, caspase inhibited lymphocytes were 6.5% AnnexinV+, 4.5% 7AAD+. rAaGroEL (20µg) stimulated lymphocyte apoptosis decreased from 26% AnnexinV+, 16% 7AAD+ to 13% AnnexinV, 10% 7AAD+ (Figure 3.6.b).

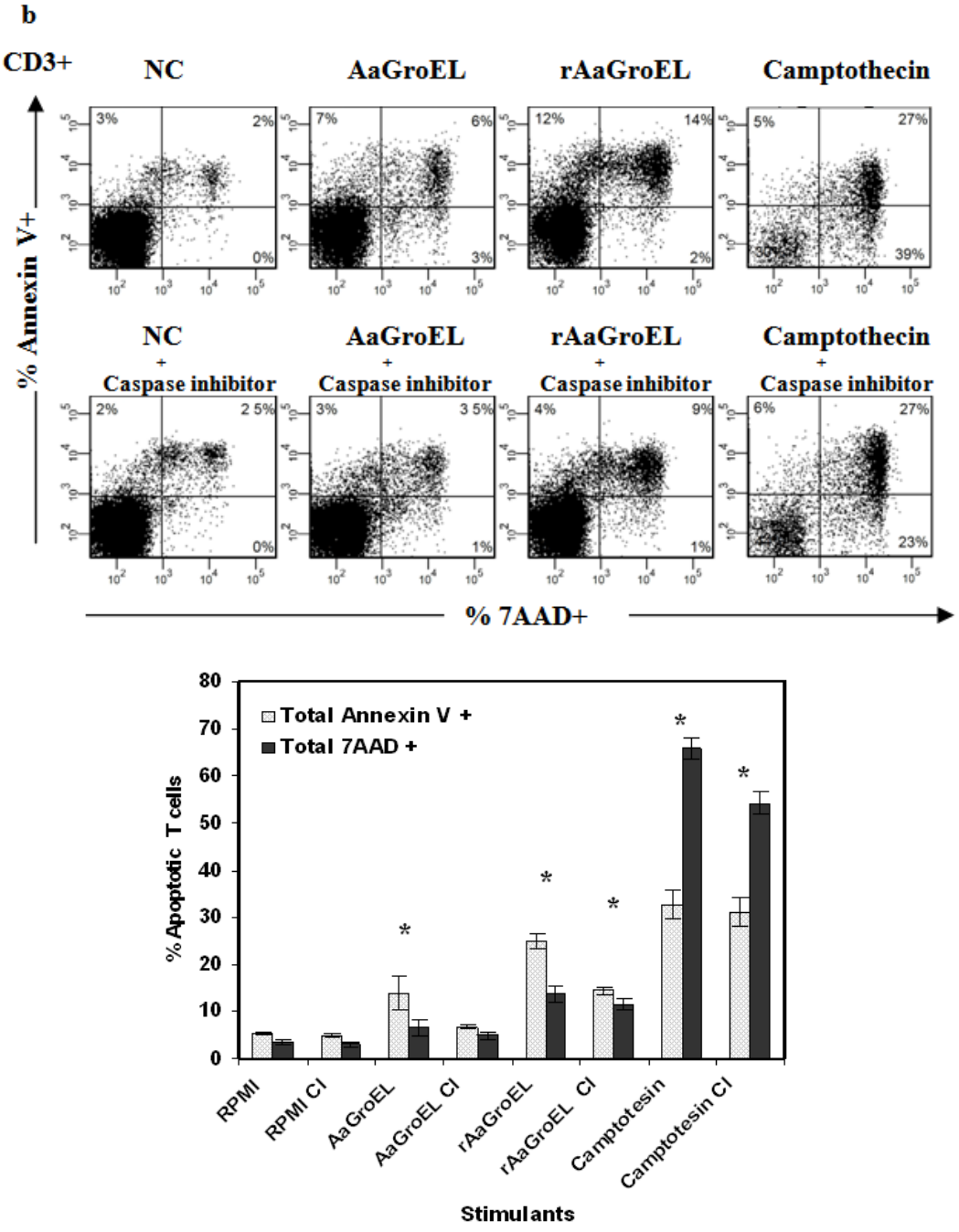

Figure 3.6. AaGroEL Mediated T cell Apoptosis Measured by Active Caspase-3 and Caspase Inhibition assay. b) Caspase Inhibition Assay. PBMCs were incubated with general caspase inhibitor (Z-VAD-FMK) for 1 h at 37°C. After the incubation, PBMCs were cultured with AaGroEL (100ng), rAaGroEL (20µg) and camptothecin (4µM) for 72 h. At the end of the culture cells were labeled with anti-CD3 antibody, AnnexinV and 7AAD and analyzed by Flow Cytometry. (*p<0.05)

3.2.6. Measurement of Apoptosis by DNA Fragmentation Assay

DNA fragmentation is the latest stage of the apoptotic process. AaGroEL mediated apoptosis induces caspase-3 activation. Active caspase-3 is the irreversible point of apoptosis and cause DNA fragmentation. PBMCs were treated with AaGroEL (100ng) and Camptothecin (4µM) for 72 h, RPMI alone used as a negative control and camptothecin used as a positive control. At the end of the incubation time cells were stained by MEBSTAIN Apoptosis detection kit, and analyzed by Fluorescent Microscopy (magnification x20). Fragmented DNA positively labeled by BrdU-FITC and AaGroEL protein stimulated apoptotic cells were detected. A great amount of increase in the BrdU+ cells has been seen when compared to negative control (Figure 3.7). Samples acquired by Flow Cytometry to analyzed apoptosis in terms of plasma membrane changes (Figure 3.7).

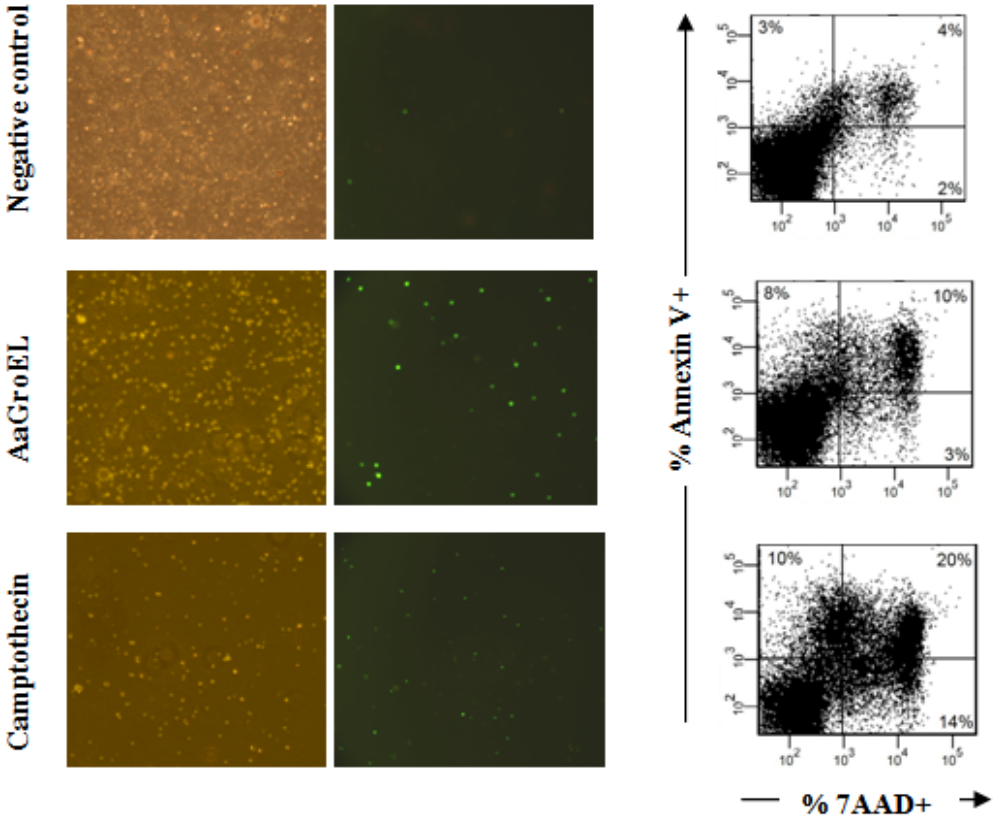

Figure 3.7. DNA Fragmentation Assay. PBMCs were treated with AaGroEL (100ng) and Camptothecin (4µM) for 72 h, RPMI alone used as a negative control. At the end of the incubation time cells were stained by MEBSTAIN Apoptosis detection kit, and analyzed by Fluorescent Microscopy (magnification x20).

3.3. Antigenic Property of Other Heat Shock Proteins on T Cells

Depending on the results up to now AaGroEL protein induces apoptosis in PBMCs and T lymphocytes. AaGroEL mediated apoptosis has been measured by plasma membrane changes, caspase-3 activation and also DNA fragmentation. In this study Hsp 65 from ***Mycobacterium bovis*** and Hsp 71 from ***Mycobacterium tuberculosis*** were investigated in terms of apoptosis to compare with antigenic activity of AaGroEL protein. PBMCs were treated with Hsp 65 from ***Mycobacterium bovis*** (500ng) and Hsp 71 from ***Mycobacterium tuberculosis*** (500ng) and RPMI alone used as a negative control. Following the culture cells labeled with AnnexinV and 7AAD and acquired by Flow Cytometer. Once its activity compared to AaGroEL protein, both Hsp 65 and Hsp 71 do not have any antigenic or apoptotic activity.(Figure 3.8)

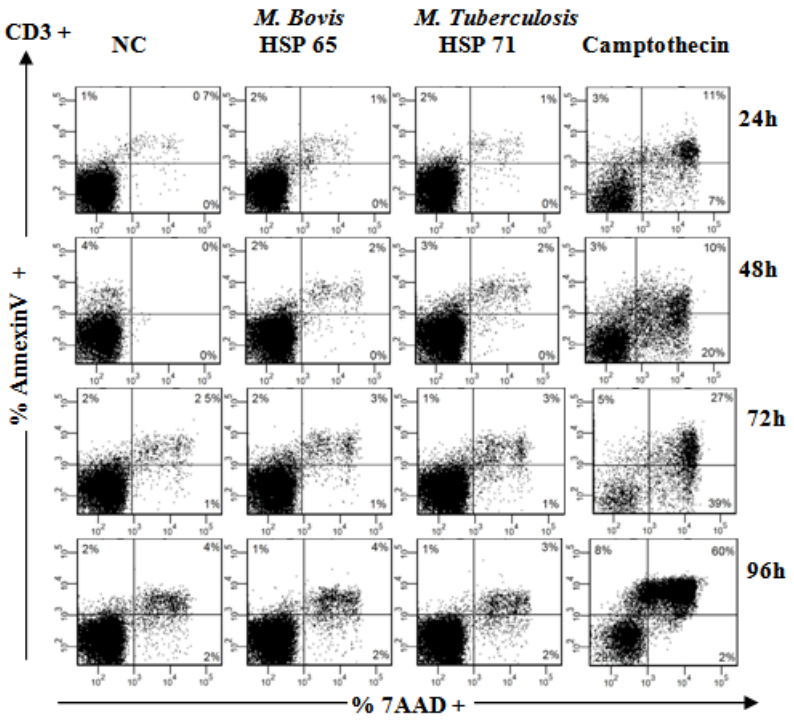

Figure 3.8. T cell Apoptosis of Other Heat Shock Proteins. PBMC were cultured with Hsp 65 (500ng), Hsp 71 (500ng) for 24-96 h. RPMI alone was used as a negative control. At the end of the incubation time cells were labeled with anti-CD3, AnnexinV and 7AAD and analyzed by Flow Cytometry. All the samples were triplicate and samples were compared to negative control by using student's t test.

CHAPTER 4

DISCUSSION

Aggregatibacter actinomycetemcomitans (Aa) is one of the most important peridonthopathogenic bacteria, which also contribute to other systemic infections (Slots 1982), (Van, et al. 2000), (Henderson, et al. 2002). Recent studies have demonstrated its virulence factors, but GroEL protein can be one of the major candidate which have an important role in pathology (Nalbant, et al. 2003). Function of GroEL protein has been proved many years ago as being a molecular chaperone. But up to now although some of the studies have referred cytotoxic property of AaGroEL, it has not been clearly identified yet. In literature, there isn't any information about AaGroEL protein in terms of Apoptosis. Depending on the literature there were some studies that infer GroEL protein can be a candidate antigen but none of the studies have showed AaGroEL mediated T cell apoptosis. It has been investigated on epithelial cells; its effect on epithelial cells goes from proliferation to cell death in a dose dependent manner (Paju, et al. 1999), (Goulhen, et al. 1998).

In this study AaGroEL protein has been purified by ATP affinity chromatography and electroeluted, its purity has been identified by mass spectrophotometry and Western Blot. Lps contamination has been removed and detected by LAL Assay. AaGroEL protein has been cultured with T cells (0-96h), it has been found that AaGroEL induces apoptosis in a dose and a time dependent manner. AaGroEL (500ng) kills 50% of T cells in 48h. This study demonstrates that AaGroEL has an important antigenic property and mediate T lymphocyte apoptosis. Our data has strong evidences that purification process has not got any side effects or contributions to its activity. In order to prove this statement BSA and *E.coli* GroEL protein has been purified at the same time, same conditions, and cultured with PBMC, neither EE-BSA nor EE-*E.coli* GroEL induce apoptosis of PBMCs or T cells, their activity is nearly same as negative control (RPMI alone). Also AaGroEL protein has been inactivated by heat to control the contamination of lps and cultured with T cells, but it lost all of the

apoptotic effect. The hypothesis has been supported by all the investigations and controls for the side effects, apoptotic property of AaGroEL protein has been proved on T cells. Apoptosis has been studied by AnnexinV-7AAD labeling, caspase 3 labeling and also DNA fragmentation.

Activation of capase-3 is induced by caspase-3 labeling, when PBMCs has been cultured with caspase inhibitor Z-VAD-FMK, caspase activation can be down regulated.

GroEL also has been studied in other pathogenic organisms, *Chlamydia trachomatis* hsp60 cause trophoblast apoptosis through TLR4 (Equils, et al. 2006). Mycobacterium tuberculosis hsp 65 play a major role in bacterial virulence (Lewthwaite, et al. 2001). *Chlamydia pneumonia* GroEL protein is related with the invasion of the bacteria to host cell (Frederick, et al. 2008).

Although AaGroEL induce apoptosis in T cells, other Hsps of pathogenic bacteria haven't got the same antigenic property. In this study Hsp 65 and Hsp 71 has been used, Hsp 65 from *Mycobacterium Bovis* and Hsp 71 *Mycobacterium tuberculosis* haven't got any effect on T cells. AaGroEL toxicity is very strong in contrast to Hsp 65 and Hsp 71, so that mechanism of AaGroEL mediated apoptosis need further investigations.

AaGroEL protein plays an important role on bacterial pathogenesis, because of its cytotoxic effect on T cells. It established the idea that it mediates bacterial-host relationship in disease pathology and cause chronic inflammation of the tissue. Our studies show that AaGroEL protein has a great importance of inducing T cell apoptosis as an antigen in vitro and regulation of T cell mediated immune response.

CHAPTER 5

CONCLUSION

AaGroEL protein has been defined in terms of its apoptotic property and regulation of T cell immune response in this study. It was determined as an antigenic protein because it induces apoptosis on T lymphoctes. AaGroEL has been studied on PBMCs and Jurkat T cell, AaGroEL stimulated cells were apoptotic even at 24 h. Depending on the results its apoptotic property must be investigated for identify its mechanism. Our results demostrated that its apoptotic activity is observed by plasma membrane changes, caspase-3 activation and DNA fragmentation but mechanism of AaGroEL mediated apoptosis has to be elucidated. It is not known which apoptotic pathway is used in AaGroEL mediated apoptosis.

In conclusion the purpuse of this to identify AaGroEL mediated T lymphocte apoptosis, it was validated. But antigenic property of AaGroEL protein needs further investigations.

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