REVIEW ARTICLE

Immunology and Genetic of *Leishmania infantum*: The Role of Endonuclease G in the Apoptosis

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Leishmania infantum is the causative agent of infantile visceral leishmaniasis (VL) in the Mediterranean region. Despite developing protective responses, the disease progresses due to many of factors. These include the action of suppressive cytokines, exhaustion of specific T cells, loss of lymphoid tissue, and defective humoral response. Genetic changes that occur inside the genome of alienated or parasite cells, along with immune responses, play an important role in controlling or progressing the disease. Proapoptotic proteins such as Smac/DIABLO, EndoG, AIF (apoptosis-inducing factor), and cytochrome C are effective in apoptosis. EndoG is a mitochondrion-specific nuclease that translocates to the nucleus during apoptosis. Once released from mitochondria, endoG cleaves chromatin DNA into nucleosomal fragments independently of caspases. Therefore, endoG represents a caspase-independent apoptotic pathway initiated from the mitochondria. A comprehensive understanding of the immune and genetic events that occur during VL is very important for designing immunotherapy strategies and developing effective vaccines for disease prevention. In this review which explained the immunological responses and also the important factors that can contribute to parasite apoptosis and are used in subsequent studies as a target for the preparation of drugs or recombinant vaccines against parasites are briefly reviewed.

Key words: Apoptosis, endonuclease G, *Leishmania infantum*, vaccine

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INTRODUCTION

Leishmaniasis is an infectious disease with worldwide spread (especially in tropical and sub-tropical areas) and is transmitted by *Leishmania* protozoa parasites (from the genus of trypanosomes), which are unicellular eukaryotes. There are various clinical signs and symptoms of this infection such as skin lesion and presentation of ulceration on the skin, mouth and mucus as well as metastatic skin and mucus diseases and fatal systemic disturbances.[1] This disease is one of the most vital cross contaminated diseases between animal and humans and major health concern in many parts of the world including the four continents, Asia, Europe, Africa, and America.[2] This parasite lives in the macrophage cells of vertebras (mammalians) and is transmitted by sandflies to animals and human beings.[2]

Visceral leishmaniasis (VL), also known as, Kala-azar, is a dangerous infectious disease that is caused by *Leishmania donovani* species. However, the main cause of VL in the Mediterranean area (Middle East) including Iran is *Leishmania infantum*.[3] In Iran, VL is mostly prevalent in children and people who live in rural areas or villages. The visceral infection can occur with or without signs

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and symptoms, and in some areas, the silent type of this disease is more frequently evident. The main cause of this illness is pancytopenia, hypergammaglobulinemia, and hypoalbuminemia. Sources of this disease in Iran include dogs and fox, and the vectors are various types of sandflies with the main one being *Phlebotomus* major.[3]

In general, VL is evidence either an infection with symptoms (acute and persistent) or without symptoms (silent) or subclinical (in patients with minor and unusual signs and symptoms).[4] The acute type is more prevalent in children under the age of 2 years, and the persistent type is more commonly seen in adults. In endemic parts of Iran, the silent types are more frequently present and are more common than the sub-clinical type. In general, in patients who are not showing symptoms or are diagnosed at late stages, there is 98% chance of death, especially in children.[5] Therefore, accurate and on-time diagnosis and finally treatment of the disease is important. There are various diagnostic methods for this infection such as bone marrow biopsy (gold standard method) for finding Leishman bodies, serology methods such as ELISA, DAT, IFA, rK39, and other molecular methods.[6]

There has been an increase in the number of incidences in the recent years due to increase in ecological and demographic changes, urbanization, increase in transplant operations and a higher number of patients who are immunocompromised.[7] Recently, VL is present endemically in 5 provinces of Iran: Ardebil, Fars, Boshehr, Azarbaijan Sharghi and Qom and sporadically in other provinces.[8]

Leishmania parasites in its asexual and sexual life cycles can have two different types: promastigote form which lives in the alimentary tract of sandflies (phelebotomine variant) and amastigote which live in the host's cells.

The promastigote form has the ability for live extracellular and in the motile form in the alimentary tract of the female sandflies and colonize and multiply and feed from the blood of infected host until they get transmitted to another person.[9]

IMMUNOLOGICAL RESPONSE AGAINST *LEISHMANIA INFANTUM*

Parasites infect the host's macrophages and in the period of 12–24 h the endosome area would contain carbon dioxide, high temperature and low pH, gets transmitted to nonmotile amastigote and therefore gets multiplied in the vesicles of macrophages.[10] Following that, the macrophages get ruptured, and consequently, infection gets transmitted to other macrophages.

Immune response to the *Leishmania* is very complicated and in some situations might even improve the healing process of the infected patient; however, immune response in some patients under especial circumstances can worsen the infection in the patient.[11] The immune responses in the patients is dependent on several factors such as the genetic composition of the patient's immune system, type and the variant of the parasite, the position of the affected macrophages, and the amount of endotoxins that has been inserted to and contaminated the macrophages.[12]

The normal immune response to the *Leishmania* would occur through Natural Killer (NK) cells, cytokines, phagocytic macrophages (with single or multiple nucleuses), and proteins complexes.[11,13] However, the role of macrophages in defending, initiating, and controlling the immune response is crucial.[14] In order for *Leishmania* to multiply successfully, these parasites most survive innate as well as acquired immune responses. Complement protein c3b is one of the most powerful opsonins of the immune system which attached to the *Leishmania* parasites, and via c3b receptors which are present on the surface of macrophages, there is an increased chance that this parasite attached to macrophages.[15] *Leishmania* parasites by having an especial glycoprotein on its surface, named c3b gp63, converts gp to ic3b which results in switching of lytic response to phagocytic response.[16] Furthermore, macrophages by producing poisonous oxygen types such as anion superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide attack the parasite,[17] on the other hand, *Leishmania* by producing acid phosphatases on its surface, prevents the reaction between the oxygen species and reduction reaction.[11,18] Moreover, when lysosomes and phagosomes are reacting with each other, macrophages use acidic enzymes to destroy parasite and use the protein pumps which are present on their surface to naturalizes the intracellular pH and prevent the enzymes from functioning.[19,20] Furthermore, lipophosphoglycan molecules of *Leishmania* have an important role in removing lysosomal enzyme.[21] At the same time infected macrophages, pick up *Leishmania* antigens and through MHC-II transfer it the receptors of the TCD + 4 of the T-cells.[22,23] Other similar molecules such as CD 80/86, which are used as intermediates to exacerbate or potentiate the number of signals that gets delivered to TCD-28 cells.[24] The interconnection between 4CD-L40 CD is essential for potentiation of the immune response via Th1 cells. The connection occurs between CD40 of the macrophages and L40 CD of the T cells and the signal transmitted through MAPK P38 (kinase proteins responsible for initiating mitogenesis) and NFκβ which potentiates in producing interleukin (IL-12).[12,24] The released IL-12 attached to IL-12 receptors on the surface of the T cells and results in an increase in production of interferon gamma (IFN-γ) (most important cytokine of the macrophages) through these cells. The produced IFN-γ on

the infected macrophages functions via attaching to the IFN-γ receptor and stimulates killing the parasites which are in the macrophages.[11] On the other hand, parasites by interrupting the copying of NFκβ and production of IL-12, decrease in MHC-II and increase in production of the regulatory cytokines such as IL-10 and TGF-β, prevents the macrophages from functioning.[11,14] With continuous life cycle inside the macrophage, *Leishmania* prevents being affected by hormonal changes of the immune system. In each of the steps that had been explained, the parasite interrupts the immune system first and therefore avoids being ingested.[25]

APOPTOSIS OF *LEISHMANIA INFANTUM*

One of the mechanisms that the cells use in order to eliminate infection is programmed cell death or apoptosis. Apoptosis is defined as a natural cellular event, and essential for more efficient and functional immune system.[26] It occurs simultaneously through particular cellular mechanism such as ROS production, vesicle formation from plasmatic matrix, lipid peroxidation, increase in cytosolic calcium, appearance of serine phosphatidyl on cellular matrix, changes in mitochondrial composition, and enzymatic breakage of chromosomal DNA.[27]

There are two general pathways in apoptosis: the external pathway, which is through activation of necrotizing receptors (for example: sub-elements of tumor necrosis factor (TNF) such as [TNF receptor 1, Fas (CD95)] and by extracellular ligands (such as FasL) as well as internal or mitochondrial pathway.[28] In mitochondrial pathway, activation occurs through interaction or getting exposed to ultraviolet radiations, or decreasing in the cells growth factors and nutrients which are essential for cellular function and growth.[28] In both pathways, apoptosis occurs through specialized caspase proteases which are present as inactive preform of procaspases and are activated through other caspases. In the places where caspases are active, catalytic cysteine amino acids are present which through peptide connection can hydrolize aspartate amino acids.

There are two types of caspases, initiators, and executioners.[29] The initiator caspases such as 2, 8, 9, and 10, by receiving apoptotic signals activated and then, executioner caspases such as 3, 6, and 7 are activated. Activation of external pathway by itself is sufficient for apoptosis[29,28] For instance, by the attachment of ligand 5 (Fas [FasL]) to extracellular Fas, procaspase 8 with the help of cofactors Fas-associated via death domain is added to the complex and due to local accumulation, it is raptured and results in the activation of caspase 8.[30] Activated Caspase 8 intern activates executioner procaspase 3.[30] This external pathway cooperates with internal mitochondrial apoptosis pathway, in such way that activated caspase 8 transforms Bid protein to tBid. Transferring tBid to mitochondria results in activation of BAXs and BAKs, which make pores on the extracellular matrix of mitochondria. Through these pores, pro-apoptotic proteins such as Smac/DIABLO, EndoG, AIF (apoptosis-inducing factor) and cytochrome C open up the space between internal matrixes of mitochondria to the cytoplasm.[27,30] In cytoplasm and via the presence of dATP, cytochrome C together with apoptotic protease activating factor 1 (Apaf-1) cooperates with procaspase 9 which results in the formation of apoptosis complex and therefore, caspase 9 activated which intern it can activate executioner caspases19.[30]

Smac protein is the second most important mitochondrial protein after cytochrome c, which in case of apoptosis, together with cytochrome c is released into cytosols. This protein by attaching to X-inked inhibitor of apoptosis protein and cellular inhibitor of apoptosis protein as well as preventing them to show their inhibitory action on caspases, plays an important role in making apoptosis to occur.[31] This protein has a role in activating procaspase 9 as well as activating executioner caspases. Furthermore in internal pathway of apoptosis, as well as damaging DNA, concentration of P53 protein is increased and this protein itself causes an increase in BAX protein, which leads the explained above pathway.[32]

Recently, it has been proven that progression of *Leishmania* infection in a mammalian host is dependent on apoptosis of promastigote parasite in the early stages of infection.[33] Apoptosis in *Leishmania* have been overlooked in different studies, and it has been determined that cellular death can occur under wide and various circumstances such as oxidative stress, heat shock, and under the influence of medications. The cellular death mechanism is resulted from changes in cellular composition, DNA denaturation, separation of phosphatidylserine, changes mitochondrial matrix permeability, activation of proteases, and finally through changes permeability of the cell.[32] Even though there have been various studies that have been carried out on the process of cellular death, the molecular changes and composition have not yet been determined.[34] Studying on *Leishmania* genome has not shown or discovered a protein that can encode proteins which can progress the apoptosis process similar to mammalian hosts (for example, BcI-2 family members and caspases which are the dominant and effective protein for apoptosis in mammalians). However, metacaspase in *Leishmania major* has been found as a suitable substitute for programming cellular death.[35] Furthermore, it has been determined promastigotes of *L. infantum* are transfected with coding sequence BLC-XL results in protection of parasite from heat shocks.[36]

ENDOG, MAIN NUCLEASE FOR APOPTOSIS OF PARASITE

Studying the *Leishmania* shows the presence of nuclease similar to caspase in mammalian (DNAaes activator) that can significantly reduce DNA breakdown during cellular death.[36] The process of cellular death in mammalian is also dependent on a secondary nuclease, named endonuclease G (EndoG), which is a mitochondrial nuclease that is transferred to the nucleus during cellular death and cause breakdown of chromatin DNA in some parts of the nucleosome in cellular death that is not dependent on caspases.[37]

EndoG is from a diverse family member of αßß metalloproteinase which their activity is dependent on cations with multiple charges such as Mn^{+2}, Co^{+2}, and Mg^{+2}, however low concentrations of single charge cations such as K^{+} and Na^{+} prevents them from functioning.[38] Analogous to these proteins which are effective in cellular death has been evidenced in *Caenorhabiditis elegans*, *Sacaromyces cerviciea*, *Trypanosome brucei* and *L. Donovani.*[33,39,40] Even though there are similarities between family members of endonucleases, their nucleoside action is not the same. For example, nuclease enzymes in *Saccharomyces cerevisiae* or *Neurospora* have 5′ exonuclease function, whereas in EndoG family this activity is not evidenced.[41] The presence of such endonuclease in mitochondria of eukaryotes that have been present in the past has also been reported.[42] Endonucleases specifically attack the nucleotide DNA sequences and prefer to break or change the sequence in a position near guanine and that the reason they have been named EndoG.[42] As well as cooperating in programmed cell death, EndoG enzymes participate in growth, development, and survival of cells.[34] Studying the catalytic functions of EndoG enzymes has shown that for it to be function, disulfide bonds present in the enzyme are essential.[43]

Results for previous studies show toxic effect of EndoG on *Escherichia coli* bacteria, while connection of this enzyme with other bacteria does not have the same effect.[44] The results for complementary studies shows that these proteins after becoming present in bacteria aggregate and precipitate and this can be the reason for being ineffective in other bacteria.[45] Recognizing ExoG as a new marker for mitochondria endo/exonuclease opens up a new window for studying the function of mitochondrial nucleases in reproduction or cellular death in eukaryotes.[42] The result of studies shows that EndoG and ExoG enzymes are complement and cooperate in some of the enzymatic reactions as because in the mouses that their EndoG gene has been destroyed, no phenotypic difference has been seen and ExoG can cover its′ function.[42] As it was mentioned, the dual action of EndoG (cooperating in growth and maintenance and programmed cellular death) has an important function in different organisms. For example, decrease in the EndoG ortholog level due to CPS-6 gene in *C. elegans* as the result of interference with RNA causes delay in cellular growth in this roundworm as its developing.[39] Similar to this finding, elimination of Nuc1P gene (EndoG) in yeasts in standard growth media results in decrease in apoptosis and death when there is increase in mitochondrial respiration.[41] Therefore due to such characteristic, this strategy can be used, which is by decreasing level of activity of LiEndoG, growth of parasites will be limited and decrease their ability to cause infections and being alive in differentiated Th1 cells.[34]

Moreover, these proteins can be considered as a new target for medications against *Leishmania* and place under trial.[34] Changing genetic make-up of eukaryotic organisms such as *Leishmania* causes changes in genotype and consequently their biological action. In study for immune response to the infection, using transgenic *Leishmania* can determine the parasites ability to cause infection, interaction with cells, as well as getting innate and acquired immune response mechanisms. This approach has been used to discover pathogenic factors of the parasite, initiating immune mechanism responses for enhancing new therapeutic pathways and innovations for leishmaniasis.[25] *Leishmania* organisms are diploid which can easily undergo homologous recombination and therefore it can cause elimination or presence of new genes in *Leishmania*.[45] Currently, transgenic *Leishmania* can be used in three research fields: 1) studying functions of the parasite's genes in involving host's cells and pathogenic causes, 2) maintaining weakened parasite for generating vaccines, 3) marking the parasites (by using reporter genes in biochemistry or fluorescence methods) for studying postinfection *in vivo* and *in vitro* conditions. Despite broad international researches, vaccination of live attenuated *Leishmania* parasite, regardless of having safety concerns, is the only accepted vaccine in humans.[45]

Taking into account different studies by using transgenic *L. major* has shown expression of immune proteins cytokine production in the host towards Th1 cells and away from disease-causing Th2.[46] For instance, in one of the researches done on transgenic *L. major* producing GM-CSF, a small number of macrophages *in vitro* survived. In addition, increase in a number of cytokines of macrophages in preinflammation stage (IL-Iβ, IL-6, and IL-18) resulted in minor damage the affected mice.[47] In another study on transgenic *L. major*, the extracellular domain of CD40-L in mice showed less damage and decrease in a number of parasite in the affected mice compared to controlled mice, as it was related to decrease in the level of IL-4 and increase in IFN-γ.[48] In another study, effect of monocyte chemotactic protein-1 (MCP-1) and IP-10 in mice as regards increase in

the damage due to leishmaniasis using transgenic *Leishmania* was examined. MCP-1 of transgenic parasite caused less damage, and ILP-10 resulted in wider damage compared to the wildtype.[49] In studies that have been done against *Leishmania* showed inclusion of different transgenic immune factors caused a positive effect in decrease progression of the disease. However, there has not been an improvement in researching on transgenic proteins that stimulate apoptosis in macrophages. With regard to increase in differentiation process and formation of a stable form of amastigotes, total dependence of parasite to the host, time for immune cellular responses against *Leishmania* as well as activation of NK cells in the first stage of *Leishmania* under influence of IL-12, IL-18, and Toll-like receptors 9.[50]

It appears that faster presence and increase in concentration of the proteins involved in apoptosis of macrophages can enhance apoptosis process and prevent proliferation and prevents reproduction of parasite and consequently progression of the disease and causing broader damages. Parasite burden refers to the number of *Leishmania* parasites present in the phagocytes and macrophages which can be counted by usual ways such as counting with Neubauer chambers or culturing a certain number and estimating the number of parasite present.[51] However in recent studies for determining parasite burden *in vivo* conditions, blood agar media is used to culture parasite in plates of 96 well and also serial dilution method is used.[52]

The main purpose of common chemical and medicinal methods such as Miltefosine and pentavalent antimonials is making apoptosis in the parasite.[53] However, they are used after the presence of damage and is also mutual with having side effects such as poisoning surrounding cells.[54] One of the strategies that *Leishmania* uses to prevent being affected by immune cells is delaying of apoptosis of macrophages. On the other hand, EndoG enzymes can enhance the apoptosis mechanism in the parasite, therefore by taking these factors into account, it appears that including such proteins in this mechanism by the parasite itself, the adverse effect on the surrounding cells can be decreased and apoptosis would occur naturally and faster as well as preventing proliferation of the parasite and causing damage.[55] EndoG gene is the coding protein which is involved in the apoptosis mechanism inside the cell and are not classified as proto-oncogenes. Therefore, including them in the cell would result in apoptosis and does not cause stimulation in the cellular cycle.[56]

CONCLUSION

Cell death by apoptosis mediates several important physiologic and pathologic processes and appears to be intrinsically programmed. Its characteristic features are distinctive morphologic changes of nucleus and cytoplasm, along with cleavage of chromatin at regularly spaced sites. EndoG is a mitochondrion-specific nuclease that translocates to the nucleus during apoptosis. Once released from mitochondria, EndoG cleaves chromatin DNA into nucleosomal fragments independently of caspases. Therefore, EndoG represents a caspase-independent apoptotic pathway initiated from the mitochondria. As a consequence, this does not result in cancer and the cell that it appears in is condemned to programmed cell death. Hence, use sequence of this nuclease only or adding to other peptides or genes is effective in internal apoptosis that the results would be directed toward apoptosis of macrophages and this is a useful hypothesis that we are doing right now by a new gene and peptide (Smac-EndoG) in *L. infantum*.

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Conflicts of interest
There are no conflicts of interest.

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