Abstract
Vacuolating cytotoxin (VacA) is one of the most important virulence factors of *H. pylori* (Hp), which is the only toxic protein that is secreted from Hp cell into the culture supernatant. The effects of VacA on eukaryotic systems is the subject of many previous and ongoing research studies. Intracellular targets for this toxin include: late endosomal and lysosomal compartments, mitochondria, cell-cell junctions and phospholipid bilayers. Its effects on these targets include vacuolation of late endosomal and lysosomal compartments, apoptosis and channel formation, which result in the increase of ion uptake specially anions. The aim of this review is to increase the perception on this toxin and its functions.

Keywords: Helicobacter pylori, Vacuolating cytotoxin, vacA, Virulence, Pathogenesis.

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**Literature Review**

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responses through specific cytokine secretion, such as IL-4 (Mohammadi et al., 1996a).

Several studies suggest that Hp adheres to gastric epithelial cells without any significant destruction of cellular membranes (Hofman et al., 2000). This bacterium is capable of residing in the gastric pH of 2-7, pertaining to luminal surface to deep epithelial layers respectively (Cover et al., 1997). Hp infection is the second most common bacterial infection after Streptococcus mutans infection that is responsible for dental cavities (Telford et al., 1997).

There are some virulence factors, which are necessary for Hp pathogenesis. These factors include: Urease, which is required for neutralization of acidic pH of the gastric lumen and initial colonization of Hp cells (Labigne et al., 1991). This enzyme is active in acidic pH and hydrolyzes urea into CO₂ and ammonia which protects Hp cells by creating a neutral microenvironment (Dunn et al., 1997). Flagellum which is the major cause of bacterial movement in the gastric mucosal layer (Leying et al., 1992). Cytotoxic-associated gene product (CagA) which is present in about 60-70% of Hp strains and is associated with exacerbation of the associated gastric inflammation (Atherton, 1997). This effect may be due to the induction of IL-8 production (Atherton, 1997). cagA gene is a genetic marker for a major gene cluster which is called cagPAI and is about 40 kb and is suggested to be acquired from other bacterial species. Apparently Hp can infect more subjects than other Helicobacter species because of producing enzymes such as catalase, oxidase, protease and phospholipase (Ilver et al., 1998).

Vacuolating cytotoxin (VacA) is one of the most important virulence factors of Hp that is extracellular and causes damage to the gastric epithelial layer as a result of vacuolation of late endosomal and lysosomal compartments of these cells.

All of the Hp strains possess the gene for vacA but only 50-60% of them manifest cytotoxic activity in vivo. This gene is composed of different regions beginning with the signal sequence (s region) followed by the mid region (m region) which display the following two genotypes respectively: s1 or s2 and m1 or m2. These genotypes are distributed differently in various parts of the world. The mosaic combination of these genotypes result in several genotypes, which include: s1m1, s1m2, s2m2 and the s2m1 genotype that has seldom been reported (Atherton et al., 1995; Letley et al., 1999; Mohammadi et al., 2003).

Pathological studies in western countries have shown that the majority of Hp strains that cause PUD are of the s1m1 or s1m2 genotype and the s2m2 genotype has little relation with PUD or gastric cancer. In our study and some eastern studies such as that from Taiwan, it was shown that s1m2 strains are more prevalent in PUD patients (Wang et al., 1998; Mohammadi et al., 2003).

VacA protein is composed of three parts: signal peptide (33 aa), mature protein (95 kDa), carboxy terminal region (45 kDa). The mature peptide has two parts with different roles in vacuolating activity of Hp. This protein is secreted from Hp cells into the gastric lumen and acidic pH of gastric juice causes activation of this protein and internalization of the protein into gastric epithelial cells. Vacuolation of these cells will occur as a result of changes in H⁺-K⁺ ATPase activity of late endosomal and lysosomal compartments.

1- vacA gene

As previously mentioned, almost all Hp strains have one copy of vacA gene but only 50-60% of them show cytotoxic activity (Cover et al., 1990; Han et al., 1998). vacA has an open reading frame (ORF) of about 3900 bp that is responsible for coding a protein with approximately 1296 aa with MW of about 140 kDa (Telford et al., 1994b). This ORF has an inverted repetitive sequence that is responsible for the stem loop conformation in mRNA (Cover et al., 1994). In the upstream of the vacA gene, AGGAA sequence (Shine Dalgarno) is located that is required for effective initiation of translation in prokaryotes (Phadnis et al., 1994). It should be noted that there are about 10 direct repeats in this coding region that most of them are translated in the same frame (Phadnis et al., 1994). Upstream, there is a region that includes promoter and other required signals for transcription and translation and -10 and -35 consensus motifs of promoter are located in this region that are separated with 15 bp. This promoter is located 100 bp upstream of coding region of vacA gene. Immediately following the coding region, there is a signal for Rho independent termination of transcription. This gene is located 224 bp downstream of the gene for cysteiny1-tRNA synthetase (Fig. 1) (Phadnis et al., 1994).

vacA gene has two different regions: signal sequence (s) and mid region (m) which are very heterogenic and vary in sequence in different strains from various locations in the world (Rudi et al., 1998).

The s region has two sub-types: s1 and s2. The s1
type is associated with the presence of cagA gene, in vitro cytotoxic activity and gastric and duodenal ulcers (PUD & DUD) (Han et al., 1998; Rudi et al., 1998). The difference between these two types is related to small insertions of 24 bp in the s2 type. Thus it seems as the s2 type does not have effective secretion of cytotoxin through bacterial plasma membrane (Rudi et al., 1998). The s1 region has been divided into two types of alleles: s1a and s1b, of which s1a is more prevalent than s1b or s2 (Han et al., 1998). In many pathogenesis studies, most of Hp isolates from patients with PUD history are s1 and most of them are cagA-positive and iceA-positive, that are stronger pathogens and have lower density than s2m2 Tox-negative strains (Atherton et al., 1995; Evans et al., 1998; Han et al., 1998; van Doorn et al., 1998, 2000). These studies were performed in western countries such as Germany, Portugal, Brazil and USA. Recently a new subtype has been found in eastern Asia, which is called s1c and is rarely found in other parts of the world (van Doorn et al., 2000).

The m region has two subtypes: m1 and m2. The m2 subtype has a 23 aa insert with a ATP/GTP binding motif which is not present in Tox+ (m1) strains (van Doorn et al., 2000). But prevalence of m1 is commonly similar to m2 and little relation has been found between the m type and presence of cagA or occurrence of PUD (Han et al., 1998; van Doorn et al., 2000). However, we have reported that the majority of the Iranian Hp strains to be of the m2 genotype and this study showed that in our country, most of PUD samples are s1m2 instead of s1m1(Mohammadi et al., 2003).

There are different combinations of s and m subtypes producing the following genotypes: s1m1, s1m2, s2m2 and the s2m1 type has yet to be detected in western countries (Atherton et al., 1995). But we (Mohammadi et al., 2003) and an African group (Letley et al., 1999) have reported this genotype in some of the studied strains that is not represented in PUD group but is found in NUD group. The absence or low occurrence of s2m1 genotype may suggest that strains with this genotype have a selective disorder or lack viability (Rudi et al., 1998).

2- VacA protein

VacA is the only toxic protein which is produced and released by Hp (Galmiche et al., 2000) and it is an extracellular toxin (Phadnis et al., 1994). The precursor of this protein has no homology to other reported proteins in the GenBank (Phadnis et al., 1994) but it is similar to some exoproteins such as IgA protease types that are produced by Neisseriae and Haemophilus species (Schmitt and Hass, 1994).

VacA is produced as a 139 kDa precursor that releases a 90 kDa mature peptide out of the cell and a 45 kDa C-terminal fragment will be retained with bacterial cell wall that consists of two fragments with MW of 33 and 12 kDa (Telford et al., 1994a) and it suggests that this fragment directs transport of the protein through the membrane (Telford et al., 1994b).

The 90 kDa monomer forms oligomers with MW of up to 1000 kDa in bacterial culture media or in vivo conditions (Cover and Blaser, 1992; Pai et al., 2000). Each oligomer consists of 6 or 7 copies of 90 kDa monomer (Lupetti et al., 1996), and converts to monomeric forms under denaturing conditions, during SDS-PAGE (Lupetti et al., 1996). In the bacterial supernatant the mature peptide (90 kDa) will be
processed mostly after alanine (at position 352) which is located in a hydrophilic region that forms a flexible loop in the protein, containing 46 amino acids (Telford et al., 1994b; Manetti et al., 1995) and results in the N-terminal 37 kDa and C-terminal 58 kDa fragments. Following cleavage, these two fragments remain associated with non-covalent interactions (Telford et al., 1994b; Manetti et al., 1995; Lupetti et al., 1996; Galmiche et al., 2000). According to Telford et al. (1994b) the mature protein has a terminal signal peptide with 33 amino acid residues that is most likely responsible for trans-membrane secretion of the protein (Fig. 2).

As mentioned above, each oligomer consists of 6 or 7 copies of 90 kDa monomers and nearly 70% of oligomeric, wild types of the toxin have heptameric radial symmetry and in mutant forms of VacA, hexameric forms are prevalent (Fig. 3) (Lupetti et al., 1996). These hexameric molecules have a deletion in amino acids of loop region and this deletion causes the two subunits come closer and favors the formation of hexameric molecules.

Oligomerization is a transient state for hiding hydrophobic regions in a protein that are required for membrane interaction of the molecule (Molinari et al., 1998). Mature peptide has about 850 aa but not all of the C-terminal amino acids of the p58 are required for vacuolation. Upon N and C terminal truncation it was found that C-terminally truncated molecule with 422 aa (amino acid 1 to 422) is the smallest fragment that can induce vacuolation in HeLa cells but non of the N-terminal truncated forms of VacA are able to induce vacuolation in the cytosol and more importantly deletion of 17 aa from the N-terminus of this protein (following signal peptide), destroys vacuolation activity. On the other hand, N-terminus of VacA protein is necessary for intracellular vacuolation (de Bernard et al., 1998; Ye et al., 1999).

In electronic micrographs, VacA oligomer appears flower-shaped and consists of two parts (Lupetti et al., 1996; Cover et al., 1997); large lobes like flower petals and ring structure toward the center of the molecule. In VacA protein, there are two cystein residues that likely have a role in secretion of vacA gene product, and are 7 or 11 aa apart (Cover et al., 1994). Expected isolectric pH (pI) of the 90 kDa mature VacA protein is 9.1, whereas the observed pI for this protein is 6.1 (Cover and Blaser, 1992). VacA protein is found in three forms (Molinari et al., 1998):

1. Natural oligomeric structure that consists of 12 VacA monomers that are associated with non-covalent forces.

2. At pH lower than 5, oligomers dissociate to monomers and represent hydrophobic regions that are necessary for effective insertion into lipid bilayers.

3. Under neutralization, the structure of VacA changes by monomers forming into oligomers with different physical and structural properties.

Native VacA has low but detectable activity and with treatment at pH lower than 5, the activity may increase which is associated with conformational changes in the molecule (de Bernard et al., 1995). This form of activation with acidic pH is related to the development of surface hydrophobicity that enables the toxin for
deep penetration into the lipid bilayers. Membrane insertion is a critical stage in the intoxication process of peptic toxins that have intracellular targets, because this involves in transport of their catalytic domains into the cytosol (Molinari et al., 1998).

Incubation of VacA with epithelial cells at differing temperatures for 10 minutes has shown that the activity of this protein is stable in temperatures up to 55°C but higher temperatures induce significant activity reduction (Yahiro et al., 1997). N-terminal segment of the protein mostly consists of hydrophobic amino acids with no charge and a PI of about 5.83 (Cover and Blaser, 1992).

Unusual stability of Hp in low pH environment and the significant induction of activity upon treatment with acidic pH, are two specific characteristics of this protein that matches the acidic environment of the stomach (Molinari et al., 1998).

This toxin has a slow rate of internalization requiring 2 to 3 hours, which has not yet been reported for other toxins (Garner and Cover, 1996) and this slow internalization is a temperature related process (Garner and Cover, 1996).

VacA protein purified from Hp culture supernatant is actually inactive, and treatment with acidic pH (below 5) causes conformational changes in the molecule that is associated with induction of vacuolating activity (de Bernard et al., 1995).

### 3 - VacA function

#### 3-1 Binding and vacuolation

Cleavage of oligomers at low pH does not inactivate the toxin but changes the conformation and will result in the exposure of hydrophobic regions, interaction with hydrocarbonic chains of membrane phospholipids. Hydrophilic regions can form channels by which the active moiety of VacA enters the cytosol (Lupetti et al., 1996). Therefore, acidic pH enables the toxin for internalization into the cytosol. It seems that amino acid sequence of p58 is responsible in binding of VacA to different cell lines (Garner and Cover, 1996) but this binding is not sufficient for successful internalization of VacA, and it is proposed that amino-terminal p37 is also required for internalization (Reyrat et al., 1999). On the other hand, it has been demonstrated that p37 domain plays an important role in channel formation (Ye et al., 1999). Toxin can bind to a cell surface glycoprotein (250 kDa) that is called P250 and is a receptor for Tyrosin Phosphatase (RPTPβ) (de Bernard et al., 1995; Yahiro et al., 1999). Subsequently it enters the cytosol of gastric epithelial cells through endocytosis and will be located in membrane of acidic compartments such as late endosomes and results in channel formation in endosomal membrane (Tombola et al., 1999b). These are anion-specific trans-membrane channels (Tombola et al., 1999a; de Bernard et al., 2000). This increases penetration of anions such as HCO₃⁻ and Cl⁻ from cytosol to endosomes and results in depolarization of cell membrane and higher activity of Vacuolar-ATPase (influx of H⁺ and Cl⁻) and accumulation of weak bases such as ammonium ions in the lumen of endosomes. This penetration causes increase of osmotic pressure in these compartments yielding influx of water and thus swelling of these organelles will take place (Garner and Cover, 1996; Tombola et al., 1999a, 1999b) and large vacuoles will be formed. These vacuoles have Rab7 and V-H-ATPase that are characteristics of late endosomal compartments (Molinari et al., 1997, 1999).
reviewed by Papini et al., 2001). Thus, VacA interferes with the controlling processes of late endosomal structures and their cycling to lysosomes or golgi apparatus (Telford et al., 1994a).

3-2 Induction of apoptosis

Intracellular expression of vacA, induces apoptosis and has been shown that p37 is the active moiety in this process (Galmiche et al., 2000). The two known biochemical events in apoptosis procedure are release of cytochrome c from mitochondria into the cytosol and activation of Caspase 3. Cellular death has most likely a critical role in the appearance of atrophic gastritis, a pre-neoplastic condition. Therefore, VacA may play a role in carcinogenesis of Hp (Shirin and Moss, 1998; Galmiche et al., 2000).

3-3 Blocking antigen presentation

VacA interferes with B-cell antigen presentation via inhibition of maturation and processing of antigens (Satin et al., 1997). Furthermore, it seems that VacA inhibits T cell proliferation, reducing immune responses in gastric mucosal level and permitting the colonization of pathogenic strains of Hp (Molinari et al., 1997).

3-4 Cellular damage

Several lines of evidence suggest that VacA causes cellular damage in gastric mucosal layers; intragastric administration of purified toxin to mice results in gastric epithelial damages and mucosal erosions (Telford et al., 1994b); experimental infection of mice with Tox⁺ strains of Hp result in peptic ulcers and epithelial vacuolation whereas infection with Tox⁻ strains does not (Marchetti et al., 1995); peptic ulcer is commonly observed in patients infected with cytotoxic strains of Hp (Atherton et al., 1995).

It is noteworthy to mention that VacA will be extremely activated at pH 1.5-6 without being affected by this range of pH. At pH 2 this protein is resistant to pepsin digestion. Some of VacA molecules that are released from Hp and are activated in the gastric juice can pass through pylorus and cause vacuolation of duodenal epithelial cells before digestion with intestinal proteases that protect other parts of intestinal lumen. Such processes can cause duodenal ulcers without actual presence of Hp (de Bernard et al., 1995).

3-5 Transepithelial electrical resistance reduction

When VacA is added to the apical layer of gastric epithelial cells, it decreases the Transepithelial Electrical Resistance (TER) which indicates the degree of sealing and through this state, permeability to small molecules and ions such as pyruvate, Ni²⁺ and Fe³⁺ that are necessary for the survival of Hp will be increased.

Thus, one of the important roles of VacA is increasing permeability of mucosal layers that is seemingly required for Hp nutrition and maintenance in the stomach (Papini et al., 1998).

4- Concluding Remarks

Several studies have been performed investigating various molecular and cellular aspects of VacA. Due to the critical roles of Hp cytotoxin in the pathogenesis of this bacterium, it is recommended as a strong vaccine candidate against Hp infection with applications in therapeutic and preventive approaches. Native VacA does not seem like an appropriate immunogen due to its pathological effects on host cells. However, a genetically detoxified or recombinant form of this protein can be used for this purpose. According to the studies about VacA binding and toxic activity, different parts of this protein have been identified and attempts are being made to use recombinant partial sequences or truncated forms for applications in therapeutic approaches such as a cocktail of Hp antigens as a complex vaccine, or in diagnostic ELISA or western blotting kits. Previous studies have shown that intragastric administration of Hp antigens, and particularly its cytotoxin, urease and the recombinant form of VacA into mice, induces protective immunity against re-infection (Mohammadi et al., 1996b; Ghiara et al., 1997; Lanzavecchia and Bellon, 1998). On the other hand, the vast heterogeneity present in vacA gene and protein sequences from Hp strains from various geographical locations, re-emphasize the need for using local strains as the source for the afore-mentioned recombinant proteins to match with the local infecting strains in the two aspects of “detection” and “prevention”. In accordance, the authors have amplified the vacA gene (related to mature peptide~90 kDa) from a local (Iranian) Hp strain, which has subsequently been cloned and sequenced. Complete sequencing confirmed the extreme heterogeneity for the vacA gene.
between Iranian and western strains (GenBank accession number=AY232454). This protein is presently being expressed and purified for future diagnostic and vaccine trials.

References


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