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Histidine-rich protein Hpn from *Helicobacter pylori* forms amyloid-like fibrils in vitro and inhibits the proliferation of gastric epithelial AGS cells

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ABSTRACT

Helicobacter pylori causes various gastric diseases, such as gastritis, peptic ulcerations, gastric cancer and mucosa-associated lymphoid tissue lymphoma. Hpn is a histidine-rich protein abundant in this bacterium and forms oligomers in physiologically relevant conditions. In this present study, Hpn oligomers were found to develop amyloid-like fibrils as confirmed by negative stain transition electron microscopy, thioflavin T and Congo red binding assays. The amyloid-like fibrils of Hpn inhibit the proliferation of gastric epithelial AGS cells through cell cycle arrest in the G2/M phase, which may be closely related to the disruption of mitochondrial bioenergetics as reflected by the significant depletion of intracellular ATP levels and the mitochondrial membrane potential. The collective data presented here shed some light on the pathologic mechanisms of *H. pylori* infections.

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1. Introduction

Abnormal protein aggregation and deposition of misfolded proteins have been recognized as a common pathological feature of the neurodegenerative diseases, such as Alzheimer's disease (AD), prion diseases, Parkinson's disease and the poly(glutamine) (polyQ) disease [1]. Alzheimer's disease is a neurodegenerative disorder of the elderly that is characterized by progressive cognitive deterioration. It has been suggested that the most toxic form to cause memory loss may be the small diffusible oligomers, or the protofibrils, rather than the mature amyloid fibers [2]. One notable characteristic of AD is the highly enriched metal concentrations in the brain: four times higher Cu^{2+} levels in the AD neuropil than in the neuropil of a healthy brain [3], which has been supported by several hot spots with highly concentrated Cu^{2+} and Zn^{2+} within Aβ-associated amyloid plaques [4].

Aβ undergoes a conformational transition from a soluble random coil to an insoluble fibrillar β-sheet rich form. The trigger to promote oligomerization and fibril formation is still a subject of debate. Zn^{2+} and Cu^{2+} can be involved in promoting [5,6] or inhibiting [7] the amyloid fibril formation. Smith et al. [8] suggested that the controversial results are possibly attributed to the Cu^{2+}/peptide molar ratio: at sub-equimolar ratios, Aβ(1–42) forms amyloid fibrils; at supra-equimolar ratios, Aβ(1–42) forms small spherical oligomers (approx. 10–20 nm in size) and large amorphous aggregates. Several animal model experiments seem to favor the role of Cu^{2+} in promoting fibril formation: (1) copper chelators can reverse the aggregation and cause solubilization of amyloid deposits from post-mortem AD brain tissue [9]; and (2) trace amounts of copper induce amyloid plaques and learning deficits in a rabbit model of AD [10].

The polyQ diseases are caused by abnormal expansions of the polyQ stretch to more than 40 glutamine residues and the subsequent aggregation of the protein [11]. One molecular dynamic study of the polyQ protein ribonuclease A indicated that the fibrillar structure is stabilized by the highly dense packing of Gln side chains, through intra-sheet hydrogen bonding interactions of the amide stacks and inter-sheet van der Waals interactions of their alkylic parts in the steric zipper [12]. Previously we hypothesized that histidine-rich proteins, such as Hpn from *Helicobacter pylori* [13], histidine-rich Ca^{2+} binding protein (HRC) from *Sarcoplasmic reticulum* [14] and imidazole glycerol-phosphate dehydratase from *Filobasidiella neoformans* [15], tend to form high order of oligomers, probably induced by aromatic stacking between intra- or inter-molecule imidazole side chains of histidine residues [13]. The experimental evidence of the direct involvement of histidine protonation in the formation of amyloid has been identified in such proteins as β-amyloid, prion protein (PrP), islet amyloid polypeptide (IAPP), β-2-microglobulin [16] and HDL-associated serum amyloid A [17].

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; $\Delta\Psi_m$, mitochondrial transmembrane potential; FBS, fetal bovine serum; *H. pylori*, *Helicobacter pylori*; HRC, histidine-rich Ca^{2+} binding protein; IAPP, islet amyloid polypeptide; JC-1, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanine iodide; LUV, large unilamellar vesicle; MALT, mucosa-associated lymphoid tissue; PBS, phosphate-buffered saline; PI, propidium iodide; polyQ, poly(glutamine); PrP, prion protein; ThT, thioflavin T; TOM, translocase of the outer membrane

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H. pylori is a Gram-negative bacteria causing gastritis and peptic ulcerations [18]. The development of gastric carcinoma and MALT (mucosa-associated lymphoid tissue) lymphoma is also strongly associated with chronic *H. pylori* infections [19,20]. *H. pylori* produces a small cytoplasmic protein, Hpn, which accounts for approx. 2% of total proteins synthesized by *H. pylori* [21] and has 28 histidine residues among its 60 amino acids. The majority of the histidine residues lie in the central part of the protein with two stretches of six and seven consecutive histidine residues. Another feature of the Hpn is the presence of 20 pairs of one specific amino acid, including 12 pairs of His, two pairs of Cys, three pairs of Glu, two pairs of Gly and one pair of Ser, which seems to suggest sequence self-repeating from an ancient monomeric origin. It is of note that among its 42 amino acids, Aβ(1–42) is also rich in pairs of one specific amino acid, i.e., one pair each of His, Phe, Ile, Gly and Val. Hpn has been proposed to play a role in nickel storage, homeostasis and detoxification [22,23]. In physiologically relevant conditions, Hpn is present in an equilibration of multimeric forms: with the 20-mers (ca. 136 kDa) being the predominant species [13], and this protein represents a novel class of metal binding proteins with the relative binding affinities: $Cu^{2+} > Ni^{2+} > Bi^{3+} \approx Zn^{2+}$ [24]. EPR data indicated that Cu^{2+} ions have a 4N (four histidines) or 3N1O/S (three histidines plus one oxygen/sulfur donor ligand) ligand environment during the binding to Hpn. Similarly, Aβ(1–42) has a picomolar affinity to Cu^{2+} with the coordinating ligands to be the three histidine imidazole nitrogens and two carboxylate oxygens [25].

Considering the various aspects in common between Hpn and amyloidogenetic peptides, we checked whether Hpn oligomers form amyloid-like fibrils in this present study. The cytotoxicity of Hpn oligomers to gastric epithelial cells was also investigated to deduce the possible biological meanings. The formation of amyloid fibrils by Hpn protein was confirmed by transition electron microscopy, Thioflavin T and Congo red binding assays. The amyloid fibrils of Hpn inhibit the proliferation of gastric epithelial AGS cells through cell cycle arrest and the disruption of mitochondrial bioenergetics. Possible involvement of Hpn amyloidogenesis in the pathologic mechanisms for *H. pylori* infections was discussed.

2. Materials and methods

2.1. Materials

Chemicals were of the highest reagent grade commercially available and used without further purification. Stock solutions were prepared by dissolving anhydrous salts in Milli-Q (Millipore) purified water and adjusted to the desired pH values with 1.0 M NaOH or HCl whenever needed. Congo red and Thioflavin T (ThT) were purchased from Sigma.

2.2. Hpn protein preparations

Recombinant Hpn protein was overexpressed and purified as described previously [13]. Briefly, a saturated overnight culture of *E. coli* BL21(DE3) transformed previously with the Hpn-expression plasmid pET-hpn was diluted into the LB media supplemented with 100 μg/ml ampicillin, 0.5 mM $NiSO_4$ and 0.5% glucose. Protein expression was induced with the addition of IPTG (0.5 mM) when an OD_{600} of 0.7 was reached, and the cultures were then incubated for another 4 h. Cells were harvested and resuspended in 10 ml of ice-cold Buffer A (20 mM sodium phosphate buffer, 500 mM NaCl, 100 mM imidazole, 1 mM PMSF, pH 7.4) per liter of cell culture. Bacteria were ruptured by sonication in the presence of 1% v/v Triton X-100. The lysates were centrifuged at 14,000 *g* for 30 min. The supernatant was loaded onto a home-made Ni-NTA resin column (1 ml, Roche). The column was washed with 10 ml of Buffer A, and then Hpn protein was eluted with 4 ml of Buffer B (20 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.4).

2.3. Hpn binding to amyloid-diagnostic dyes

Hpn aggregates were tested for Congo red binding by the spectroscopic band-shift assay as described [26]. Proteins were diluted to a final concentration of 10 μM in a buffer containing 20 mM Tris (pH 7.2) and 10 μM Congo red, and incubated for 30 min at ambient temperature. For the ThT assay, Hpn proteins were diluted to 10 μM with 20 mM Tris buffer (pH 7.2), and a final concentration of 5 μM ThT was added. The fibrillation kinetics was followed with a Shimadzu RF5301 spectrophotometer at an excitation wavelength of 450 nm and an emission wavelength of 482 nm.

2.4. Transmission electron microscopy

Samples (10 μl) were absorbed on 400-mesh copper grids for 2 min, washed twice with deionized water and negatively stained with 1% (wt/vol) uranyl acetate for 90 s. Fibril aggregates were visualized at 100 kV on a Philips Tec-Nai 10 microscope.

2.5. Proliferation assay

Gastric epithelial AGS cells were maintained at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Around 5000 cells/well were grown in 96-well plates and incubated overnight. The cells were then treated with various concentrations of Hpn fibrils of 5-day age for 8 h. MTT (Sigma) labeling reagent was added into each well, and the cells were incubated for another 4 h. The supernatant was removed, and 100 μl of hydrochloric acid (40 mM) in isopropanol was added to each well. The optical densities were measured at 590 nm with a reference filter at 620 nm.

2.6. Cell cycle analysis

Cells either treated with 4.2 μM Hpn fibrils or untreated (buffer only without Hpn fibrils) for 8 h were harvested, washed twice with cold PBS, fixed by vortexing in 70% ice-cold ethanol, treated with RNase, stained with propidium iodide (PI) and then analyzed by FACStar Plus flow cytometry, as previously reported [27]. The WinMDI 2.9 software was used to calculate the percentage of the populations in sub-G0/G1, G0/G1, S and G2/M phases.

2.7. Mitochondrial transmembrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential was measured by the lipophilic cationic probe, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) as described previously [27,28]. Cells were incubated with 5 μM JC-1 for 30 min and then washed with PBS. Mitochondrial membrane potential depletion was observed under a fluorescence microscope. A green filter was used for green-fluorescent monomer at depolarized membrane potential and a red filter for orange-fluorescent J-aggregate at hyperpolarized membrane potentials.

2.8. ATP assay

Cells were seeded into 96-well plate without or with the treatment of 4.2 μM Hpn fibrils. At time intervals, cells were harvested and washed twice in cold TE buffer (20 mM Tris–HCl, pH 7.8, 2 mM EDTA), and resuspended in hot TE buffer for 20 min. ATP levels were determined for the supernatant after centrifugation of the cell suspension, with a luciferase–luciferin enzymatic assay kit from Molecular Probes. The

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