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Immunosuppressive Proteins Isolated from Spiral and Coccoid Cytoplasmic Solutions of *Helicobacter pylori*

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Abstract: The aim of this study was to investigate the antiproliferative proteins that probably have a role in *Helicobacter pylori* evade of immune response and cause chronic infection disease and also to see if coccoid form had a role in its chronicity. *H. pylori* strain VacA s2/m2 positive and CagA negative, from a gastric biopsy of a patient with peptic ulcer disease, was isolated and cultured in brucella agar. Both spiral and coccoid forms were harvested and ruptured by sonication. The cytoplasmic solutions of both forms were collected and their fractions obtained by gel chromatography and preparative polyacrylamide gel electrophoresis. The fractions were analyzed by MTT assay for their antiproliferative activity. We isolated two proteins with a significant dose dependent antiproliferative activity that analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, one of them that was urease positive showed two bands with 61 and 27 kDa, which is resumed to urease of *H. pylori*, another consist of 57 and 63 kDa. *Helicobacter pylori* secret some proteins like urease that inhibit immune cells proliferation response against its antigens.

Key words: *Helicobacter pylori*, spiral and coccoid forms, immunosuppressive, MTT

INTRODUCTION

Helicobacter pylori is a gram negative and spiral bacterium that has infected more than 50% of world's population. *H. pylori* infection is related to gastric ulcer and MALT lymphoma (Kimmel *et al*., 2000).

Although, *H. pylori* are noninvasive bacteria, it induces chronic infection (Marshall, 2002). *H. pylori* infection is almost asymptomatic and less than 10% of the infected individuals have ulcer or gastric associated diseases. These patients are in high risk of stomach cancer. Relationship between *H. pylori* infection and gastric cancer is so significant that World Health Organization (WHO) has organized *H. pylori* as a type I carcinogen. It is postulated that long-term inflammation induced by *H. pylori* has an important role in gastric cancer development (Bah *et al*., 2001).

Helicobacter pylori releases factors that are responsible for colonization and pathogenesis (Franco *et al*., 2008). It has been shown by Ibraghimov and Jacques (2000) that *H. pylori* secrets a protein that inhibits epithelial and lymphocyte cell lines proliferation and that its effects is dose dependent. It was also shown that urease, VacA and CagA have cytotoxic effects on cells. In the stomach, mostly spiral forms of *H. pylori* were found and coccoid forms were in the more damaged regions of gastric mucosa (Saito *et al*., 2003).

It has been shown that chronic inflammation promotes cancer (Shacter *et al*., 2002). Despite local and systemic response to *H. pylori* infection (Bhat *et al*., 2005), *H. pylori* induces chronic gastric inflammation. Epidemiological studies have shown that gastric cancer and MALT lymphoma are related to *H. pylori* infection (Feldman, 2001). *H. pylori* is acquired in childhood (Mitchell *et al*., 2003), but gastric cancer is seen mostly after the six decade of life. It is postulated that chronic infection with *H. pylori* is necessary for gastric cancer (Feldman, 2001).

-It has been shown that lymphocytes from *H. pylori*, that infected individuals sometimes shows a decrease in proliferation, in response to *helicobacter* antigens *in vitro*. This had revealed that *H. pylori* might down regulate the cellular immune responses toward itself (Windle *et al*., 2005). It is possible that *H. pylori* secrets proteins that inhibit immune response to eradicate the infection locally (Ibraghimov and Jacques, 2000). The aim of this study was to isolate the cytoplasmic proteins of *H. pylori* responsible for the inhibition of T cells proliferation.

In this study, we isolated different fractions of both spiral and coccoid forms of *H. pylori* and evaluated their antiproliferative activity.

MATERIALS AND METHODS

Bacterial isolation and culture conditions: The *H. pylori* strain was isolated from gastric biopsy of a patient suffering from a gastric ulcer. Present study was

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performed in 2006 and it took 7 mounts. The cells were grown on brucella agar (Merck, Germany) supplemented with 10% sheep blood, vancomycin (10 mg mL^{-1}), nystatin (1 mg mL^{-1}) and trimethoprim (5 mg mL^{-1}) and incubated for 48 h in CO_2 incubator (10% CO_2, 5% N_2 and 85% O_2) (Ramarao and Meyer, 2001). Bacterial cells were harvested after the second and 13th days of incubation and suspended in PBS (pH 7.2). To assess the relative percentages of coccoid and bacillary forms, Gram-staining was used for each suspension. The Gram staining showed that the suspension of 2nd day culture, contained only spiral forms and 13th day culture contained coccoid forms.

Preparation of cytoplasmic solutions: The cells were collected and washed three times with 20 mL of PBS. The packed cells were frozen at -80°C before use. The cells were sonicated on ice (eight times for 30 sec each time). Unbroken cells and debris were precipitated by centrifuge at 4,000 x g for 10 min at 4°C. The supernatants were collected and centrifuged at 45,000 x g for 20 min at 4°C. The pellets contained cell walls with outer membrane protein (Ge *et al*., 2001) and the supernatants contained cytoplasmic proteins.

Chromatography: The cytoplasmic solutions obtained from spiral and coccoid forms were concentrated with freeze-drying and then dialyzed against PBS for three times. The concentrated solutions of spiral and coccoid forms (7 and 3 mg mL^{-1}) were applied in gel filtration column using S-200 and eluted with sodium phosphate buffered saline, pH 7.2, (0.02 M sodium phosphate, 0.15 M NaCl). Optical density was measured at 280 nm and fractions with maximum OD were pooled.

Preparative PAGE: Fractions with antiproliferative activity obtained by chromatography were used in preparative PAGE to isolate proteins with antiproliferative effect. A piece of the gel was cut from length and stained by silver stain. According to the stained gel, the protein bands were cut from wide of the remained gel and cut into pieces in eluting solution (0.1 M Tric-HCl; pH 7) and then centrifuged at 3200 x g for 15 min after shaking. The supernatants were collected and stored at 4°C for 30 min. Proteins were precipitated by acetone and the solution was stirred magnetically for 15 min at 20°C. After being centrifuged at 3200 x g, the acetone was removed and the proteins were diluted in PBS. The isolated proteins were dialyzed against PBS and prepared for proliferation test. The concentration of proteins was determined by Bradford assay (Kruger, 2002). The presence of urease activity in the stomach biopsies and the isolated proteins was determined with a Christensen urease medium. A rapid color change suggests the presence of urease activity.

Cell viability assay: The antiproliferative effect of the fractions on Jurkat cell lines, a $CD4^+$ leukemia T-cell line, were determined by MTT assay described by Young *et al.* (2005). The cells were maintained in the culture medium in 10 mL culture flasks at 37°C in a 7% CO_2 humidified atmosphere. Cell viability was assessed by trypan blue and the exclusion was over 90%. The cells were seeded in 96-well microtiter plates at a density of approximately 4×10^6 cells/well containing 100 µL RPMI 1640 (Gibco, UK), 10% heat-inactivated Fetal Calf Serum (FCS), 100 U L^{-1} penicillin and 100 mg L^{-1} streptomycin (Gibco, Scotland). Each fraction was added in various concentrations and some wells were leaved without samples as controls. All MTT assays were measured in triplicate. 10 µL of the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium-bromide, 5 mg mL^{-1}; Sigma) solution was added to each well and incubated for 4 h until a purple precipitate was visible. The developed crystals were dissolved in 100 µL of 0.04 M HCl in isopropanol and the OD was read in a microplate spectrophotometer at a wavelength of 570 nm.

The stimulation index was calculated by dividing OD at 570 nm with sample by OD at 570 nm without samples. Student's t-test was utilized for the statistical analysis of the samples. Differences between the treatment means were considered statistically significant if $p<0.05$.

RESULTS AND DISCUSSION

Isolation of antiproliferative proteins from cytoplasmic solutions: After chromatography of cytoplasmic solutions, various fractions were pooled as S1, S2 and S3 for spiral forms and C1, C2 and C3 for coccoid forms (Fig. 1a, b). The fractions with antiproliferative activity (S2 and C2) were applied in preparative PAGE for isolation of proteins with antiproliferative activity. The preparative PAGE (Fig. 2) was cut in 6 pieces (P1, P2, P3, P4, P5 and P6) from length in both S2 and C2. P5 band was not seen in preparative PAGE of C2. Both P4 and P5 consisted of one band in preparative PAGE. The P5 from S2 with urease activity showed 61 and 27.5 kDa bands in SDS-PAGE, P4 showed two bands of 63 and 57 kDa (Fig. 3a, b).

Antiprolifrative activity of the isolated proteins: After gel chromatography, the fractions were analyzed by MTT assay for their antiproliferative activity (Fig. 4a). The cells treated with S2 and C2 showed lower OD than negative controls ($p<0.05$). No statistical difference was observed between other fractions.

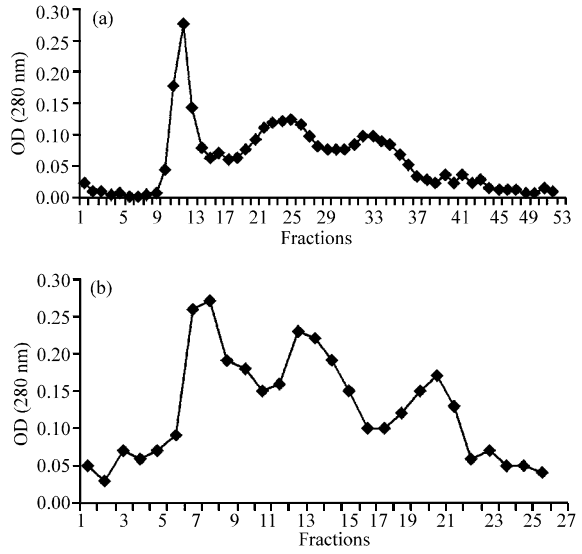

Fig. 1: Cytoplasmic solutions of (a) spiral and (b) coccoid forms were applied in gel filtration column, using S-200. The 1-17, 18-30 and 31-56 fractions were pooled as S1, S2 and S3 for Spiral forms and 1-11, 12-17 18-23 as C1, C2 and C3 for coccoid forms, respectively

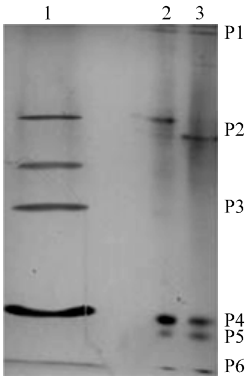

Fig. 2: PAGE analysis of spiral and coccid forms fractions 2, S2 and C2, obtained by gel chromatography. Lane 1. C2, Lane 2 and 3. S2. The preparative PAGE of S2 and C2 were cut in 6 pieces (P1-P6), from its wide

The P1 to P6 fractions obtained by preparative PAGE were analyzed by MTT assay (Fig. 4b). The P4 and P5 fractions showed significant dose dependent antiproliferative activity on Jurkat cells.

CagA, VacA and urease are antiproliferative proteins (Takagi *et al*., 2000) but VacA s2/m2 does not have the cytotoxic effect that is due to defect in N-terminal of this protein. For inhibiting the interference of CagA and VacA

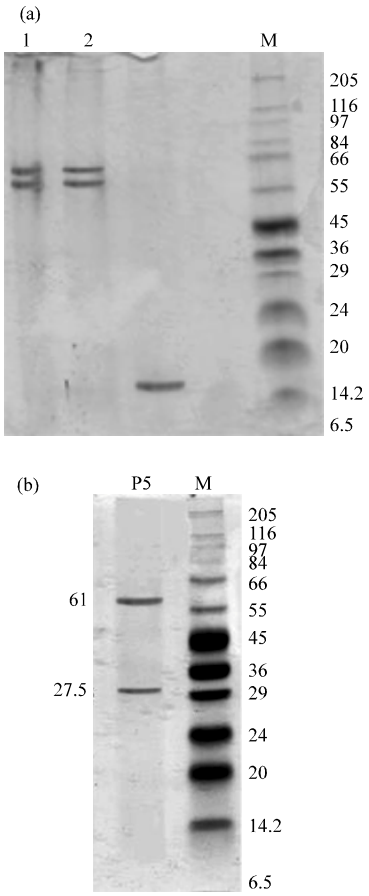

Fig. 3: SDS-PAGE analysis of fractions isolated from preparative PAGE (a) Lane 1. P4 isolated from S2, lane 2. P4 isolated from C2, (b) lane 1. P5 isolated from preparative PAGE of S2

cytotoxic effect the selected strain was urease positive, VacA s2/m2 positive and CagA negative.

Spiral forms had two proteins with antiproliferative activity ($p<0.05$): P5 with urease activity in SDS-PAGE showed two bands, 61 and 27.5 kDa, the other (P4) showed two bands of 63 and 57 kDa. It is likely that P5 protein and its subunits that were shown in SDS-PAGE are similar to urease protein of *H. pylori* (Figueroa *et al*., 2002). It has been shown that in coccoid forms, urease mRNA does not exist (Bah *et al*., 2001). We have shown that in coccoid forms, only P4 was expressed and urease was not expressed.

The isolated protein (P4) showed a dose dependent antiproliferative activity that is necessary be cloned for more recognition and sequencing.

Relation between *H. pylori* and gastric cancer is so certain that WHO has classified *H. pylori* in class I carcinogens (Romero-Gallo *et al*., 2008). Chronic

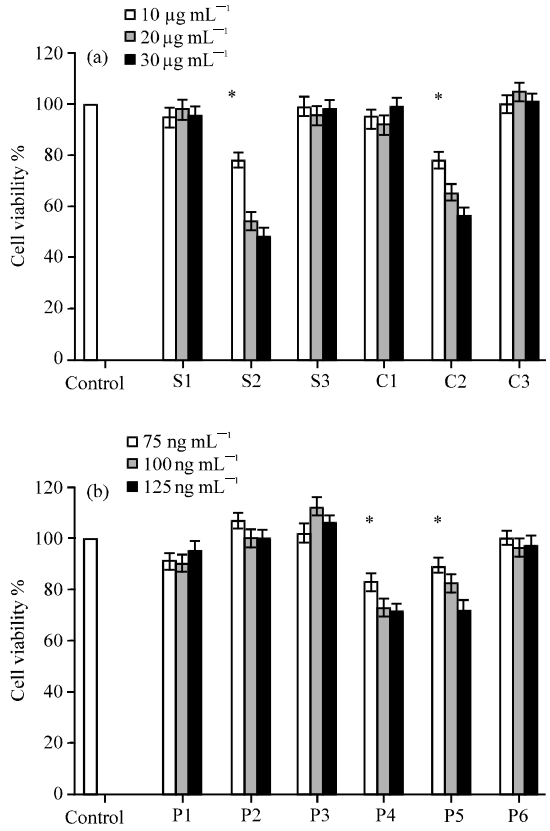

Fig. 4: *In vitro* influence of different concentrations of *H. pylori* fractions on proliferation of Jurkat T cell was estimated by MTT assay. Experiments were performed in triplicate and at least 3 separate experiments, the Means±SD of the values are indicated. (A) S1, S2 and S3 from spiral forms and C1, C2 and C3 from coccoid forms, obtained by gel filtration (10, 20 and 30 µg mL^{-1}) and (B) P1, P2, P3, P4, P5 and P6 obtained by preparative PAGE (75, 100, 125 ng mL^{-1}) were utilized in MTT assay. Untreated control cells were given the value of 1 (100% SI). *Indicates a significant difference in comparison with control as shown by Student t-test, at $p<0.05$

inflammation alone is not sufficient to induce gastric cancer. *H. pylori* requires a direct effect of such mutagens to induce tumorogenes. *H. pylori* induces progression of carcinoma, but it may have direct effect on gastric to induce carcinoma.

Helicobacter pylori has a role in development of gastric carcinoma. It is postulated that *H. pylori* induces mutation in cells and inhibits proliferation of immune cells and also induces initiation and progression of carcinoma (Leon-Barua *et al*., 2006).

CONCLUSION

In this study it was shown that both spiral and coccoid forms of *H. Pylori* have two antiproliferative proteins that one of them had urease activity and the other showed two bands of 63 and 57 kDa. It is recommended to evaluate the effect of the unknown protein on immune response since it perhaps have a role in the chronic infection and also stomach cancer.

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