ROLE OF TUMOUR NECROSIS FACTOR-ALPHA
EXPRESSION IN \textit{H. pylori} INFECTION

3.1 INTRODUCTION

The role of infectious agents in carcinogenesis has commanded significant scientific interest culminating in 5 nobel prizes in the 20\textsuperscript{th} century (Kuper \textit{et al.}, 2000). Infection can cause cancer by a variety of mechanisms including direct transformation of cells, induction of immunosuppression with consequent reduced cancer immunosurveillance, or by causing chronic inflammation. The latter is becoming increasingly recognized as an essential component of many epithelial cancers by virtue of its combined effects of generating genotoxic by-products and increased cellular proliferation, thus maximising the potential for DNA damage. Gastric cancer is a major global health problem that claimed 800,000 lives in 1998. It remains the world’s second commonest malignancy, having only been overtaken by lung cancer in the late 1980’s. In 1994, the IARC declared \textit{H. pylori} as a group 1 carcinogen (IARC monographs 1994). This bold statement was met with considerable scepticism but in the ensuing 8 years, evidence from epidemiological and inventional studies in humans as well as experiments with rodents has convinced many that this bacterial infection is indeed the key factor in the initiation of the neoplastic process in the stomach.
*H. pylori* infection is acquired during childhood, most probably via the fecal oral, or gastric/oral routes, and if not treated with antibiotics will persist throughout life. Although the bacteria mainly reside on the surface of mucus gel layer with little invasion of the gastric glands, the host responds with an impressive and cell-mediated immune response. However, immune response is largely ineffective as most infections become chronically established with little evidence that spontaneous clearance had occurred.

*H. pylori* has to survive in one of the harshest and least hospitable niches in the human body. Despite this, *H. pylori* seems well equipped and adapted for habitation within this harsh environment. In order to understand how this bacterium can predispose to gastric cancer, it is necessary to understand the basic pathophysiologic consequence of its presence within the human stomach.

### 3.2 *HELIcobacter pylori* and Chronic GASTRIC INFLAMMATION

The key pathophysiologic event in *H. pylori* infection is initiation of an inflammatory response (Israel *et al.*, 2001). This response is most probably triggered by the bacterium's lipopolysaccharide, urease and/or cytokines. Inflammation is mediated by cytokines, the cytokine repertoire comprising of a multitude of pro and anti inflammatory mediators whose function is to co-ordinate an effective immune/inflammatory response
against invading pathogens without causing undue damage to the host. In addition to their pro or anti-inflammatory properties, some *H. pylori*-induced cytokines have direct effects on gastric epithelial cells that have a profound effect on gastric physiology. The pro inflammatory cytokine interleukin-1 beta (IL-1 beta) is the most potent of known agents that are gastric cytoprotective, antinuclear, antisecretory, and inhibitors of gastric emptying (Robert *et al*., 1991).

Wolfe and Nompleggi (1992) estimated on molar basis, that IL-beta is 100 times more potential than both prostaglandins and the PPI omeprazole is 6000 times more potent than cimetidine in inhibiting acid secretion. Another important pro-inflammatory cytokine that is up-regulated by *H. pylori* infection is the tumor necrosis factor alpha (TNF-alpha), which also inhibits gastric acid secretion, but to a lesser extent than IL-1 beta (Beales and Calam, 1998).

3.3 ASSOCIATION OF TNF-α IN *H. PYLORI* MEDIATED GASTRIC CARCINOGENESIS

*H. pylori*-associated gastroduodenal diseases are characterized by the severe infiltrations of neutrophils, lymphocytes, monocytes and plasma cells in the gastric mucosa. Indeed, cure of the infection results in a notable reduction of these cells in the gastric mucosa (Ando *et al*., 1998). The accumulation and activation of inflammatory cells have been induced by the local production of cytokines (Lindholm *et al*., 1998) and cytokines are
proposed to play an important role in the pathogenesis of *H. pylori*-associated gastroduodenal diseases. Many investigators have examined the relation between *H. pylori* and mucosal cytokines (Crabtree *et al.*, 1995; Karttunen *et al.*, 1997; Moss *et al.*, 1994; Noach *et al.*, 1994).

Several potential virulence factors derived from *H. pylori* are considered to stimulate the cytokine induction in the gastric mucosa, through attracting and activating neutrophils and mononuclear cells (Huang *et al.*, 1995; Rieder *et al.*, 1997). However, it remains unclear how these non-invasive bacteria residing in the gastric mucous layer, produce the inflammation and cause the damage to the underlying epithelial tissue. Furthermore, the specific *H. pylori* protein responsible for stimulating the cytokine induction has not been identified, although the Cag pathogenicity island was reported to be closely related to IL-8 production (Censini *et al.*, 1996).

Gastric mucosal damage in *H. pylori* infection depends on specific virulence factors of *H. pylori* including enhanced motility (Hazell *et al.*, 1986) and production of enzymes, such as urease (Marshall *et al.*, 1990), catalase (Hazell *et al.*, 1991) and phospholipase (Crabtree *et al.*, 1991). Specific adherence to gastric epithelial cells (Dunn, 1993), production of a vacuolating cytotoxin (Vac A) by some *H. pylori* strains (Leunk *et al.*, 1988) and the presence of bacterial gene cluster (Cag PAI) (Censini *et al.*, 1996) are also important in the inflammatory reaction of the host, which is modulated by secretion of various cytokines, like IL-8 (Crabtree, 1991).
gamma interferon (IFN-γ) (Karttunen et al., 1995) and tumour necrosis factor alpha (TNF-α) (Crabtree et al., 1991).

TNF-α is a key mediator in host response against gram-negative bacteria and in the septic shock syndrome induced by either lipopolysaccharide (LPS) or bacterial superantigens (Beutler and Cerami, 1988). Secretion of TNF-α from LPS-activated mononuclear phagocytes or antigens stimulated T cells can be enhanced by IFN-γ. In H. pylori gastritis, the cytokine response is of the Th 1 type since IFN-γ but not IL-4 is predominant (Lindholm et al., 1998). In mice lacking interferon regulatory factor 1, the defective Th 1 response was associated with the total lack of gastritis and atrophy disease after severe colonization with H. pylori (Takagi et al., 2000). The multiple biological activities of TNF-α such as stimulation of expression of adhesion molecules, activation of leukocytes and T-lymphocytes, stimulation of the production of cytokines by macrophages and monocytes (Hatz et al., 1997; Talmadge et al., 1998) and induction of apoptosis (Kim et al., 2000), (Tartaglia et al., 1993). These activation and stimulation facilitate the extravasation of neutrophils into the lamina propria of mucosal tissues, which is mediated by two district cell surface receptors, Tumour necrosis factor receptor 1 (TNF-R1) binding TNF-α and lymphotoxin alpha (LT-α) = (TNF-β), (Erikstein et al., 1991).
3.4 WORKING HYPOTHESIS

The immune response to *H. pylori* is considered to be a major factor contributing to gastric mucosal damage (Crabtree, 1998). Recent *in vitro* studies showed that *H. pylori* stimulates human blood monocyte and possibly mucosal macrophage production of inflammatory cytokines, including interleukin IL-1β, IL-6 and tumor necrosis factor (TNF)-α (Harris *et al.*, 1996), but the inflammatory responses within the gastric mucosa *in vivo* are not known. Therefore, in this study we hypothesise that the expression of tumor necrosis factor alpha induced by *H. pylori* infection is involved in the tumor initiation and promotion of gastric carcinogenesis. Thus sensitive molecular methods such as RT-PCR, western blotting and immunohistochemistry were employed. These findings may help in the early prevention of the disease.

3.5 STUDY DESIGN

This study was carried out in two hundred gastric specimens, consisting of normal gastric mucosa (n=20), mucosa with chronic gastritis (n=63), atrophic gastritis (n=20), intestinal metaplasia (n=11) and gastric adenocarcinoma (n=86), in which the *H. pylori* status have been analysed already (Chapter II). Histopathological grading of specimens were carried out by haematoxylin and eosin staining according to revised Sydney system. The expression of TNF-α was studied at mRNA as well as protein level
using RT-PCR and western blotting respectively. The localization of TNF-α was also studied semiquantitatively by immunohistochemistry.

The data obtained were subjected to statistical analysis for the association of TNF-α between histological progression and *H. pylori* status.

### 3.6 METHODOLOGY

Gastric biopsy samples were processed as given in Chapter-II. From each case, one biopsy sample was used to prepare frozen sections for histology and immunohistochemistry. 2 biopsies were used for western blotting analysis and one biopsy was used for RNA extraction and subsequent RT-PCR.

#### 3.6.1 RT-PCR analysis of TNF-α expression

A fresh biopsy sample was snap frozen in liquid nitrogen and RNA was extracted using standard TRIZOL protocol. The extracted total RNA was reverse transcribed to cDNA by using reverse transcriptase. PCR was performed on the cDNA using standard primers published already.

Primers

**Forward 5'-TTC TGC CTG CTG CAC TTT GGA CTC AT-3’**

**Reverse 5'-TTG ATG GCA GAG AGG AGG TTG ACC TT-3’**

(Detailed protocol is given in Appendix).
3.6.2 Immunoprecipitation and Western Blotting

The tissue lysates prepared from the biopsies were immunoprecipitated with TNF-α mouse monoclonal antibody using protein A sepharose. The immunocomplex was then resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane. The transferred protein was then hybridized using TNF-α mouse monoclonal antibody and the expression was identified using antimouse secondary antibody tagged with HRP. DAB substrate was used as a chromogen (see Appendix for detailed protocol).

3.6.3 Immunohistochemistry and immunofluorescence

The TNF-α protein expression was semiquantitatively analysed by immunohistochemistry and further confirmed by immunofluorescence analysis. The cryostat sections were used for the analysis. After airdrying and fixation in acetone, the tissue sections were treated with 1% NP40 for membrane lysis and treated with TNF-α mouse monoclonal antibody. After appropriate incubation and washing, the sections were treated with HRP conjugated antimouse secondary antibody (or) FITC tagged antimouse secondary antibody in case of immunofluorescence. DAB chromogen was used in immunohistochemistry, counter stained with haematoxylin and examined under light microscope. In case of immunofluorescence, the sections were directly analysed under Axioscope-II fluorescence microscope using appropriate filters (for detailed protocol see Appendix).
Sections of tonsillitis tissue were used as positive control. Sections without primary antibody treatment were used as negative control.

3.6.4 Assessment of immunoreactivity

Immunoreactivity of TNF-α was graded based on the intensity of the brown DAB colour developed and the cells showing positivity. At least 10% of the cells in the field showing positivity was taken as significant TNF-α expression, 10-25% cells mild expression, 26-50% moderate, 51% and more were taken as intense expression (Table 3.1).

Table 3.1 Assessment of Immunoreactivity

<table>
<thead>
<tr>
<th>Immunoreactive cells</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10%</td>
<td>Negative</td>
</tr>
<tr>
<td>10-25%</td>
<td>Mild</td>
</tr>
<tr>
<td>26-50%</td>
<td>Moderate</td>
</tr>
<tr>
<td>51% and above</td>
<td>Intense</td>
</tr>
</tbody>
</table>

3.6.5 Data analysis

χ² analysis were used to identify the association of TNF-α with histopathological grade and with *H. pylori* status. The expression of TNF-α was analysed using three techniques, RT-RCR and western blotting for qualitative analysis and immunohistochemistry for semiquantification. The
patients were considered TNF-α positive, if they were detected positive by both RT-PCR and western blot.

3.7 RESULTS

3.7.1 TNF-α Expression in the Study Population

RT-PCR experiments were done to characterise the presence of TNF-α mRNA transcripts in gastric biopsy samples. A representative RT-PCR result is shown (Plate 2.1a) The presence of 580 bp transcript determines the expression.

Western blotting was done to identify the presence of TNF-α protein in gastric biopsy sample. A representative western blot is shown in Plate 2.1b. The detection of 19 kDa protein band showed the presence of TNF-α.

Out of 200 cases analysed for TNF-α expression by RT-PCR and WB, (only 41 cases: 20.5%) were negative for TNF-α and the remaining 159 cases (79.5%) were expressing TNF-α. In that, out of 20 normal patients 18 (90%) were not expressing TNF-α mRNA and only 2 (10%) showed mRNA expression of TNF-α. The expression of TNF-α in gastric preneoplastic lesion, chronic gastritis, atrophic gastritis, intestinal metaplasia were 51/63 (81%), 20/20 (100%) and 11/11 (100%) respectively. In gastric adenocarcinoma, out of 86 patients 65 showed (75.6%) TNF-α expression.
A Proteins were electrophoresed on SDS-PAGE (5% and 10%) and visualised by CBB staining

B Proteins were immuno precipitated and electrophoresed on SDS-PAGE (5% and 10%) and visualised by CBB staining

C The proteins were transferred to nitrocellulose membrane and incubated with anti TNF-Alpha MAb followed by a second antimouse peroxidase coupled antibody, visualised by diaminobenzidine staining.

D RT-PCR analysis of TNF-Alpha expression. *H. pylori* positive specimens showing transcript 580 bp, in contrast to normal showing no transcript.

E The quality and abundance of the cDNA were confirmed using β-actin specific PCR.
M-100bp ladder 1. Normal Stomach  2. Chronic Gastritis  3. Atrophic Gastritis  
4. Intestinal Metaplasia  5. Adeno carcinoma
Figure 3.1 Expression of TNF-alpha in the study population
Figure 3.2 TNF-Alpha expression in relation to study population
These results indicate the steady increase in the expression of TNF-α from normal to intestinal metaplasia. When the data were analysed for association with progression, the significant association between TNF-α and histology was observed (P<.001 \( \chi^2 = 55.842 \)), Fig.3.1).

3.7.2 **Association of *H. pylori* infection with TNF-α Expression**

Out of 57 *H. pylori* negative patients 28 cases (49%) were positive for TNF-α expression and 29 cases (51%) were not expressing TNF-α. Out of 143 *H. pylori* positive cases only 22 cases 22/143 (15.4%) were negative for TNF-α, while the remaining cases 121/143 (84.6%) were expressing TNF-α. There is a highly significant association between *H. pylori* infection and TNF-α. (p <0.0001 and \( \chi^2 = 27.025 \)). The data were summarised in the Table -3.3. These results suggest that TNF-α expression plays an important role in the *H. pylori* mediated pathogenesis, Fig. 3.2.

3.7.3 **Immunohistochemical Localisation of TNF-α in Study Population**

A distinct membranous or extracellular immuno reactivity for TNF-α was judged as positive. The expression of TNF-α was represented in 5 groups. The immuno reactivity assessment was carried out as mentioned earlier in the methodology.
PLATE 3.2

Immunohistochemical analysis of TNF-α

(A) Normal stomach mucosa (40 x magnification)
(B) Chronic gastritis (40 x magnification)
(C) Gastric Atrophy (40 x magnification)
(D) Intestinal Metaplasia (40 x magnification)
(E) Cancer stomach (40 x magnification)
PLATE-3.2

Immunohistochemical Expression of TNF-α in various stages of *Helicobacter pylori* Mediated Gastric Carcinogenesis
PLATE 3.3

Immunofluorescence analysis of TNF-α

(A) Normal stomach mucosa (20 x magnification)
(B) Chronic gastritis (20 x magnification)
(C) Gastric Atrophy (20 x magnification)
(D) Intestinal Metaplasia (20 x magnification)
(E) Cancer stomach (20 x magnification)
PLATE-3.3

Immunofluorescent Analysis of TNF-α in various stages of *Helicobacter pylori* Mediated Gastric Carcinogenesis
Figure 3.3 Immunohistochemical expression of TNF-alpha in different Histopathological Stages

- Normal (n=20)
- Gastritis (n=63)
- Atrophy (n=20)
- IM (n=11)
- Adenocarcinoma (n=86)

Histopathological Stages

Levels of TNF-alpha expression:
- Negative
- Mild
- Moderate
- Intense
The histopathological grades were assigned into 5 groups as mentioned earlier, and the TNF-α expression was correlated with these histopathological stages in the same 200 study population. The expression of TNF-α in relation to the histopathological stages are shown in Plate 3.2, 3.3. There was significant expression of TNF-α increase in the precancerous condition from chronic gastritis to intestinal metaplasia.

The data for TNF-α immuno reactivity among the 5 histopathological stages are given in figure 3.4. We observed that all the 20 normal samples analysed were negative for TNF-α expression (100%). In chronic gastritis cases, only 14 (22%) were negative for TNF-α, 43 (65.5%) were mild and 6 (9.5%) were moderate. No intense staining was noticed in this group. In atrophy, no negative staining were observed, 7/20 (35%) were mild, 9/20 (45%) were moderately and 4/20 (20%) were stained intensely. In intestinal metaplasia, no negative staining were observed, 2/11 (18.2%) mild, 5/11 (45.5%) moderate and 4/11 (36.4%) intensely stained. Of the 86 gastric cancer patients 18/86 (20.9%) were negative, 29/86 (33.7%) were mild, 31/86 (36.0%) were moderately stained and 8/86 (9%) were intensely stained for TNF-α. When the data were analysed for the association between expression and histopathological stages, highly significant association was observed. p<0.0001 and χ²=115.28, Fig.3.3.
3.7.4 Association of immunoreactivity of TNF-α and *H. pylori* Infection

The immuno reactivity of TNF-α in relation to *H. pylori* status was analysed in the study population. Table 3.5 explains the data analysis.

Among the 57 *H. pylori* negative cases 26/57 (45.6%) were negative, 12/57 (21%) were mild, 16/57 (28%) were moderate and 3/37 (53%) were intense. Whereas among 143 positive cases, only 26/143 (18.2%) were negative, remaining 69/143 (48%) were mild, 35/143 (24.5%) were moderate and 13/143 (9%) were intensely stained for TNF-α expression. Highly significant association p<0.0001 and $\chi^2=20.193$ (Fig.3.4) was observed. This indicates the close association between the *H. pylori* infection and TNF-α expression in gastric carcinogenesis.

3.8 DISCUSSION

Several studies have indicates that infection with *H. pylori* induces the expression and production of various cytokines in gastric mucosa, and it is known that cytokines contribute to pathogenesis of *H. pylori* gastro duodenal diseases. (Ando, 1998; Crabtrec *et al.*, 1995 ; Moss *et al.*, (1994). As reported previously, an inflammatory cell infiltrate, composed mainly of neutrophils is present in *H. pylori* associated gastro duodenal disease. The continued accumulation of neutrophils at the site of inflammation could be an important pathogenic mechanism (Jone *et al.*, 1984). Also histological
Figure 3.4 Immunohistochemical expression of TNF-alpha in relation to H. pylori

H. pylori Positive

H. pylori negative

TNF-Alpha Expression

80 70 60 50 40 30 20 10 0
association between *H. pylori* and the inflammatory infiltrate is indicative of recruitment by inflammatory mediators.

Our study shows the increased expression of TNF-α in the sequential evolution of gastric cancer. The gastric precancerous conditions such as chronic gastritis, atrophy and intestinal metaplasia showed the high expression of TNF-α. The results are consistent with the previous studies (Fan *et al.*, 1995). The enhanced gastric epithelial cell apoptosis in *H. pylori* infection has been suggested to play an important role in the pathogenesis of chronic gastritis, peptic ulcer and gastric neoplasia. There are a number of mechanisms that may be involved, including the direct cytotoxic effects of the bacteria, as well as inflammatory responses elicited by infection (Rudi *et al.*, 1998; Wagner *et al.*, 1997) Recent studies have suggested that T helper type 1 (Th1) cells are selectively increased during *H. pylori* infection (Barnford *et al.*, 1998; Lindholm *et al.*, 1998). Th1 cytokines such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) can increase the release of proinflammatory cytokines, augmenting apoptosis induced by *H. pylori* (Wagner *et al.*, 1997). Suginuma *et al.*, (1999), demonstrated that TNF-α is the first instigator in tumor promotion and the sequence of cytokine network for tumor promotion appears to be from TNF-α through IL-1 and IL-6. In another study Suginuma *et al.*, (2001) proposed the new carcinogenic mechanism that the expression of TNF-α protein induced by HP-MP1 and urease B, resulted in the cell transformation in cooperation with viral Ras protein. The results of our study suggest that the
increased expression and accumulation of TNF-α may be involved in tumor initiation which show the highly significant association with the histopathological progression (P<0.0001). The *H. pylori* infection and TNF-α expression in the sequential evolution too showed the highly significant association (P<0.001).

Mononuclear phagocytes participate in innate response to microorganisms by providing rapid anti-microbial activity presenting the antigens to the acquired immune system response and directing Th cells towards a cell-mediated pathway. Mononuclear phagocytes also produce soluble mediators, including IL-1β, IL-6 and TNF-α, which causes the release of the acute-phase reactants, promote inflammation and enhance functions of other effector cells. Thus, the ability of *H. pylori* to induce the expression of macrophage-derived cytokines in the gastric tissue of infected patients demonstrates that this non-invasive bacterium evokes an innate response in the stomach, elucidating the macrophage response and the role of TNF-α in directing the adaptive insight into the development and maintenance of the chronic inflammation induced by *H. pylori*.

In conclusion, our observations implicate the role of inflammatory cytokines in the pathogenesis of *H. pylori* associated gastro duodenal diseases. The emerging understanding of the potential role of TNF-α in *H. pylori* mediated gastric carcinogenesis may provide a new basis for the design of anti-inflammatory agents.
Chapter-IV