Regulatory effects of pro-inflammatory cytokines on genes associated with gastric carcinogenesis

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Als meus pares i al meu iaio

Al Ramon pel seu suport incondicional

Gràcies a tots els companys i amics que he fet durant aquests anys a la UBCM i a l'IMIM per moments inoblidables dins i fora del laboratori.

Abstract

In the gastric carcinogenetic process the specific expression pattern of glycosyltransferases and Lewis antigens displayed by the normal gastric mucosa is lost. We detected that changes in the expression of Lewis antigens induced by the transfection of FUT1 cDNA in HT-29/M3 cells determined a less invasive and metastatic phenotype.

Chronic gastritis caused by *H. pylori* infection is a major determinant in the pathogenesis of gastric cancer, and present increased levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, which can regulate the expression of genes involved in the gastric neoplastic transformation. We analysed the effect of pro-inflammatory cytokines on the expression of glycosyltransferases and Lewis antigens in gastric cancer cells. IL-1β treatment increased the expression of type 2 Lewis antigens, and, in addition, we observed that gastric tumours with chronic inflammation displayed increased levels of type 2 glycan structures, suggesting that specific glycosyltransferases may be regulated by inflammation.

Intestinal-type gastric adenocarcinomas develop from successive pre-cancerous lesions that lead to an intestinal transdifferentiation of the gastric mucosa. In this process many intestinal genes are activated in gastric cells, such as the intestinal mucin MUC2 and MUC4 genes and the transcription factor CDX2. We studied the effect of pro-inflammatory cytokines and their associated signalling pathways on the expression of these genes. IL-6 regulated the expression of MUC4 through the gp130/STAT3 signalling pathway in gastric cancer cell lines, while MUC2 expression was induced by TNF-α through the NF-κB signalling pathway. No effects on CDX2 expression were observed after cytokine treatment in gastric tumour cells. Finally, we found that the differences observed in the expression of the intestinal mucins MUC2 and MUC4 in gastric tumours could be explained by differences in the predominant type of inflammation present in gastric adenocarcinomas.

In conclusion, our results suggest that inflammation and its associated signalling pathways modulate the expression of genes associated with gastric carcinogenesis.

CONTENTS

PUBLICATIONS

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ABBREVIATIONS

AMOP, adhesion-associated domain in MUC4 and other proteins

APC, adenomatous poliposis coli

Cag PAI, Cag Pathogenicity Island

CDX, caudal-related homeobox protein

CK, cystine knot

COX-2, cyclooxigenase 2

EGF, epidermal growth factor

ER, endoplasmic reticulum

ERK, extracellular signal-regulated kinase

FGF, fibroblast growth factor

Fuc, fucose

FUT, fucosyltransferase

GalNAc, N-acetylgalactosamine

GlcNAc, N-acetylglucosamine

GSK, glycogen synthase kinase

H. pylori, *Helicobacter pylori*

HCl, hydrogen chloride

HDGC, hereditary diffuse gastric carcinoma

ICAM, intercellular adhesion molecule

IFN, interferon

IGF, insuline-like growth factor

IL, interleukin

IL1R, interleukin-1 receptor

iNOS, inducible nitric oxide synthase

JAK, janus kinase

LOH, loss of heterozygosity

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MMP, matrix metalloproteinase

MUC, mucin

NF-κB, nuclear factor κB

NeuAc, sialic acid

NIDO, nidogen homology region

PMA, phorbol 12-myristate 13-acetate

RAR, retinoic acid receptor

RUNX, runt related transcription factors

s-Lewis, sialyl-Lewis antigen

SEA, sea urchin sperm protein enterokinase and agrin

SOCS, suppressor of cytokine signalling

SPEM, spasmolytic polypeptide expressing metaplasia

ST, sialyltransferase

STAT, signal transducer and activator of transcription

TCR, T-cell receptor

TFFs, trefoil factors

TGF, tumour growth factor

Tip, tumour necrosis factor inducing protein

TLR, toll-like receptor

TNF, tumour necrosis factor

TNFR, tumour necrosis factor receptor

TRAF, tumour necrosis factor receptor-associated factor

VEGF, vascular endothelial growth factor

VNTR, variable number of tandem repeats

vWF, von Willebrand factor

WHO, World Health Organization

INTRODUCTION

1. Anatomy and histology of the stomach

The stomach is an expanded region of the digestive tube anatomically divided into three major areas: fundus, corpus or body, and antrum (Figure 1). The stomach is located between the oesophagus and small intestine, to which it is connected by the cardia and the pylorus, respectively.

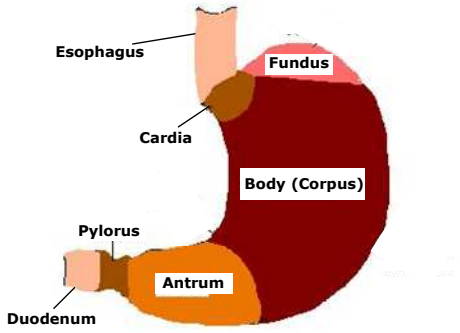


Figure 1. Main anatomic regions of the stomach.

The gastric wall presents five different layers: the mucosa, which is the inner lining that contains the glands; the submucosa which is underneath the mucosa and next to the layer of muscle; the subserosa and the serosa, which is the outer layer (Figure 2).

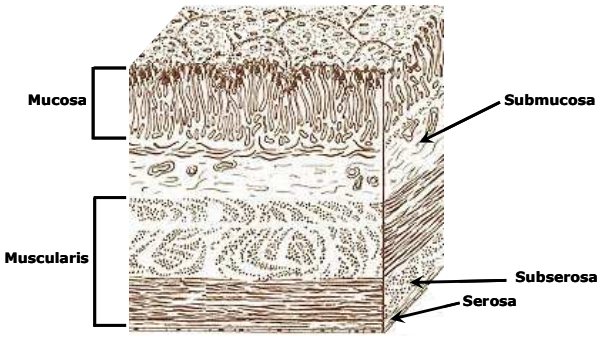


Figure 2. Schematic representation of the gastric wall.

The gastric mucosa is formed by packed tubular glands located beneath a surface of a simple columnar epithelium of mucous-secreting cells. The surface is indented into numerous gastric pits, which open to the lumen and have a distinctly

glandular appearance. Mucus-secreting cells are shed continuously and replaced by cells that migrate from the gastric pits. Other mucus-secreting cells in the necks of the gastric glands, called neck mucous cells secrete less viscous mucus which may protect the gland duct from autodigestion.

The mucosa of the stomach presents three distinctly different histological zones: the cardia, a small area of predominantly mucus-secreting glands surrounding the entrance of the oesophagus, the mucosa of the fundus and body, which forms the major histological region and consists of glands which secrete gastric juices as well as some protective mucus, and the antrum, that has a different glandular conformation: the glands secrete mucus, and associated endocrine cells secrete the hormone gastrin.

The gastric glands in the mucosa of the fundus and body of the stomach contain a mixed population of cells of three main types (Figure 3): mucus-secreting cells that cover the luminal surface of the stomach and line the gastric pits; hydrochloric acid-secreting cells, called parietal cells or oxyntic cells that are distributed along the length of the glands, but tend to be most numerous in the middle portion and pepsin-secreting cells, called peptic, chief or zymogenic cells, which tend to be clustered at the base of the gastric glands.

The antral glands are branched and composed almost exclusively of mucus-secreting cells. The function of the mucus secreted is to lubricate and protect the entrance to the duodenum. In addition to mucus-secreting cells, endocrine secretory cells or G cells are found scattered throughout the pyloric mucosa. G cells secrete the peptide hormone gastrin, which induces the gastric glands of the body to secrete acid and pepsin (1).

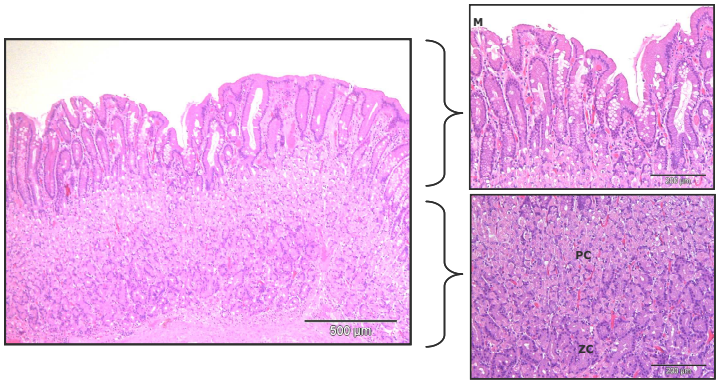


Figure 3. Gastric mucosa. Mucus-secreting cells (**M**), parietal cells (**PC**) and chief or zymogenic cells (**ZC**) are shown.

2. Gastric cancer

Gastric cancer remains the fourth most common cancer and the second leading cause of cancer-related death worldwide (2). Gastric cancers are malignancies arising in any part of the stomach. Several different types of cancer can occur in the stomach, however adenocarcinomas account for 95% of gastric tumours. Lymphoma, arising in the B and T cells of the lamina propria, sarcomas, arising from the cells of the muscle layer, and carcinoid tumours, believed to arise from neuroendocrine cells, comprise the remaining less common neoplasms of the stomach (3,4).

Gastric adenocarcinomas can be classified according to the site of tumour origin and the pathological appearance of the lesion.

Macroscopically, the most used classification system is that of Borrmann, developed in 1926 and characterised by the shape of the tumour on the gastric mucosa and its pervading style in the gastric wall (5). Tumours can be divided into four groups:

- Type I polypoid: well circumscribed polypoid tumours.
- Type II fungating: polypoid tumours with marked central infiltration.
- Type III ulcerated: ulcerated tumours with infiltrative margins.
- Type IV infiltrating: linitis plastica.

Microscopically, gastric cancer can present different histological patterns and several classifications have been proposed based on the morphologic features of gastric tumours. The most recommended, however, is the histological classification proposed by the World Health Organization (WHO) in 2000 (6). According to the WHO, carcinomas can be classified in the following subtypes:

- Adenocarcinoma:
  - Diffuse type
  - Intestinal type
- Papillary adenocarcinoma: exophytic lesions with elongated slender or plump finger-like processes, in which fibrovascular cores and connective tissue support cells.
- Tubular adenocarcinoma: presenting well-defined glandular lumens.
- Mucinous adenocarcinoma (greater than 50% mucinous): also referred to as colloid carcinomas. Contain abundant mucin secreted by the tumour cells and they are defined by the large amount of extracellular mucin retained within the tumour.

• Signet-ring cell carcinoma (greater than 50% signet-ring cells): composed of cells containing unsecreted mucus in the cytoplasm and nucleus located at the edge of the cell.

• Adenosquamous carcinoma: this rare epithelial tumour usually invades deeply into the muscular layer (7).

• Squamous cell carcinoma: proposed to develop from two possible mechanisms: from overgrowth of the squamous component in a primary adenocarcinoma or from malignant transformation of squamous metaplasia of the stomach (8).

• Small cell carcinoma: unusual and rare neoplasm in the stomach. The stages in its development have not been well defined (9).

•Undifferentiated carcinoma: containing no glandular structures or other features such as mucous secretions.

• Other.

Another widely used classification is the DIO system firstly proposed by Laurén in 1965 also based on tumour histology. According to this system gastric adenocarcinomas are classified as diffuse (D), intestinal (I) or other (O) (10). The former two groups account for 90% of all stomach cancers. Intestinal tumours are usually well differentiated and tend to form structures resembling functional glands of the gastrointestinal tract. By contrast, the diffuse-type adenocarcinomas exhibit low cell cohesion and tend to replace the gastric mucosa by signet-ring cells (11) (Figure 4).

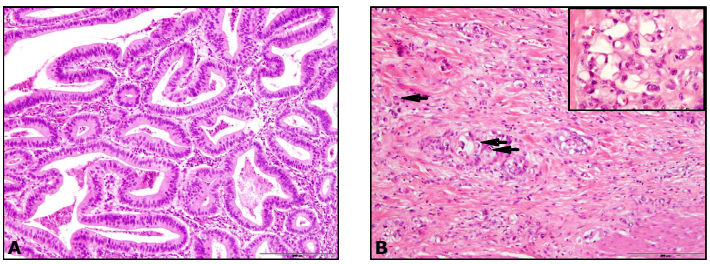


Figure 4. Morphology of intestinal-type tumours (**A**) and diffuse-type gastric tumours (**B**). Arrows indicate signet-ring cells.

Two different carcinogenesis pathways have been suggested in relation to these two histological types of gastric cancer, with different molecular changes being present or predominant. Intestinal tumours evolve through a number of sequential steps, firstly described by Correa et al. (12). The first step is gastritis

which progresses to mucosal atrophy (atrophic gastritis) followed by intestinal metaplasia, dysplasia and carcinoma. No preceding steps have been identified in the pathogenesis of diffuse tumours other than the chronic gastritis associated with *Helicobacter pylori* (*H. pylori*) infection.

2.1 Diffuse-type adenocarcinomas

Diffuse-type gastric tumours presumably arise from single-cell changes in the mucous-neck region of the gastric glands. These cells may proliferate and invade out from the crypt into the lamina propria.

The molecular mechanisms involved in gastric carcinogenesis of diffuse-type include abnormalities of oncogenes, tumour suppressor genes and cell cycle regulators, as well as genetic instability and alterations in growth factors and cytokines (Figure 5).

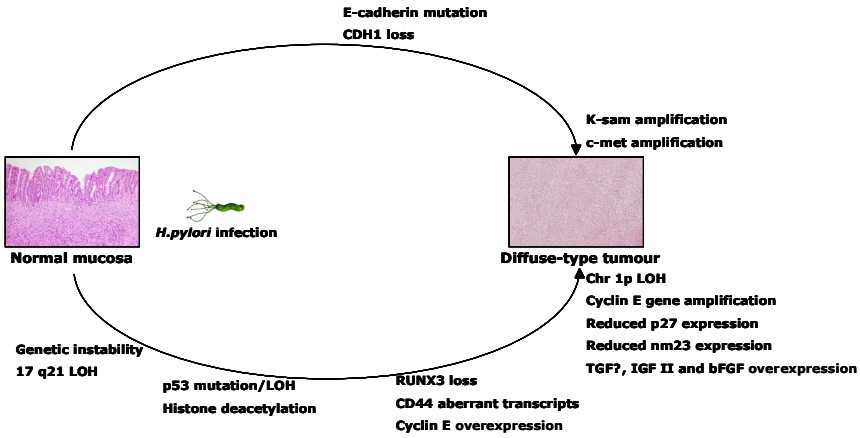


Figure 5. Genetic and epigenetic alterations during carcinogenesis of diffuse-type gastric adenocarcinomas.

Many proto-oncogenes are activated in diffuse-type gastric carcinoma. The tyrosine kinase receptor gene c-met, encoding for hepatocyte growth factor receptor is amplified in 39% of diffuse-type gastric tumours (13). The Type II K-sam oncogene, which encodes a receptor for keratinocyte growth factor, is also frequently activated in gastric carcinomas, and is preferentially amplified in 33% of

advanced diffuse-type gastric tumours. Overexpression of this gene is associated with poorer prognosis (14).

The E-cadherin tumour suppressor gene plays an important role in the carcinogenesis of the diffuse-type carcinomas. Somatic mutations in the E-cadherin gene CDH1 have been identified in 40 to 83% of sporadic diffuse tumours, but not in intestinal-type gastric cancer (15). Furthermore, in mixed gastric carcinomas loss of E-cadherin expression has been only detected in the diffuse component of the tumour, suggesting that E-cadherin loss might be the genetic basis for the divergence between diffuse and intestinal tumours (16). Most adenocarcinomas of the stomach occur sporadically, however a small proportion of gastric cancers arise in a clearly inherited predisposition syndromes. One of these syndromes is the hereditary diffuse gastric carcinoma (HDGC), which is an autosomal dominantly inherited gastric cancer susceptibility syndrome caused by germline mutations in the CDH1 gene (17). HDGC presents 70% penetrance and it has also been associated with an increased risk of breast cancer (18). A further tumour suppressor altered in diffuse-type adenocarcinomas is the p53 gene, which is frequently inactivated in gastric carcinoma by loss of heterozygosity (LOH), missense mutations and frame-shift deletions, being GC-AT transitions common in diffuse-type carcinomas (19). Additional alterations in tumour suppressor genes include those affecting 1p loci, loss of RUNX3 transcription factor and histone H4 deacetylation (4).

LOH on chromosome 17q21, including the BRCA1 gene (4), and abnormalities in cell-adhesion molecules and metastasis-related genes (aberrant CD44 transcripts (20) and reduced expression of nm23, involved in c-myc transcriptional activation (21), are also frequent in diffuse tumours, in addition to altered expression pattern of cell cycle regulators such as gene amplification or overexpression of Cyclin E and reduction in the CDK inhibitor p27 (4) and overexpression of growth factors such as TGF-β, IGF II and bFGF (22).

2.2 Intestinal-type adenocarcinomas

There are three possible routes leading to intestinal-type gastric carcinoma development: firstly, progression through pre-cancerous lesions of intestinal metaplasia, dysplasia and finally adenocarcinoma; secondly, intestinal metaplasia may proceed directly to carcinoma; and the third route involves the development of *de novo* gastric carcinoma with no preceding stage (22).

The progression to adenocarcinoma through a succession of tissue changes is known as the Correa pathway (Figure 6). These pre-cancerous lesions are: chronic active nonatrophic gastritis, multifocal atrophic gastritis, intestinal metaplasia (first complete, then incomplete), dysplasia and invasive carcinoma (23).

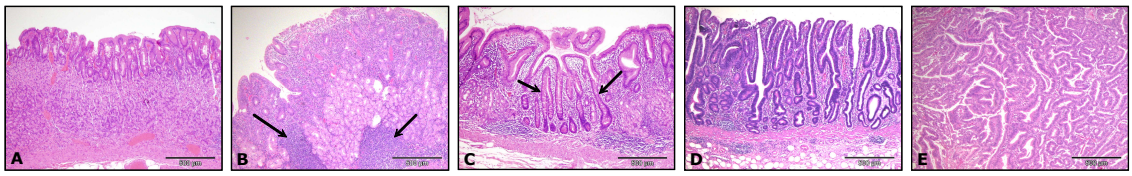


Figure 6. Histological progression to intestinal type gastric cancer. **A)** Normal gastric mucosa. **B)** Gastritis. **C)** Intestinal metaplasia. **D)** Dysplasia. **E)** Intestinal type adenocarcinoma.

- **Chronic active non-atrophic gastritis**

Chronic active non-atrophic gastritis is usually triggered by *H. pylori* infection. This lesion is characterized by diffuse infiltration of the gastric mucosa by white blood cells, namely lymphocytes, plasma cells, and macrophages (chronic inflammation). In addition, scattered eosinophils and mast cells can be observed. Polymorphonuclear neutrophils, representing acute inflammation, are also found (23) (Figure 7A), and they can form small aggregates either in the stroma or the epithelial layer. This phase does not show loss of glands (atrophy). In most cases, non-atrophic gastritis is localized predominantly in the antrum. However, in patients receiving anti-acid medication, especially proton pump inhibitors, gastritis becomes prominent in the corpus mucosa.

Different studies suggest that cytokines such as IFN-γ, TNF-α and IL-1β that are released during gastritis, initiate the cellular changes observed in this phase (24,25,26).

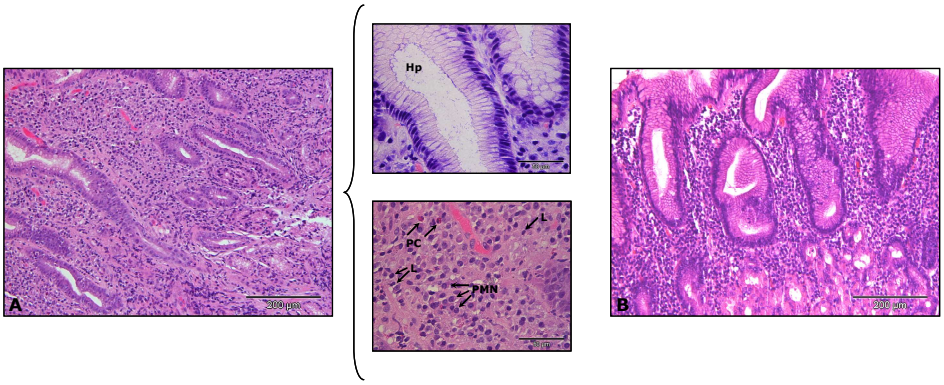


Figure 7. Gastritis. **A)** Chronic active non-atrophic gastritis. Lymphocytes (**L**), plasma cells (**PC**) and polymorphonuclear (**PMN**) cells are shown. The presence of *H. pylori* can be also observed (**Hp**). **B)** Chronic atrophic gastritis.

- **Multifocal atrophic gastritis**

The second phase of the intestinal-type gastric carcinogenetic process is characterized by alterations in the epithelial cell cycle such as changes in proliferation and apoptosis. These changes result in focal loss of glands (atrophy), and the acid-secreting parietal cells are replaced with mucous secreting cells (Figure 7B). The mechanism of cell loss appears directly related to effects of *H. pylori* products and the cytokine milieu within the gastric mucosa. Atrophy and progression to severe disease have been strongly associated with virulence of the bacterial strains and permissiveness of the host immune response (23).

H. pylori-host interactions and the inflammatory response triggered by the infection will be addressed in more detail in section 3.

- **Intestinal Metaplasia**

Proliferation of the mucous cells with an intestinal phenotype can lead to intestinal metaplasia, which is the major precursor lesion for gastric cancer. Classically intestinal metaplasia is classified as Type I, complete, Type II, incomplete and, sometimes, a third category, Type IIa or III, is also included in the incomplete type. In the complete metaplasia absorptive enterocytes with a well-developed brush border, globet cells containing mucins and Paneth cells, harbouring eosinophilic granules in their cytoplasm and located tipically at the base of the glands, are detected (Figure 8A). This type of metaplasia is called complete

because the normal digestive enzymes such as sucrase, trehalase and alkaline phosphatase are secreted (23). In incomplete intestinal metaplasia the metaplastic cells lose the small intestinal phenotype observed in the complete type and acquire morphologic features of the large intestine. Enterocytes and goblet cells secreting sulphomucins, which are no detected in complete metaplasia, are present (Figure 8B).

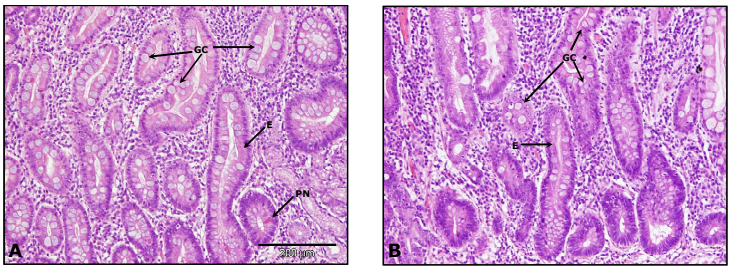


Figure 8. Intestinal metaplasia. **A)** Type I or complete intestinal metaplasia. Enterocytes (**E**), goblet cells (**GC**) and Paneth cells (**PN**) are shown. **B)** Type II or incomplete intestinal metaplasia. Enterocytes (**E**) and goblet cells (**GC**) are observed.

The expression of specific intestinal genes has been detected in goblet, absorptive and Paneth cells present in intestinal metaplasia. Goblet cells, for instance, express the intestinal mucins MUC2 and MUC4 (27,28). Absorptive cells have been described to contain sucrase and intestinal-type alkaline phosphatase activity and to express the structural protein villin, and Paneth cells are reactive with anti-defensin (29) and lysozyme antibodies (30).

Lately, a further metaplasia in the stomach is growing in importance: the so called spasmolytic polypeptide expressing metaplasia (SPEM) lineage. SPEM is characterized by the expression of trefoil factor family 2 (TFF2; spasmolytic polypeptide), and is associated with loss of parietal cells (31). This type of metaplasia occurs characteristically in the fundus of the stomach (32) and its development is accelerated as a consequence of defective epidermal growth factor receptor (EGFR) signalling. SPEM has been strongly associated with both chronic *H. pylori* infection and gastric adenocarcinoma, potentially representing another pathway to gastric neoplasia (33).

The development of intestinal metaplasia has been associated with the deregulation of different genes, such as p27, cyclin D2, telomerases, c-myc or cyclooxygenase 2 (COX-2), mutations in p53 protein or in adenomatous polyposis coli (APC) gene, loss of heterozygosity, microsatellite instability and *H. pylori*

infection (34,35,36). However, other genes have lately acquired relevant importance for intestinal metaplasia development, such as CDX1, CDX2, PDX1, Sox2, OCT1, TFF3 and RUNX3 (37). Specifically, caudal-related homeobox genes CDX1 and CDX2 are aberrantly expressed in areas of the stomach which contain intestinal metaplasia (38) and their expression may contribute to the intestinal phenotype (39). CDX1 participates in the transdifferentiation of the gastric mucosa to an intestinal type (40), but the maintenance of intestinal differentiation appears to depend on the presence of CDX2 (38). Furthermore, by using a transgenic mice model, Silberg et al. showed that the ectopic expression of CDX2 in the gastric mucosa was enough to activate the intestinal differentiation program (41).

- **Dysplasia**

Intestinal metaplasia can progress to dysplasia. This lesion is recognised by cellular atypia with nuclear pleomorphism, cellular undifferentiation and abnormal distribution of glands and crypts (Figure 9). Dysplasias are classified as low- or high-grade, depending on the degree of nuclear atypia and architectural distortion (23). At the molecular level, dysplasia is characterized by genetic alterations similar to that observed in gastric tumours, such as loss of heterozygosity in the APC gene (42). p21, p53 and Bcl-2 genes, which are overexpressed in gastric cancer, have been also detected in dysplasia and can be considered an early event in the development of lesions that can lead to gastric cancer (35).

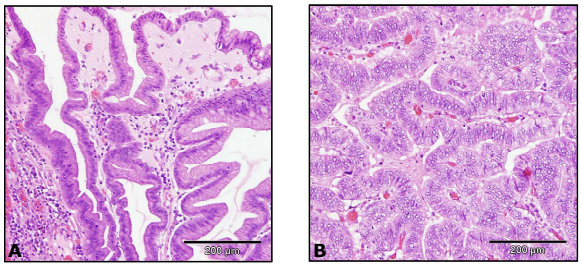


Figure 9. **A)** Low-grade dysplasia. **B)** High-grade dysplasia. Nuclei of the dysplastic cells are enlarged, irregular in shape, and devoid of polarity. The architecture is irregular and packed tubular structures with irregular lumens can be observed.

- **Adenocarcinoma**

Numerous genetic and epigenetic alterations in oncogenes, tumour-suppressor genes, cell-cycle regulators, cell adhesion molecules, DNA repair genes and genetic instability as well as telomerase activation are detected in intestinal-type gastric adenocarcinomas (Figure 10).

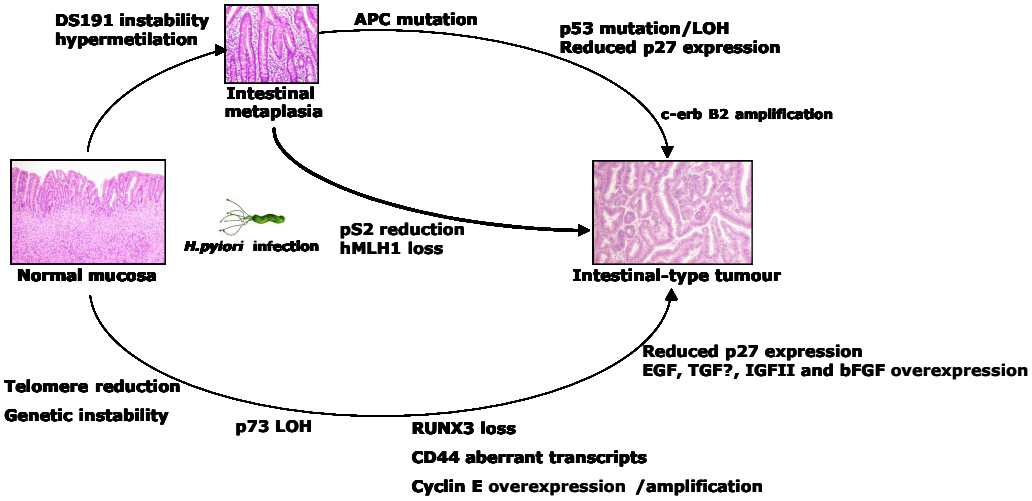


Figure10. Genetic and epigenetic alterations during carcinogenesis of intestinal-type gastric adenocarcinomas.

Reduced expression of the tumour suppressor genes p53, p73, TFF1 and APC (Adenomatous polyposis coli) gene is usually detected in intestinal-type cancers. p53 and p73 are inactivated by LOH, and mutations usually at A:T sites and frame shift deletions have been also described to inactivate p53 gene in this type of tumours (4). Inactivation of the TFF1 (pS2) gene, a gastric specific trefoil factor, results in dysplasia, adenoma and carcinoma in mice (43). In intestinal metaplasia and gastric adenomas, loss or reduction of TFF1 due to DNA methylation in its promoter region have been observed, suggesting an important role for TFF1 in intestinal-type adenocarcinoma development (22). APC gene missense mutations are also common in intestinal-type tumours (44), and the inactivation of APC enhances the expression of β-catenin, which acts as an oncogene. Other genes affected are RUNX3 and FHIT.

The proto-oncogene c-erbB2 is amplified in intestinal-type cancers and its overexpression has been correlated with poorer prognosis (45). The same occurs

with the cell cycle regulator cyclin E, which amplification or overexpression is associated with aggressiveness and lymph node metastasis (46). Reduced p27 expression, aberrant CD44 transcripts, epigenetic inactivation of the mismatch repair gene hMLH1, microsatellite instability of the D1S191 locus and overexpression of the growth factors of the EGF family (EGF, TGF-α, IGF II and bFGF) are also hallmarks of intestinal-type gastric adenocarcinomas.

3. Inflammation and Gastric Cancer

Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, survival, proliferation, invasion, angiogenesis, and metastasis (47). The association between chronic inflammation and cancer has been described particularly in the digestive tract, where the risk for carcinogenesis increases in the presence of chronic inflammatory conditions such as esophagitis, gastritis, colitis, pancreatitis, and hepatitis (48). Accumulating evidence indicates that gastric atrophy linked to chronic gastritis is much more consistently associated with gastric cancer than other precursor lesions as intestinal metaplasia. In this regard, a number of mouse models have shown that inflammation triggers neoplastic transformation (49,50,51). For instance, mice expressing mutated IL-6 receptor, $gp130^{Y757F/Y757F}$, present over-stimulation of the IL-6/STAT3 pathway that results in development of antral gastric tumours (49). STAT3 hyper-activation also occurs in gastrin deficient mice, which are hypochlorydric and present bacterial overgrowth and chronic gastritis (51). Furthermore, transgenic mice expressing COX-2 and microsomal prostaglandin E synthase (m-PGES)-1 in the gastric epithelium develop intestinal metaplasia, hyperplasia and cancer triggered by macrophage infiltration (50).

The chronic inflammation of the stomach is usually caused by *H. pylori* infection, which confers a significantly increased risk of developing gastric cancer (52), and cytokines released during bacterial infection play a pivotal role in triggering cellular changes that contribute to gastric mucosal damage and gastric neoplastic transformation.

3.1 *Helicobacter pylori* infection

H. pylori is a Gram-negative, spiral shaped, microaerophilic bacilli that colonizes the gastric epithelium and represents the most common bacterial infection worldwide (4). All *H. pylori* strains cause chronic gastric mucosal inflammation, characterized by the presence of infiltrating macrophages, B and T lymphocytes, polymorphonuclear cells and plasma cells (53). Gastritis is the basic process that mediates *H. pylori*-induced damage, and its extension and distribution determine the clinical outcome. *H. pylori* infection can lead to three main pathological gastric phenotypes, each of which is associated with a set of pathophysiologic abnormalities responsible of inducing a certain outcome (Figure 11).

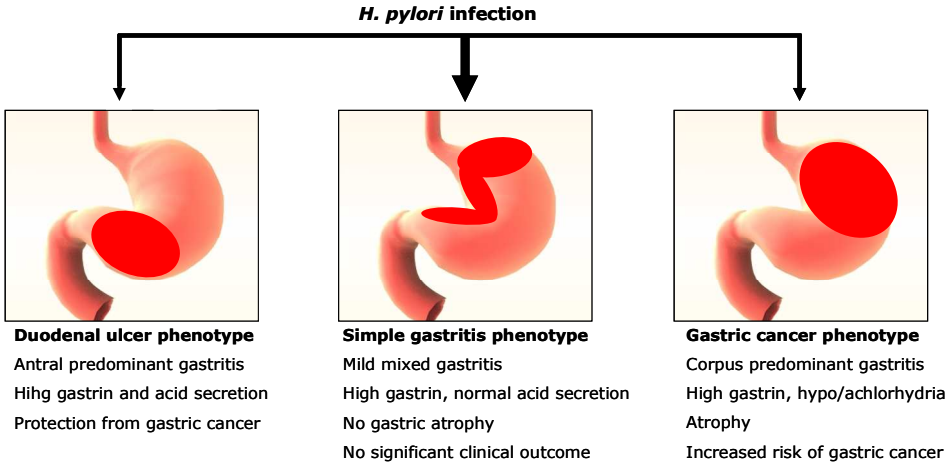


Figure 11. Divergent responses to *H. pylori* infection.

The most common pathological phenotype is benign gastritis, characterized by mild pan-gastritis with little disruption of gastric acid secretion. No symptoms are associated with this type of gastritis that does not develop to serious gastrointestinal disease (54). Around 15% of infected subjects, particularly in Western countries, develop the so-called duodenal ulcer phenotype. Gastritis is localized characteristically in the antrum and is associated with high levels of gastrin, a relatively healthy corpus mucosa and hyperchlorhydria (55). These pathophysiologic abnormalities contribute to the development of duodenal and pre-pyloric peptic ulcers. The third phenotype is called the gastric cancer phenotype, and it is characterized by corpus-predominant gastritis, multifocal gastric atrophy and hypo- or achlorhydria (56). In addition low acid secretion, high gastrin and low pepsinogen I and pepsinogen I/II ratio are observed. Approximately 1% of infected subjects present these abnormalities, which develop as a result of the chronic inflammation induced by the infection. Increased risk for gastric cancer has been associated with this phenotype (57). Interestingly, patients that develop duodenal ulcers are protected from developing gastric cancer, suggesting that these two outcomes are mutually exclusive (58).

It is clear that *H. pylori* infection leads to different clinical outcomes and the coordinated interaction between pathogen and host is responsible of increasing the risk for developing gastric cancer (Figure 12).

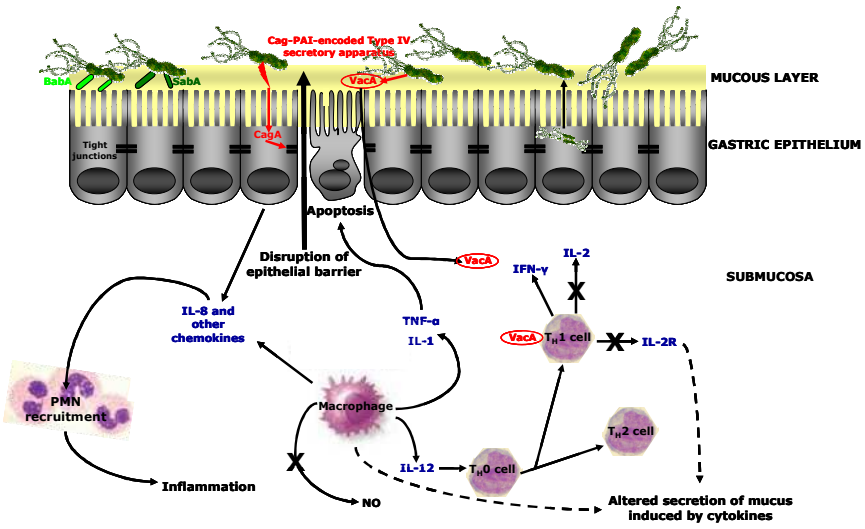


Figure 12. Interaction between *H. pylori* factors and the host response. Adapted from Monack et al. (59).

These interactions are dependent on strain-specific bacterial factors and on the inflammatory responses determined by host genetic diversity. In the following sections, bacteria-host interactions and role of bacterial and host genetic factors in *H. pylori*-induced disease will be discussed in more detail.

- **Bacteria-host interactions**

Approximately 20% of *H. pylori* in the stomach are found adhered to the surfaces of mucus epithelial cells. The adhesion of the bacteria to the epithelial layer is assured by a large family of 32 related outer membrane proteins that include the adhesins (60). One of the best characterized is **BabA**, encoded by the strain-specific gene babA2. BabA binds the Lewis b histo-blood group antigen on gastric epithelial cells (61) forming a scaffold apparatus that allows bacterial proteins to enter host epithelial cells. Strains possessing the babA2 gene adhere more tightly to epithelial cells, promote a more aggressive phenotype and are associated with higher incidence of gastric adenocarcinoma (60). The outer membrane protein **SabA** binds sialyl-Lewis x (s-Lewis x), and this interaction also contributes to *H. pylori* adherence, allowing close interactions with the gastric epithelium (62). s-Lewis x antigen is a well established tumor antigen and marker of gastric dysplasia which expression as a dimeric form (s-dimeric-Lewis x) is up-regulated by *H. pylori*-induced inflammation and interactions between this molecule and SabA are amplified. Moreover, SabA has been described to be required for

nonopsonic activation of neutrophils (63), providing another example of the different coordinated and dynamic interactions existing between host and *H. pylori* in relation to pathogenesis. The distribution of Lewis x mirrors the expression of TFF1 and MUC5AC, which serve as a binding factor for *H. pylori* (64,65).

- **Role of *H. pylori* virulence factors in the progression to disease**

Different bacterial factors have been described to have the capacity to induce epithelial responses with carcinogenic potential. The most well-characterized virulence factor is the **Cag pathogenicity island** (Cag PAI). Cag PAI is a 40 kb region of DNA that contains 31 genes, and encodes a type IV secretion system that translocates **CagA**, the product of the terminal gene in the island, into gastric epithelial cells (66). Once translocated into the epithelial cells CagA is phosphorylated at specific tyrosine residues contained within the EPIYA amino acid motif by members of the Src family of kinases, which have been implicated in many human malignancies (67,68). Once phosphorylated CagA remains near the plasma membrane where it interacts with a number of host proteins, such as SHP-2 phosphatase and phosphorylated c-met (69,70), triggering signals that resemble the activation of receptor-tyrosine kinase growth factors (Figure 13). In this way, CagA affects the proliferative activities, adhesion, and cytoskeletal organization of epithelial cells. CagA can also activate the ERK/MAPK pathway and EGF receptor, leading to epithelial morphological changes similar to that induced by uncontrolled stimulation of growth factors (71,72,73).

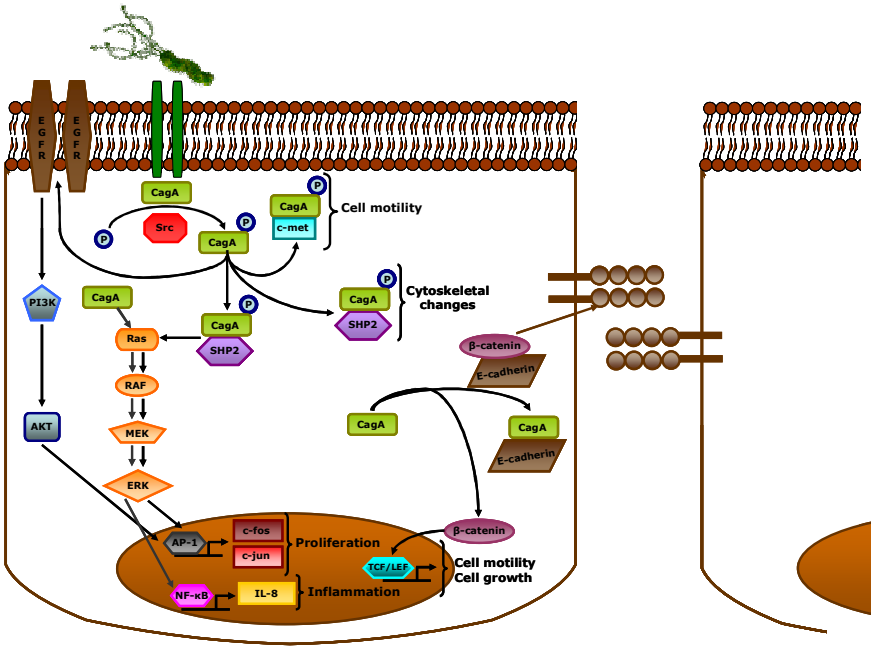


Figure 13. Molecular alterations induced by intracellular delivery of *H. pylori* **CagA**.

Another important cellular pathway targeted by CagA *in vivo* are the epithelial junctions, in particular the E-cadherin/β-catenin pathway, which regulates epithelial cell adhesion, junction formation and control of cell growth (74,75). In addition, CagA has also powerful pro-inflammatory effects because it can activate the nuclear factor (NF)-κB transcriptional response leading to activation of pro-inflammatory signals and IL-8 secretion (76,77).

The second major virulence determinant of *H. pylori* is the secreted protein **VacA**. VacA is a pore-forming cytotoxin first made as a large 140-kilodalton polypeptide that is trimmed at both ends during secretion from the bacterial cell. The amino terminus contains a signal sequence, "s" region of the gene that shows allelic variability. *H. pylori* strains harbouring s1 types of VacA secrete active toxin and are more highly associated with both ulcers and gastric cancer (78,79). The carboxyl end of the pro-VacA peptide, involved in auto-transport of the toxin out of the bacteria, is also removed from the mature protein (80). The middle region ("m" region) shows also allelic variation presenting the m1 subtypes the stronger vacuolating activity. Most biochemical studies have been performed with s1m1 variants of VacA. VacA has several toxigenic properties that can alter the outcome of infection and colonization by *H. pylori*. The best known is VacA's effect on endosomal maduration leading to vacuolation of epithelial cells (Figure 14). VacA binds the plasma membrane of cells, is internalized by cells, forms anion-selective channels in endosomal membranes and vacuoles arise due to swelling of endosomal compartments (81). This effect alters cellular functions as antigen presentation, which depends on proper function of endosomal trafficking (82). VacA also induces host-cell death through apoptosis probably as a consequence of pore formation in mitochondrial membranes (83) and, indirectly, due to the activation of pro-apoptotic signalling molecules (84). In addition, VacA can disrupt the barrier function of tight junctions without affecting junction integrity, and in this way *H. pylori* can acquire nutrients across an intact epithelial barrier (85). Recent studies have focused on the potential VacA pro-inflammatory effects as well as on its effects on the immune system. In this regard, VacA has been found to block efficiently T cells activation and proliferation and it can also interfere with signalling downstream of the TCR and IL-2 receptor (86,87).

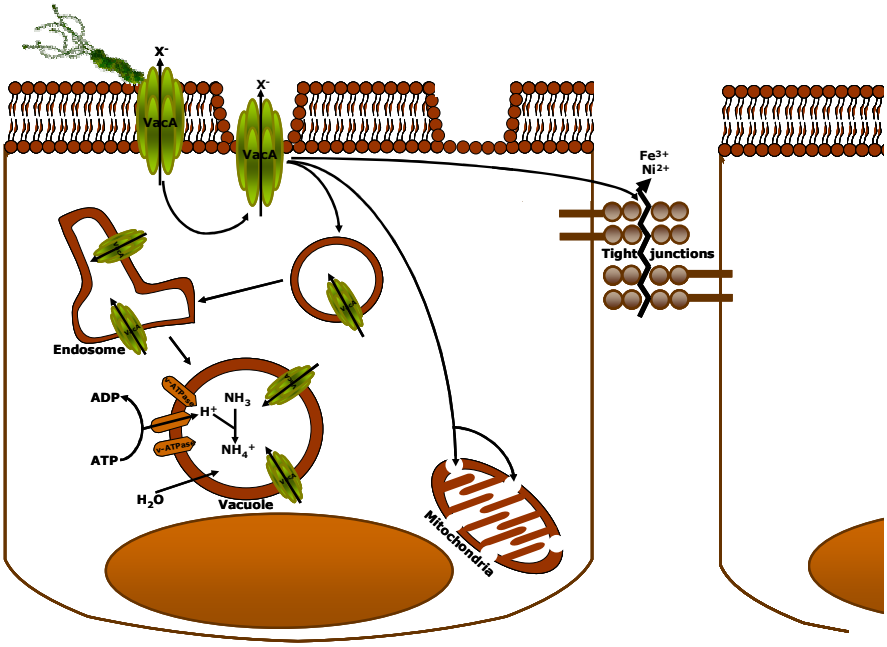


Figure 14. Molecular effects induced by toxigenic *H. pylori* **VacA**.

- **Role of host genetic factors in *H. pylori* induced-disease**

As previously mentioned, *H. pylori* induces its damage by initiating chronic inflammation in the gastric mucosa that is mediated by different pro- and anti-inflammatory cytokines. Several data suggest that host genetic polymorphisms are of crucial importance in determining progression to gastric cancer, specifically those involving genes implicated in the immune response to *H. pylori* and in the resulting inflammation. The observation that patients who progressed to atrophy and cancer secreted lower levels of gastric acid when compared to patients of duodenal ulcers, led to one of the first genetic studies focusing on the pro-inflammatory cytokine IL-1β, which is also a potent inhibitor of acid secretion. Several studies have shown that IL-1 gene cluster polymorphisms increase the risk of gastric cancer and precursor lesions (88,89,90,91). Individuals with the IL-1β-511T and IL-1β-31C genotypes present more risk of developing hypochlorhydria, gastric atrophy and gastric cancer compared to subjects who have the less pro-inflammatory genotypes.

Polymorphisms in TNF-α and IL-10 genes have been also reported as independent additional risk factors for non-cardia gastric cancer (92). The pro-inflammatory cytokine TNF-α is produced in the gastric mucosa in response to *H. pylori* infection and, as IL-1β, it has an acid inhibitory effect, although much weaker

(93). The TNF-A-308 G>A polymorphism is involved in a number of inflammatory conditions and its role in increasing the risk of gastric cancer has been also described (94). IL-10 is an anti-inflammatory cytokine that downregulates IL-1β, TNF-α, IFN-γ and other pro-inflammatory cytokines. Homozygosity for the low-IL-10 ATA haplotype (based on three promoter polymorphisms at positions -592, -819 and -1082) increases the risk of noncardia gastric cancer (92). Polymorphisms in TNF-α, IL-10 and their combination with pro-inflammatory IL-1β gene cluster polymorphism have been described to result in a high-risk genotype, presenting a 27-fold or greater risk of developing gastric cancer (92).

Another important cytokine that plays a central role in the pathogenesis of *H. pylori* is IL-8. IL-8 is a potent chemo-attractant for neutrophils and lymphocytes, and studies on IL-8 promoter have identified the -251T allele as significantly associated with an increased risk of gastric cancer (95,96).

Genetic polymorphisms affecting genes of the innate immune response play also an important role in *H. pylori*-induced disease. Specifically, Toll-like receptor 4 (TLR4) polymorphism (TLR4+896A>G) has been associated with increased risk of gastric cancer and precursor lesions (97).

- **Inflammatory response induced by *H. pylori*.**

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria including *H. pylori*, is the main cause of inflammation in Gram-negative infections. LPS binds the transmembrane pattern recognition receptor TLR4, expressed on macrophages and monocytes (98,99), and signal transduction is activated trough MyD88 (100), interleukin-1 receptor associated kinase (IRAK) and TRAF6 (Figure 15). *H. pylori* has been recently reported to increase growth of gastric tumours via LPS-TLR4 signalling (101), that is able to activate MAPK and NF-κB pathways (102). Activated NF-κB translocates to the nucleus and induces an increase in IL-8 mRNA levels and protein expression (103) and synthesis and release of other pro-inflammatory cytokines, such as IL-1 and TNF-α. Different chemokines such as GROα, IP10, MIG, MIP-1α, MIP-1β and iNOS are also synthesized (4). iNOS has been described to promote oncogenesis (104) and nitric oxide generated by iNOS can be converted to reactive nitrogen species, which can produce oncogenic effects such as DNA and protein damage, inhibition of apoptosis, promotion of angiogenesis and mutations in proteins involved in repair functions as p53 (105). Moreover, NF-κB activation is known to regulate cell adhesion molecules as ICAM-1, whose expression has been correlated with an increase in *H. pylori*

induced gastritis (106). *H. pylori* infection also induces the expression of antibacterial peptides and pro-inflammatory Cyclooxigenase enzyme (COX-2), which has been described to inhibit apoptosis, to maintain cell proliferation and to stimulate angiogenesis within cancer cells, facilitating tumour growth (107). In addition, proteins of the TNF-α inducing protein (Tipα) gene family in *H. pylori* genome contribute to the inflammatory response inducing the expression of TNF-α and NF-κB activation, and act as new carcinogenetic factors of *H.pylori* (108).

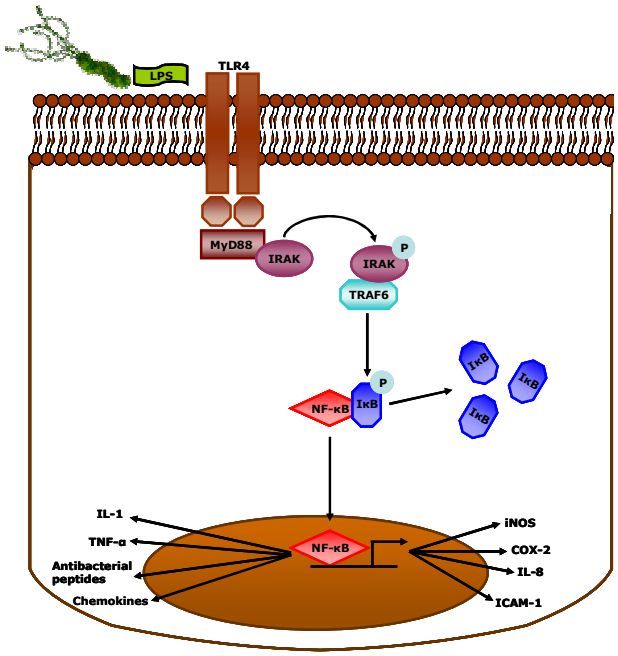


Figure 15. *H. pylori*-induced inflammatory response.

3.2 Inflammatory pathways associated with gastric cancer progression

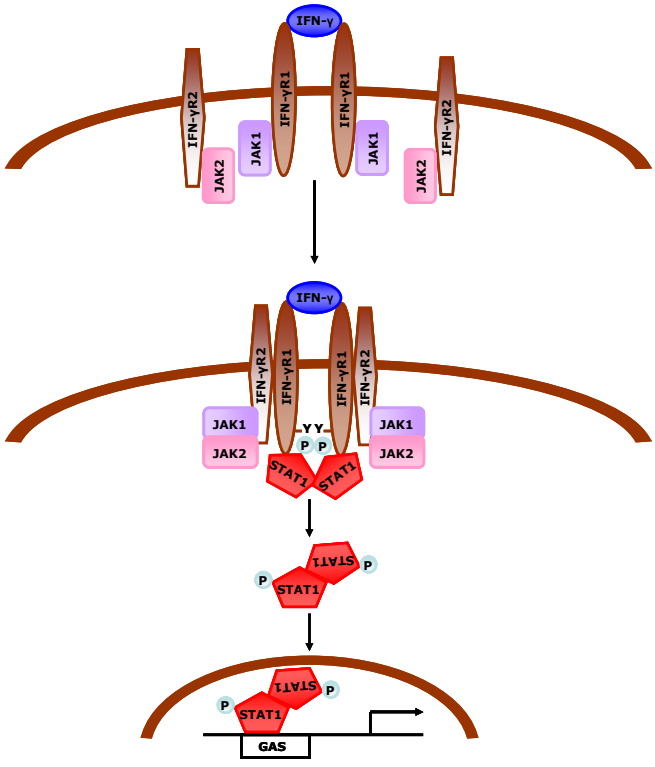
Chronic inflammation develops through the action of several inflammatory molecules such as pro-inflammatory cytokines, chemokines and small chemical mediators. Among them, pro-inflammatory cytokines TNF-α, IL-1, IL-6 and IFN play essential roles (109). The molecular pathways associated with these cytokines as well as their role in gastric cancer development and progression will be discussed in detail in the next sections.

- **IFN-γ**

In the Th1 immune response induced by *H. pylori* IFN-γ-expressing T lymphocytes are primarily recruited (110). IFN-γ has been suggested to have an important role in initiating the mucosal damage observed during gastritis due to the lack of response to *H. pylori* infection detected in a IFN-γ null mice model (111). Furthermore, exogenous infusion of IFN-γ on the gastric mucosa of mice induced inflammation, development of mucous gland metaplasia, hypergastrinemia and reduced somatostatin expression (112). IFN-γ also stimulates the release of the pro-inflammatory cytokines TNF-α and IL-1β and the hormone gastrin (113).

IFN-γ signalling (Figure 16A) is mainly mediated through the Janus tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (114). In particular, IFN-γ induces the phosphorylation of STAT1, which translocates into the nucleus and binds to the γ-IFN-activated sequence (GAS) of target genes (115).

A



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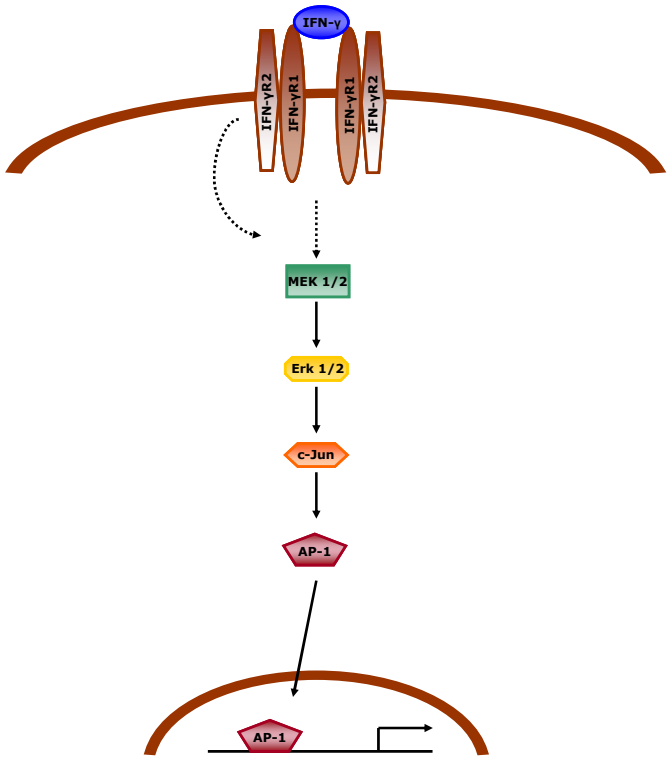


Figure 16. Schematic representation of IFN-γ-induced activation of the JAK/STAT (**A**) and MEK/ERK/AP-1 (**B**) pathways.

STAT1 has been reported to mediate IFN-dependent tumour suppressor activity by promoting apoptosis, cell cycle arrest, and tumour surveillance (116,117). However, more recently, using a mouse model, Ernst et al. have shown that germline mutation of Stat1 partially suppressed the growth of gastric tumours, reduced gastric inflammation and activated STAT3 expression (118), suggesting a new role for STAT1 in promoting gastric disease. These opposite functions described for STAT1 illustrate the paradoxical roles of the immune system in cancer development.

IFN-γ can also regulate several genes in a STAT-independent fashion (Figure 16B). For instance, IFN-γ can activate AP-1 DNA binding via c-Jun, independently of JAK1 and STAT1. This pathway includes the MEK1/2-ERK1/2 module of the MAP kinase cascades (119). The transcription factor AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis (120).

- **TNF-α and IL-1**

TNF-α is a strong tumour promoter (121), mainly produced by activated macrophages and lymphocytes during inflammation. In *H. pylori* patients increased levels of this pro-inflammatory cytokine have been detected (24,25), and its expression can be induced by urease, CagA, *H. pylori*-membrane protein-1 (HP-MP1) and Tipα protein (26,122,7,108). Recently, Oguma et al. have shown that TNF-α derived from activated macrophages promotes the Wnt/β-catenin signalling activation in gastric cancer cells through the suppression of GSK3β, contributing in this way to gastric cancer development (123). However, TNF-α regulatory effects are mainly mediated through NF-κB transcription factor.

IL-1 is a potent pro-inflammatory cytokine inhibitor of gastric acid secretion that contributes to the initiation and amplification of the inflammatory response to *H. pylori* infection (94). To analyse the etiological role of IL-1β in gastric carcinogenesis a transgenic mouse model overexpressing IL-1β in the stomach has been recently established (124). IL-1β transgenic mice developed spontaneous inflammation, metaplasia, dysplasia and carcinoma of the stomach, demonstrating that increased levels of IL-1β can be sufficient to induce neoplasia. Furthermore, IL-1β may also play an important role in metastasis. In this sense IL-1β has been reported to be able to enhance invasiveness of gastric cancer cells through NF-κB activation (125).

The NF-κB family of transcription factors consists of five members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel. After TNF-α or IL-1β binding TRAF2 or TRAF6 are, respectively, recruited to TNFR1 or IL1R receptors. TRAF/RIP complexes trigger IKK activation leading to phosphorylation, ubiquitination and degradation of IκB proteins (Figure 17), which are associated with inactivated NF-κB. NF-κB dimers free of IκB translocate to the nucleus and bind to κB sites within promoters of target genes, and gene transcription is regulated through the recruitment of co-activators and co-repressors (126). A large number of anti-apoptotic factors such as cIAPs, c-FLIP, A20 and $BclX_L$ (127) as well as angiogenic factors (VEGF) (128) and proteins involved in invasion and metastasis (MMP-2 and MMP-9) (129) are activated by NF-κB, providing an important role for NF-κB in tumour progression and metastasis.

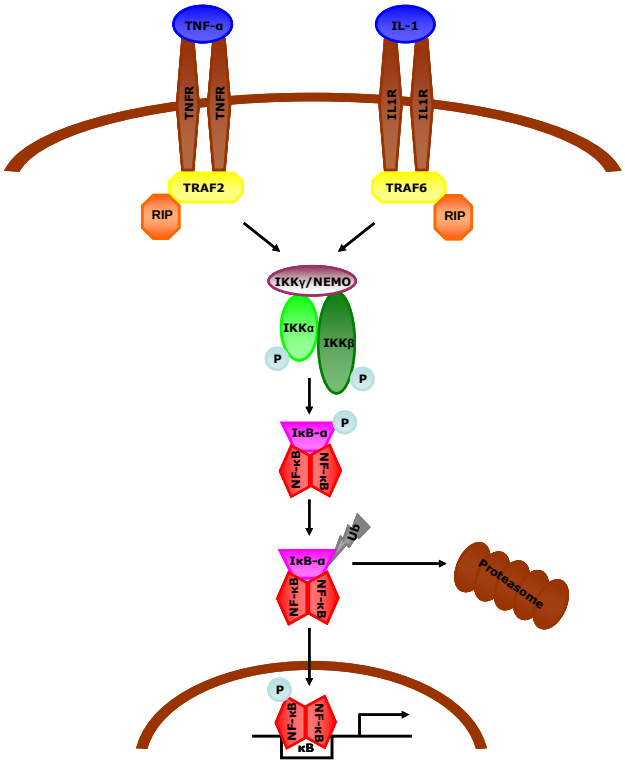


Figure 17. Schematic representation of NF-κB signalling pathway activated by TNF-α and IL-1.

In stomach, NF-κB activation is an important event for the progression from chronic inflammation to carcinogenesis. In this sense, the degree of gastritis has been described to be modulated by NF-κB activation induced by *H. pylori* infection (130,131). In addition, in gastric tumours NF-κB (p65) has been observed to be constitutively activated (132,133,134), and in a recent report NF-κB activation in late-stage gastric carcinoma has been correlated to the survival time of patients after chemotherapy (135).

- **IL-6**

IL-6 is a pleiotropic cytokine that is important for immune responses, cell survival, apoptosis and proliferation (136). In gastric carcinogenesis, IL-6 has been associated with the disease status and outcomes of gastric tumours (137,138). There is not definitive evidence for a causal role for IL-6 in gastric cancer progression; however, deregulation of its associated signalling pathways has been described to promote tumour development.

IL-6 initiates signalling by binding its specific receptor (IL-6Rα) that associates with and induces the homo-dimerization of the transducing receptor subunit gp130. As a result the JAK/STAT and the SHP-2/ras/MAPK/ERK1/AP-1 signalling cascades (Figure 18), which are in homeostatic balance under normal conditions, are activated (139). The JAK/STAT pathway is activated after ligand binding due to autophosphorylation of the Janus kinases JAK1, JAK2 and Tyk2. Subsequently, the JAKs phosphorylate several specific tyrosine residues on the intracellular domain of gp130 that act as docking sites for molecules encompassing a Src-homology-2 (SH-2) domain, particularly STAT transcription factors 1 and 3. STAT proteins are also tyrosine phosphorylated, form homo- or heterodimers and translocate to the nucleus where they activate the transcription of target genes (140). IL-6 signalling is mainly mediated by STAT3. A significant increase in STAT3 activation has been detected in *H. pylori* infected patients as well as in adenocarcinoma (141), and it has been proposed as a prognostic factor for poor survival of gastric cancer patients (142). STAT3 regulates the expression of genes involved in suppressing apoptosis (Bcl-2), promoting angiogenesis (Vegf, Mmp9) and inducing proliferation (c-myc) (109).

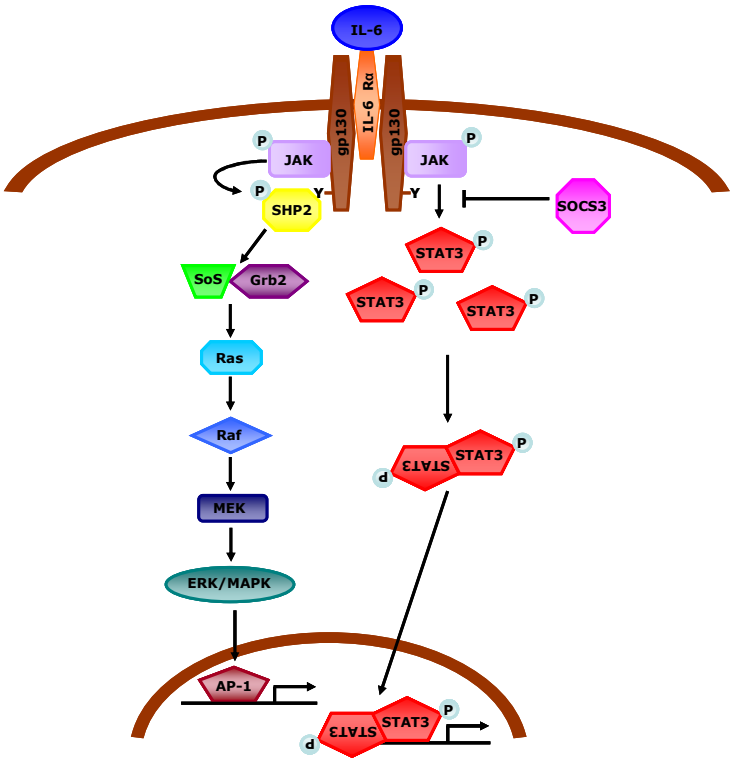


Figure 18. Schematic representation of IL-6 induced signalling pathways.

IL-6 can also regulate other genes though activation of the tyrosine phosphatase SHP-2, which is recruited to tyrosine 759 of gp130 receptor and phosphorylated by JAK1/2, leading to binding of the adapter molecules Grb2 and SoS and recruitment of Ras. A series of MAP kinases are activated ending in gene regulation by transcription factors as AP-1 (143). The negative regulator of STAT3 activation, suppressor of cytokine signalling 3 (SOCS3), also binds the same tyrosine residue on gp130 as SHP-2 contributing to the balance between the two pathways activated by IL-6.

To study the consequences of disrupting the balance between the STAT1/3 and SHP2/Erk pathways a transgenic mice model bearing mutated gp130 receptor was generated (144). Transgenic mice developed distal stomach tumours as a consequence of the loss of gp130-dependent SHP-2/Erk signalling and increased STAT3 signalling, which was accompanied by a downregulation of the stomach-specific tumour suppressor gene TFF1. Using this genetic background several mutant mice for other members of the IL-6 cytokine family have been obtained ($gp130^{Y757F/Y757F}$ $IL\text{-}6^{-/-}$; $gp130^{Y757F/Y757F}$ $Stat3^{+/-}$; $gp130^{Y757F/Y757F}$ $IL\text{-}11ra1^{-/-}$; $gp130^{Y757F/Y757F}$ $Stat1^{-/-}$; $gp130^{Y757F/Y757F}$ $Stat1^{+/-}$) with the purpose of elucidate the contribution of IL-6 members to gastric cancer development and progression (145,146,147,118).

4. Mucins

4.1 Definition, classification and distribution

Mucins are high molecular weight extracellular glycoproteins that maintain epithelial integrity and lubricate and protect epithelial surfaces. From sequence data more than 20 different human mucins have been described (148). Mucins can be classified into two main categories: membrane-associated and secreted. Membrane-tethered mucins are bound to cells by an integral transmembrane domain, have relatively short cytoplasmic tails that associate with cytoskeletal elements and cytosolic adaptor proteins and can participate in signal transduction (149). They can be further classified into two distinct groups (Table 1): small (MUC1*, MUC13, MUC14, MUC15, MUC18 and MUC20), and large mucins (MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC16 and MUC17) (150). Secreted mucins are entirely extracellular and are subdivided in two groups: gel-forming (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) and non-gel-forming mucins (MUC7, MUC8 and MUC9).

Mucin genes expression is tightly regulated, exhibiting a highly ordered tissue distribution (Table 1). Some mucins, such as MUC1 and MUC4, are present in multiple tissues, while others have a more limited localization, as MUC2, which is predominantly expressed in the intestine. The respiratory tract produces the largest variety of mucins (151), providing a good example of cell-specific expression. The expression of mucins is also regulated during development. They arise at a specific developmental stage and continue in the adult (152,153). However, there are some embryonic tissues that express certain mucins that are lost in the adult. That is the case of MUC4 in skin, which is present in the embryo before cornification, but not in the adult (154).

An altered expression of mucins has been observed in tumours from epithelial origin (155) and in pre-cancerous lesions. For instance, MUC1 expression is a hallmark of most breast carcinomas (156), MUC4 of pancreatic cancer (157), MUC16 of ovarian cancer (158) and MUC2 of intestinal metaplasia. Also, inflammatory responses are accompanied by deregulated expression of mucins (159).

* Human mucins are designated in capitals (MUC1, MUC4…), while rodent mucins are indicated as Muc (Muc1, Muc4…).

Human mucin	Approved Gene Name	Chromosome	Tissue distribution	Reference
Small membrane-bound mucins				
MUC1	Mucin1, cell associated	1q21	Most epithelia	(160)
MUC13	Mucin 13, cell associated	3q13.3	Intestine, trachea	(161)
MUC14	EMCN, endomucin	4q22.1	Endothelia	(162)
MUC15	Mucin 15, cell associated	11p14.3	Placenta, salivary gland, trachea, intestine	(163)
MUC18	MCAM, melanoma cell adhesion molecule	11q23.3	Respiratory tract	(164)
MUC20	Mucin 20, cell associated	3q29	Kidney	(165)
Large membrane-bound mucins				
MUC3A	Mucin 3A, cell associated	7q22	Intestine	(166)
MUC3B	Mucin 3B, cell associated	7q22	Intestine	(167)
MUC4	Mucin 4, cell associated	3q29	Respiratory tract, intestine	(168)
MUC11	Withdrawn and substituted by MUC12			(169)
MUC12	Mucin 12, cell associated	7q22	Colon	(169)
MUC16	Mucin 16, cell associated	19p13.2	Ovary	(158)
MUC17	Mucin 17, cell associated	7q22	Colon, pancreas	(170)
Secreted gel-forming mucins				
MUC2	Mucin 2, oligomeric mucus/gel forming	11p15.5	Intestine	(171,172)
MUC5AC	Mucin 5AC, oligomeric mucus/gel forming	11p15.5	Respiratory tract, stomach	(173)
MUC5B	Mucin 5B, oligomeric mucus/gel forming	11p15.5	Respiratory tract, salivary gland, cervix	(174)
MUC6	Mucin 6, oligomeric mucus/gel forming	11p15.5	Stomach, biliary gland	(175)
MUC19	Mucin 19, oligomeric	12q12	Salivary gland, trachea	(176)
Secreted non-gel-forming mucins				
MUC7	Mucin 7, secreted	4q13.3	Salivary gland	(177)
MUC8	Mucin 8	12q24.3	Respiratory tract	(178)
MUC9	Oviductal glycoprotein 1, 120kDa (mucin 9, oviductin)	1p13.2	Reproductive tract	(179)

Table 1. Human mucin genes: classification, chromosomal localization and tissue distribution.

4.2 Sequence and structure

The structural feature shared with all mucins is the tandem-repeat domain, containing tandem repeats of identical or highly similar sequences rich in serine, threonine and proline residues. Variation in the specific sequence and number of tandem repeats is found among different mucins and among orthologus mucins from different species (155). The repetitive domain is characterized by an inter-individual variable number of tandem repeats (VNTR) (180). The VNTR polymorphism is caused by the instability of the number of repetitions from generation to generation and can be detected at the genomic level, the RNA level (181) and the protein level (182). In addition, O-glycosylation with complex oligosaccharides occurs on serines and threonines at tandem-repeat domains, and it is crucial for mucin structure and function.

- **Membrane-bound mucins**

Two dissimilar dimers compose membrane-tethered mucins that are held together by non-covalent, sodium dodecyl sulphate (SDS)-labile bonds. The larger subunit is extracellular and heavily glycosylated, and almost entirely composed by the VNTR domain. The smaller subunit consists of an integral transmembrane domain, a short cytoplasmic tail, extracellular domains that are released from the cell surface, and proteolytic cleavage domains (183). In most of the membrane-tethered mucins cleavage is mediated in the SEA domain (184,185) by an unidentified intracellular protease .

In addition, most membrane-bound mucins have juxtamembrane domains with homology to the EGF family (186). These domains allow cell-surface mucins to interact with members of the EGF receptor family (ERBB), and in this way mucins can participate in the regulation of different processes such as growth, motility, differentiation and inflammation (187). Some conservation among membrane-associated mucins is found in the number and general arrangement of EGF domains. MUC3A, MUC3B, MUC4, MUC12, MUC13 and MUC17 have two or three EGF domains, which are separated by the SEA domain for MUC3A, MUC12, MUC13 and MUC17, but not MUC4. One of the EGF domains is located on the extracellular region that contains the tandem-repeat domain, and a second (in some cases a third) EGF domain is located on the extracellular side of the membrane-associated subunit, proximal to the cell surface.

SMALL MEMBRANE-BOUND MUCINS

MUC1

Distinct MUC1 glycoforms are expressed in different tissues, varying in their molecular weight from 250 to 1000 kDa. The larger MUC1 subunit comprises the N-terminal sequence and the VNTR domain made up by 20-100 repeats of a 20-amino acid sequence (Figure 19) (188). The smaller fragment consists of an extracellular stem region of 58 residues, containing multiple O- and five N-glycosylation sites, a short hydrophobic transmembrane domain and a 72-amino acid phosphorylated cytoplasmic tail (156). Cleavage of the MUC1 precursor polypeptide occurs through autoproteolysis immediately following translation (189,190). The site of cleavage is found in the SEA domain (191), and the two parts obtained remain together associated via non-covalent forces (192). The localization of the mature protein is directed to the apical membrane by a signal peptide at the N-terminus of the molecule (193). Once at the plasma membrane the MUC1 extracellular domain can be shed into the lumen by the action of different proteases, such as TACE/ADAM17 and ADAM9 (194), which only act on the SEA domain of MUC1 after autoproteolysis.

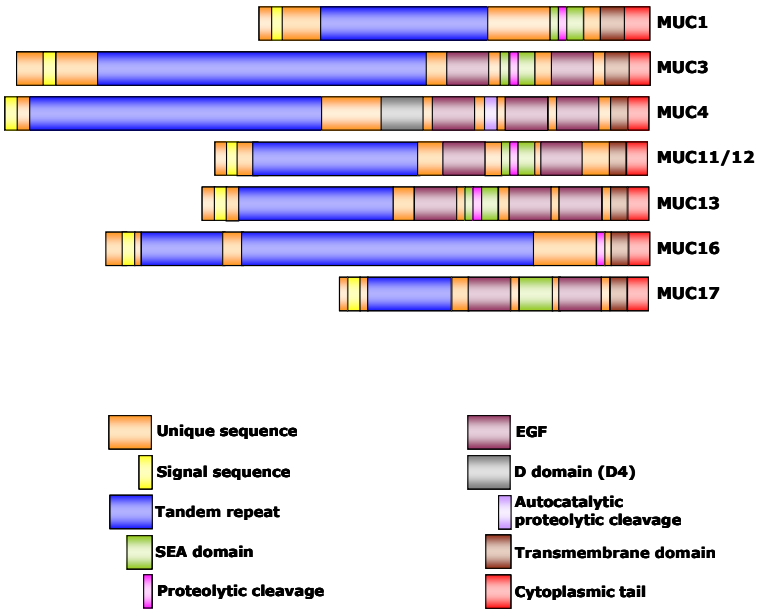


Figure 19. Schematic representation of the main domains present in membrane-associated mucins.

MUC13

MUC13 presents at the N-terminus a signal peptide for the secretory pathway. The signal peptide is followed by a serine- and threonine-rich domain involved in O-glycosylation and consisting of 10 degenerate tandem repeats (Figure 19). Following this mucin domain (MUC13α), there are two cystein-rich domains containing EGF-like motifs, which are separated by the SEA module. The first cystein-rich domain contains one EGF-like sequence, and the second presents two EGF-like sequences. The third EGF-like domain is followed by a 23-amino acid transmembrane domain and a 69-amino acid cytoplasmic tail. The cytoplasmic tail contains a protein kinase C consensus phosphorylation motif and eight serine and two tyrosine residues that can be phosphorylated. In addition, MUC13 presents six extracellular and one intracellular consensus N-glycosylation sites (161).

MUC14, MUC15, MUC 18 and MUC20

The N-terminus of MUC14 is followed by a putative cleavage site. The transmembrane domain is flanked by the extracellular and cytoplasmic domains. The extracellular domains are rich in serine, threonine and proline that are potential sites for O-glycosylation. No tandem repeat domains are observed in MUC14, and its cytoplasmic domain contains three potential phosphoryltaion sites for protein kinase C (162).

The coding sequence of MUC15 contains a signal peptide, an extracellular serine-, threonine-, proline-, leucine-, and asparagine-rich domain, containing ten N-glycosylation motifs and numerous O-glycosylation sites, a transmembrane domain and a short cytoplasmic tail. No tandem repeat regions are localized in MUC15 protein backbone (163).

MUC18 extracellular part contains five domains all homologous to each other and to immunoglobulin-like domains. Eight sites for N-glycosylation are also located in the extracellular region of the molecule. As the other membrane-associated mucins MUC18 possesses a membrane-spanning region, but it does not present a tandem repeat domain (195).

MUC20 gene is located very close to MUC4 and its protein backbone contains a 19-amino acid tandem repeat sequence consisting of many serine, threonine and proline residues (196).

LARGE MEMBRANE-BOUND MUCINS

MUC3A and MUC3B

MUC3A and MUC3B repetitive serine-, threonine-, and proline-rich domain is followed by two EGF modules flanking a SEA domain, a putative transmembrane motif and an intracytoplasmic tail (Figure 19). The fact that BLASTing of the MUC3A and MUC3B sequences against the human genome sequence predicted that the two sequences belonged to the same genomic unit suggests that they may arise from the same unique gene (150).

MUC4

MUC4 heterodimer is derived from a single gene that is post-translationally processed into two subunits: the O-glycosylated extracellular MUC4α subunit (600-800 kDa) and a largely N-glycosylated subunit MUC4β (120 kDa). MUC4α contains a N-terminal sequence followed by 3000-7300 amino acids comprising four different structures (Figure 19): three imperfect repeats of 126-130 residues, a unique sequence, the 16-residue serine- and threonine-rich VNTR (145-395 repeats) and a cystein-rich domain followed by the proteolytic cleavage site (197). MUC4β is rich in N-glycosylation sites and presents up to three EGF-like domains, the transmembrane domain and a cytoplasmic tail of 22 amino acids that contains a single tyrosine residue (198). Cleavage of MUC4 into its two component fragments requires an unknown serine protease (199). MUC4 is the only membrane-tethered mucin without a SEA domain (200). In addition, it contains three domains that are not present in the other membrane-bound mucins: a nidogen homology region (NIDO), the adhesion-associated domain (AMOP) and a von Willebrand factor type D sequence (VWD), which is also found in gel-forming mucins and may mediate their polymerization (201,202,200).

MUC11 and MUC12

Two partial cDNAs named MUC11 and MUC12 were reported in 1999 and believed to belong to two different mucins (169). However, subsequent BLAST searches showed that they were two sequences from a unique mucin gene that now is referred as MUC12 according to the HUGO GeneNomenclature Committee. MUC12 also presents a large serine-, threonine-, and proline-rich region followed by two EGF motifs flanking a SEA module, a putative transmembrane peptide and a short 75-amino acid intracytoplasmic tail (Figure 19) (150).

MUC16

MUC16 transmembrane-bound protein was also called CA125 (158), which was known as a marker for ovarian cancer. The N-terminal subunit is a typical, heavily O-glycosylated mucin domain (Figure 19). The C-terminal portion consists of more than 60 tandem repeats of 156 amino acids, a transmembrane region, and a 32-residue cytomplasmic tail with several tyrosine, serine, and threonine sites for potential phosphorylation (203,204). A proteolytic cleavage site is located 50 amino acids N-terminal to the transmembrane region (204). MUC16 has 16 SEA domains (205), but only the second resembles that of MUC1 and other mucins, and may provide a site of proteolytic cleavage.

MUC17

MUC17 also has a serine-, threonine-, and proline-rich region, and this mucin-like domain is followed by two EGF modules flanking a SEA domain, a putative 22-amino acid transmembrane sequence and a 78-amino acid intracytoplasmic tail (Figure 19) (206).

- **Secreted mucins**

GEL-FORMING MUCINS

The most well-known group of secreted mucins is that of gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6). They share some sequence homology, are clustered on chromosome 11p15 and are believed to have arisen by duplication from a common ancestor (207). The core proteins of secreted gel-forming mucins are very large and complex due to their ability to oligomerize. Oligomerization is mediated by D domains, which are named because of their homology with the dimerization (D) domains of von Willebrand factor (201).

MUC2

The central domain of MUC2 is composed of two highly repetitive sequences. The first is characterized by the repetition of a 23-amino acids motif. The second is composed of an irregular sequence repeated in tandem with a unit of 347 amino acids (172). These two sequences are rich in serine, threonine and proline, being sites for O-glycosylation. The N-terminal domain, made up of 347 amino acids, is flanked by two regions rich in cysteine, called Cys domains (Figure 20). In addition, MUC2 presents five D domains. D1, D2, D’ and D3 domains are located in its N-

terminal part, whereas the D4 domain is localized in the C-terminal region. Downstream of the D4 domain three other sequences show similarity with domains of the von Willebrand factor, one domain C, one domain B, and one domain CK (Cystine Knot), that are also found in other secreted proteins (208). Towards the C-termius MUC2 contains an autocatalytic protein-cleavage site at the motif GDPH (cleavage between the D and P residues) (209). Upon cleavage a subunit, which was previously characterized as a "link protein" and believed to have a role in establishing higher order structures in the gel-forming mucins (210), is produced.

MUC5AC

MUC5AC central domain is composed of 17 major domains. Nine are cysteine-rich domains that exhibit high sequence similarity to the cysteine domains described for MUC2 and MUC5AC. Cys1 to Cys5 are interspersed by domains rich in serine, threonine and proline residues (Figure 20). Domains Cys5 to Cys9 are interspersed by four domains composed of various numbers of MUC5AC-type repeats (211). In the N-terminus four D domains (D1, D2, D' and D3) are localized, while the von Willebrand-like domains D4, C, B and the CK domain are located in the C-terminus. The GDPH motif found in MUC2 is also present in MUC5AC.

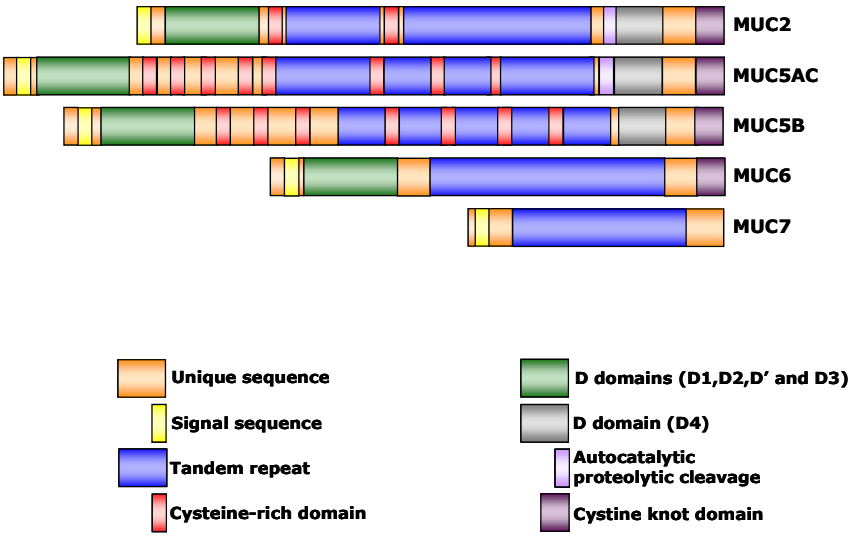


Figure 20. Schematic representation of the main domains present in secreted mucins.

MUC5B

MUC5B presents seven cysteine-rich domains (Cys1 to Cys7) that have a structure similar to the Cys domains of MUC2 (Figure 20). It also has five subdomains composed by the imperfect repetition of 29 amino acids and three subdomains with a unique sequence rich in serine, threonine and proline residues (212). The central domain of MUC5B is composed of four super-repeats of 528 amino acids. Similarly to MUC2 and MUC5AC, D domains (D1, D2, D' and D3) are located in the N-terminal part of MUC5B (213), while in the C-terminus D4, C, B and CK domains are localized (214).

MUC6

In addition to the tandem repeat region, containing a repeat unit of 169 amino acids, MUC6 also presents D1, D2, D' and D3 domains at its N-terminal part (Figure 20) In the C-terminus a CK domain is located, but no D4 domain is found (215).

MUC19

MUC19 does not belong to the same cluster as MUC2, MUC5AC, MUC5B and MUC6. MUC19 sequence includes a signal peptide, a large tandem repeat region, von Willebrand factor type C and D domains, a trypsin inhibitor-like cysteine-rich domain, and a C-terminal CK domain (216).

NON-GEL-FORMING MUCINS

MUC7, MUC8 and MUC9 fall into this class. MUC7 mucin domain shows variation in length (Figure 20), with two major alleles containing five or six repeats (217). MUC8 presents a mucin domain with a tandem repeat unit but its sequence remains incomplete (218,178). No von Willebrand-like domains are observed in these two mucins. MUC9 sequence contains serine- and threonine-rich repeated units clustered in its C-terminal portion (179).

4.3 Biosynthesis and secretion

The synthesis of mucins follows the general scheme of all secreted and cell-surface glycoproteins. Mucin transcripts, most of which are extremely large, are translated on endoplasmic reticulum-associated ribosomes and transported into the

the endoplasmic reticulum (ER) lumen. Co-translationally, N-glycosides are added, and the signal sequence specifying ER binding is removed by proteolysis. Next, a "copy-editing" step involving deglucosylation, glucosylation and proteosomal degradation eliminates misfolded proteins (219). In the case of gel-forming mucins, oligomerization by disulfide bonding and packaging into granules also occur in the ER (220). Oligomerization is essential for escape from the ER because monomers do not enter the Golgi. In the Golgi apparatus, O-glycosylation and further processing of N-glycosides takes place, before mucin transit to the cell membrane.

The exocytosis process by which mucins are secreted is not completely known. Delivery of mucins to the exterior occurs via storage granules. This process may be essential to concentrate mucins to an optimal physical state or can be used to delay targeting choice between retention in the granule mass or secretion. Mucins stored in central granules seem to be released by compound exocytosis, a regulated event that requires secretagogue stimulation such as cholinergic agonists, proteases, arachidonic acid metabolites, secreted inflammatory cell products and pathogens, while single granules located at the periphery are released in a constitutive manner (221).

4.4 Glycosylation

Although in their primary sequence potential sites for N-glycosylation can be found, the main form of mucin glycosylation is O-glycosylation.

O-glycosylation is initiated in the cis-Golgi by addition of of N-acetylgalactosamine (GalNac) from UDP-GalNac to the hydroxyl groups in serine and threonine residues located in the tandem repeat regions of mucins. This reaction is catalyzed by a large family of up to 20 different polypeptide GalNAc-transferases (222,223), and results in the formation of the GalNAcα1-Serine/Threonine or Tn antigen (Figure 21). Most O-glycan structures found in mucins are based on the Core I structure or T antigen formed by addition of galactose (Gal) in a β1-3 linkage to GalNAc. There is only one Core I galactosyl-transferase in mammals (core 1 β3Gal-T, T-synthase) (224), which is under the control of a specific chaperone (Cosmc) (225). In a recent report Ju et al. have shown that mutations in Cosmc induced loss of T-synthase activity and were the responsible for the increased levels of Tn and sTn observed in tumour cells (226). Core I structures can be further elongated by the action of different glycosyltransferases that add different monosaccarides such as Gal, N-acetylglucosamine (GlcNAc), fucose (Fuc) or sialic acid (NeuAc). Two main

intermediary O-glycan structures are found in mucins: Type 1 (GlcNAcβ1-3 Galβ1-3)$_n$ and and Type 2 chains (GlcNAcβ1-3 Galβ1-4)$_n$. When Fuc or NeuAc are added by fucosyltransferases or sialyltransferases, repectively, the glycosydic chains cannot be elongated any more.

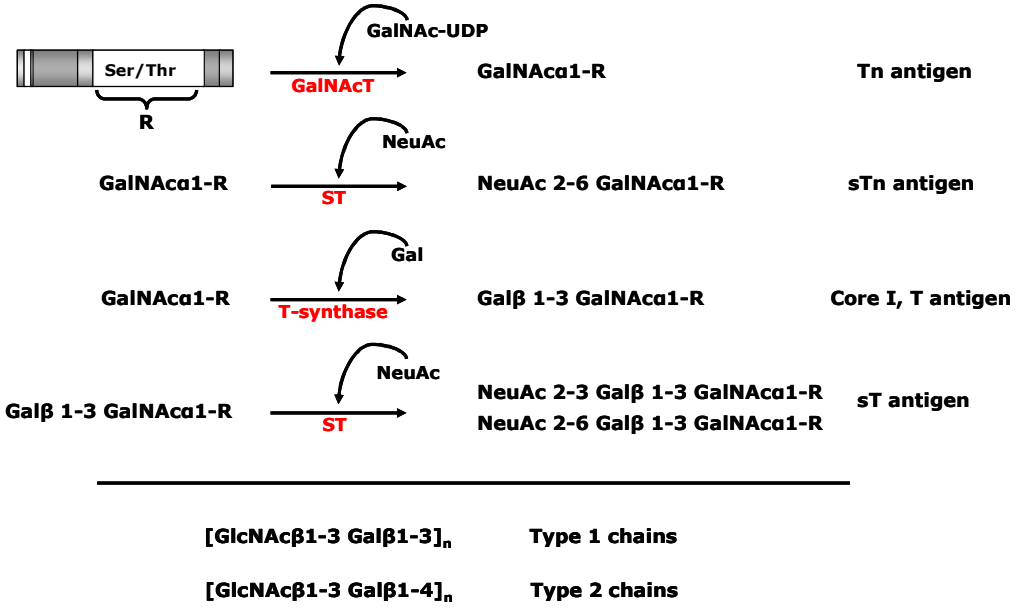


Figure 21. Mucin-type O-glycans.

Fucosyltransferases catalyze the final step in the synthesis of the terminal fucosylated structures Lewis antigens. To date 11 fucosyltransferase genes have been cloned (FUT1-FUT11) (227,228), and their encoded proteins show a complex tissue- and cell type-specific expression pattern that varies during development and malignant transformation. For instance, in stomach, cells in the superficial epithelium express FUT2, while deep gland cells express FUT1 (28). Lewis antigens constitute the Lewis histo-blood system and can be found associated with glycoproteins and glycolipids (229). These oligosaccharides are generally present in hematopoietic as well as in epithelial cells (230,231,232), and in the epithelia, mucins are the major carriers of Lewis antigens. They are synthesized by the sequential action of specific glycosyltransferases on precursor type 1 or type 2 chains (Figure 22).

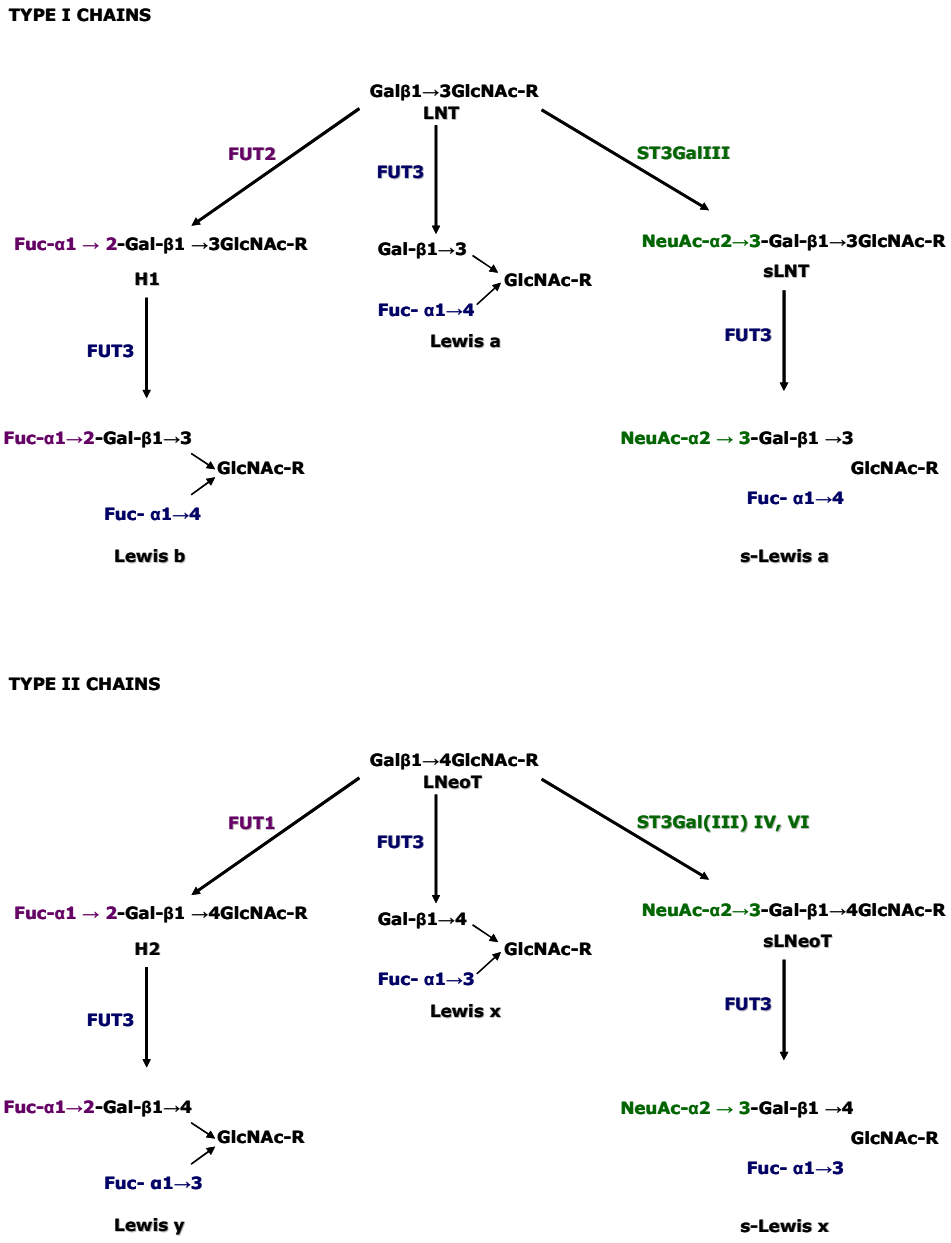


Figure 22. Schematic representation of Lewis antigens biosynthesis.

The cell surface fucosylated oligosaccharides participate in several biological processes such as implantation, embryogenesis, tissue differentiation, tumour metastasis, inflammation and bacterial adhesion (233,234,235,236). In stomach, *H. pylori* BabA-mediated adhesion to the gastric mucosa has been reported to be influenced by the expression of Lewis b (237), while *H. pylori* SabA binds s-Lewis x (62). In addition, s-Lewis antigens are overexpressed in gastric tumours and they

have been associated with increased invasion and metastasis (238,239,240,241), due to their capacity to bind to selectins (242).

Aberrant glycosylation of mucins has been detected in a number of tumours. In particular, MUC1 is aberrantly O-glycosylated in most adenocarcinomas, including breast, ovarian and pancreatic cancers, and this fact has been used in different approaches for the development of MUC1 cancer vaccines (243)

4.5 Regulation of mucin expression

Multiple mechanisms have been involved in the regulation of mucins such as transcriptional modulation, transcript stabilization, post-translational control and epigenetic regulation. Most of the studies to date have been done on the membrane mucins MUC1 and MUC4 and on the gel-forming mucins, and several signalling pathways have been demonstrated to be implicated, such as the canonical Erk/MAPK pathway or the cytokine-JAK-STAT pathway.

- **Transcriptional regulation of mucins**

TRANSCRIPTION FACTORS

The diversity of mucin gene expression patterns can be explained by two aspects of transcriptional regulation: specific, unique promoter sequences in the MUC genes, and differential, tissue-specific expression and regulation of transcription factors. Different studies have focused on describing promoter sequences, which have served to identify specific transcription factors involved in MUC gene regulation (Table 2) and to characterize their interactions with defined promoter elements (244,245). However, a better understanding of the regulation and activation of these transcription factors may be needed.

Transcription Factor	Mucin gene regulated	References
CDX family	MUC2, MUC4	(246,247,248)
FOX family	MUC4, Muc2	(248,249)
GATA family	MUC1, MUC4, MUC5B	(250,251,252)
NF-κB	MUC1, MUC2, MUC5AC,MUC5B, MUC6, MUC7	(253,254,255,252,256,257)
Sp1 family	MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6	(258,259,251,255,252,256)
STATs	MUC1, MUC4	(260,245)

Table 2. Transcription factors implicated in mucin gene regulation.

DIFFERENTIATION FACTORS

Mucin production is affected by factors that promote differentiation. It is not clear if these differentiation factors (Table 3) increase mucin expression by increasing the number of goblet cells expressing the mucin or by directly affecting mucin gene expression. An example occurs in the airway epithelia, wich in response to irritants increases the number of goblet cells secreting MUC5AC (151). However, studies in cell culture models also suggest that mucin expression can be directly modulated by differentiation factors.

One of the first differentiation factors studied was retinoic acid, which acts via specific nuclear receptors RAR and RXR that activate gene transcription (261). For instance, retinoic acid has described to induce MUC4 expression in pancreatic tumour cells via its receptor RARα (262). This induction was mediated by TGFβ, which surprisingly had been reported to repress Muc4 expression in other systems (263,264). Therefore, TGFβ seems to be able to modulate MUC4 by a number of different mechanisms depending on cell context.

Phorbol esters, that are differentiation factors and tumour promoters, are able to modulate mucin expression. One example is phorbol 12-myristate 13-acetate (PMA), which increases the transcription of MUC2 in colon cancer cells (265). PMA acts via PKC that activates Ras-Erk pathway. NF-κB was also observed to be implicated in the transcriptional regulation of MUC2 by PMA.

Butyrate acts as differentiation agent for a number of cell types, including those of the colon. MUC2 expression has been described to be activated by butyrate

in colon cancer cells (266), and this activation involved Erk signalling pathway and inhibition of histone H3 deacetylation (267).

Differentiation factor	Mucin	References
Retinoic acid	MUC2, MUC4, MU5AC,MUC5B	(268,262,269)
Phorbol esters	MUC1, MUC2, MUC3, MUC5AC, MUC5B	(270,265,271,272,273)
Butyrate	MUC2, MUC3, MUC5B	(267,266)

Table 3. Differentiation factors involved in mucin expression regulation.

CYTOKINES

Cytokines bind to specific receptors to activate associated tyrosine kinases that initiate downstream signalling (see Figures 15, 16 and 17), regulating in this way the transcription of target genes.

IFN-γ is able to regulate the expression of MUC1 in a number of cell types (274,275,276), likely through STAT binding to a specific site in MUC1 promoter (260). Recently, MUC4 has been also described to be activated by IFN-γ in pancreatic cells (277). This activation occurs through direct binding of STAT1 to IFN-γ-activated sites in the MUC4 promoter.

TNF-α is able to enhance MUC1 expression in multiple cell types (253,278,279,276). In airways epithelial cells MUC5AC expression has been described to be regulated by TNF-α and IL-1β via pathways requiring Erk and p38 MAP kinases (280,281). A similar mechanism requiring Erk and p38 is responsible for MUC2 activation upon IL-1β treatment in cells from the respiratory tract (281), and in colon cancer cells MUC2 regulation by TNF-α occurs via NF-κB (282).

IL-4 and IL-13, which have been implicated in goblet cell metaplasia, can regulate the expression of MUC2 and MUC5AC (283). These cytokines act via a common receptor IL-4R to activate STAT6. It is not clear if increased mucin production induced by IL-4 and IL-13 occurs though direct regulation of mucin gene transcription or through an increase in the number of goblet cells. Distinct results have been obtained in different cell and animal models (284,285,286,287) that cannot be explained due to the little information available about the pathways and mechanisms involved in the effects of these cytokines.

Cytokine	Mucin	References
IFN-γ	MUC1, MU4	(253,275,245)
TNF-α	MUC1, MUC2, MUC5AC, MUC7	(279,282,280,257)
IL-1β	MUC1, MUC2, MUC4, MUCAC, MUC8	(288,281,289,280,290)
IL-4	MUC2, MUC4, MUC7	(291,292,293)
IL-13	MUC2, MUC5AC, MUC7, MUC8	(291,294,293,286)
IL-6	MUC1, MUC5AC, MUC5B, MUC6	(288,295,296)

Table 4. Cytokines implicated in the regulation of mucin expression.

BACTERIAL PRODUCTS

Different bacterial products can alter the expression of mucins. One of the most well-known is LPS present in Gramm-negative bacteria. LPS can induce the expression of MUC2 via Src-dependent activation of Ras, Erk and p90rsk. As a result, NF-κB translocates to the nucleus and binds to MUC2 promoter sequence (254). The expression of MUC5AC and MUC5B is also regulated by LPS in intestinal cells, but here the mechanism of activation involves the IL-8 signalling pathway.

By mechanisms still unknown *H. pylori* has been described to up- and downregulate MUC6 expression in the gastric epithelium (297,298). In addition, infection of gastric cancer cells by *H. pylori* has been recently reported to increase the expression levels of MUC2 and MUC5AC (299).

- **Post-transcriptional regulation of mucins**

Rapid transcript turnover is one of the earliest control mechanisms after transcription and is regulated by specific sequences of the transcript which bind stabilizing proteins. MUC5AC and MUC4 are mucins regulated by this mechanism. MUC5AC and MUC4 transcript stabilization occurs through binding of neutrophil elastase that increases mucin levels in airway cells (300,301).

Mucins can also be regulated post-translationally. That is the case of Muc4. Muc4 is cleaved in the ER to be functional. TGF-β represses this cleavage in the rat mammary epithelial cells and in this way the synthesis of Muc4 is blocked (264). The most probable mechanism for this effect is that the precursor fails to pass the "copy-editing" step and is transported to the proteosome for degradation.

- **Epigenetic regulation of mucin genes**

DNA methylation, associated with histone deacetylation is a common mechanism used by cancer cells to inhibit the expression of tumour suppressor genes and genes involved in tumour formation (302,303). MUC2, MUC5AC, MUC5B and MUC6 mucin genes were described to be located in a hot spot of methylation in the genome (207). In the case of MUC2 gene, methylation of its promoter was found to repress its expression in non-mucinous colon carcinoma cells (304,305). Following studies in pancreatic carcinoma cells showed that *de novo* expression of MUC2 was triggered by promoter demethylathion (306), and in mucinous gastric carcinomas aberrant MUC2 expression was induced by promoter hypomethylation (307). Also MUC5B was reported to be regulated by DNA methylaion. In gastric cancer cells the presence of methylated cysteins in its promoter induced MUC5B repression (308). Vincent et al. have studied the epigenetic regulation of the 11p15 mucin genes, and have demonstrated that MUC2 and MUC5B are highly submitted to DNA methylation and histone modifications, whereas MUC5AC is rarely influenced by epigenetic regulation, and MUC6 expression is not modulated by epigenetic mechanisms (309). Finally, and more recently, MUC1 has been also described to be regulated by DNA methylation and histone H3 lysine 9 modification in cancer cells (310), and MUC4 promoter methylation has been described to contribute to its regulation in breast, lung, pancreas and colon cancer cells (311).

4.6 Normal functions of mucins

Mucins are known to play a central role in the protection, lubrication and hydratation of the external surfaces of epithelial tissues. They have been also suggested to be implicated in other important biological processes such as epithelial cell renewal, differentiation, cell signalling and cell adhesion (187,312).

In a typical aerodigestive epithelium the secreted mucin layer might be in contact through diverse interactions with membrane-associated mucins near the cell surface, and both layers will contribute to **physicochemical protection** and **maintenance of environment** of the epithelial surfaces. One good example is found in the stomach. Gastric mucus, which is composed mainly by MUC5AC and MUC6 (313,314), forms a protective layer over the surface of the gastric epithelium and acts as a selective diffusion barrier for HCl. Gastric epithelial cells secrete bicarbonate that is held in the mucus layer and creates a pH gradient (from pH 2 at the stomach lumen to pH 6-7 at the epithelial cell surface). Depending on the pH

HCl interacts with the gastric mucus layer in different ways. When the pH is above 4, HCl passes through channels formed in the mucus layer. In contrast, when pH is below 4 HCl does not penetrate the mucus layer (315). In this way HCl secreted from the gastric glands can pass through the mucus layer to the lumen, but at the same time the mucus layer represents a barrier that does not allow the secreted HCl to approach the epithelial cell surface.

Mucin gels have been described to **capture and hold** different molecules, the best characterized of which are trefoil factors. TFFs (TFF1, TFF2 and TFF3) are small peptides usually co-expressed with mucins in the respiratory tract, salivary glands, gastrointestinal tract, ocular surface and uterus (316). Following their release, TFFs promote wound healing and mucosal restitution at sites of epithelial damage (317). They are bound to mucins in the region of the D domains (318), form part of the mucus gel and protect the epithelial cells by contributing to mucus viscosity (319) and by their anti-apoptotic properties and their motogenic activity modulating cell migratory processes (320). It has been hypothesized that mucins can also bind cytokines, growth factors, differentiation factors and mediators of inflammation. In this sense, it has been shown that human interleukins IL-1, IL-4, IL-6 and IL-7 have specific lectin sites that allow them to bind oligosaccharides expressed on mucin tandem repeats (321). However, direct molecular interactions explaining the presence of these cytokines in mucus gels have not been demonstrated.

Recent studies have suggested the participation of cell-surface-associated mucins in **signal transduction** events, and it is thought that in response to alterations in the mucin layer or local microenvironment mucins could provide signals to epithelial cells that would alter their proliferation, differentiation or cell-cell adhesion status (155).

4.7 Roles of mucins in cancer

The deregulated expression of mucins has been associated with various types of pathological conditions, including malignant transformation and inflammatory disorders (155). In cancer, the aberrant expression of different mucins has been found. For instance, MUC4 is ectopically expressed in pancreatic tumours contributing to proliferation, invasion and metastasis (322).

Cancer cells use mucins for **protection** from adverse conditions and to **control the local molecular microenvironment**. During invasion and metastasis, expression of mucins by cancer cells would allow them to survive and

proliferate, since mucus can act as a shield for cytotoxic compounds, and in this way mucins can provide resistance to chemotherapy. In addition, the capture of cytokines or growth factors in the mucus secreted by tumour cells might contribute to the growth of the tumour (155).

Cell-surface mucins, specifically MUC1 and MUC4, contribute to the regulation of **differentiation** and **proliferation** of tumour cells**.** The cytoplasmic tail of MUC1 can bind and signal through the MAP kinase and β-catenin pathways (323,324,325,326,327) (Figure 23). β-catenin binds to a MUC1 serine-rich motif (323) and this binding is increased by activated EGF receptor, c-Src (325,326) or PKCδ (328). In contrast, β-catenin/MUC1 interaction is decreased by the activity of GSK3β in some cells (324). MUC1 might affect β-catenin signal transduction by two mechanisms: MUC1 might be cleaved and a fragment of its cytoplasmic tail is transported to the nucleus in association with β-catenin, influencing transcription through TCF/LEF and/or other transcription factors (329), or might sequester β-catenin in the cytoplasm and therefore prevent it from interacting with cadherins or other complexes influencing in this way WNT signalling pathway (327,329). MUC1 can also interact with ERBB1, and MUC1/ERBB1 interactions have been suggested to increase ERK1/2 phosphorylation through the MAPK pathway (330). MUC4 can interact with ERBB2 through an EGF-like motif (Figure 23) enhancing ERBB2 phosphorylation, and in this way it is postulated to induce cellular differentiation signals through p27 (331).

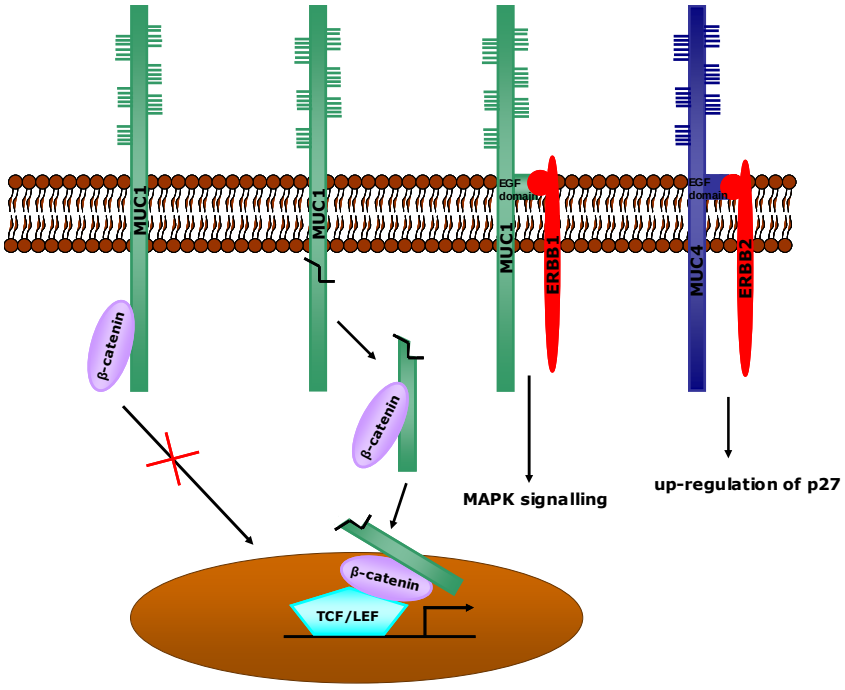


Figure 23. Signal transduction by membrane-associated mucins.

MUC2 has been proposed to be a **tumour suppressor** gene due to the observation that Muc2 deficient mice developed tumours in the small intestine, colon and rectum within one year of birth (332). However, the role of MUC2 in tumour suppression is not fully understood.

Mucins, particularly MUC1 and MUC4, are also involved in processes of **invasion** and **metastasis** due to their adhesive and anti-adhesive properties. For instance, increased MUC1/β-catenin interactions might reduce the pool of cytoplasmic β-catenin, weakening the intercellular adherence (333,334,335), conferring anti-adhesive properties to MUC1. On the other hand, MUC1 also has adhesive functions due to the presence of mucin-associated antigens, as s-Lewis x, on MUC1 surface (336). These antigens can bind adhesion molecules such as E-selectin, P-selectin and ICAM-1, favouring the extravasation of tumour cells and their dissemination to distant metastatic sites (337). MUC4 has been also associated with tumour invasion and metastasis. In pancreatic tumour cells, MUC4 expression has been associated with changes in actin organization resulting in increased cell motility. In addition, MUC4 interferred tumour cell-extracellular matrix interactions, in part, by inhibiting the integrin-mediated cell adhesion and as a result invasiveness was increased (322).

5. Mucin expression in normal gastric epithelium, pre-cancerous lesions and gastric tumours

5.1 Mucin expression in the normal stomach

Synthesis of mucins in the human stomach starts between 8 to 12 weeks of gestation. MUC1, MUC3, MUC4, MUC5AC, MUC5B and MUC6 are expressed in the embryonic stomach (338,153).

Normal adult gastric mucosa expresses MUC1, MUC5AC and MUC6 in a characteristic zonal pattern (Figure 24). MUC1 is expressed in the surface, foveolar epithelium and mucous neck region cells (339,340,27). MUC5AC is limited to the foveolar epithelium and mucous neck cells throught the stomach (341,314) and MUC6 is expressed in the antral pyloric glands and also in mucous neck and chief cells of the gastric body (341,340,342).

MUC2, MUC3 and MUC4 are generally absent in normal gastric specimens, while MUC13 is present at moderate levels in surface epithelium and deep glands of the stomach (161).

The expression of MUC5AC and MUC6 has been associated with the expression of FUT2 and type 1 Lewis antigens and FUT1 and type 2 Lewis antigens, respectively (28).

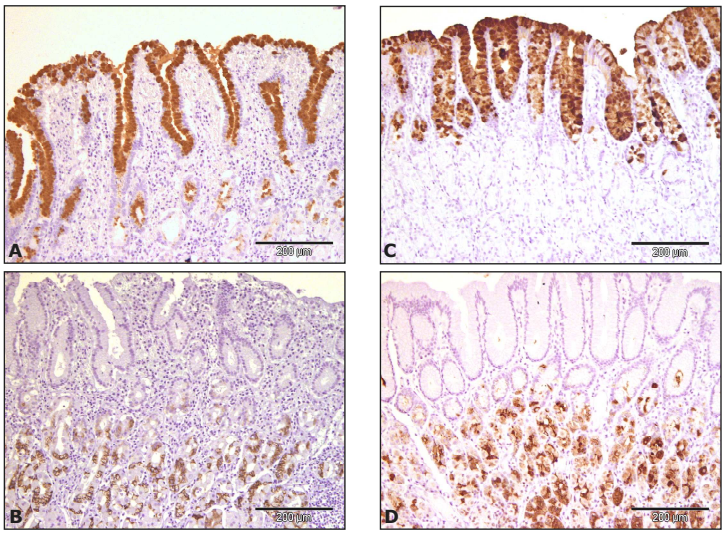


Figure 24. Expression of MUC5AC (A), MUC6 (B) and its respectively associated Lewis antigens Lewis a (C) and H type 2 (D).

5.2 Mucin expression in gastric pre-cancerous lesions

• Mucins in chronic gastritis

H. pylori infection induces degradation of the mucus layer (343) and alteration of mucin glycosylation (344), in addition to changes in the expression pattern of mucins. MUC6 in surface mucous cells is increased whereas there is a decrease in the expression of MUC5AC and MUC1 (345). *H. pylori* is very closely associated with extracellular MUC5AC and epithelial cells that produce it, indicating that MUC5AC plays a role in the adhesion of *H. pylori* to the gastric mucosa (346,347).

In atrophic gastritis altered expression of MUC5AC and MUC6 is observed in the surface columnar cells (297), in some cases MUC4 can be detected in the

superficial epithelium (28) and MUC2 is not expressed (297). No changes in the expression of carbohydrate structures have been found (348,28).

- **Mucins in intestinal metaplasia**

Based on the expression profile of mucins in intestinal metaplasia two main types of intestinal metaplasia are distinguished. Type I or complete intestinal metaplasia is characterized by the presence of absorptive cells, Paneth cells and goblet cells that secrete sialomucins. Incomplete intestinal metaplasia (type II and type II) is characterized by the presence of columnar and goblet cells that secrete sialo and/or sulphomucins (23). In complete intestinal metaplasia MUC1, MUC5AC and MUC6 are not or barely expressed. *De novo* expression of MUC2 and MUC4 (Figure 25) is remarkable in goblet cells (297,27) and high levels of MUC3 are found (340).

Incomplete metaplasia type II and type III share an identical pattern of mucin expression. MUC1 and MUC5AC are expressed in both goblet cells and columnar cells. MUC6 expression is found in the lower crypt and glandular epithelium but not at the same levels observed in the normal gastric mucosa. Also MUC2 and MUC4 are expressed in goblet cells. The characteristic mucin/Lewis antigen association observed in the normal gastric epithelium is not observed in metaplasia. Metaplastic cells co-express gastric and intestinal mucins associated with both types of Lewis antigens (349), in addition to increased levels of T, Tn and s-Tn antigens (348).

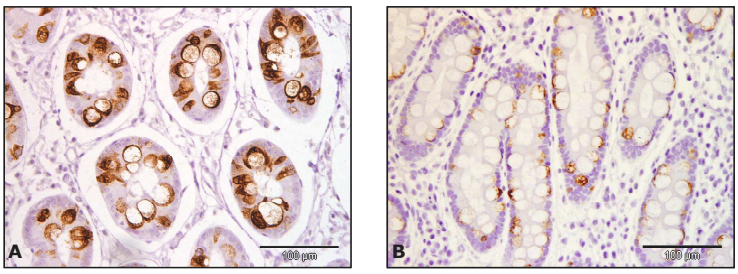


Figure 25. *De novo* expression of the intestinal mucins MUC2 **(A)** and MUC4 **(B)** in intestinal metaplasia samples.

5.3 Mucin expression in gastric adenocarcinoma

Intestinal-type gastric tumous display decreased levels of MUC1 and gastric mucins MUC5AC and MUC6 and increased levels of the intestinal mucins MUC2 and MUC4 (Figure 26). Ectopic expression of MUC3 is also detected in this type of gastric adenocarcinomas, and the association between gastric mucin genes, fucosyltransferases and Lewis antigens is totally lost (340,349). Aberrant carbohydrate structures such as the T, Tn and s-Tn antigens are found in intestinal-type tumours. Also high levels of s-Lewis x (Figure 26) have been detected and related to increased risk for gastric cancer and metastasis (238,239).

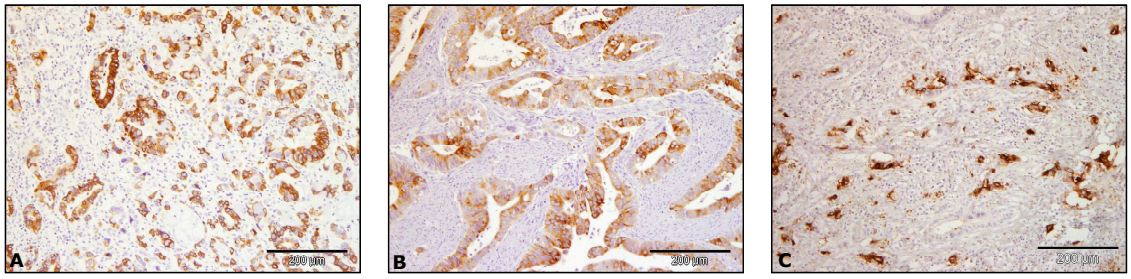


Figure 26. Expression of the intestinal mucins MUC2 (A), MUC4 (B) and the s-Lewis x antigen (C) in an intestinal-type gastric tumour.

Diffuse tumour cells express high levels of gastric mucins (MUC5AC and MUC6) whereas in general they lack MUC2 expression. Altered expression of Lewis antigens and glycosyltransferases is also found (349).

Recently, a new classification of gastric carcinomas based on mucin expression has been proposed. Gastric tumours are classified as gastric phenotype or intestinal phenotype according to the mucin expression by surface mucous cells, glandular mucous cells and intestinal goblet cells (350,351,342). MUC2 is employed as a marker of intestinal goblet cell differentiation while MUC5AC and MUC6 are employed as markers of the gastric phenotype.

Mucin expression has been shown to be correlated with different clinico-pathological characteristics of gastric tumours. For instance, MUC5AC has been associated with antral carcinoma and MUC2 with cardial carcinomas (342). Moreover, MUC1 and MUC2 expression was positively correlated to tumour size, depth of invasion, presence of lymph node metastasis and clinical stage (352), being the combined evaluation of MUC1 and MUC2 clinically useful to predict the outcome in patients with gastric cancer (353). In addition, MUC2 has been also

considered as intestinal metaplasia marker and may also be used for early detection in *H. pylori* infected pre-neoplastic gastric epithelium (297).

OBJECTIVES

In the gastric carcinogenetic process the specific expression pattern of glycosyltransferases and Lewis antigens displayed by the normal gastric mucosa is lost.

Our **first objective** was to study how changes in the expression of Lewis antigens induced by the transfection of the human FUT1 gene affected the invasive and metastatic capacities of cancer cells.

Chronic gastritis caused by *H. pylori* infection is a major determinant in the pathogenesis of gastric cancer, and increased levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 have been observed in *H. pylori*-infected patients, which can regulate the expression of genes involved in the gastric neoplastic transformation.

Our **second objective** was to analyse the effect of pro-inflammatory cytokines on the expression of glycosyltransferases and Lewis antigens in gastric cancer cells and to study if inflammation could modulate their expression in gastric tumours.

Intestinal-type gastric adenocarcinomas develop from successive pre-cancerous lesions that lead to an intestinal transdifferentiation of the gastric mucosa. In this process many intestinal genes are activated and ectopically detected in gastric cells.

Our **third objective** was to study the effect of pro-inflammatory cytokines and their associated signalling pathways on the expression of the intestinal mucins MUC2 and MUC4 and the intestinal transcription factor CDX2 in gastric cancer cells.

Our **fourth objective** was to evaluate if the predominant type of inflammation present in intestinal-type gastric adenocarcinomas could explain the differences observed in the expression of the intestinal mucins MUC2 and MUC4 in gastric tumours.

RESULTS

Chapter 1

Mejías-Luque R, López-Ferrer A, Garrido M, Fabra A, de Bolós C.
Changes in the invasive and metastatic capacities of HT-29/M3 cells induced by the expression of fucosyltransferase 1.
Cancer Sci. 2007 Jul;98(7):1000-5. Epub 2007 Apr 23.

Chapter 2

Regulatory effects of pro-inflammatory cytokines on glycosyltransferases and Lewis antigens expression in gastric cancer cells

Introduction

The normal gastric mucosa displays a specific expression pattern of glycosyltransferases and Lewis antigens. FUT1 and H type 2 glycans are expressed in cells of deep glands, FUT2 and H type 1 glycans in the superficial cells, and FUT3 in both populations of cells (28). This characteristic expression pattern is lost during the gastric carcinogenetic process and in gastric tumours both types of Lewis antigens can be detected (28). Increased expression of sialylated structures such as s-Tn and s-Lewis x antigens has been also found in gastric tumours (349) as a consequence of increased expression of sialyltransferases such as ST3Gal III, ST3Gal IV or ST6 Gal I (354,355). Inflammation of the gastric mucosa usually associated with *H. pylori* infection is the first step in the progression to gastric cancer. Gastritis is accompanied by the release of different pro-inflammatory cytokines, being especially important TNF-α, IL-1β and IL-6 (24,25,94,356), which have been suggested to trigger the activation of genes involved in the gastric neoplastic transformation. Several studies have shown that TNF-α, IL-1β, and more recently, IL-6 can modulate the expression of various glycosyltransferases in different cell types and tissues (357,358,359,360), but no data regarding this possible regulation in gastric models have been published to date.

MKN45 and GP220 gastric cancer cell lines were subjected to different TNF-α, IL-1β and IL-6 treatments (20 ng/ml and 40 ng/ml for 20 hours), and the expression of the fucosyltransferases FUT1, FUT2 and FUT3 and the sialyltransferases ST3Gal III, ST3Gal IV and ST6Gal I, which are detected in the stomach as well as in gastric tumours (28), was evaluated by semi-quantitative RT-PCR. Also, the expression of Lewis antigens product of the reaction catalyzed by the fucosyl- and sialyltransferases examined were analysed by flow cytometry. In addition, in a panel of intestinal-type gastric tumours classified according to their inflammatory score the expression of Lewis antigens was evaluated.

Materials and Methods

Reagents and antibodies

TNF-α and IL-1β were purchased from R&D Systems, Inc (MN, USA) and IL-6 from PreproTech EC (London, UK). The primary antibodies used in this study are listed in Table 1.

Antibody	Specificity	Reference
T.174	Lewis a	(361)
T.218	Lewis b	(361)
19-0LE	H type 2	(362)
77/180	Lewis y	(341)
57/27	s-Lewis a	(341)
Cslex-1	s-Lewis x	(363)
KM93	s-Lewis x	Chemicon International (CA, USA)
B72.3	s-Tn	(364)
M8	MUC1	(365)
B12	Synthetic dextran molecule	Dr. Castro (Barcelona, Spain)

Table 1. Antibodies used in this study.

Cell culture and treatments

MKN45 cells (ATCC), expressing FUT1, FUT2, ST3Gal III, ST3Gal IV and ST6Gal I, and GP220 cells (366) expressing FUT1, FUT2, FUT3, ST3Gal III, ST3Gal IV and ST6Gal I, were cultured in DMEM supplemented with 10% FCS and maintained at 37ºC in 5%CO_2 atmosphere. Cells were routinely checked for *Mycoplasma* contamination.

For cytokine treatments semi-confluent cells were rinsed in phosphate-buffered saline (PBS) and incubated for 20 hours with 20 ng/ml and 40 ng/ml TNF-α, IL-1β or IL-6 diluted in DMEM.

Semi-quantitative RT-PCR

Total RNA extraction was carried out from control and cytokine-stimulated cells using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis. MO). After rDNAse I (Ambion, Austin, TX) treatment, fucosyltransferases FUT1, FUT2 and FUT3 and sialyltransferases ST3Gal III, ST3Gal IV and ST6Gal I were amplified by primers and conditions previously described (28,367,368). As a control

for mRNA levels β-actin cDNA was also amplified (369). The size of the products was: FUT1 198 bp, FUT2 582 bp, FUT3 265 bp, ST3GalIII 300 bp, ST3Gal IV 458 bp, ST6Gal I 371 bp and β-actin 349 bp.

Flow cytometry

Cultured untreated and TNF-α, IL-1β or IL-6-treated cells were trypsinized and counted. A total of $5x10^5$ viable cells were incubated for 30 minutes at 4ºC with primary antibodies diluted in PBS containing 1% bovine serum albumine (BSA). Cells were rinsed in PBS-1%BSA and incubated with the secondary antibody Alexa Fluor 488 (Invitrogen) for 30 minutes at 4ºC. After washing, fluorescent analysis was performed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). As positive and negative controls M8 and B12 antibodies were respectively used.

Immunohistochemistry

Stomach cancer tissue samples (n=49) were obtained from the paraffin-embedded tissue bank of the Servei d'Anatomia Patològica at the Hospital del Mar (Barcelona, Spain). The study was approved by the Ethics Committee of our Institution (IMIM-Hospital del Mar). Tissue samples were processed in 4 μm sections and haematoxylin-eosin stained to be used for diagnosis and classification by the inflammatory component.

Monoclonal antibodies T-218, T-174, 77/180, 57/27, Cslex-1, M8 and B12 were used as a supernatant at a 1/2 dilution in PBS-BSA 1%. MoAb 19-0LE was used as ascites diluted at a 1/1000. Indirect immunoperoxidase technique was performed as described (28) and sections were developed using DAB.

Statistical analysis

To statistically compare the differences observed in the expression of Lewis antigens due to cytokine treatment the Student's t-test was used. The association between the expression of the different Lewis antigens and the inflammatory score of the samples was evaluated using Mann-Whitney U-test for continous variables and Pearson's Chi-square test for categorical variables. Statistical analysis was performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was established when $p \leq 0.05$.

Results

The expression of fucosyl- and sialyltransferases is not modulated by TNF-α, IL-1β or IL-6

To analyse the effect of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 on the expression levels of FUT1, FUT2, FUT3, ST3Gal III, ST3Gal IV and ST6Gal I mRNA in gastric cancer cell lines, MKN45 and GP220 cells were incubated with different doses (20 ng/ml and 40 ng/ml) of the three cytokines for 20 hours. No significant differences in mRNA levels of the fucosyl- and sialyltransferases studied were observed after 20 ng/ml (data not shown) or 40 ng/ml TNF-α, IL-1β and IL-6 treatment by semi-quantitative RT-PCR (Figure 1).

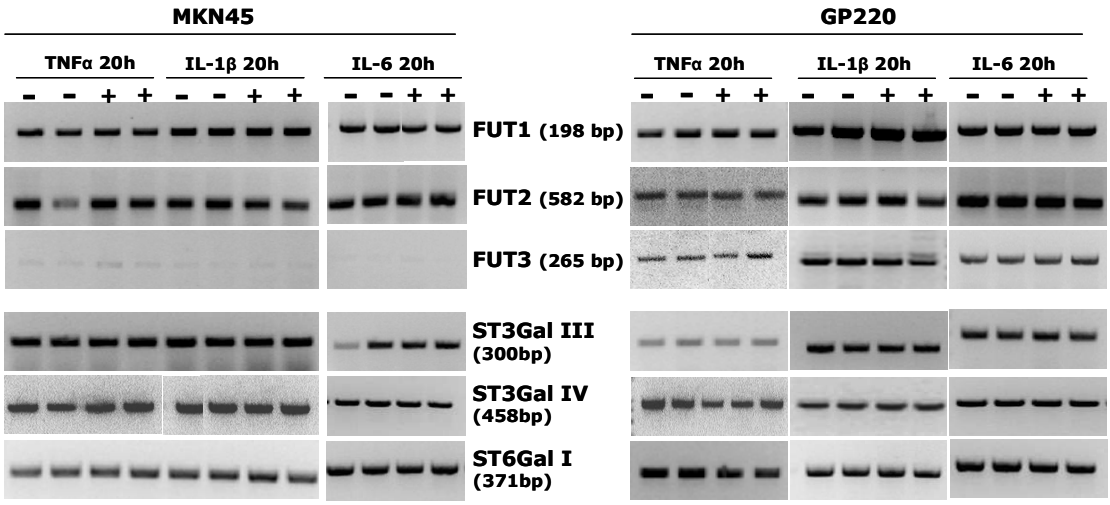


Figure 1. Expression of fucosyl- and sialyltransferases in MKN45 and GP220 cells after TNF-α, IL-1β and IL-6 treatment.

Changes in the expression of Lewis antigens induced by TNF-α, IL-1β and IL-6

The expression of Lewis antigens in the surface of MKN45 and GP220 cells after TNF-α, IL-1β or IL-6 treatment (20 ng/ml and 40 ng/ml) was evaluated by flow cytometry. The basal levels of Lewis antigens observed in MKN45 cells were lower than in GP220 cells, probably due to the fact that MKN45 cells lack FUT3.

Regarding MKN45 cells, 20 hours TNF-α treatment induced a decrease in the levels of s-Lewis x: 51.7% of positive untreated cells vs 45.3% and 37.3% of positive cells (Table 2A) after 20 ng/ml and 40 ng/ml TNF-α, respectively. The differences observed between untreated and 40 ng/ml TNF-α-stimulated cells were statistically significant ($p \leq 0.05$). IL-1β treatment induced an increase in type 2

Lewis antigens. 9.9% of untreated cells expressed the precursor structure H type 2 while 20.8% of cells were positive after 20 hours and 40 ng/ml IL-1β stimulation; however these differences were not statistically significant. The expression of Lewis y changed from 4.3% in untreated cells to 8.2% and 15.0% after 20 hours 20 ng/ml and 40 ng/ml IL-1β treatments, respectively. The difference between untreated and 20 ng/ml IL-1β-treated cells was marginally significant (p=0.059), whereas the difference between untreated and 40 ng/ml IL-1β-stimulated cells was statistically significant (p≤0.05). The percentage of s-Lewis x positive cells also increased after IL-1β treatment, from 19.2% (untreated cells) to 25.3% (20 ng/ml IL-1β) and 36.0% (40 ng/ml IL-1β) of positive cells. These differences were statistically significant (p≤0.05). When MKN45 cells were treated with IL-6 decreased levels of H type 2, s-Lewis a, and s-Tn antigens were found. In the case of H type 2 antigen 23.8% of untreated MKN45 cells were positive, while 19.6% and 8.8% of positive cells were detected after 20 ng/ml and 40 ng/ml IL-6 treatments, respectively. s-Lewis a expression was also reduced from 95.1% of positive untreated cells to 86.4% and 55.2% of positive cells after the 20 ng/ml and 40 ng/ml IL-6 treatments. Finally, decreased levels of positive cells for s-Tn were also found after IL-6 treatment: 14.2% of positive untreated cells, 7.6% of positive 20 ng/ml IL-6-treated cells and 4.9% of positive 40 ng/ml IL-6-treated cells. All the differences observed after IL-6 treatment were statistically significant (p≤0.05). A summary of the results obtained for MKN45 cells is shown in Table 2 and Figure 2.

	TNF-α 20 h			IL-1β 20 h			IL-6 20 h		
	-	**20ng/ml**	**40ng/ml**	**-**	**20ng/ml**	**40ng/ml**	**-**	**20ng/ml**	**40ng/ml**
Lewis a	7.0%	7.8%	8.0%	5.4%	8.5%	5.2%	5.7%	6.4%	3.0%
Lewis b	99.7 %	99.6%	99.5%	97.4%	99.0%	98.5%	98.7%	98.5%	97.0%
H type 2	20.9 %	20.1%	23.9%	9.9%	14.7%	20.8%	23.8%	19.6%	8.8%
Lewis y	6.1%	4.3%	4.6%	4.3%	8.2%	15.0%	4.6%	7.9%	1.4%
s-Lewis a	64.1 %	71.9%	66.6%	65.6%	66.2%	78.5%	95.1%	86.4%	55.2%
s-Lewis x	51.7 %	45.3%	37.3%	19.2%	25.3%	36.0%	46.2%	42.6%	36.2%
s-Tn	11.1 %	10.1%	13.8%	-----*	-------	-------	14.2%	7.6%	4.9%

Table 2. Expression of Lewis antigens in MKN45 cells. *s-Tn was not evaluated after IL-1β treatment. Values statistically significant are presented in red.

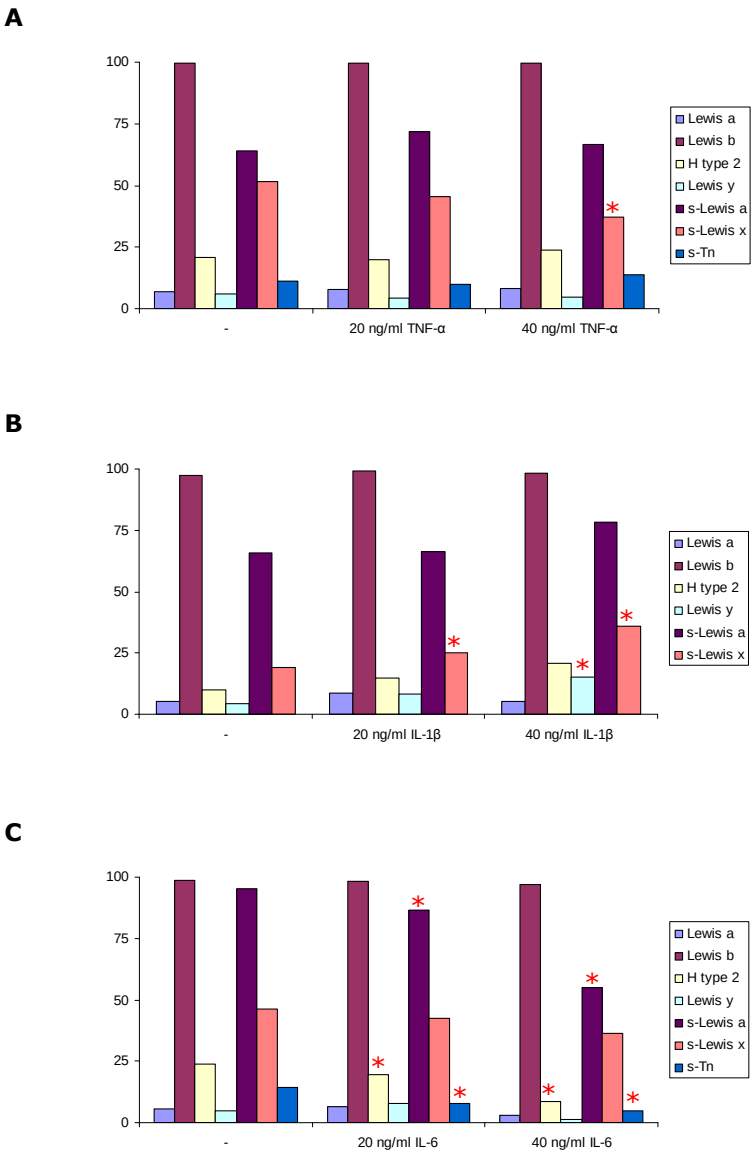


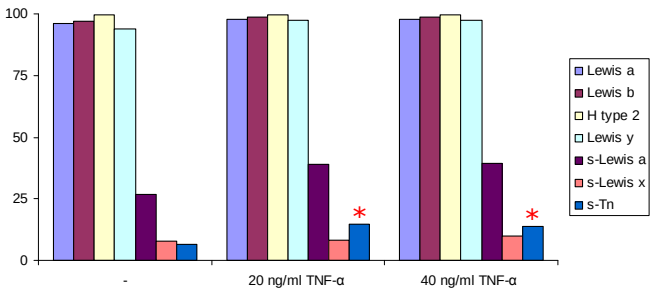
Figure 2. Lewis antigens expression in MKN45 cells after TNF-α **(A)**, IL-1β **(B)** and IL-6 **(C)** treatments. *Significant differences observed when compared with untreated cells.

GP220 cells were treated with TNF-α and IL-1β (20 ng/ml and 40 ng/ml) for 20 hours. After TNF-α stimulation an increase in the expression of s-Tn was detected. 6.5% of GP220 untreated cells expressed s-Tn while 14.8% and 13.7% of positive cells were positive after 20 ng/ml and 40 ng/ml TNF-α treatment, respectively. These differences were statistically significant ($p \leq 0.05$). No other relevant differences in the expression of Lewis antigens after cytokine treatment were observed. A summary of the results obtained for GP220 cells are presented in Table 3 and Figure 3.

	TNF-α 20 h			IL-1β 20 h		
	-	20ng/ml	40ng/ml	-	20ng/ml	40ng/ml
Lewis a	95.9%	97.9%	97.7%	94.4%	95.9%	95.9%
Lewis b	97.0%	98.9%	98.7%	97.1%	96.9%	97.1%
H type 2	99.4%	99.5%	99.6%	99.7%	99.7%	99.7%
Lewis y	94.0%	97.6%	97.3%	98.3%	99.1%	99.5%
s-Lewis a	27.0%	39.1%	39.2%	20.8%	36.4%	29.3%
s-Lewis x	7.9%	8.3%	10.0%	-----*	-------	-------
s-Tn	6.5%	14.8%	13.7%	-----*	-------	-------

Table 3. Expression of Lewis antigens in GP220 cells. *s-Lewis x and s-Tn were not evaluated after IL-1β treatment. Values statistically significant are presented in red.

A



B

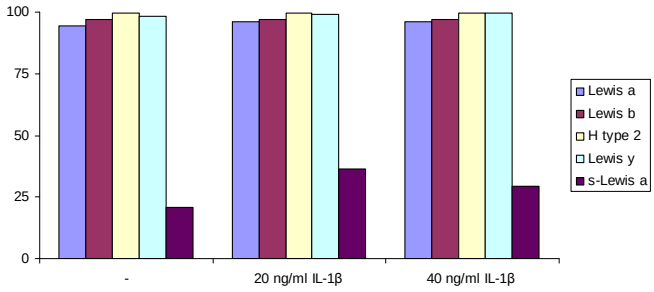


Figure 3. Lewis antigens expression in GP220 cells after TNF-α **(A)** and IL-1β **(B)** treatments. *Significant differences observed when compared with untreated cells.

Classification of intestinal-type gastric tumours according to their inflammatory score

To study if inflammation could induce changes in the expression of Lewis antigens *in vivo*, intestinal-type tumour samples were classified according to the predominant cell type present in their inflammatory component. Two groups were established: LP Group, with ≥50% of lymphoplasmocytic inflammatory infiltrate (n=30) corresponding to chronic inflammation; and PMN Group with ≥50% of polymorphonuclear cells (n=19), corresponding to acute inflammation (Figure 4).

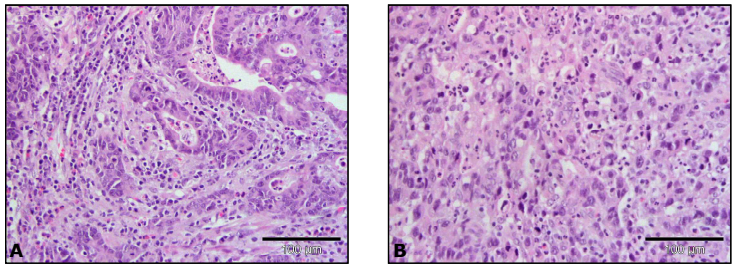


Figure 4. A) Intestinal-type gastric tumour with predominant lymphoplasmocytic inflammatory infiltrate, corresponding to chronic inflammation. **B)** Intestinal-type gastric tumour with predominant polymorphonuclear inflammatory infiltrate, representing acute inflammation.

Expression pattern of Lewis antigens and its association with inflammation and clinico-pathological characteristics of the tumours

The expression of Lewis antigens in intestinal-type gastric tumours and its possible association with the inflammatory score was analysed. Differences in the expression of the type 2 Lewis antigens (Lewis y and s-Lewis x) were observed between the two groups of tumours (Figure 5, Table 4 and Figure 6). In the LP Group 63.5% of cells and 27/30 cases expressed Lewis y antigen. In the PMN Group, all the tumours expressed Lewis y, but at lower levels (51.3% of positive cells). When statistically compared the difference observed in the expression of Lewis y was marginally significant (p=0.059). Also, the difference in the expression of s-Lewis x between the two groups of tumours (39.9% of positive cells in the LP Group vs 23.4% in the PMN Group) was statistically marginally significant (p=0.074). These results suggest that chronic inflammation may be inducing the activation of specific glycosyltransferases involved in the synthesis of type 2 Lewis antigens.

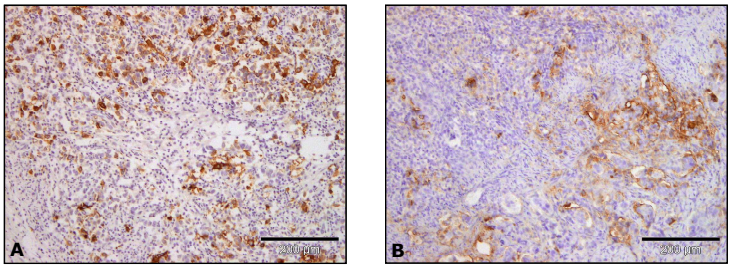


Figure 5. Expression pattern of s-Lewis x antigen in an intestinal-type gastric tumour with lymphoplasmocytic inflitrate **(A)** and with polymorphonuclear infiltrate **(B)**.

Inflammatory score	Lymphoplasmocytic infiltrate n=30 ≥50% Lymphocytes/Plasmatic cells	Polimorphonuclear infiltrate n=19 ≥50% Polymorphonuclear cells
Lewis a	48.0%* (24/30)**	35.8% (12/19)
Lewis b	49.3% (28/30)	51.6% (16/19)
H type 2	63.1% (27/30)	54.9% (19/19)
Lewis y	63.5% (27/30)	51.3% (19/19)
s-Lewis a	39.8% (22/30)	34.9% (14/19)
s-Lewis x	39.9% (26/30)	23.4% (12/19)

Table 4. Expression of Lewis antigens in the two groups of tumours. *Percentage of positive cells. **Number of cases.

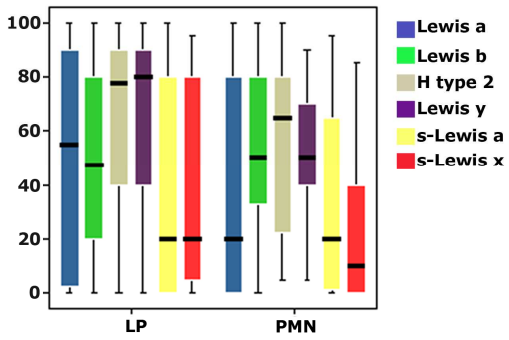


Figure 6. Expression levels of Lewis antigens in tumours of the LP and PMN Groups.

The association between the expression of Lewis antigens and different clinico-pathological characteristics of the tumours was also evaluated (Table 5). No significant differences were observed in the expression of Lewis antigens that could be explained by the characteristics of the tumours.

Inflammatory score	Lymphoplasmocytic infiltrate n=30 ≥50% Lymphocytes/Plasmatic cells	Polimorphonuclear infiltrate n=19 ≥50% Polymorphonuclear cells
Age	75.3%±9.2	75.2%±7.2
Sex		
M	20* (66.7%)**	10 (52.6%)
F	10 (33.3%)	9 (47.4%)
Histological grade		
I	3 (10.0%)	0 (0.0%)
II	22 (73.3%)	11 (57.9%)
III	3 (10.0%)	4 (21.1%)
IV	2 (6.7%)	4 (21.1%)
pT		
pT1	2 (6.7%)	6 (31.6%)
pT2	12 (40.0%)	6 (31.6%)
pT3	13 (43.3%)	5 (26.3%)
pT4	3 (10.0%)	2 (10.5%)
pN		
pN0	13 (43.3%)	10 (52.6%)
pN1	12 (40%)	6 (31.6%)
pN2	4 (13.3%)	1 (5.3%)
pN3	1 (3.3%)	2 (10.5%)
Vascular invasion	14 (46.7%)	8 (42.1%)
Perineural invasion	6 (20.0%)	11 (57.9%)
H. pylori	14 (46.7%)	11 (57.9%)
Intestinal Metaplasia	25 (83.3%)	18 (94.7%)

Table 5. Clinico-pathological characteristics of the two groups of patients classified by the inflammatory cells infiltrating the tumour. *Number of cases. **Percentage of cases.

Association between inflammation and clinico-pathological characteristics of gastric tumours

We also analysed the relation between the type of inflammatory infiltrate present in the tumour and its clinoco-pathological characteristics (Table 5). We observed that in the LP Group the 83.3% (25/30) of the tumours were well differentiated (Grade I and II), whereas only the 16.7% (5/30) were moderate or poorly differentiated (Grade III and IV). In contrast, in the PMN Group the two

grades of differentiation were equally represented (57.9% (11/19) well differentiated vs 42.2% (8/19) poor differentiated). When statistically analysed the differences observed were statistically marginally significant ($p=0.052$), suggesting an association between inflammation and the histological differentiation grade of the tumours. In addition, tumours of the PMN Group presented more perineural invasion (57.9% of the cases (11/19)) when compared to tumours of the LP Group (20.0%, 6/30 cases), and this difference was statistically significant ($p\leq0.05$). No other relevant differences in the clinico-pathological characteristics of the tumours were observed.

Mejías-Luque R, Peiró S, Vincent A, Van Seuningen I, de Bolós C. *IL-6 induces MUC4 expression through gp130/STAT3 pathway in gastric cancer cell lines.*
Biochim Biophys Acta. 2008 Oct;1783(10):1728-36. Epub 2008 Jun 4.

GASTRIC CANCER

Activation of MUC2 mediated by NF-κB is independent of CDX2 expression in gastric cancer cells

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Background and aims: Pro-inflammatory cytokines released in response to *Helicobacter pylori* infection, can regulate genes associated to gastric carcinogenesis. The aim of the present study was to analyse the effect of TNF-a / NF-κB signalling pathway on the expression of the intestinal mucin MUC2 and the intestinal transcription factor CDX2, which are ectopically detected in gastric tumours as well as at earlier stages as intestinal metaplasia.

Methods: To study the regulatory effects of TNF-a on MUC2 and CDX2 expression, gastric cancer cell lines were subjected to different TNF-a treatments. The expression of MUC2 and CDX2 was evaluated by quantitative RT-PCR, immunocytochemistry and western blot. Luciferase reporter assays were performed to analyse MUC2 and CDX2 promoter activity after TNF-a stimulation. The levels of MUC2, CDX2 and p65 were also assessed in intestinal metaplasia and gastric tumour samples by immunohistochemistry, and co-expression was evaluated by double labelling immunofluorescence.

Results: TNF-a induced MUC2 expression in gastric cancer cell lines whereas no effects were observed in CDX2 levels. Three of the four selected gastric cancer cell lines presented high mRNA levels of CDX2, but no protein was detected. Decreased levels of MUC2 were observed after blocking the TNF-a / NF-κB pathway at the level of IκB phosphorylation, and MUC2 but not CDX2 promoter activity was increased after TNF-a treatment. In intestinal metaplasia and tumour samples MUC2 was associated both to CDX2 and p65 expression.

Conclusions: MUC2 can be activated by NF-κB and expressed independently of CDX2 in gastric cancer cell lines, in intestinal metaplasia and in gastric tumours.

Chronic inflammation has been linked to tumorigenesis particularly in the digestive tract, where the risk for carcinogenesis increases in the presence of chronic inflammatory conditions as the gastritis caused by *Helicobacter pylori*[1] (*H. pylori*). *H. pylori* infection induces the release of pro-inflammatory cytokines such as IL-6, TNF-α or IL-1β[2,3], which may regulate the expression of genes activated during the gastric neoplastic transformation. In the intestinal transdifferentiation process, preceding the development of an intestinal-type adenocarcinoma, the ectopic expression of intestinal genes such as the secreted mucin MUC2, the membrane-bound mucin MUC4, and CDX1 and CDX2 transcription factors has been detected.[4,5,6,7] We have recently reported the regulation of MUC4 by IL-6 through gp130/STAT3 pathway in gastric cancer cell lines[8], whereas no activation of MUC2 expression was observed. MUC2 has been described to be regulated by the pro-inflammatory cytokines IL-1β and TNF-α in the respiratory tract and in colon cancer cell lines.[9,10,11] TNF-α can modulate the expression of target genes through the NF-κB signalling pathway, which has been shown to be constitutively activated in gastric tumours.[12] The NF-κB family comprises five different members NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. Rel proteins form homo- or heterodimers with p50 and p52, and the most common dimer is the p65/p50 heterodimer.[13] After TNF-α binding TRAF2 is recruited to the TNF receptor TNFR1. TRAF/RIP complexes trigger IKK activation leading to phosphorylation, ubiquitination and degradation of IκB proteins that are associated with inactivated NF-κB. NF-κB free of IκB translocates to the nucleus, where it activates the transcription of target genes. Transcriptionally, MUC2 can be regulated by NF-κB[14] among other transcription factors including Sp1 family of transcription factors[15], p53[16], GATA-4[17] and Foxa1 and Foxa2.[18] However, in gastric cancer cell lines, MUC2 expression has been reported to be regulated by the intestine-specific transcription factor CDX2.[19] CDX2 is a homeobox gene that has been demonstrated to play an important role in the development of the small and large intestine in mammals and in the differentiation of intestinal epithelial cells.[20] The ectopic expression of CDX2 in the gastric mucosa of transgenic mice is enough to activate the intestinal differentiation program[21] and it has been ectopically detected in intestinal metaplasia, dysplasia and intestinal-type adenocarcinomas.[4,22,5]

In the present study we demonstrate that TNF-α induces the expression of MUC2 through NF-κB pathway and it occurs independently of CDX2 expression. In addition, the expression of MUC2, CDX2 and the NF-κB subunit p65 has been also examined in tissue samples from intestinal metaplasia and intestinal-type gastric tumours and co-expression of p65 and MUC2 has been detected.

Abbreviations: *H. pylori*, *Helicobacter pylori*; TNF-a, tumour necrosis factor a; CDX2, caudal-related homeobox protein 2, NF-κB, Nuclear factor-κB.

MATERIALS AND METHODS

Reagents and antibodies

TNF-α was purchased from PreproTech EC (London) and Dubelcco's modified Eagle's medium (DMEM) from Invitrogen (Carlsbad, CA). The specific inhibitor of IκB phosphorylation, panepoxydone, was obtained from Alexis Biochemicals (San Diego, CA). Anti-IkBα and anti-p-IkBα (Ser32/36) antibodies were purchased from Cell Signaling (Danvers, MA), anti-CDX2 was obtained from Biogenex (San Ramon, CA) and anti-p65 from Santa Cruz Biotechnology (Santa Cruz, CA). For MUC2 detection monoclonal antibody LDQ10 recognizing the tandem repeat sequence[23] was used. Anti-mouse or anti-rabbit HRP-conjugated antibodies and anti-rabbit EnVision-HRP were purchased from Dako Cytomation (Glostrup), and for fluorescent analysis anti-mouse Alexa Fluor 555 and 488 (Molecular Probes, Leiden) and anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, Inc., Cambridgeshire, UK) were used.

Cell culture and treatments

MKN45 and NUGC-4 human gastric cancer cell lines were obtained from ATCC. St2957, St23132 and St3051 were characterised in Dr. Peter Vollmers' laboratory[24], and GP220 cells were established in Dr Sobrinho-Simões' laboratory.[25] All cell lines were maintained at 37ºC in CO_2 atmosphere in 10% FCS supplemented DMEM. For TNF-α treatment semi confluent cells were rinsed in phosphate-buffered saline (PBS) and incubated for 10 minutes, 1, 5, 10 or 20 hours with 40 ng/ml, 70 ng/ml or 100 ng/ml of the cytokine diluted in DMEM. To prevent NF-κB activation, cells were incubated with 70 ng/ml or 100 ng/ml of TNF-α in combination with 5 µg/ml of the specific inhibitor of IκB phosphorylation, panepoxydone for 10 minutes, 10 or 20 hours.

Semi-quantitative RT-PCR

Total RNA extraction was carried out from control and TNF-α stimulated cells using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis. MO). After rDNAse I (Ambion, Austin, TX) treatment, MUC2 was amplified by primers 5'-CTT CGA CGG ACT CTA CTA CAG C–3' (sense) and 5'-CTT TGG TGT TGT TGC CAA AC–3' (antisense)[26], and CDX2 by primers 5'-GCC GAG CTA GCC GCC ACG C-3' (sense) and 5'-TGC AGG GAA GAC ACC GGA CTC AAG-3' (anti-sense). As a control for mRNA levels β-actin cDNA was also amplified.[27] Amplification conditions for MUC2 were: 94ºC 1', 58ºC 30" and 72ºC 30" for 35 cycles, and for CDX2 94ºC 15", 65ºC 30" and 68ºC 30" for 40 cycles The size of the products was 387 bp for MUC2, 218 for CDX2 and 349 bp for β-actin.

Quantitative RT-PCR

Quantitative determination of MUC2 and CDX2 mRNA levels was performed in triplicate using QuantiTect SYBR green reverse transcription-PCR (Qiagen GmbH, Germany). MUC2 and CDX2 were amplified by the same primers used for semi-quantitative RT-PCR. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA (GeneCards database, NCBI36:X) was analysed as an internal control by using oligonucleotides 5'-GGCCAGACTTTGTTGGATTTG-3' (sense) and 5'-TGCGCTCATCTTAGGCTTTGT-3' (antisense). RT-PCR and data collection were performed on the ABI Prism 7900HT system. All quantifications were normalized to the endogenous control (HPRT). At least three independent experiments were performed.

Cell lysates and Western Blot Analysis

Cytoplasmic cell extracts were obtained for MUC2 detection by lysing the cells in 50 mM Tris pH8, 62.5 mM EDTA and 1% Triton X-100 lysis buffer. western blot was performed on 2% SDS-agarose gels as previously reported [28]. LDQ10 antibody was incubated for 2 hours and bound secondary antibody was detected using the ECL Western Blotting Substrate (Pierce, Rockford, IL).

For CDX2 analysis nuclear extracts were obtained as previously described[8], and for IkBα and p-IkBα detection cellular pellets were solubilized in 2X SDS gel sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris-HCl pH 6.8, 10% glycerol, and bromophenol blue) and sonicated using 3 bursts of 10 seconds each. Lysates were boiled at 95ºC for 5 minutes and immediately cooled on ice. Protein extracts were electrophoresed on 10% SDS-polyacrylamide gels. Separated proteins were blotted onto nitrocellulose membranes (Protran, Dassel), blocked for 1 hour at RT, and incubated overnight with the specific primary antibodies following manufacturer's instructions. Bound secondary antibody was detected by ECL Western Blotting Substrate.

Immunocytochemistry, Immunohistochemistry and Double Labelling Immunofluorescence

Pellets from untreated and 100 ng/ml 20 hours TNF-α treated cells were fixed in formol for 48 hours, and after dehydrating were paraffin embedded.

Tissue samples of intestinal-type gastric adenocarcinoma (n=18) and intestinal metaplasia (n=17) from the same patient were obtained from the paraffin-embedded tissue bank of the Servei d'Anatomia Patològica at the Hospital del Mar (Barcelona, Spain) and used for immunohistochemistry. The study was approved by the Ethics Committee of the institution (IMIM-Hospital del Mar). LDQ10 antibody, recognizing MUC2, was used as ascites diluted at 1/500 in PBS-1%BSA, and indirect immunoperoxidase technique was performed as described.[6] For CDX2 and p65 detection slides were boiled for 2 or 15 minutes, respectively, in 10mM sodium citrate pH 6. For CDX2 staining, sections were then incubated in 3% hydrogen peroxide in methanol for 10 minutes. After washing, slides were blocked using 5% horse serum diluted in PBS-0.1%Tween or PBS-1% BSA for 1 hour. Undiluted CDX2 antibody and p65 diluted at 1/2000 in PBS-1% BSA were incubated o/n at 4ºC. Samples were rinsed and incubated with anti-mouse HRP-conjugated antibody for 1 hour (for CDX2) or anti-rabbit EnVision-HRP for 30 minutes. Sections were developed using DAB (Dako, Carpinteria, CA).

In selected cases (n=4) immunofluorescent double labelling was performed. Briefly, after blocking the samples in 5% horse serum - 0.3% triton, they were incubated o/n at 4ºC with anti-p65 or anti-CDX2 antibodies. For p65/MUC2 detection, slides were successively incubated with anti-rabbit Cy3, LDQ10 antibody and anti-mouse Alexa 488. For CDX2/MUC2 detection samples were incubated with LDQ-biotin conjugated antibody and streptavidin Alexa 488. Following antibody incubation, samples were rinsed and mounted using Fluoromount reagent.

Luciferase Reporter Assays

pGL3-MUC2 promoter constructs 1852 and 1985, covering the -2627/-1[29] and the -371/+27[19] regions of MUC2 promoter, respectively, and pGL3-CDX2 871 promoter construct covering the -871/-1[30] region of CDX2 promoter, were used for luciferase reporter assays. Cells were cotransfected with 200 ng and 800 ng of the pGL3 construct of interest and 1 ng of simian virus 40-Renilla luciferase plasmid as the control for transfection efficiency. 48 hours post-transfection cells were incubated with 100 ng/ml of TNF-α for 1 hour. The expression of Firefly and Renilla luciferases was analysed according to manufacturer's instructions.

Statistical analysis

For statistical analysis the Student's t-test was used. Statistical significance was established when $p \leq 0.05$.

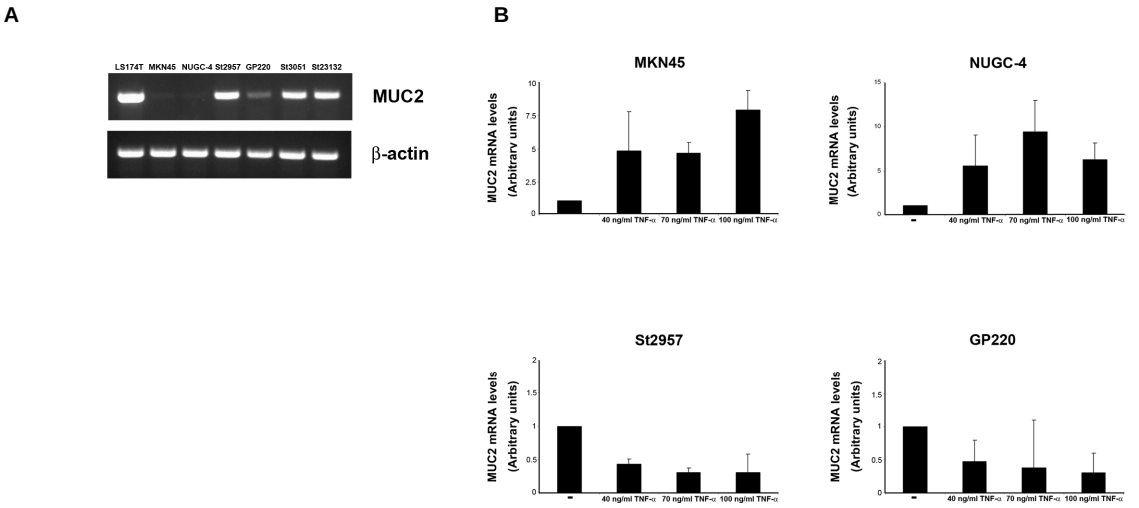

Figure 1 TNF-α induces the expression of MUC2 mRNA in MKN45 and NUGC-4 gastric cancer cell lines. A) MUC2 mRNA expression in gastric cancer cell lines. LS174T colon cancer cell line was used as a positive control. β-actin was used as a loading control. B) Quantitative analysis of MUC2 mRNA levels after 20 hours and 40 ng/ml, 70 ng/ml and 100 ng/ml TNF-α treatment. Results are presented as the average ± S.D. (error bars) from three independent experiments.

RESULTS

TNF-α induces MUC2 expression

MUC2 mRNA levels were analysed in six gastric cancer cell lines, including MKN45, NUGC-4, St2957, GP220, St3051 and St23132 (Figure 1A). MKN45 and NUGC-4 cells that do not express MUC2, and GP220 and St2957 expressing MUC2 mRNA at different levels, were selected to study the effect of TNF-α on MUC2 expression.

After 20 hours of 40, 70 and 100 ng/ml TNF-α treatment MUC2 transcription was induced in MKN45 and NUGC-4 cells, whereas no differences were detected in GP220 and St2957 cells (Figure 1B). The levels of the intestinal mucin MUC4 were also analysed after TNF-α stimulation, but no induction of its expression was observed (data not shown).

Protein levels of MUC2 were assessed in untreated and TNF-α- stimulated cells (100 ng/ml) by immunocytochemistry. No MUC2 apomucin was detected in untreated MKN45 and NUGC-4 cells. After 20 hours TNF-α treatment positive MKN45 and NUGC-4 cells were observed (Figure 2A), whereas no differences in MUC2 positive cells were observed after treating GP220 and St2957 cells.

The levels of MUC2 apomucin were also analysed by western blot in MKN45 cells. After 20 hours TNF-α induced the expression of MUC2 (Figure 2B).

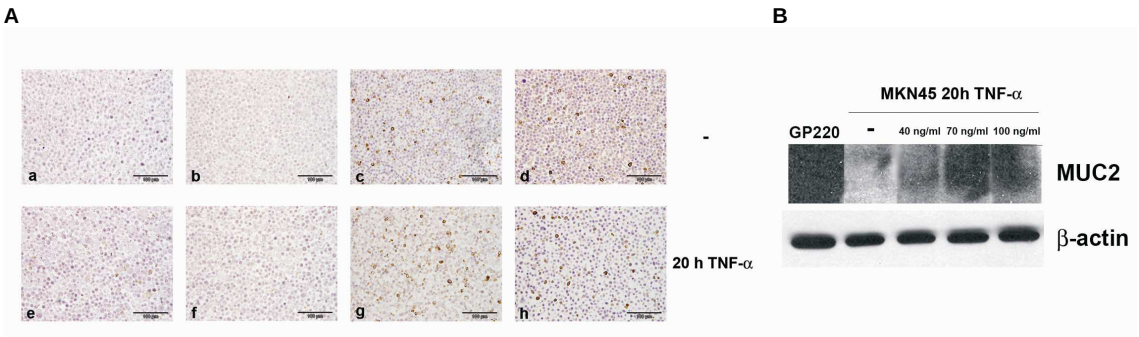

Figure 2 MUC2 protein expression is induced after TNF-α treatment. A) MUC2 apomucin expression detected by immunocytochemistry in untreated (a-d) and in 20 hours and 100 ng/ml TNF-α -treated (e-f) gastric cancer cells. MKN45 (a) and NUGC-4 (b) untreated cells do not express MUC2, whereas GP220 (c) and St2957 (d) express MUC2 at different levels. The expression of MUC2 was induced after TNF-α stimulation in MKN45 (e) and in NUGC-4 (f) cells. No differences in the levels of MUC2 expression were detected in GP220 (g) and St2957 (h) TNF-α-treated cells. B) MUC2 protein expression in MKN45 untreated and 20 hours TNF-α-stimulated (40 ng/ml, 70 ng/ml and 100 ng/ml) cells detected by western blot. GP220 cells were used as a positive control.

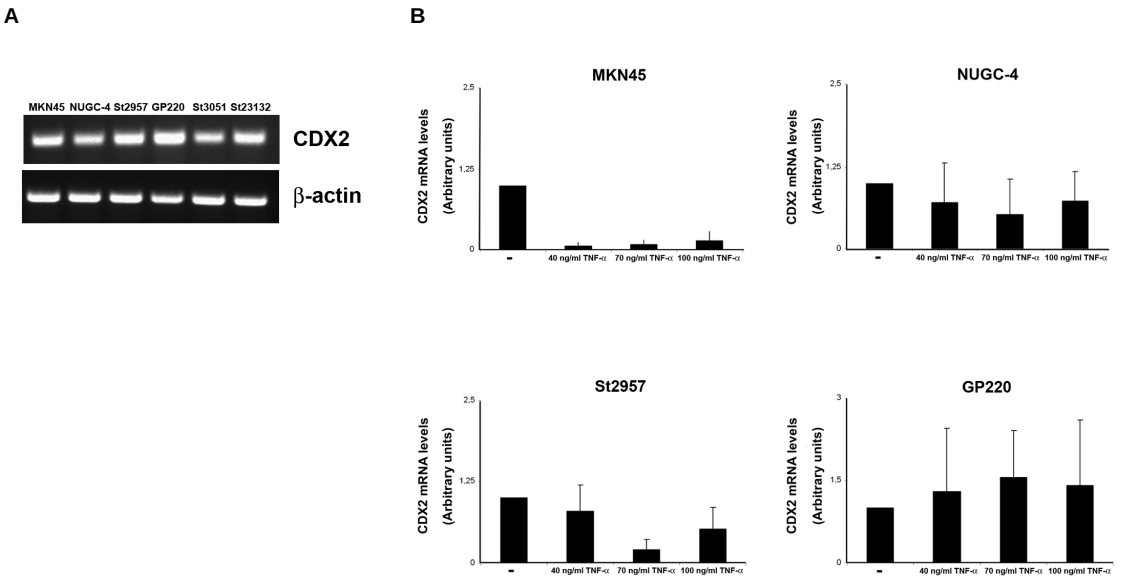

Figure 3 CDX2 mRNA expression in untreated and TNF-α treated cells. A) CDX2 mRNA expression in gastric cancer cells. β-actin was used as a loading control. B) Quantitative analysis of CDX2 mRNA levels after 20 hours and 40 ng/ml, 70 ng/ml and 100 ng/ml TNF-α treatment. Results are presented as the average ± S.D. (error bars) from four independent experiments.

CDX2 expression is not modulated by TNF-α

CDX2 mRNA was assessed in the same six gastric cancer cell lines (Figure 3A) and it was detected in all of them. To determine the effect of TNF-α in CDX2 expression, cells were treated with different concentrations of the cytokine for 20 hours and the levels of CDX2 mRNA were analysed by quantitative RT-PCR. CDX2 expression was detected in MKN45, NUGC-4, St2957 and GP220 cells and no changes were observed after treating the cells with different concentrations of TNF-α (40 ng/ml, 70 ng/ml and 100 ng/ml) for 20 hours (Figure 3B).

Surprisingly, when the levels of CDX2 protein were analysed by immunocytochemistry no positive cells were observed in MKN45 (data not shown), NUGC-4 (data not shown), and St2957 cells, and no changes were detected after 20 hours and 100 ng/ml TNF-α treatment (Figure 4A). In GP220 cells the number of CDX2 positive cells did not change. The same results were obtained by western blot. No protein was detected in MKN45, NUGC-4 and St2957 untreated and in 10 minutes (data not shown) and 20 hours TNF-α-treated cells (Figure 4B). CDX2 was only detected in GP220 cells and no changes in the levels of expression were observed after being TNF-α-stimulated.

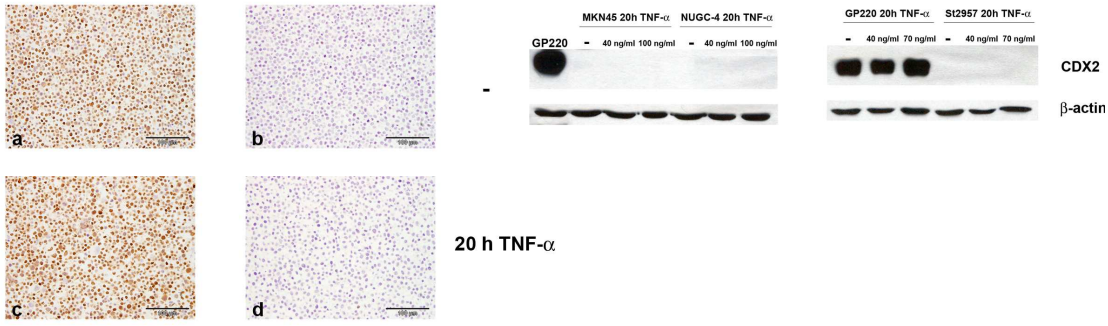

Figure 4 CDX2 protein expression in gastric cancer cell lines. A) CDX2 protein expression detected by immunohistochemistry. No changes in CDX2 levels were detected in GP220 cells (a) after 20 hours and 100 ng/ml TNF-α treatment (c). In St2957 cells that do not express CDX2 (b) no induction of its expression was observed after the treatment (d). B) The levels of CDX2 protein (40 kDa) were analysed by western blot. No CDX2 protein was detected in MKN45, NUGC-4 and St2957 cells even when they were treated with different concentrations of TNF-α (40 ng/ml and 100 ng/ml) for 20 hours. GP220 expressed CDX2 and no differences in its levels of expression were observed after the treatments. β-actin (45 kDa) was used as a loading control.

TNF-α induces MUC2 expression through NF-κB pathway

To analyse the activation of the NF-κB signalling pathway after TNF-α treatment, western blot against p-IκBα and IκBα was performed. After 10 minutes of TNF-α stimulation, phosphorylation of Iκα was observed in MKN45 and NUGC-4 cells. As expected, a decrease in IκBα protein levels was detected after TNF-α treatment (Figure 5A). Levels of nuclear p65 were also assessed by immunocytochemistry in MKN45 and NUGC-4 untreated and TNF-α treated cells. Only nuclear staining was observed in TNF-α stimulated cells (data not shown).

To determine the direct involvement of the NF-κB signalling pathway in the activation of MUC2 transcription, MKN45 cells were treated with the specific inhibitor of IκBα phosphorylation panepoxydone. MUC2 mRNA levels were analysed after 10 and 20 hours of TNF-α or TNF-α / panepoxydone treatment. Decreased levels of MUC2 after TNF-α stimulation were observed in the cells incubated with panepoxydone (Figures 5B). IκBα phosphorylation was also analysed by western blot, and as expected, the cells treated with panepoxydone presented lower levels of phosphorylated IκBα (Figure 5C).

Induction of MUC2 transcription by TNF-α is independent of CDX2

To demonstrate that TNF-α activates MUC2 but is not able to induce CDX2 transactivation, luciferase reporter assays were carried out. MKN45 cells were transfected with MUC2 wild type promoter (MUC2 wt Pr) or with a MUC2 mutant promoter containing two CDX2 but no NF-κB binding site (MUC2 mut Pr). Increased promoter activity was observed in cells transfected with the MUC2 wild type promoter and treated with 100 ng/ml of TNF-α for 1 hour (p=0.001) (Figure 6A). In contrast, no effect on MUC2 promoter activity was detected in cells transfected with the MUC2 promoter construct lacking the NF-κB binding site (p=0.365).

Cells were also transfected with CDX2 wild type promoter 871. No differences in the promoter activity were observed between non-stimulated cells and cells stimulated with 100 ng/ml of TNF-α for 1 hour (Figure 6B). The same results were obtained when cells were transfected with 800 ng of CDX2 construct and were treated with increasing amounts of TNF-α (70 ng/ml and 100 ng/ml) for 5, 10 and 20 hours (data not shown), indicating that TNF-α induces MUC2 promoter activity independently of CDX2 transactivation.

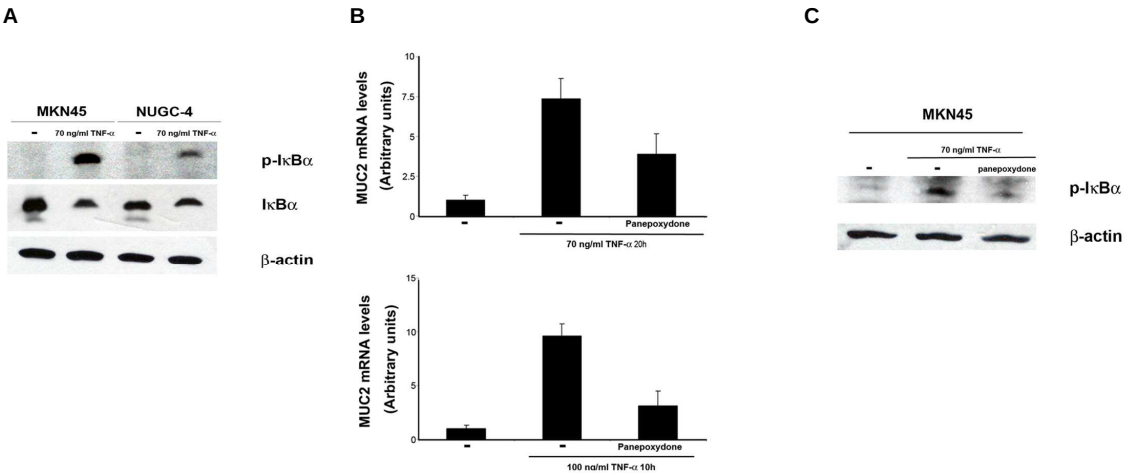

Figure 5 MUC2 expression is induced through the NF-κB signalling pathway after TNF-α stimulation. A) Levels of IκBα (39 kDa) and p-IκBα (40 kDa) in untreated and 70 ng/ml and 10 minutes TNF-α treated MKN45 and NUGC-4 cells analysed by western blot. Phosphorylation of IκBα was only observed in TNF-α treated cells. A decrease in the levels of total IκBα was detected. β-actin (45 kDa) was used as a positive control. B) MUC2 mRNA levels analysed by quantitative RT-PCR in MKN45 cells. MUC2 expression was induced after treating the cells with TNF-α (70 ng/ml for 20 hours or 100 ng/ml for 10 hours). Decreased levels of MUC2 were observed when cells were incubated with panepoxydone (5 µg/ml), a specific inhibitor of IκBα phosphorylation. C) Levels of IκBα phosphorylation after 10 minutes and 40 ng/ml TNF-α treatment. MKN45 cells incubated also with the specific inhibitor panepoxydone (5 µg/ml) presented lower levels of p-IκBα (40 kDa). β-actin (45 kDa) was used as a positive control.

MUC2, CDX2 and NF-κB expression in intestinal metaplasia and intestinal-type gastric tumours

The expression of MUC2, CDX2 and p65 was analysed in intestinal-type adenocarcinoma (n=18) and in intestinal metaplasia (n=17) from the same patient. For CDX2 and p65 only nuclear staining was considered as positive. In 17/17 (100%) intestinal metaplasia samples MUC2 expression was detected with a range from 70% to 100% of positive metaplastic cells. In these samples the levels of expression of CDX2 ranged from 20% to 100% of positive cells, and the levels of p65 ranged from 2.5% to 30% of positive cells. Almost half of the tissue samples, 8/17 cases (47.05%) were positive for MUC2, CDX2 and p65. Lower number of cases, 6/17 samples (35.29%), were positive for MUC2 and p65 (Figure 7A), whereas only 2/17 of the cases (11.76%) were positive for MUC2 and CDX2 but not for p65. Only in one case MUC2 expression was not accompanied either by CDX2 or p65.

Lower levels of MUC2 were detected in 14 of the 18 tumour samples (77.77%), ranging from 5% to 80% of positive cells. The expression levels of CDX2 ranged from 20 to 100% of positive cells, and the levels of p65 ranged from 2.5% to 35% of positive cells. 6/18 cases (33.33%) expressed MUC2, CDX2 and p65 (Figure 7), and 7/18 tumour samples (38.88%) expressed MUC2 and p65 but not CDX2. No cases expressing only MUC2 and CDX2 were detected. Again, in one sample the expression of MUC2 was not correlated to CDX2 or p65 expression.

Double labelling immunofluorescence was performed to analyse co-expression of CDX2 and p65 with MUC2 in gastric tumours (Figure 8) and in intestinal metaplasia (data not shown). MUC2/CDX2 and MUC2/p65 co-

expression was detected both in intestinal metaplasia and in gastric tumours, suggesting that MUC2 expression can be modulated by CDX2 as well as by the NF-κB signalling pathway in vivo.

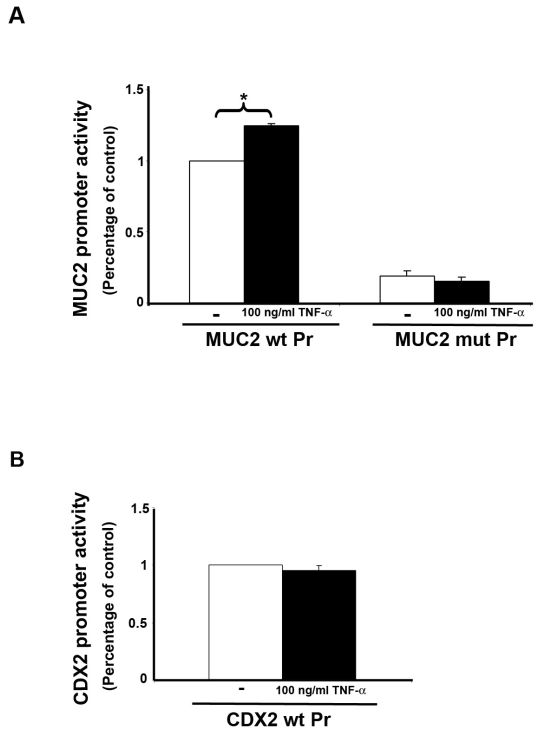

Figure 6 MUC2 wild type and MUC2 mutated (A) and CDX2 wild type **(B)** promoter activity was analysed in MKN45 untreated cells and TNF-α stimulated cells (100 ng/ml for 1 hour) by luciferase reporter assays. The value obtained in untreated cells transfected with the wild type promoter was referred as to 1. Means±S.D. of two independent experiments are shown.

DISCUSSION

Intestinal-type gastric tumours develop from a succession of histological changes starting with gastritis usually associated to *H. pylori* infection.[31] The pro-inflammatory cytokines released as a result of chronic inflammation of the gastric mucosa can initiate the neoplasic transformation and modulate the expression of intestinal genes activated during this process. Recently, we have described the regulatory effect of IL-6/gp130/STAT3 pathway on MUC4 expression in gastric cancer cell lines, whereas no effects on MUC2 expression were detected[8]. By contrast, MUC2 has been reported to be regulated by TNF-α in colon cancer cell lines.[9] Here, we have demonstrated the induction of MUC2 expression by TNF-α in gastric cancer cells and we have further analysed the possible pathways involved in its activation.

MUC2 has been detected associated with the expression of the intestine-specific transcription factor CDX2[4], in intestinal metaplasia and in gastric tumours. Moreover, the stable expression of CDX2 in gastric cells induced the activation of MUC2 transcription[19], indicating that CDX2 can regulate the expression of MUC2. To analyse if the induction of MUC2 by TNF-α in gastric cancer cells occurred through a TNF-α-mediated up-regulation of CDX2 expression, we first analysed the levels of CDX2 mRNA and protein in untreated and TNF-α-treated cells.

Interestingly, we observed that CDX2 mRNA levels in untreated cells did not correlate with the expression of CDX2 protein, and, in addition, MUC2 was detected independently of CDX2, as it occurs in the St2957 cells. Moreover, TNF-α treatment did not induce changes in CDX2 expression, suggesting that an alternative pathway might be involved in the activation of MUC2 expression by TNF-α.

TNF-α mediates its regulatory effects mainly through the NF-κB signalling pathway, which we have found to be activated in TNF-α-treated gastric cancer cell lines, and also in intestinal metaplasia and gastric tumour samples we have detected the activated form of the NF-κB subunit p65. In MUC2 promoter a NF-κB site, located at basepairs -1441 to -1452[32], has been described. Here, we have shown, by luciferase reporter assays, that TNF-α treatment increased MUC2 promoter activity. In contrast, no effect on CDX2 promoter was observed in TNF-α-stimulated cells. In addition, in intestinal metaplasia and gastric cancer tissue samples MUC2 and CDX2 expression were not always correlated, and MUC2 was also co-detected with activated p65. These results support the idea that MUC2 transcription can be modulated independently of CDX2 expression, and its activation occurs through the NF-κB signalling pathway.

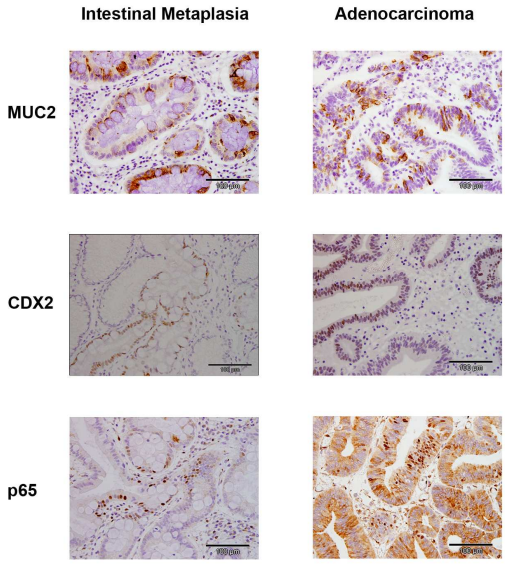

Figure 7 Expression of MUC2, CDX2 and p65 in intestinal metaplasia and intestinal-type adenocarcinoma samples.

The fact that the expression of CDX2 mRNA did not correlate with the expression of the protein, suggests that CDX2 may be regulated at the level of translation. The most common mechanisms of translational control affecting the 3'UTR are polyadenilation at the 3' end of the mRNA, and binding of microRNAs.[33]. No CPEs or HEXA sequences, involved in the polyadenilation process, are found in the 3' UTR of CDX2 gene (http://genome.imim.es/CPE/server.html)[33]. In contrast, different microRNAs (miRNAs) target sites are present in human CDX2 (miRanda web server, http://cbio.mskcc.org/mirnaviewer), indicating that translational regulation of CDX2 is likely to occur through this mechanism. However, further analysis is needed in order to elucidate the regulatory mechanisms involved in CDX2 translation in gastric cancer cells and their possible implication in the gastric carcinogenesis process.

In summary, our results demonstrate that MUC2 is activated by TNF-α through the NF-κB signalling pathway independently of CDX2 expression in gastric cancer cells. Moreover, the reported data regarding the expression

patterns of MUC2 and the transcription factors CDX2 and p65 in intestinal metaplasia and gastric tumour tissues support the hypothesis that different mechanisms might be involved in the activation of specific genes, as the intestinal mucin MUC2, being particularly important the signalling pathways associated with the inflammatory component present in tumours.

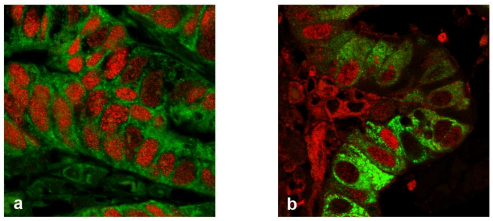

Figure 8 Immunofluorescent double labelling showing co-expression of MUC2 (green) and CDX2 (red) (a) and MUC2 (green) and p65 (red) (b) in gastric tumours.

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GASTRIC CANCER

Inflammation modulates the expression of the intestinal mucins MUC2 and MUC4 in gastric tumors

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Background and aims: The inflammatory response associated to the infection of gastric mucosa by *Helicobacter pylori* can act as initiator of the carcinogenetic process through the regulation of genes by inflammatory cytokines. TNF-a, IL-1β, and IL-6 induce the activation of signalling pathways that regulate the expression of genes, as MUC2 and MUC4 mucins, ectopically detected in gastric tumors.
Methods: Seventy-eight human gastric adenocarcinomas were classified, according to the infiltrating inflammatory cells, in two groups: tumors with lymphoplasmocytary cells (n=53) and tumors with polymorphonuclear cells (n=25), corresponding to chronic and acute inflammation. In these tumors, the expression pattern of the intestinal mucin genes, MUC2 and MUC4, and the transcription factor STAT3, implicated in the activation of genes through the IL-6 pathway, was analysed by immunohistochemistry and results related to specific clinico-pathological characteristics.
Results: Tumors with lymphoplasmocytic component were predominantly well differentiated (Grades I and II: 36/53) comparing to tumors with polymorphonuclear infiltrate (14/25 and 14/25, respectively). In addition, different expression levels of MUC2 (28.58% vs. 13.20% of positive cells) were detected in tumors with chronic and acute inflammation, whereas no differences were found in MUC4 (20.75% vs. 24.4%) and STAT3 (31.66% vs. 37.89%). Also, the expression of Muc2, Muc4 and Muc5AC was assessed in normal and tumoral gastric tissues from *gp130*$^{+/+}$ (n=3), *gp130*$^{Y757F/Y757F}$ (n=4), and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ (n=3) mice by quantitative RT-PCR and immunohistochemistry. In normal stomach only Muc5AC was found, and Muc4 was detected in the gastric tumors developed in the *gp130*$^{Y757F/Y757F}$, with hyperactivated STAT3 pathway.
Conclusions: Signalling pathways associated to the inflammatory response can modulate the expression of the intestinal mucin genes, MUC2 and MUC4 in human and mouse gastric tumors.

Several types of cancer have been associated to chronic infection and inflammation,[1] and numerous data linking inflammation and cancer have been recently reported. The inflammatory cells infiltrating the tumor prompt the presence of proinflammatory cytokines, such as IL-1β, TNF-α and IL-6, that can induce the activation of genes related to the neoplastic transformation. IL-1β and TNF-α can activate the signalling pathways mediated by the NF-κB transcription factor,[2] involved in tumour progression by the activation of anti-apoptotic [3] and angiogenesis related genes.[4] The IL-6 cytokine family activates genes that can regulate gastric homeostasis through the SHP2/Erk and STAT1/3 pathways, upon binding to gp130 receptor. To analyze the consequences of disrupting the balance between the two pathways, several mouse models have been developed. Transgenic mice with mutated gp130 receptor develop distal gastric tumors as a consequence of increased STAT3 activation.[5,6]

The infection of the stomach mucosa by *Helicobacter pylori (H. pylori)* can initiate the gastric carcinogenesis process by the associated inflammatory response. (Reviewed in [7]) In the normal mucosa, gastric mucins (MUC5AC, MUC6) are detected in a specific expression pattern,[8] that is lost in gastric adenocarcinomas and during the first stages of the neoplastic transformation in which intestinal mucins, MUC2 and MUC4, can be detected.[9,10,11] MUC4 is a membrane-bound mucin that has been involved in tumor progression potentiating cell proliferation, survival and invasion.[12] The activation of these specific intestinal mucin genes could be regulated by the inflammatory cytokines induced by the response associated to *H. pylori* colonisation of the gastric mucosa. *H. pylori* infection is characterised by the massive recruitment of polymorphonuclear and mononuclear cells, and, as a consequence, increased levels of proinflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-8 are found in the stomach mucosa.[13] Recently, we have demonstrated that IL-6 induces the expression of MUC4, but not MUC2, through the activation of the gp130/STAT3 signalling pathway in gastric cancer cell lines *in vitro*.[14]

The presence of specific inflammatory cell types characterizes the acute and chronic inflammation, and these different inflammatory cells determine specific patterns of cytokines expression. The predominant cells infiltrating the gastric mucosa in chronic inflammation are lymphocytes, plasma cells and macrophages; whereas in the acute or active inflammation the polymorphonuclear neutrophiles are the main infiltrating cells. In order to evaluate the implication of the inflammatory cells in the expression of intestinal markers associated to gastric cancer, we have classified a group (n=78) of intestinal gastric carcinomas according to their inflammatory score, chronic or acute, and we have analysed the expression of the intestinal mucin genes (MUC2 and MUC4) and STAT3 transcription factor. Results were related to specific clinico-pathological characteristics of the patients. To confirm the activation of the intestinal mucins *in vivo* through the gp130/STAT3 signalling pathway, the expression of Muc2, Muc4, and Muc5AC has been examined in normal and tumor stomach samples from *gp130*$^{+/+}$, and transgenic *gp130*$^{Y757F/Y757F}$ and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ mice.

Key words: Intestinal gastric cancer, inflammatory cells, intestinal mucins, IL-6 pathway, gp130/STAT3.

MATERIALS AND METHODS

Human tissue samples. Histological and clinico-pathological characteristics of the patients

Stomach cancer tissue samples (n=78) were obtained from the paraffin-embedded tissue bank of the Servei d'Anatomia Patològica at the Hospital del Mar (Barcelona, Spain). The study was approved by the Ethics Committee of the Institution (IMIM-Hospital del Mar). Paraffin-embedded tissues were processed in 4 µm sections and haematoxylin-eosin stained to be used for diagnosis and classification by the inflammatory component.

Mouse gastric tissues

Stomachs from 14 week old mice of *gp130*$^{+/+}$ (n=3), *gp130*$^{Y757F/Y757F}$ (n=4), and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ (n=3) mice [5,6] were harvested, fixed over night in 4% paraformaldehyde and paraffin-embedded. Stomachs from wild type mice were normal, the *gp130*$^{Y757F/Y757F}$ mice developed large gastric tumors, and the *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ mice presented an intermediate phenotype with mainly normal epithelium and small adenomas, as it has been previously reported.[6]

Antibodies and immunohistochemical assays

To detect human intestinal mucins, monoclonal antibody LDQ10, recognizing MUC2, was used as ascites at 1/500 dilution, and rabbit polyclonal antibody anti-MUC4 was used at 1/80 dilution. The characterization and specificity of these antibodies had been previously reported.[9,15] Indirect immunoperoxidase technique was performed on paraffin-embedded sections as described.[9] For mouse mucins detection the antibodies used were: the polyclonal antibodies MM2-2 for Muc2 [16] and hHA1-B-1 against the C-terminal sequence of Muc4 (a kind gift from Prof. K. Carraway, Miami, USA) and the 45M1 monoclonal antibody recognizing Muc5AC.[17] The specificity of these antibodies on mouse tissues had been described previously [18]. Antigen retrieval used for the detection of the different antibodies was either: 1) 10 mM citric acid, pH 6 at 95ºC for 20 min followed by 10 mM 1,4-dithiothretiol in 0.1 M Tris/HCl buffer, pH 8 at 37℃ for 30 min and then 25 mM iodoacetamine in the dark for 30 min (MM2-2); 2) High pH Antigen Retrieval Solution (Dako, hHA1-B-1); or 3) Rodent decloaker (Biocare Medical, 45M1) at 80ºC for 2 hours. Sections were then treated with 3% (v/v) hydrogen peroxide, washed, and blocked by protein block (Dako, MM2-2 and hHAI-B-1) or rodent block M (Biocare Medical, 45M1) for 30 min. The primary antibody was diluted in Antibody Diluent (Dako, MM2-2 5 µg/ml, hHAI-B-1 1:200 and 45M1 1:1000) and incubated for 1 hour. Then secondary anti-rabbit horseradish peroxidase conjugated antibody (MM2-2 and hHAI-B-1) or MM HRP-Polymer (Biocare Medical, 45M1) were incubated for 20 min. Bound antibody was visualized with diaminobenzidine and the sections were counterstained with Harris's haematoxylin.

Intestinal sections were used as positive controls for MUC2 and MUC4 human and mice antibodies.

Double labelling immunofluorescence

After antigen retrieval (10mM sodium citrate, pH 6), samples were blocked for 1 hour in 5% horse serum diluted in PBS-Triton and anti-STAT3 and anti-MUC4 antibodies were incubated o/n at 4ºC. Slides were rinsed and incubated for 2 hours with anti-mouse Alexa 488 and anti-Rabbit Alexa 546 antibodies and nuclei were stained with Hoechst.

Quantitative RT-PCR

Total RNA was extracted from snap-frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To eliminate any contaminating genomic DNA, on-column DNase digestion was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from 1µg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) following manufacturer's instructions. Quantitative RT-PCR (Q-PCR) gene expression analyses were performed on triplicate samples with SYBR Green (Invitrogen) using the 7900HT Fast RT-PCR System (Applied Biosystems, Foster City, CA) over 40 cycles (95℃/15sec, 60℃/1min), following an initial denat uration step at 95℃/10min. Primers to specifically amplify mouse 18S were used to normalize cDNA concentrations of target genes. The Muc4/18S Q-PCR was done using TaqMan primer/probe sets (ABI), and the Muc2/18S Q-PCR was performed using SYBR Green with the following primers: 18S forward 5'-GTAACCCGTTGAACCCCATT-3' 18S reverse 5' CCATCCAATCGGTAGTAGCG-3' Muc2 forward 5' CCCAGAAGGGACTGTGTATG-3' Muc2 reverse 5'-TGCAGACACACTGCTCACA-3'. Data acquisition and analyses were performed with the Sequence Detection System Version 2.3 software (Applied Biosystems).

Statistical analysis

The association between the expression of the different molecular markers and the inflammatory score of the samples was evaluated using Mann-Whitney U-test or Student's t-test for continuous variables and Pearson's Chi-square test for categorical variables.

Positive (r^2>0) or negative (r^2<0) correlations were established using the Spearman's rho correlation coefficient (r^2).

Statistical analysis was performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Statistical significance was established when p≤0.05.

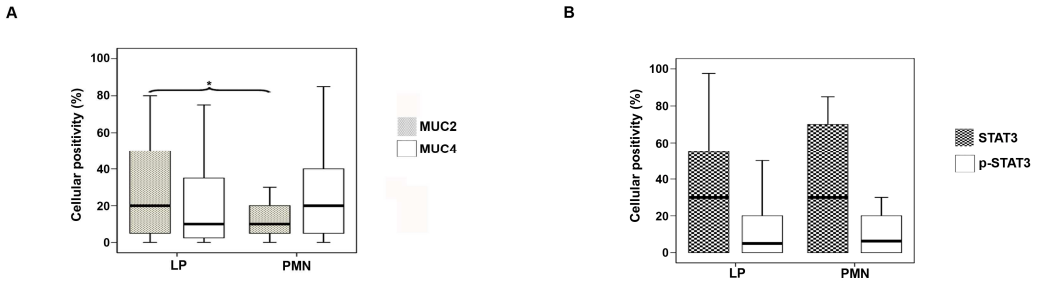

Figure 1 Expression levels of MUC2 and MUC4 mucins (A) and STAT3 and p-STAT3 (B) in human stomach tumors classified by the inflammatory cell type. (* p = 0.029).

RESULTS

Classification of the tumors by the inflammatory infiltrate

Two groups of intestinal gastric cancer samples were established according to the predominant cell type present in their inflammatory component: LP Group, with ≥50% of lymphoplasmocytic inflammatory infiltrate (n=53) corresponding to chronic inflammation; and PMN Group with ≥50% of polymorphonuclear cells in the inflammatory component (n=25), which corresponds to acute or active inflammation (Figure 1A and 1B). The different number of tumors in each group indicated that chronic inflammation is most frequently associated to this tumor type.

Relation between clinical characteristics of the patients and inflammatory infiltrate

The most relevant clinical characteristics of the patients are shown in Table 1.

Regarding the association between specific clinical features and the inflammatory cells infiltrating the tumors, it is remarkable that in the LP Group the 67.9% (36/53) of the tumors were well differentiated (Grade I and II) and only 32.0% (17/53) were moderate or poorly differentiated; whereas in the PMN Group, the number of well differentiated tumors and poorly differentiated tumors was similar: 56.0% (14/25) and 44.0% (11/25), respectively. These data suggest a trend in the association between the inflammatory score of the gastric tumors and their histological grade.

No other relevant clinical characteristics were different between both groups of gastric tumors, as it is shown in Table 1.

Expression patterns of the intestinal mucins MUC2 and MUC4 in human gastric tumors

MUC2 and MUC4, that have been described to be ectopically detected in intestinal gastric cancer and in the precursor phases of this tumor type,[9] were analysed in the two groups of gastric cancer to detect if their expression could be related to the inflammatory characteristics of the tumor.

The expression of MUC2 was 28.58% and 13.20% of positive cells and 44/53 and 19/25 positive cases in the LP and PMN Groups, respectively. These results indicated that in tumors with acute inflammatory infiltrate, that have predominance of polymorphonuclear cells, the levels of MUC2 positive cells were lower and this difference was statistically significant (p=0.029).

By contrast, no significant differences were found in the detection of MUC4: 20.75% and 24.40% of positive cells and 40/53 and 20/25 positive cases for LP and PMN Groups, respectively (p=0.472).

In summary, these data showed that tumors displaying chronic inflammation (LP) expressed higher levels of MUC2 than tumors with acute inflammation (PMN), whereas no differences in the levels of MUC4 were detected. Results are shown in Figures 1 and 2.

Table 1. Clinico-pathological characteristics of the two groups of patients classified by the inflammatory cells infiltrating the tumor.

Inflammatory score	Lymphoplasmocytic (LP) n = 53 ≥ 50% Lymph/Plasmatic cells		Polymorphonuclear (PMN) n = 25 ≥ 50% Polymorphonuclear cells	
Age	74.96 ± 10.96		75.76 ± 6.76	
Sex				
M	31*	(58.5%)**	14	(56.0%)
F	22	(41.5%)	11	(44.0%)
Histological Grade				
I	5	(9.4%)	2	(8.0%)
II	31	(58.5%)	12	(48.0%)
III	11	(20.8%)	6	(24.0%)
IV	6	(11.3%)	5	(20.0%)
pT				
pT1	9	(17.0%)	8	(32.0%)
pT2	20	(37.7%)	9	(36.0%)
pT3	18	(34.0%)	6	(24.0%)
pT4	6	(11.3%)	2	(8.0%)
pN				
N0	21	(39.6%)	12	(48.0%)
N1	21	(39.6%)	8	(32.0%)
N2	8	(15.1%)	3	(12.0%)
N3	3	(5.7%)	2	(8.0%)
Vascular inv.	31	(58.5%)	12	(48.0%)
Perineural inv.	29	(54.71%)	14	(56.0%)
H. pylori	25	(47.1%)	12	(48.0%)
Intestinal Metaplasia	45	(84.9%)	23	(92.0%)

* Number of cases ** Percentage of cases

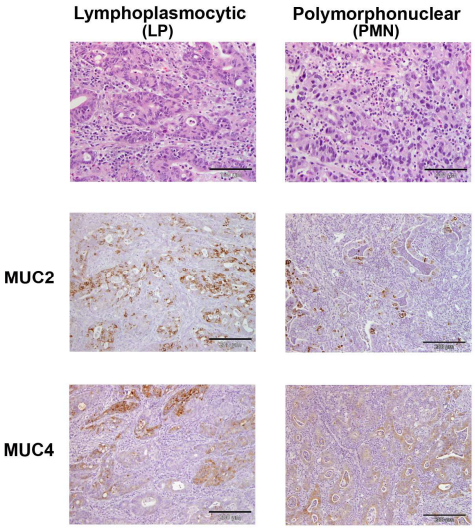

Figure 2 Pattern of expression of MUC2 and MUC4 intestinal mucin genes detected by immunohistochemistry in human gastric tumors with acute (PMN) and chronic (LP) inflammation.

Detection of STAT3 transcription factor

In gastric cancer cell lines we have reported that MUC4 was activated through STAT3 by the direct binding of the active form p-STAT3 to the MUC4 promoter. [14]

The expression of STAT3 was evaluated in the two groups of stomach cancer tissues as total STAT3 (n=48) and activated p-STAT3 (n=48). STAT3 was detected in the cytoplasm and focally in the nucleus of the tumor cells, whereas p-STAT3 staining was exclusively nuclear. STAT3 was expressed in 31.67% and 37.89% of the tumor cells, and in 20/30 and 14/18 tumor samples of the LP and PMN Groups, respectively. The active form of STAT3, p-STAT3, was positive in 11.17% of the cells and in 17/30 cases in the LP tumors, and in 9.87% and 12/18 in the PMN Group. A weak negative correlation was found between the expression of MUC2 and STAT3 (r^2 = -0.091), whereas MUC4 and STAT3 expression correlated positively (r^2 = 0.145). These results are shown in Figure 1.

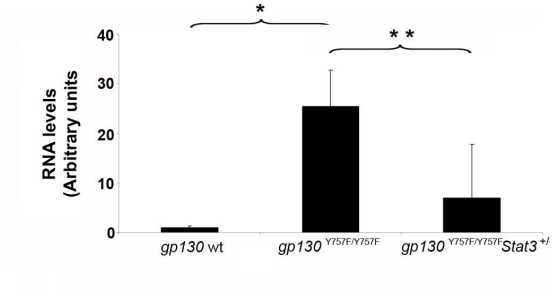

Figure 4 Muc4 mRNA levels analysed by quantitative RT-PCR in gastric samples from *gp130*$^{+/+}$ (n=3), *gp130*$^{Y757F/Y757F}$ (n=4), and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ (n=3) mice. Results are presented as the average±SD (error bars). * p= 0.02; **p= 0.042.

To asses the co-expression of STAT3 and MUC4, double immunofluorescence labelling was performed inrformed in selected tumor cases (n=5) in which both molecules had been previously detected. Results indicated that STAT3/MUC4 were co-detected in all the cases with different number of cells co-expressing both molecules, as it is shown in Figure 3.

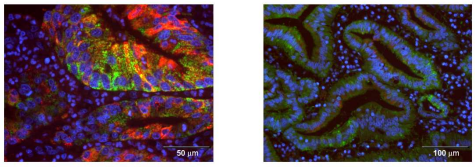

Figure 3 Detection of MUC4 (red) and STAT3 (green) by double labelling immunofluorescence in two human tumor samples.

Detection of Muc2 and Muc4 gastric tissues from gp130$^{+/+}$, gp130$^{Y757F/Y757F}$, and gp130$^{Y757F/Y757F}$ Stat3$^{-/+}$ mice

In the *gp130*$^{Y757F/Y757F}$ mice, hyperactivated STAT3 signalling pathway caused spontaneous development of gastritis and gastric tumors.[6] Expression of the intestinal mucins, Muc2 and Muc4, and the gastric mucin Muc5AC was analysed in the normal gastric mucosa of the *gp130*$^{+/+}$, *gp130*$^{Y757F/Y757F}$, and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ mice specimens at the mRNA and protein levels. Quantitative RT-PCR to detect Muc2 and Muc4 mRNA was performed. Muc2 was not detected in either normal or tumoral stomach samples (data not shown). By contrast, Muc4 transcripts were absent in gastric tissue from the *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ mice, but were detected in tumor-bearing *gp130*$^{Y757F/Y757F}$ stomach specimens. The differences in Muc4 expression between *gp130*$^{+/+}$ and *gp130*$^{Y757F/Y757F}$ mice, and between *gp130*$^{Y757F/Y757F}$ and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ mice were statistically significant (p=0.02 and p=0.042, respectively) (Figure 4).

By immunohistochemistry, Muc5AC was detected in the mucus-secreting cells of the normal superficial epithelium in all the mice: wild type, *gp130*$^{Y757F/Y757F}$, and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ as expected (Figure 5). The 14 weeks old *gp130*$^{Y757F/Y757F}$ mice, had large stomach tumors that covered the whole antrum and part of the corpus. In these tumors Muc5AC expression was decreased by 90% (Figure 5). The intestinal mucin, Muc2 was absent from the gastric tissue, including the tumors, in all the 3 genotypes (data not shown), although the goblet cells of the duodenum were positive, demonstrating that the immunohistochemical stain worked. The intestinal mucin, Muc4 was absent or very weakly expressed in the normal antrum of mice of all genotypes and only one of the normal corpus samples was weakly positive for Muc4 (Figure 5). In contrast, Muc4 was detected at varying levels in tumors from all the *gp130*$^{Y757F/Y757F}$ mice (Figure 5). These data confirm the results obtained by quantitative RT-PCR.

DISCUSSION

The inflammatory cells infiltrating the gastric mucosa as a response to *H. pylori* infection, can modulate the presence of specific cytokines.[19] These cytokines activate signalling pathways that regulate the expression of genes involved in the gastric carcinogenetic process. Among them, glycosylation enzymes, mucins, and trefoil factors have been reported to be aberrantly expressed during the acquisition of an intestinal phenotype in gastric carcinogenesis.

Here, we report that MUC2 was significatly more detected in gastric tumors displaying chronic inflammation (lymphoplasmocytic cells) than in tumors with acute inflammation (polymorphonuclear cells). By contrast, no differences were found in the expression of MUC4 or the

transcription factor STAT3, which can activate MUC4, but not MUC2. [14] MUC2 is a secreted mucin than can be regulated through different pathways such as MAPK, NF-KB and Ras/MEK in colon cancer cells.[20,21,22] Muc2$^{-/-}$ mice present increased proliferation and migration and decreased apoptosis of intestinal epithelial cells and develop spontaneous tumors that progress to invasive carcinomas, suggesting a role for Muc2 in tumor suppression.[23] MUC4 is a membrane-bound mucin that has been involved in tumor growth and metastasis through several mechanisms related to its cytoplasmic tail, which has been implicated in cell signalling events (reviewed in [24]). The transcriptional induction of MUC4 by STAT3 agrees with the fact that no differences in MUC4/STAT3 detection were found in the two groups of tumors, suggesting that the initial acute inflammation should trigger the activation of the gp130/STAT3 signalling pathway, and this activation may promote the apparition of chronic inflammatory cells, as it has been recently described in the *gp130*$^{Y757F/Y757F}$ mouse model. In these mutant mice, the clearance of polynuclear cells associated to an increased number of mononuclear cells is regulated through the gp130-mediated STAT3 signalling in induced acute peritoneal inflammation.[25]

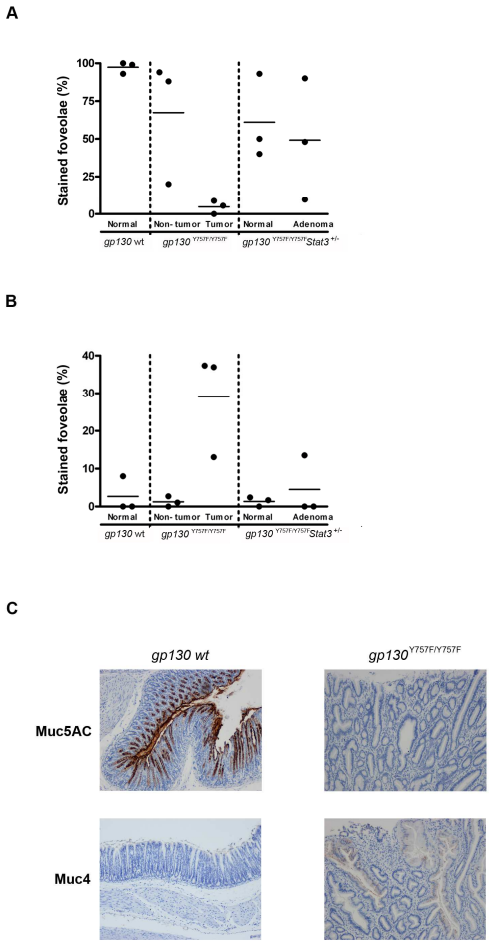

Figure 5 A) Average Muc5AC scores of antrum and corpus. B) Average Muc4 scores of antrum and corpus. C) Muc5AC and Muc4 in normal gastric mucosa of wild type mice and in gastric tumors of *gp130*$^{Y757F/Y757F}$ transgenic mice. (Original magnification: x20).

These *gp130*$^{Y757F/Y757F}$ mutant mice with inhibited SHP2-Ras-ERK signalling pathway, develop spontaneous gastric hyperplasia and gastric tumors as a consequence of hyperactivated STAT3.[5,6] Here, we show that, in the tumor-free stomach from *gp130*$^{+/+}$, *gp130*$^{Y757F/Y757F}$ and *gp130*$^{Y757F/Y757F}$ *Stat3*$^{-/+}$ mice, no differences were found in the expression of the Muc5AC gastric mucin, and the intestinal mucins Muc2 and Muc4. Only the *gp130*$^{Y757F/Y757F}$ mice develop gastric tumors expressing Muc4, which is not detected in the normal gastric mucosa, suggesting that it must be activated due to the hyperactivation of the STAT3 signalling pathway. The Muc4 expression in these mice tumors agrees with the results reported in human gastric cell lines [14] and suggests an active role of gp130/STAT3 pathway in the regulation of genes associated to the gastric neoplastic transformation. In gastric cancer, the expression of molecules associated to this pathway has been related to a worse prognostic, to a VEGF over expression,[26] and with a shorter survival post-surgery.[27] Also, the activation of STAT3 and ERK 1/2 is increased in stomach samples of patients infected with *H. pylori,* and these increases are likely driven in an IL-6 dependent fashion, whereas IL-11 is up-regulated in gastric adenocarcinoma regulating epithelial cell turnover.[28]

The inflammatory cells infiltrating human colorectal tumors have been recently characterized in a large cohort of samples, and in these tumors the type, density, and location of the immune cells have a prognostic value superior to the histopathological methods currently used to stage colorectal cancer.[29] Although a larger number of samples is needed to be evaluated, here we have observed that the classification of gastric tumors according to the predominant infiltrating inflammatory cells in the two groups, chronic (LP) or acute/active (PMN), can be correlated with their histological grade: Tumors with lymphoplasmocytic infiltration are more differentiated (Grade I and II) and this characteristic has been associated with a better prognostic. Furthermore, these tumors present higher levels of MUC2 agreeing with the hypothesis that Muc2 can act as a tumor suppressor gene.[23] Together these data suggest that inflammatory cells infiltrating the intestinal-type gastric tumors can modulate the transdifferentiation process by regulating signalling pathways implicated in the expression of specific genes involved in the gastric carcinogenesis process.

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DISCUSSION

1. Changes in the expression pattern of glycosyltransferases and Lewis antigens induce changes in the invasive and metastatic behaviour of tumoral cells

The gastric mucosa displays a specific expression pattern of mucins, glycosyltransferases and Lewis antigens that is lost at the earliest stages of the gastric carcinogenesis process. Previous data of our group showed that in intestinal metaplasia, for instance, downregulated expression of gastric mucins (MUC5AC and MUC6) was accompanied by an increase in the expression of intestinal mucins (MUC2 and MUC4) associated with both types of Lewis antigens (28). In addition, the glycosylation pattern observed in mucins was demonstrated not to be determined by the primary sequence of the protein but by the set of glycosyltransferases expressed in a given cell (370). In that study the model used was HT-29/M3 cells, which express MUC5AC, fucosyltransferase 2 and type 1 Lewis antigens as it occurs in the epithelial cells of the gastric surpeficial epithelium. HT-29/M3 cells were transfected with human FUT1 and the obtained clons expressed *de novo* type 2 Lewis antigens. The tumorigenic capacity of parental and FUT1-transfected cells was evaluated after intrasplenically injection in BALB/c nude mice, and no differences in tumorigenicity were observed (370). However, we further analysed this model and we found differences in the invasive and metastatic capacities between parental and FUT1-transfected cells. These differences were induced by the different expression pattern of s-Lewis x antigen displayed by the cells, that correlated with the levels observed in the derived tumours. s-Lewis x has been described to be expressed in a number of human carcinomas including colon, ovary, breast, prostate, uterine and gastric cancer (371,241,239,238). s-Lewis x serves as a ligand for endothelial E-selectin and in this way, cancer cells can be extravasated and travel through the bloodstream, where they can interact with blood cells and finally adhere to the endothelium and form metastatic tumours. The contribution of s-Lewis x and E-selectin to metastasis has been reported in gastric cancer models, where they have been described to mediate the adhesion of gastric tumour cells to endothelial cells (372). In summary, our results show that transfection of human FUT1 produces an imbalance in the biosynthesis of Lewis antigens (Figure 27) that results in decreased levels of s-Lewis x. As a consequence, the *in vitro* capacity of these cells to bind E-selectin is significantly reduced and their metastatic properties *in vivo* modified.

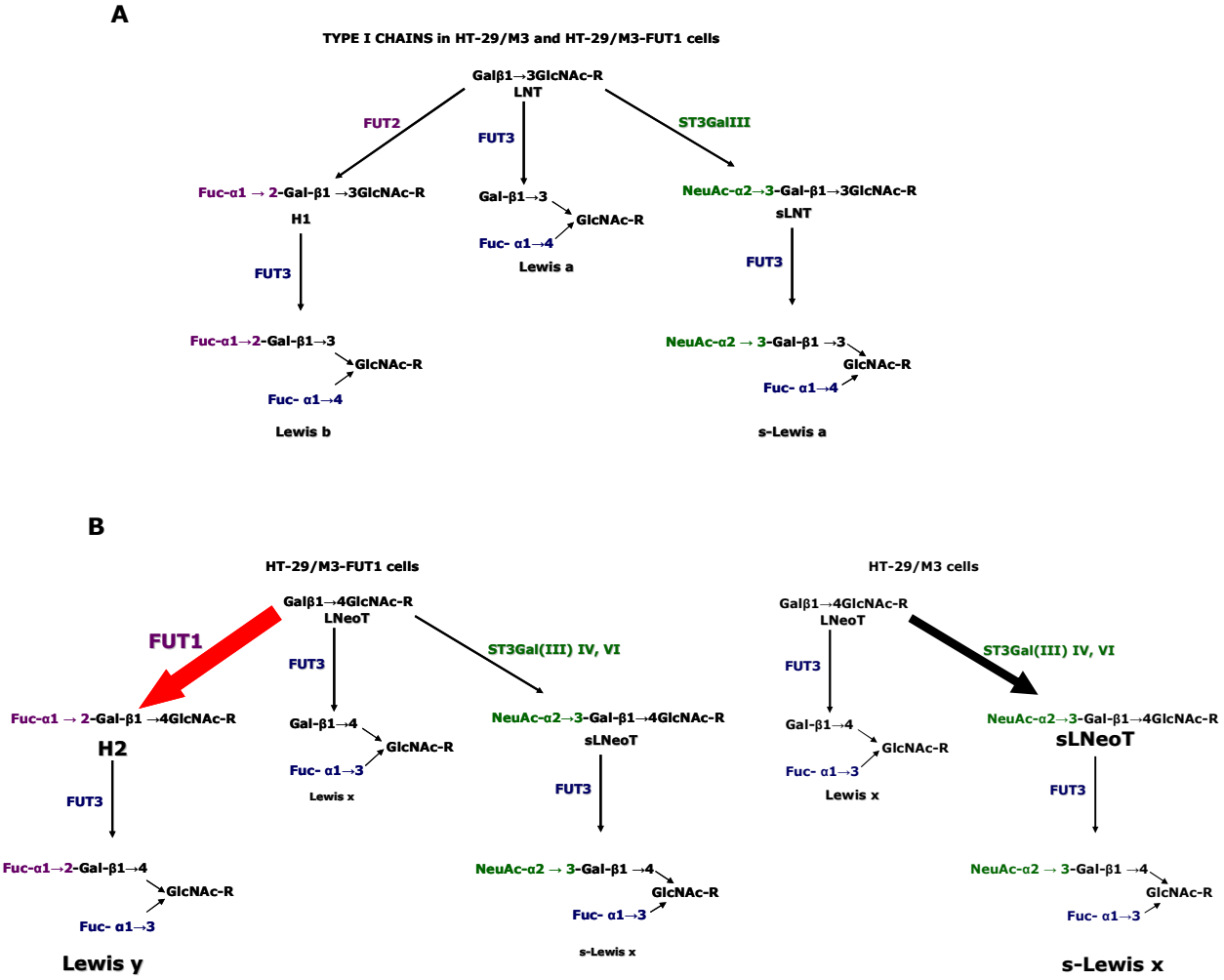

Figure 27. A) HT-29/M3 and HT-29/M3-FUT1 express type 1 Lewis antigens at similar levels. **B)** Transfection with human FUT1 imbalances the synthesis of type 2 Lewis antigens, inducing de novo expression of h type 2 precursor (H2) and Lewis y and decreased levels of s-Lewis x antigen.

Our results show that changes in the expression pattern of specific glycosyltranseferases not only modify the type of glycosidic structures present in a specific cell type or tissue, but also alter the invasive and metastatic behaviour of the cells. Lewis antigens are glycosidic structures attached to different glycoproteins and glycolipids, being especially important for their participation in invasion and metastasis MUC1 and CD44. Presence of s-Lewis x confers adhesive properties to MUC1 (336), and fucosylated and sialylated structures of CD44v isoforms have been described to function as high-affinity ligands for E-selectin in colon cancer cells (373). In gastric tumours, overexpression of CD44, and specifically the CD44v6 isoform has been correlated with higher lymph node

metastasis and invasion, and can be used as a prognostic factor (374,375). Further analysis of MUC1 and CD44 glycosylation pattern and possible differences induced by FUT1 transfection would be useful in order to elucidate if the changes in the metastatic capacities observed in our model are associated with changes in the O-glycosylation pattern of these glycoproteins.

2. Effect of pro-inflammatory cytokines on the expression of fucosyl- and sialyltransferases in gastric cancer cells

Altered expression of glycosyltransferases and Lewis antigens has been observed in gastric cancer and pre-cancerous lesions that can induce changes in the metastatic capacities of tumoral cells, as we have previously discussed. Different fucosyl- and sialyltransferases contribute to the synthesis of Lewis antigens and their expression condition the type of Lewis structures observed in a specific cell type or tissue. In different cellular models the expression of some glycosyltransferases has been described to be regulated by inflammatory cytokines. We have analysed the effect of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 on the expression of fucosyl- and sialyltransferases implicated in the synthesis of Lewis antigens in gastric cancer cell lines (FUT1, FUT2, FUT3, ST3Gal III, ST3Gal IV and ST6Gal I). These cytokines seem not to induce changes in the mRNA levels of fucosyl- and sialyltransferases in the two cell lines analysed. However, a much more quantitative approach should be addressed, as well as the analysis of other gastric cancer cell lines. In addition, the analysis at the protein level would be useful to determine changes in the expression of the glycosyltransferases studied.

Regarding the expression of Lewis antigens, increased levels of type 2 structures (H type 2, Lewis y and s-Lewis x) have been observed in cells treated with IL-1β, suggesting that this cytokine is probably modulating the expression of glycosyltransferases involved in the synthesis of type 2 glycans. When the expression of Lewis antigens was evaluated in intestinal-type gastric tumours classified according to their inflammatory score, tumours with chronic inflammation (lymphoplasmocytic inflammatory infiltrate) presented higher levels of the type 2 antigens Lewis y and s-Lewis x, supporting the hypothesis that cytokines related to chronic inflammation as IL-1β may be modulating the expression of glycosyltransferases in gastric cancer cells.

As previously discussed, s-Lewis x is implicated in the process of metastasis, for this reason tumours expressing higher levels of s-Lewis x would be expected to be more metastatic. However, we could not analyse the incidence of metastasis in our retrospective study of gastric tumour samples since this information was lacking for many of the cases.

Differences in the histological differentiation grade and the perineural invasion between tumours with chronic inflammation and tumours with acute inflammation were observed, although these differences were not related to the different expression pattern of Lewis antigens detected in the two groups of tumours, but to the different expression of intestinal mucins, as it will be discussed in the next section.

3. Effect of pro-inflammatory cytokines on the expression of the intestinal mucins MUC2 and MUC4 in gastric cancer

Ectopic expression of the intestinal mucins MUC2 and MUC4 has been detected in gastric tumours as well as in pre-cancerous lesions (28). These mucins have been reported to be modulated by different inflammatory cytokines in various cell types. Specifically, MUC2 expression was observed to be regulated by TNF-α and IL-1β in colon cancer and airways cells, respectively (282,281,376). In the case of MUC4 recent studies have shown that its expression can be stimulated by IFN-γ in pancreatic cells (277), and by IL-1β in nasal polyps (289). However, no data regarding regulatory effects of pro-inflammatory cytokines on intestinal mucin genes had been reported in gastric models.

We first analysed the effects of IL-6 on MUC2 and MUC4 expression. No changes in MUC2 levels were observed, whereas MUC4 was modulated by IL-6. Regulation of MUC4 by IL-6 occurred through the gp130/STAT3 pathway, and we demonstrated that STAT3 can bind directly to a STAT binding site on MUC4 promoter (Figure 28). The fact that no STAT binding sites have been detected in the MUC2 promoter (377) can explain why IL-6 is not able to regulate MUC2 expression.

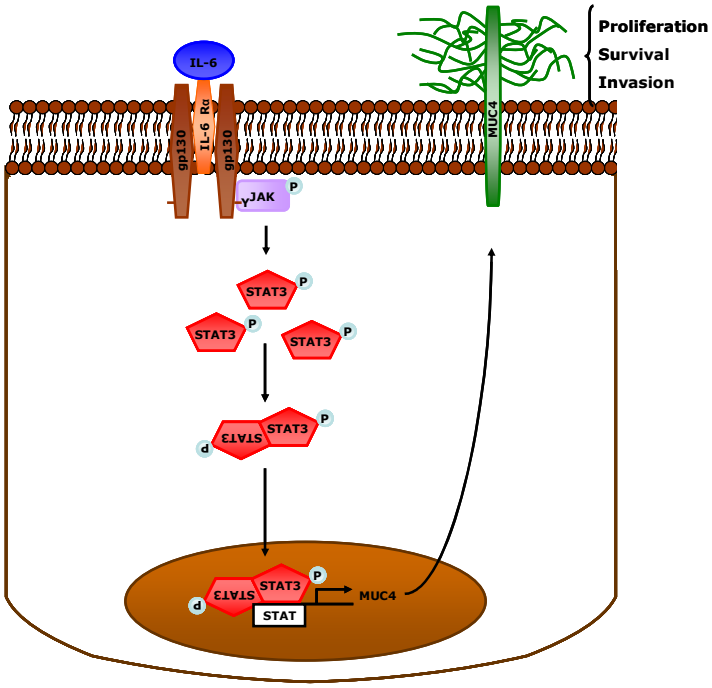

Figure 28 . IL-6-mediated regulation of MUC4 expression in gastric cancer cells. IL-6 induces STAT3 activation and translocation into the nucleus where it binds a STAT binding site in MUC4 promoter. Expression of MUC4 has been related to proliferation, survival and apoptosis of tumour cells.

Deregulation of IL-6-associated signalling pathways is implicated in gastric tumours development (378). In particular, mutation of the transmembrane receptor gp130 in mice induced hyperplasia and development of gastric adenomas due to hyperactivated STAT3 signalling (144). In gp130$^{Y757F/Y757F}$ mutant mice, impaired activation of the SHP2-Erk pathway resulted in attenuated expression of TFF1, which is considered to be a gastric tumour suppressor gene (43). In addition increased levels of STAT3 in these mutant mice were responsible for the activation of genes associated with proliferation, tumour progression and angiogenesis, such as Bcl2l1, Myc and Vegfa (146). MUC4 has been described to be involved in tumour growth and metastasis. Through its EGF domains, MUC4 can interact with ErbB2 (HER2), and, in rat, it has been reported that Muc4 phosphorylates ErbB2 and in this way induces cell transformation (379). Moreover, MUC4 expression has been related to proliferation, survival and invasive properties of pancreatic tumour cells (322). Our *in vitro* study showed that MUC4 is a target gene of STAT3, and we confirmed it *in vivo*. We analysed the expression levels of Muc4 in gp130 wild type, gp130$^{Y757F/Y757F}$ mutant mice, and gp130$^{Y757F/Y757F}$ /Stat3$^{+/-}$, and we observed that Muc4 was only significantly expressed in tumour gastric samples from

gp130$^{Y757F/Y757F}$ mice. Taken together these results provide a novel mechanism of mucin gene regulation and point out a new target gene for STAT3 that can contribute to gastric malignant transformation.

As previously mentioned IL-6 did not regulate the expression of MUC2 in gastric cancer cell lines, and this was also confirmed *in vivo*, since no expression of Muc2 was detected in gp130$^{Y757F/Y757F}$ mutant mice. In contrast, we found that MUC2 expression was induced by IL-1β and TNF-α, which mediate chronic inflammation. In this regard, we observed that intestinal-type gastric tumours presenting lymphoplasmocytic inflammatory infiltrate (corresponding to chronic inflammation) displayed significantly higher levels of MUC2 than tumours infiltrated with polymorphonuclear cells (corresponding to acute inflammation). This observation supported the results obtained *in vitro* suggesting that chronic inflammation, translated into IL-1β and TNF-α expression, modulates the expression of MUC2 in gastric cancer cells.

The intestinal transcription factor CDX2 has been ectopically detected in intestinal metaplasia as well as in gastric tumours. In transgenic mice ectopic expression of CDX2 in the gastric mucosa was able to induce intestinal differentiation (41). In addition, in gastrointestinal cells MUC2 expression was reported to be regulated by CDX2 (246). These data pointed out CDX2 as a possible transcription factor responsible for MUC2 induction after TNF-α treatment; however we observed that CDX2 expression was not modulated by TNF-α and in some gastric cancer cell lines MUC2 was detected independently of CDX2 expression. In contrast, we found that NF-κB was the responsible of MUC2 activation after TNF-α treatment, providing an alternative mechanism of MUC2 regulation in gastric cancer cells. The NF-κB signalling pathway has been shown to be constitutively activated in gastric tumours (132,133,134). Furthermore, NF-κB activation has been suggested to be important for the progression from *H. pylori*-associated chronic inflammation to carcinogenesis (130,131). We have also detected activated NF-κB in intestinal-type gastric tumours as well as in intestinal metaplasia samples, which was co-expressed with MUC2. In these samples co-expression of MUC2 and CDX2 was also observed, supporting the hypothesis that MUC2 can be regulated by at least two different mechanisms in gastric cancer cells.

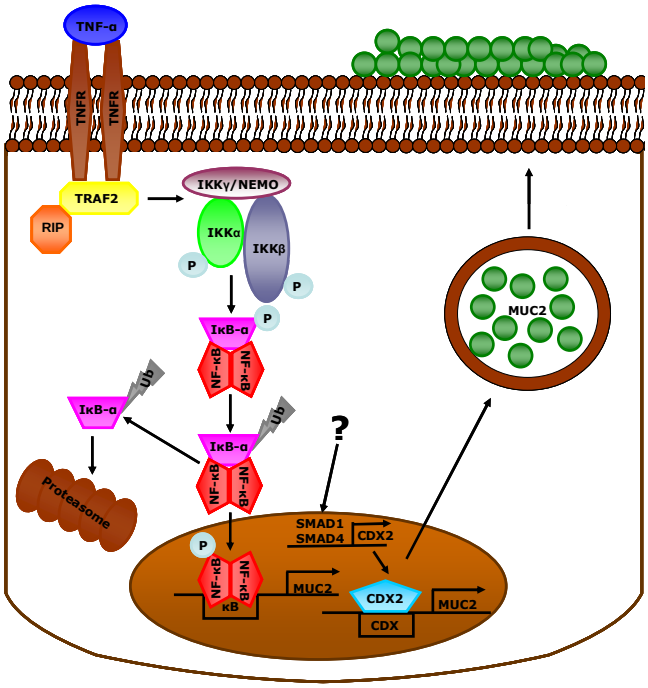

Figure 29. Molecular mechanisms involved in MUC2 activation in gastric cancer cells. TNF-α activates the NF-κB signalling pathway. Activated NF-κB binds a NF-κB site in MUC2 promoter and induces its transcription. MUC2 can be also regulated by CDX2 binding to CDX sites in MUC2 promoter. The mechanisms involved in CDX2 activation remain unknown.

We have observed that in some gastric cell lines the expression of CDX2 mRNA did not correlate with the expression of the protein, suggesting that CDX2 may be regulated at the level of translation. Translational control is a fundamental mechanism for moderating cellular events. When analysing the possible mechanisms controlling CDX2 translation we observed that up to eleven microRNAs (miRNA) target sites were present in CDX2 mRNA (miRanda web server, http://cbio.mskcc.org/mirnaviewer). miRNAs generally bind to the 3' untranslated region (UTR) of their target mRNAs, leading most often to a decrease of the target protein by either degradation of the target mRNA or translational repression (380). Alterations in the 3' polyadenylation tail of mRNAs induced by binding of cytoplasmic polyadenilation element-binding proteins (CPEB) can also control the translation of mRNAs presenting CPE sequences at their 3'UTR (381). No CPEs sequences were detected in the 3' UTR of CDX2 (http://genome.imim.es/CPE/server.html) (382), indicating that the translational control of CDX2 is likely to occur through binding of miRNAs. However, further analysis is needed in order to determine if CDX2 is regulated at the translational

level, the possible mechanisms involved in this kind of regulation and their implication in gastric carcinogenesis.

The study of intestinal-type gastric tumours classified by their inflammatory score not only confirmed that inflammation can modulate the expression of the intestinal mucins MUC2 and MUC4, but also suggested that some clinico-pathological characteristics of the tumours can be directly related to the type of inflammation present. In this regard, we observed that tumours with chronic inflammation (predominant lymphoplasmocytic inflammatory infiltrate) were more differentiated than tumours with acute inflammation and this characteristic has been related to a better prognosis. Also, tumours with chronic inflammation expressed higher levels of MUC2, which has been associated with a favourable outcome in patients with gastric cancer (383), establishing again a link between inflammation, mucin expression and tumour prognosis. In human colorectal cancer samples the type, density and location of the immune cells infiltrating the tumours were found to be a better predictor of patient survival than the histopathological methods currently used to stage colorectal cancer (384). Our results also suggest that the analysis of tumour-infiltrating inflammatory cells may be a valuable tool in the management of gastric cancer, although the analysis of a larger number of cases would be needed.

Taken together our results indicate an important role for inflammation in the activation of different genes associated with the intestinal transdifferentiation process occurring during gastric carcinogenesis.

CONCLUSIONS

1. The altered expression pattern of Lewis antigens induced by the transfection of the human FUT1 gene causes changes in the invasive and metastatic properties of HT-29/M3 colon cancer cells.

2. Inflammation and its associated signalling pathways modulate the expression of genes associated with gastric carcinogenesis.

    2.1. IL-1β treatment increases the expression of type 2 Lewis antigens in gastric cancer cells.

    2.2. Tumours with chronic inflammation display increased levels of type 2 glycan structures.

    2.3. IL-6 regulates the expression of MUC4 through the gp130/STAT3 signalling pathway in gastric cancer cell lines.

    2.4. MUC2 expression induced by TNF-α is regulated through the NF-κB signalling pathway independently of CDX2 in gastric tumour cells.

    2.5. Inflammation modulates the expression of the intestinal mucins MUC2 and MUC4 in gastric tumours.

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APPENDIX

Review

Inflammatory cytokines pathways as potential therapeutic targets for gastric cancer

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Abstract

Gastric cancer is the fourth most common cancer and the second most common cause of cancer deaths worldwide. The 90% of gastric cancers are adenocarcinomas and two main types may be distinguished, diffuse and intestinal. The process preceding the diffuse-type carcinomas is not well known whereas the precursor stages of the intestinal-type are histologically well identified. The colonization of the gastric mucosa by *Helicobacter pylori* and the associated inflammatory response, induced by the presence of inflammatory cytokines, have been postulated as initiators of the neoplasic transformation. TNF-α, IL-1β, IL-6, and IFN-γ cytokines have been detected in *H. pylori*-infected stomachs, and can activate specific transcription factors that would regulate the expression of genes implicated in the transformation of the gastric mucosa. In vivo mouse models of gastric cancer have been recently developed to analyse the implication of the inflammatory cytokines in the regulation of specific genes involved in the gastric neoplasic transformation. The signalling pathways activated by proinflammatory cytokines represent new valid approaches for antitumor therapies, and the specific transcription factors activated are potential targets for inhibition. STAT3 and NF-kappaB that are hyperactivated in many human tumors, are implicated in the transcription of genes that promote oncogenesis and metastasis by the participation in cell proliferation, apoptosis, migration, angiogenesis and immune evasion processes. Different approaches as peptidomimetics and small molecule inhibitors have been synthesised. Their potential antitumoral effects are being analysed in different tumor models and some data from pre-clinical assays are available. The association between inflammation and gastric cancer development makes this human tumor type a potential target for the use of these therapeutic approaches.

Key words: gastric cancer, *Helicobacter pylori*, inflammation, IFN-γ, IL-1, TNF-α, and IL-6.

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Inflammation and gastric cancer

Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, survival, proliferation, invasion, angiogenesis, and metastasis [1]. The association between chronic inflammation and cancer has been described particularly in the digestive tract, where the risk for carcinogenesis increases in the presence of chronic inflammatory conditions such as esophagitis, gastritis, colitis, pancreatitis, and hepatitis [2]. In particular, the chronic inflammation of the stomach caused by *Helicobacter pylori* infection confers a significantly increased risk of developing gastric cancer [3], and cytokines released during the inflammatory response may contribute to gastric mucosal damage by activating the expression of different genes involved in the gastric neoplasic transformation.

Gastric cancer remains a major cause of mortality worldwide and the incidence of gastric tumors is predicted to increase as a result of population growth. Approximately 95% of malignant tumors of the stomach are adenocarcinomas, which, histologically, can be classified into intestinal and diffuse subtypes [4]. Different carcinogenesis pathways have been suggested in relation to these two histological types of gastric cancer, with different molecular changes being present or predominant.

Intestinal-type tumors develop from a succession of histological changes (Figure 1) that have not been identified in diffuse tumors (Figure 2), although they have been also related to *H. pylori* infection and to its associated chronic inflammation. The histological changes preceding the development of an intestinal gastric tumor are known as the Correa's pathway and are: chronic active nonatrophic gastritis, multifocal atrophy, intestinal metaplasia (first complete, then incomplete), dysplasia and invasive carcinoma [5].

Chronic active nonatrophic gastritis is usually triggered by *H. pylori* infection, and this phase is characterized by infiltration of the gastric mucosa by white blood cells, namely lymphocytes, plasma cells, macrophages, and polymorphonuclear neutrophils, representing acute inflammation [5]. Different studies suggest that cytokines such as IFN-γ, TNF-α and IL-β that are released during gastritis, initiate the cellular changes observed in this phase [6,7,8].

The second phase of this gastric carcinogenetic process is characterized by alterations in the epithelial cell cycle such as changes in proliferation and apoptosis. These changes result in focal loss of glands (atrophy), and the acidsecreting parietal cells are replaced with mucous secreting cells. The mechanism of cell loss appears directly related to effects of bacterial products and the cytokine milieu within the gastric mucosa. Atrophy and progression to severe disease have been strongly associated with virulence of the bacterial strains

Figure 1. Histological changes observed in the gastric mucosa preceding the apparition of an intestinal-type gastric tumor. The presence of *H. pylori* is indicated with arrows.

and permissiveness of the host immune response [5]. Subsequently, proliferation of the mucous cells with an intestinal phenotype can lead to intestinal metaplasia, which is the major precursor lesion for gastric cancer [9].

Classically intestinal metaplasia is classified as Type I, or complete, where enterocytes and globet cells are detected, usually accompanied by Paneth cells, or as Type II, incomplete, where all the cells contain mucin. Sometimes a third category, Type IIa or III, is also included, where the predominant mucin is acid sulphomucin rather than sialomucin [10]. The expression of specific intestinal genes has been detected in globet cells, absorptive cells and Paneth cells present in intestinal metaplasia. Globet cells, for instance, express the intestinal mucins MUC2 and MUC4 [11,12]. Absorptive cells have been described to contain sucrase and intestinal-type alkaline phosphatase activity and to express the structural protein villin, and Paneth cells are reactive with anti-defensin [13] and lysozyme antibodies [14]. Lately, SPEM, another type of metaplasia in the stomach, is growing in importance due to its characteristic expression of trefoil factor family 2 (TFF2; spasmolytic polypeptide). This type of metaplasia is associated with loss of parietal cells [15]. SPEM has been also strongly associated with both chronic *H. pylori* infection and gastric adenocarcinoma, potentially representing another pathway to gastric neoplasia [16]. The development of intestinal metaplasia has been associated to the deregulation of different genes, such as p27, cyclin D2, telomerases, c-myc or cyclooxygenase 2 (COX-2), mutations in p53 protein or in adenomatous polyposis coli (APC) gene, loss of heterozygosity, microsatellite instability and *H. pylori* infection [17,18,19]. However, other genes have lately acquired relevant importance for intestinal metaplasia development, such as the transcription factors CDX1, CDX2, PDX1, Sox2, OCT1, TFF3 and RUNX3 [10]. In this sense, CDX2 has been described to be one of the main genes responsible for the initial transdifferentiation step to intestinal metaplasia. Using a transgenic mice model Silberg et al. have shown that the ectopic expression of
CDX2 in the gastric mucosa is enough to activate the intestinal differentiation program [20].

Intestinal metaplasia can progress to dysplasia. This lesion is recognised by cellular atypia with nuclear pleomorphism, cellular undifferentiation and abnormal distribution of glands and crypts. Dysplasias are classified as low-or high-grade, depending on the degree of nuclear atypia and architectural distortion [5]. At the molecular level, dysplasia is characterised by genetic alterations similar to that observed in gastric tumors, such as loss of heterozygosity in the APC/MCC gene [21]. p21, p53 and Bcl-2 genes, which are over expressed in gastric cancer, have been also detected in dysplasia and can be considered an early event in the development of lesions that can lead to gastric cancer [18].

Numerous genetic and epigenetic alterations in oncogenes, tumorsuppressor genes, cell-cycle regulators, cell adhesion molecules, DNA repair genes and genetic instability as well as telomerase activation are detected in gastric tumors [22].

Different studies have tried to correlate the molecular events observed during the gastric carcinogenetic process with *H. pylori* presence. For instance, p16, Bcl-2 and COX-2 expression have been found to be associated with *H. pylori* infection [23]. Moreover, the cytokines released during the inflammatory response to *H. pylori* infection can modulate the expression of genes involved in the neoplasic transformation of the gastric mucosa. For example, IL-6 may lead to mucosal damage and gastric carcinogenesis through transcriptional repression of trefoil factor 1 (TFF1), considered to be a stomach-specific tumor suppressor gene [24].

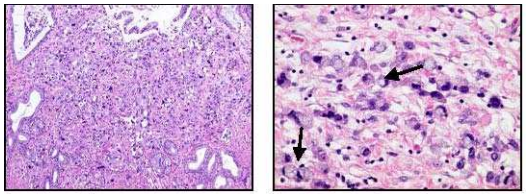

Figure 2. Diffuse-type gastric tumor, characterised by the presence of signet ring cells (→).

Helicobacter pylori infection: interacting bacterial and host genetic factors.

Chronic gastritis is usually caused by *H. pylori*, which is a major factor both in the induction of atrophic gastritis and histological progression to gastric cancer. However, only a small percentage of infected people develop neoplasia, suggesting that specific coordinated interactions between pathogen and host are responsible of increasing the risk for developing gastric cancer. These interactions are dependent on strain-specific bacterial factors, and on the inflammatory responses determined by host genetic diversity [3].

Different bacterial factors have been described to have the capacity to interact with host molecules which, in turn, can induce epithelial responses with carcinogenic potential. The most well-characterized virulence factor is the cag pathogenicity island (cag PAI). CagA, the product of the terminal gene in the island, once translocated into the epithelial cells and phosphorylated is able to activate SHP-2 phosphatase, ERK/ Mitogen-activated protein kinase (MAPK) pathway and EGF receptor (Figure 3), leading to epithelial morphological changes similar to that induced by uncontrolled stimulation of growth factors [25,26,27].

Other *H. pylori* virulence determinants are the secreted toxin VacA, and the adhesins BabA and SabA. VacA

proteins have been associated with augmented inflammation and higher risk of developing gastric cancer [28,29,30]. VacA can block efficiently T cells proliferation and might also interfere with signalling downstream of the TCR and IL-2 receptor [31]. The presence of the strain specific gene babA2, which encodes for the outer membrane protein BabA, is associated with cagA and vacA s1 alleles. Bacterial strains presenting these three genes have been described to induce the highest risk for gastric cancer [32]. SabA adhesin binds the sialyl-Lewis x antigen that is a well established tumor antigen and marker of gastric dysplasia [33]. The expression of sialyl-dimeric-Lewis x is up-regulated by *H. pylori*-induced inflammation and interactions between this molecule and SabA are amplified. Moreover, SabA has been described to be required for nonopsonic activation of neutrophils [34], providing another example of the different coordinated and dynamic interactions existing between host and *H. pylori* in relation to pathogenesis.

Several data suggest that host genetic factors are of crucial importance in determining progression to gastric cancer. Patients who progress to atrophy and cancer present lower levels of gastric acid when compared to patients of duodenal ulcers. This observation led to the study of IL-1β in families with an increased incidence of precancerous lesions, due to the fact that the proinflammatory cytokine IL-1β is a potent inhibitor of acid secretion. Several studies have shown a strong association between proinflammatory IL-1β polymorphisms and increased risk for gastric cancer [35,36,37,38]. Genetic polymorphisms in TNF-α and IL-10, and their combination with proinflammatory IL-1β gene cluster polymorphism have been described to result in a high-risk genotype, presenting a 27-fold or greater risk of developing gastric cancer [39]. Moreover, recent studies on IL-8 promoter have identified the -251T allele as significantly associated with an increased risk of gastric cancer [40,41].

Inflammatory response induced by *Helicobacter pylori*

Lipopolysaccharide (LPS) is the main cause of inflammation in Gramnegative infections. LPS binds the transmembrane pattern recognition receptor Toll-like receptor 4 (TLR4), expressed on macrophages and monocytes [42], and signal transduction is activated trough MyD88, interleukin-1 receptor associated kinase and TRAF6 (Figure 3). Then, the NF-κB and MAPK pathways are activated [43,44] and lead to the synthesis and release of inflammatory cytokines such as IL-1, IL-8 and TNF-α as well as different chemokines and antibacterial peptides [45]. Mutagenic substances such as metabolites of inducible nitric oxide synthase (iNOS) are also released due to *H. pylori* presence. iNOS has been described to promote oncogenesis [46], and nitric oxide generated by iNOS can be converted to reactive nitrogen species, which can produce oncogenic effects such as DNA and protein damage, inhibition of apoptosis, promotion of angiogenesis and mutations in proteins involved in repair functions as p53 [47].

H. pylori infection also induces the expression of proinflammatory Cyclooxigenase enzyme (COX-2), which has been described to inhibit apoptosis, to maintain cell proliferation and to stimulate angiogenesis within cancer cells, facilitating tumor growth [48].

Proteins of the TNF-α inducing protein (Tipα) gene family in *H. pylori* genome also contribute to the

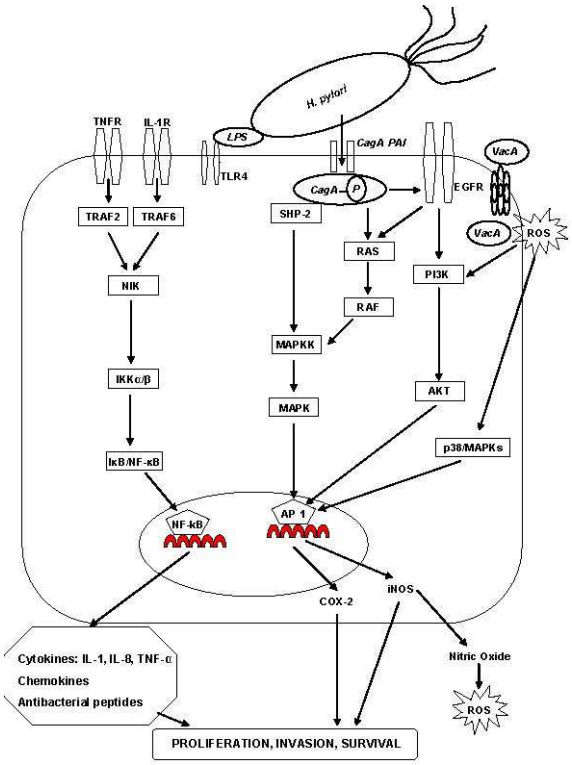

Figure 3. Molecular pathways activated by *H. pylori*.

inflammatory response induced by *H. pylori* and they have been described to strongly induce the expression of TNF-α and NFκB activation, acting as new carcinogenetic factors of *H.pylori* [49].

Inflammatory pathways associated with gastric cancer progression

Chronic inflammation develops through the action of several inflammatory mediators such as proinflammatory cytokines, chemokines and small chemical mediators. Among them, proinflammatory cytokines TNF-α, IL-1, IL-6 and IFN play essential roles [50]. The molecular pathways activated by these cytokines are discussed in more detail as well as the possible therapeutic targets for the treatment of gastric cancer. The molecular pathways activated by these cytokines are discussed in more detail as well as the therapeutic strategies, which essentially are based on blocking protein-protein interactions and on inhibiting the binding of transcription factors to DNA, for the treatment of gastric cancer.

IFN-γ

In the Th1 immune response induced by *H. pylori* IFN-γ-expressing T lymphocytes are primarily recruited [51]. IFN-γ has been suggested to have an important role in initiating the mucosal damage observed during gastritis due to the lack of response to *H. pylori* infection detected in a IFN-γ null mice model [52]. It has been also described that IFN-γ induces the release of the proinflammatory cytokines TNF-α and IL-1β and stimulates the release of gastrin [53]. IFN-γ signalling (Figure 4) is mainly mediated through the Janus tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) pathway [54]. In particular, IFN-γ induces the phosphorylation of Stat1, which translocates into the nucleus and binds to the γ-IFN-activated sequence (GAS) of target genes [55]. STAT1 has been reported to mediate IFN-dependent tumor suppressor activity by promoting apoptosis, cell cycle arrest, and

tumor surveillance [56,57]. However, more recently, using a mouse model, Ernst et al. have shown that germline mutation of Stat1 partially suppressed the growth of gastric tumors, and a reduction in gastric inflammation and STAT3 activation was observed [58], suggesting a new role for STAT1 in promoting gastric disease.

IFN-γ can also regulate several genes in a STAT-independent fashion. For instance, IFN-γ can activate AP-1 DNA binding via c-Jun, independently of JAK1 and STAT1. This pathway includes the MEK1/2-ERK1/2 module of the MAP kinase cascades [59]. The transcription factor AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis [60].

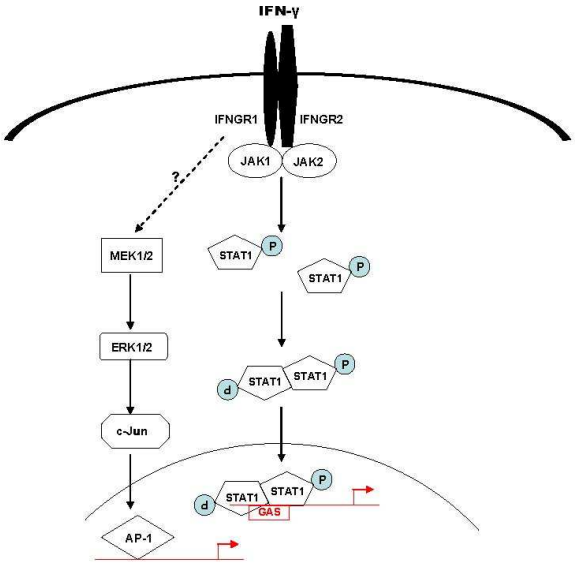

Figure 4. Schematic representation of IFN-γ-induced signalling

Different therapeutic approaches have been developed against components of the IFN-γinduced signalling pathways:

1) Raf-MEK-ERK signalling pathway has important roles in the development of several cancer types and some small molecules, inhibitors of protein kinases of the MAPK pathway have been developed to inactivate the Raf-MEK-ERK cascade in cancer cells [61,62].

-Raf inhibitors: Among them, sorafenib (BAY 43-9006) has been described as a potent Raf inhibitor and has been assayed in cancer animal models [63,64] and in several clinical trials in patients with different solid tumors such as breast, ovarian, prostate and pancreas cancer [65,66,67]. The inhibitory effect is produced through its union to the ATP-binding pocket of the Raf kinase domain preventing the binding of the substrate [68].

-MEK inhibitors: Several highly selective MEK inhibitors have been generated: PD98059, U0126, and CI-1040 [69,70,71]. Phase I and Phase II clinical trials have been performed with the CI-1040 molecule to define its toxicity and pharmacodynamics [72] and the efficacy in patients with advanced carcinoma [73]. Recently, a second generation of MEK inhibitors has been produced and some of them are under clinical studies. The most relevant are: PD0325901 [74] and AZD6244 [75] that are in Phase II studies in patients with breast, colorectal, lung, and pancreas carcinoma.

2) The JAK/STAT pathway activated by IFN-γ induces the phosphorylation of STAT1, which is lower in cancer cells. In tumors associated with inflammation the blocking of STAT1 will reduce inflammation but, at the same time, will improve tumor cell survival and will decrease the antitumoral activity. This dual behaviour makes this signalling pathway not a good target for cancer therapy.

TNF-α and IL-1

TNF-α is a strong tumor promoter [76], mainly produced by activated macrophages and lymphocytes during inflammation. In *H. pylori* patients increased levels of this proinflammatory cytokine have been detected [6,8], and its expression can be induced by urease, cagA, H. pylori-membrane protein-1 (HP-MP1) and Tipα protein [7,77,78,49]. Recently, Oguma et al. have shown that TNF-α derived from activated macrophages promotes the Wnt/β-catenin signalling activation in gastric cancer cells through the suppression of GSK3β, contributing in this way to gastric cancer [79]. However, TNF-α regulatory effects are mainly mediated through the NF-κB pathway.

IL-1β is a potent proinflammatory cytokine that contributes to the initiation and amplification of the inflammatory response to *H. pylori* infection [80]. IL-1 gene cluster polymorphisms have been reported to increase the risk of gastric cancer [35]. IL-1, up-regulated in *H. pylori* infection, is a potent acid inhibitor and induces several proinflammatory effects basically through NF-κB signalling.

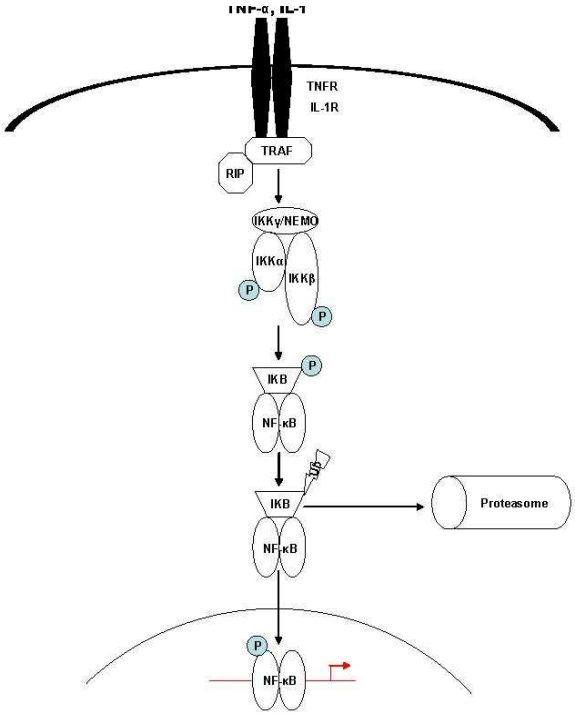

Figure 5. Schematic representation of NF-κB signalling pathway activated by TNF-α and IL-1.

After IL-1 and TNF-α binding TRAF6 or TRAF2 are, respectively, recruited to IL-1R or TNFR1. TRAF/RIP complexes trigger IKK activation leading to phosphorylation, ubiquitination and degradation of IκB

proteins that are associated with inactivated NF-κB. NF-κB free of IκB translocates to the nucleus, where it activates the transcription of target genes [81] (Figure 5). A large number of antiapoptotic factors such as cIAPs, c-FLIP, A20 and BclXL[82] as well as angiogenic factors (VEGF) [83] and proteins involved in invasion and metastasis (MMP-2 and MMP-9) [84] are activated by NF-κB. In gastric tumors constitutive activation of NF-κB has been detected [85].

Two different approaches can be assayed to inhibit the NF-κB pathway: 1) To inhibit upstream the NF-κB pathway, and 2) to directly inactivate the binding of NF-κB to DNA [86,87,88]. Numerous pre-clinical trials have been approved to test the inhibition of NF-κB in cancer cells.

1) Several strategies cab be used to inhibit upstream the NF-κB pathway:

-Suppression of the IKK – IκBα activation. The suppression of the IKKIκBα activation can be achieved by using small molecules, as the NBD peptides, to block the formation of the IKK complex. The NBD peptides inhibit the interaction of IKK-β with NEMO (NF-κB essential modulator) that corresponds to the IKK-γ binding domain. Some of these NBD peptides have been already tested in several murine models of inflammatory diseases such as bowel inflammatory disease, diabetes, and muscular dystrophy, using Protein Transduction Domains (PTDs) to deliver the NDB into the cell [89]. Also, some non steroidal anti-inflammatory drugs (NSAIDs) have been reported to be effective in the prevention and treatment of several cancer types by promoting apoptosis through direct interaction and inhibition of IKK-β kinase activity [90]. Among them, sulindac enhances the TNF-α mediated apoptosis in several human carcinoma cell lines [91], and in the MKN45 gastric cells the in vivo tumor growth was much more strongly inhibited by a combination of sulindac and TNF-α [92]. Sulfasalazine, used to treat inflammatory bowel disease, has been assayed in clinical trials in patients with malignant gliomas [93].

-Proteasome inhibitors. The inhibition of the proteasome function prevents the IκB degradation that is essential for the NF-κB nuclear translocation. A variety of peptide aldehydes have been designed to inhibit the protease activity of the proteasome. Among them, borzetomib, formerly PS-341, has been used in lung cancer and myeloma patients in phase II studies [94]. Also Cyclosporin A acts as an inhibitor of proteasome activity, preventing NF-κB activation [95].

-IκB-α-super-repressor SN50 prevents the nuclear translocation of NFκB. This repressor consists of a peptide containing the nuclear localisation sequence (NLS) of the p50 NF-κB subunit [96]. The adenoviral delivery of this IκB-α-super-repressor to chemoresistant tumors in mice induces the cells to undergo apoptosis resulting in tumor regression [97]. Recently, it has been suggested that SN50 inhibits invasion and migration of gastric cancer cell lines probably by the reduction of uPA and MMP9 proteins expression [98].

2) To block the binding of NF-κB to DNA, inducing a reduction in the transcriptional activity. The inhibition of NF-κB activity by glucocorticoids was firstly described by Auphan in 1995 [99]. Glucocorticoids as dexametasona and prednisone, interact with the steroid receptor to downregulate the expression of specific genes. The most consistent mechanism of action of glucocorticoids is the induction of the IκBα mRNA synthesis and, as a consequence, p65 is retained in the cytoplasm [99,100]. Nowadays, the design of new drugs, which selectively block NF-κB activity by binding the GR (glucocorticoid receptor) without affecting other glucocorticoid-regulated genes, is under study.

Synthetic double-stranded oligodeoxynucleotides (ODN), as decoy cis elements, have been developed to block the binding of NF-κB to the promoter regions of its target genes. The injection of decoy ODNs in mice tumor models reduced cachexia and inhibited hepatic metastasis [101]. In 5-FU resistant gastric tumor, decoy ODNs increases sensitivity to apoptosis and reduces the 5-FU resistance [102]. Finally, Rel A antisense oligonucleotides have been reported to inhibit tumorigenesis in nude mice [103].

NF-κB has a dual effect in immune response and in tumor promotion. As a consequence, its prolonged inhibition causes immunosuppression as well as tumor growth inhibition. Thus, a balance between the two effects must be considered in the design of therapeutic strategies, and the of NF-κB inhibitors probably should be used as adjuvants with other antitumoral treatments [104].

IL-6

IL-6 is a pleiotropic cytokine that is important for immune responses, cell survival, apoptosis and proliferation [105]. IL-6 initiates signalling by binding its specific receptor (IL-6Rα) that associates with and induces the homodimerization of the transducing receptor subunit gp130. As a result the JAK/STAT and the SHP-2/ras/MAPK/ERK1/AP-1 signalling cascades (Figure 6), which are in homeostatic balance under normal conditions, are activated [106]. The JAK/STAT pathway is activated after ligand binding due to autophosphorylation of the Janus kinases JAK1, JAK2 and Tyk2. Subsequently, the JAKs phosphorylate several specific tyrosine residues on the intracellular domain of gp130 that act as docking sites for molecules encompassing a Srchomology-2 (SH-2) domain, particularly STAT transcription factors 1 and 3. STAT proteins are also tyrosine phosphorylated, form homo-or heterodimers and translocate to the nucleus where they activate the transcripition of target genes [107]. A significant increase in STAT3 activation has been detected in *H. pylori* infected patients as well as in adenocarcinoma [108], and it has been proposed as a prognostic factor for poor survival of gastric cancer patients [109]. Elevated STAT3 phosphorylation correlates with overexpression of its target genes involved in suppressing apoptosis (Bcl2l1), promoting angiogenesis (Vegfa, Mmp9) and proliferation (Myc) [110]. IL-6 can also regulate other genes though activation of the tyrosine phosphatase SHP-2, which is recruited to tyrosine 759 of gp130 receptor and phosphorylated by JAK1/2, leading to binding of the adapter molecules Grb2 and SoS and recruitment of Ras. A series of MAP kinases are activated ending in gene regulation by AP-1 transcription factors [111]. The negative regulator of STAT3 activation, suppressor of cytokine signalling (SOCS3), also binds the same tyrosine residue on gp130 as SHP-2 contributing to the balance between the two pathways activated by IL-6. Different mouse models have been developed to study the consequences of disrupting the balance between the STAT1/3 and SHP2/Erk pathways [58,112,113,24]. Transgenic mice presenting mutated receptor gp130

developed distal stomach tumors as a consequence of the loss of gp130-dependent SHP-2/Erk signalling and increased STAT3 signalling, which was accompanied by a downregulation of the stomach-specific tumor suppressor gene TFF1 [24]. Recently, IL-6 has been also reported to regulate the expression of the intestinal mucin MUC4 in gastric cancer cells [114], which have been described to be involved in cancer cell signalling, tumor growth and metastasis [115].

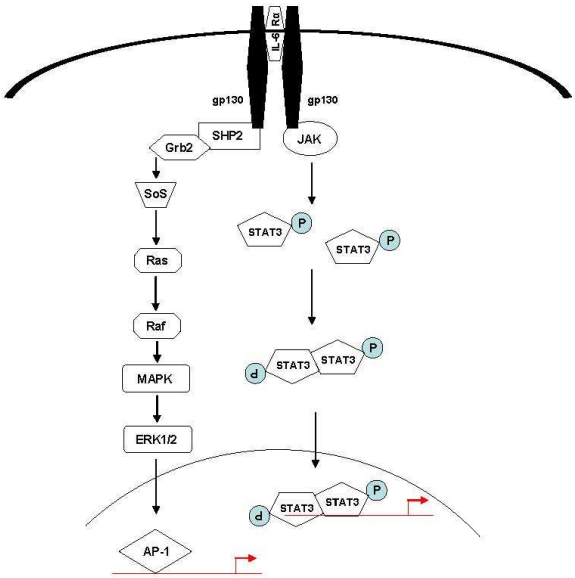

Figure 6. Schematic representation of IL-6-induced signalling pathways.

The inhibition of STAT3 signalling, aberrantly hyperactivated in tumors, induces tumor cell apoptosis and tumor regression, and can be used as therapeutic tool in cancer. The approaches to inactivate STAT3 are directed at different levels of the STAT3 activation pathway, as: 1) to inactivate c-Src kinase, 2) to inhibit PKC activity, 3) to prevent STAT3 dimerization, and 4) to block the STAT3-DNA binding.

1-Indirubin is the active compound of a chinese herbal medicine, and it has been reported as inhibitor of cyclin-dependent kinases [116] and used in the treatment of the chronic myelogenous leukaemia [117]. Several indirubin derivatives (IRD) [118] have been obtained by adding different substituents to a common core structure. Among them, E804 and E728 with an unbranched short-chain oxime ethers with one or two hydroxyl groups, have been demonstrated to block the Stat3 signalling by the induction of apoptosis in breast and prostate cancer cell lines [118]. Moreover, it has been suggested that E804 blocks Stat3 signalling by inhibiting up-stream c-Src kinase activity as it occurs with the PD180970 IRD [119]. The antiapoptotic genes, Mcl-1 and Survivin, have been suggested to be the Stat3 target genes inactivated by the E804 treatment.

2-Resveratrol, trans-3,5,4'-trihydroxystilbene, is a natural phytoalexin abundant in red grape products with antioxidant and antiinflammatory properties [120]. In cancer cells, it acts as antiproliferative and pro-apoptotic agent and it is able to inhibit tumor growth in animals [121]. Although the mechanisms involved in these biological effects have not been fully characterised, it has been reported that resveratrol acts in the cellular signalling pathways through the inhibition of the PKC activity, especially PKCα. In gastric cells, it has been shown that resveratrol induces changes in the expression of PKC α and δ, upregulation of p53 and p21 as well as cell cycle arrest and apoptosis [122]. Recently, these effects have been associated, in part, to the inhibition of Src tyrosine kinase and the repression of downstream aberrant Stat3 activation in malignant cells with constitutively active Stat3 [123]. In gastric cancer cell lines, there are some studies suggesting that the apoptotic signals engaged by this molecule may be related to their differentiation status [124]. Moreover, resveratrol has been reported to be effective in the inhibition of *H. pylori* replication, adding an additional reason for its use in the prevention and treatment of gastric cancer [125]. However, additional work is needed to fully evaluate the antitumoral effects of the resveratrol.

3-Peptidomimetic inhibitors of Stat3 are small molecules able to bind the SH2 Stat3 domain, which have been designed using the x-ray structural data and computational modelling. The dimerization of the STAT3 molecules is a critical step in STAT3 activation, and it takes place between the SH2 domain of one monomer and a consensus sequence in the other monomer. The peptidomimetic ISS 610 has been shown to have good selectivity against Stat3, inhibiting signalling in Src-transformed mouse fibroblast and in human breast and lung carcinoma cells. In vivo, it induces growth inhibition and apoptosis [126]. Also, the S3I-201 peptidomimetic induced the same biological effects and caused human breast tumor regression in mouse xenograft models [127]. New approaches to target the homodimerization by using peptidomimetics inhibitors at low micromolar concentrations are now being developed [128].

G-quarter oligonucleotides are G-rich oligodeoxynucleotides, which form intramolecular G-quartet structures, and have been developed as inhibitors of Stat3. Once delivered in the cytoplasm and nucleus of the cancer cells, they interact with the SH2 domain of Stat3 inhibiting its dimerization and the binding to DNA [129]. G-quartet oligodeoxynucleotides (GQ-ODN) have been assayed in prostate, breast [130] and non-small cell lung cancer [131] tumor mouse models, demonstrating the capacity to suppress tumor growth through the induction of apoptosis.

4-Cisplatin and novel platinum complexes have been used as active antitumor molecules due to their alkylating effects on DNA and their possible interactions with proteins. Cisplatin and other platinum complexes have been reported to be able to modulate signal transduction pathways, being the mechanism underlying their biological effects a persistent activation of both JNK1 and ERK1/2 [132,133]. Recently, two platinum (IV)-containing complexes, CPA-1 and CPA-7, and platinum (IV) tetrachloride (Pt(IV)Cl4) have been identified to interfere with Stat3, disrupting its ability to bind to DNA in vitro [134]. As a consequence, the tumor cells that harbour constitutively active Stat3 show growth inhibition and apoptosis, indicating in part the inhibition of the Stat3 signalling. As a consequence, regression of mouse colon tumors with constitutively active Stat3 upon platinum (IV) compounds treatment has been observed [134].

Conclusion

The link between *H. pylori* infection, inflammation and gastric cancer has open new perspectives in the prevention and treatment of gastric cancer. The characterisation of the activation pathways that lead to the expression of genes involved in this carcinogenetic process must be useful in the design of new therapeutical targets for gastric tumors. The eradication of *H. pylori* and the associated gastritis are the most useful strategy in the prevention of gastric cancer. However, in advanced gastric pathologies and gastric carcinomas, the targeting of the signalling pathways activated by inflammatory cytokines in the steps that precede the development of tumors is, without doubt, important to generate therapeutic tools for gastric cancer.

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