Inmunohistochemical expression of p53, Bcl-2, COX-2, C-erb-B2, EPO-R, β-catenin, and E-cadherin in non tumoral gastric mucous membrane

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The stomach possesses a highly specialized mucous membrane lined with a simple prismatic epithelium which forms mucosal glands. Depending on the glandular microscopic structure, the types of secreting mucus cells, and their distribution in the glands, three gastric regions are distinguished: cardia, fundus and pylorus. Cells in the lining epithelium and cells in the pits are similar, they being specialized in different neutral mucins. In the stomach glands, four different types of cells are found: neck mucous cells, secreting neutral and acid mucins (sialomucins); parietal cells that produce chlorhydric acid and intrinsic factor; chief cells (or zymogen cells) involved in pepsinogen production; and cells producing different kinds of hormones, such as gastrin, serotonin and somatostatin, called endocrine cells (Owen, 1997). In the last decade, different authors have investigated the immunohistochemical expression of some proteins in the adenocarcinoma of the stomach, including cell cycle regulators proteins like p53 and Bcl-2; growth factors (c-erb-B2 and EPO-R); angiogenesis-related markers such as COX-2; and cellular adhesion molecules (i.e. E-cadherin and β-catenin) (Wang, 2002; Nagashima, 2005; Kopp, 2005; Liu, 2005; Cheng, 2005), and their expression in tumor specimens has been found to correlate with several clinical and pathological features (Owen, 1997).

While these proteins have been studied in gastric adenocarcinoma, their immunophenotyping in non tumoral gastric mucous membrane remains unexplored. Very few studies have addressed the immunostaining of these molecules in non tumoral gastric epithelium (Krishnadath, 1995; Saegusa, 1999; Ohgushi, 2005; Krajewski, 1994; Wallace, 2005; Hull, 1999; Chailer, 2005).

In the present study, we investigated the expression, function and behavior of these proteins in normal gastric mucous membrane to contribute to gain further knowledge on the significance of their loss or overexpression in malignant gastric tumors.
Materials and Methods

Patient selection and clinical data
In this study, 44 surgical specimens from partial or total gastrectomy were selected at the Department of Pathology of Hospital Universitario La Paz (Madrid, Spain) between January 2000 and July 2003. The patients’ mean age was 62.5 years, and sex distribution showed male predominance (75% vs. 25%).

Tissue processing and histopathological analysis
All the gastrectomy specimens were grossly described by two expert pathologists. The specimens were fixed in 10% neutral formalin for 24 hours. After fixation, representative samples of gastric mucous membrane were embedded with paraffin and serially sectioned at 5-µm. Then, three sections from each block were stained with haematoxylin and eosin, PAS and Giemsa. The remaining sections were reserved for immunohistochemistry.

Immunohistochemistry
Seven antibodies among several commercially available antibodies were selected. Table 1 shows the characteristics of antibodies used in the present study. Slides were treated with acid buffer to recover the antigen activity. As a second antibody, a polyvalent serum with labelled biotin (Vector Burlingame, Ca, USA) was used. For second antibody, biotin and avidin-biotin-phosphatase alkaline complex was utilized (Vectorstain ABC kit, Vector Burlingame, Ca, USA) in β-catenin and E-cadherin. For the other antigens under study, complex avidin-biotin-peroxydase was utilized (Vectastain Universal Quick Kit, Vector Burlingame, Ca, USA). The immunostaining of the different antibodies was described in gastric mucous membrane. Two independent pathologists evaluated selected samples. In all cases, the built-in negative control was done in each case by omission of the primary antibody p53. Positive cell number was determined by counting 100 cells within 5 areas. p53 overexpression in breast cancer sample was used as positive control (Yamashita, 2006). Positive immunostaining was considered when more than 25% of nuclei showed positive stain. Intensity of reaction was categorized in weak positive (+), moderately positive (++) and strongly positive (+++) (Krishnadath, 1995).

Bcl-2. The criterion for positivity was as follows: positive nuclear staining of bcl-2 in at least 25% of cells. Positive cell number was determined by counting 100 cells within 5 areas (Krajewski, 1994; Saegusa, 1995). Mucous lymphoid follicules were considered positive control (Zafiridis, 2005).

Cox-2. Positive staining was considered when more than 50% of cells in gastric mucous membrane was found. Positive cell number was determined by counting 100 cells within 5 areas (Wallace, 2005). A breast carcinoma sample with COX-2 overexpression was used to positive control (Nakopoulou, 2005).

C-erb-B2. The criterion for positivity for this antibody was as follows: positive membrane staining but with no stain in cytosol. When 25% of cells in gastric mucous membrane showed a positive staining, it was considered to be positive. Positive cell number was determined by counting 100 cells within 5 areas (Hanby, 2005). Slides of breast carcinoma with an intense staining of HER-2 (3+, FISH positive) was used as positive control (Barrett C).

EPO-R. Positivity was considered when more than 50% of gastric mucous membrane cells were stained. EPO-R intensity expression differed for different cell types: weakly positive (+), moderately positive (++) and strongly positive (+++). Positive cell number was determined by counting 100 cells within 5 areas. Slides of kidney was used as positive controls (Sasaki, 2003).

β-catenin and E-cadherin. Positive immunostaining was considered when more than 50% of mucous membrane cells were positive in both cases. Intensity expression was not evaluated because it was similar in all cases. Positive cells number was determined by counting 100 cells within 5 areas (Mizuno, 2001). E-cadherin and β-catenin conserved expression in gastric carcinoma mucosa were used as positive controls (Nakamura, 2005).

Results
Different cell types were identified in the 44 samples of gastric mucous membrane. Non-significant histological features were not considered. Every type of mucus-secreting cells (surface epithelial, pit, and mucous neck cells) showed similar immunostaining. Neuroendocrine cells were not studied. Samples of normal gastric mucosa after hematoxilin and eosin staining, and after PAS reaction are given in Figure 1A, B and Figure 1C, respectively. Table 2 shows the results from immunohistochemi-
Figure 1. A: Gastric mucosa 4x. HE. B: Gastric mucosa 40x. HE. C: Gastric mucosa 20x. PAS. D: Bcl-2 immunostaining 20x. E: p53 immunostaining 40x. F: COX-2 immunostaining 40x. G, H, I: C-erb-B2 immunostaining 4x, 20x superficial glands, 20x deep glands, respectively.
Bcl-2 (Figure 1D). None mucus-secreting cells showed positive immunostaining for Bcl-2. However, cells in supportive tissues, such as capillary endothelium, exhibited positive cytoplasmatic immunostaining with similar intensity. In addition, lamina propria lymphocytes showed intense cytosolic stain.

p53 (Figure 1E). It was negative in the immunochemical analysis in all gastric mucous membrane cells, but, in a limited area in the neck of the mucus-secreting gland, a weak focal nuclear staining was noted. Other cell types in gastric mucous membrane, lamina propria or submucosa showing positive staining were not found with this antibody.

COX-2 (Figure 1F). Immunohistochemical staining for COX-2 was very interesting. All gastric mucous membrane cells show positive stain, as did mucus-secreting, parietal and chief cells. Basal cytoplasm in mucus-secreting cells showed positive immunostaining as did secreting canaliculi in parietal cells, this being highly specific for this protein. Macrophage cells in areas with inflammatory reaction showed weak stain for cytosal as did endothelial cells.

C-erb-B2 (Figure 1G-I). Cytosol immunostaining was found in some cells in gastric mucous membrane, notably in mucus-secreting and chief cells. Selectively, parietal cells were negative for this antibody. However, connective tissue cells, endothelial cells, and lymphocytes were negative.

EPO-R (Figure 2A-C). All cells in gastric mucous membrane showed positive immunostaining with different intensity. Mucus-secreting cells exhibited weak stain (+), and parietal cells were moderately (++) stained, whereas chief cells showed strong immunostaining (+++). Macrophage cells and fibroblasts were not stained for this antibody. Endothelial cells and cytosal neurons displayed moderate stain for EPO-R.

E-cadherin (Figure 2D-F) and β-catenin (Figure 2G-I) Both proteins showed a similar immunohistochemical expression. All cells in gastric mucous membrane were stained. No differences in intensity expression were found. In this case, endothelium also showed positive immunostaining.

**Discussion**

Gastric adenocarcinoma is one of the most common malignant gastric tumors and one of the leading causes of death from cancer worldwide. A number of authors have reported a correlation between immunoeexpression of Bcl-2, p53, COX-2, c-erb-B2, E-cadherin and β-catenin and several clinicopathological variables in gastric adenocarcinoma.

While medical literature includes a large number of studies addressing immunoeexpression of these antibodies in tumoral mucous membrane, studies on immunostaining patterns in non tumoral mucous membrane are scanty (Owen, 1997; Nagashima, 2005). The behavior of these proteins in the gastric epithelium is of interest in order to better understand their behavior in pathological conditions. Thus, it has been found not only quantitative differ-
Figure 2. A, B, C: EPO-R immunostaining 4x, mucosa cells 40x, chief cells 40x, respectively. D, E, F: E-cadherin immunostaining 4x, 20x and 40x, respectively. G, H, I: β-catenin immunostaining 4x, 20x and 40x, respectively.
ence in staining intensity between the normal and
tumoral gastric mucous membrane but also differ-
ences in location (membrane vs. cytoplasm), as is
the case for E-cadherin and β-catenin.
In the present study, regulatory proteins for the
cell cycle (i.e., p53 and Bcl-2) showed, respectively,
a nuclear and cytoplasmatic staining pattern. For
the former, increased staining of the cells located at
the secretory gland neck was noted, this being an
area characterized by highly secretory activity,
since in this area mucus-secreting cell replication
takes place, which would explain the high activation
of p53. This fact underscores the important role
played by this protein in the gastric mucous mem-
brane renewal. Bcl-2 is a key protein for cell apo-
ptosis. It exerts antiapoptotic activity, thereby
increased Bcl-2 activity would promote uncon-
trolled cell proliferation. As it was the case for p53,
this antibody did not stain non tumoral gastric
mucous membrane cells; however, it did stain some
cells in supportive tissues, such as the endothelium.
From our findings, it would seem that Bcl-2 pres-
ence in the gastric mucous membrane plays an
important role in the neovessel formation that
accompanies mucosal epithelium proliferation.
Cyclooxygenase-2 (COX-2) is a protein of
increasing interest for histopathologists. This pro-
tein is included in the cyclooxygenase family, which
is implicated in the transformation of arachidonic
acid into the different tissue prostaglandins. Within
this family, three homologue proteins have been
described (COX-1, COX-2, and COX-3). COX-1,
COX-2, and COX-3 share a homologue structure in
60%, and are synthesized by highly regulated
genes. COX-1 expression is constituent and allows
the maintenance and integrity of gastric epithelium
that is subject to continual regeneration. In con-
trast, COX-2 shows an expression that is inducible
in response to growth factors (EGF, VEGF, FGF2),
cytokines (TNF, IL-6) and tumor promoters (via
Wnt, HER-2, v-src, ect). Activated macrophages, as
well as synoviocytes (cells actively involving in
inflammatory processes) express the latter enzyme
all the time. Apart from pathologic conditions,
COX-2 plays a key role in normal physiologic
processes, such as ovulation, implantation, perinatal
kidney development, and ductus arteriosus remod-
eling (Honjo, 2004; Leung, 2003).

The relationship of COX-2 to gastric carcinogen-
thesis has been extensively studied, and tumors
expressing high levels of this enzyme have been
reported to be more aggressive (Leung, 2003;
Koga, 2004; Honjo, 2004). However, studies on this
molecule expression in the non tumoral gastric
mucous membrane are scarce. In the present study,
it was found a predominant membrane staining in
all gastric mucous membrane cells: mucus-secret-
ing cells (basal cytoplasm), parietal cells (including
their secretory caniculum), and chief cells. This
finding contrasts with the cytoplasmatic expression
of this antibody in tumor cells, in which is overex-
pressed. This fact has been observed not only in gas-
tric cancer but also in some cases of breast cancer.
In a recent study, other authors have addressed
COX-2 expression in breast cancer and surrounding
tissue without tumor cell (Koga T, 2004), and they
found differences in staining pattern, this being
membranic in the normal tissue and cytoplasmatic
in the tumor. An explanation for this finding would
be that pathologic overexpression in the tumor is
higher than that in non tumoral tissue, which would
lead to an accumulation of the excess of protein in
the cytoplasm. However, in the non tumoral mucous
membrane, this increment in expression would be
caused by inflammatory processes which would not
induce such a marked COX-2 accumulation. In fact,
some authors have found that this protein overex-
pression increases in parallel to malignancy grade
of the lesion under study. Thus, in non tumoral
epithelium, COX-2 overexpression would be lower
than that in metaplasia, and, in the latter, would be
lower than that in dysplasia and adenocarcinoma

In a similar way to that for Bcl-2, the endothel-
ium was stained focally. This phenomenon would
reflect the key role played for this protein in physi-
ologic angiogenesis. Several authors have studied
the correlation of COX-2 and angiogenesis mecha-
nisms, which would explain our finding (Leung,

One of the proteins studied in the present investi-
gation was HER-2. This protein is included in the
family of growth factors with tyrosine-kinase activ-
ity, which also includes the epidermal growth factor
receptor (EGFR). All mucosal cells in the gastric
epithelium showed weak stain of the basal cyto-
plasm (1+) and moderate stain of chief cells (2+),
whereas parietal cells in a selective manner were
not stained for this antibody. These findings suggest
that Her-2 overexpression may play an important
role in chief cell function in relation to specialized
protein synthesis. Some authors believe that this
marker overexpression in the tumoral gastric mucous membrane may be of interest from a prognostic point of view, as is the case in breast cancer (Koga, 2004).

EPO-R role in gastric cancer has been hardly studied. In the present investigation, all normal gastric mucous membrane cells were positively stained for EPO-R. Nevertheless, each component showed different intensity. Thus, mucosal cells showed weak stain (1+), parietal cells moderate stain (2+), and chief cells intense stain (3+). Such a difference in immunostaining is likely to be due to protein content of each type of cell, which, in turn, may be related to differential underlying complexity of cell types. Thus, chief cells are the most highly specialized cells in the gastric mucous membrane, which would account for the fact that they were the most intensely stained for EPO-R, and this would hold true for other markers under study, such as HER-2.

On the contrary, epithelial cells are the least complex ones structurally and functionally, which would explain the fact that these cells were stained less intensely.

Some authors suggest the existence of a relationship between EPO-R expression and tumor angiogenesis. Thus, Ribatti et al., showed that EPO-R expression and tumor angiogenesis are related in different pathologic conditions. Some studies have suggested that EPO-R expression is increased in the gastric mucosa of patients with inflammatory conditions such as ulcerative colitis and Crohn's disease. However, the studies of EPO-R expression in normal and tumor gastric mucosa are contradictory. Some authors have found increased EPO-R expression in gastric tumors, while others have found no significant differences.

The expression of the adhesion molecules β-catenin and E-cadherin has been extensively studied in both normal and tumor gastric mucous membrane. E-cadherin is a protein involved in intercellular adhesion. Its intracellular domain anchors in the cytoskeleton thanks to the action of catenins. Within the family of catenins, the most important one is β-catenin, whose cytosolic fraction has the ability to act as a transcriptional factor when accumulated in the cytoplasm. The gastric mucous membrane epithelium, on the contrary, shows a preserved membrane expression, as several authors have demonstrated. This finding underscores the importance of these proteins for normal tissue architecture maintenance. In fact, normal architecture loss have been related to mechanisms of carcinogenesis and tumor progression. Therefore, while β-catenin disturbances have been associated with carcinogenesis through a dual mechanism (i.e., transcriptional factor and adhesion loss) (Koga, 2004), decreased E-cadherin expression has been implicated in tumor progression. The results from several studies on gastric cancer indicate that E-cadherin loss or disturbed β-catenin expression (decreased, cytoplasmatic, or nuclear) are associated with the diffuse subtype of gastric cancer.

In summary, in the present investigation we have carried out a descriptive study of the immunohistochemical expression in the normal gastric mucous membrane epithelium of various proteins implicated in carcinogenesis and tumor progression. Our findings suggest that the immunostaining of such proteins in the gastric mucous membrane depends on functional factors, which allows further insights in their behavior in the pathologic tumor or inflammatory mucous membrane. Further investigation is needed in order to better understand pathophysiology of gastric mucous membrane.

References


