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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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 (β-catenin and E-cadherin). While these proteins have been studied in gastric adenocarcinoma, their immunophenotyping in non tumoral gastric mucous membrane remains unexplored. 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.

Key words: inmunohistochemical expression, non tumoral, 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: cardias, 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 clorhydric 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 required (Vectastain 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 (Zafirellis, 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-

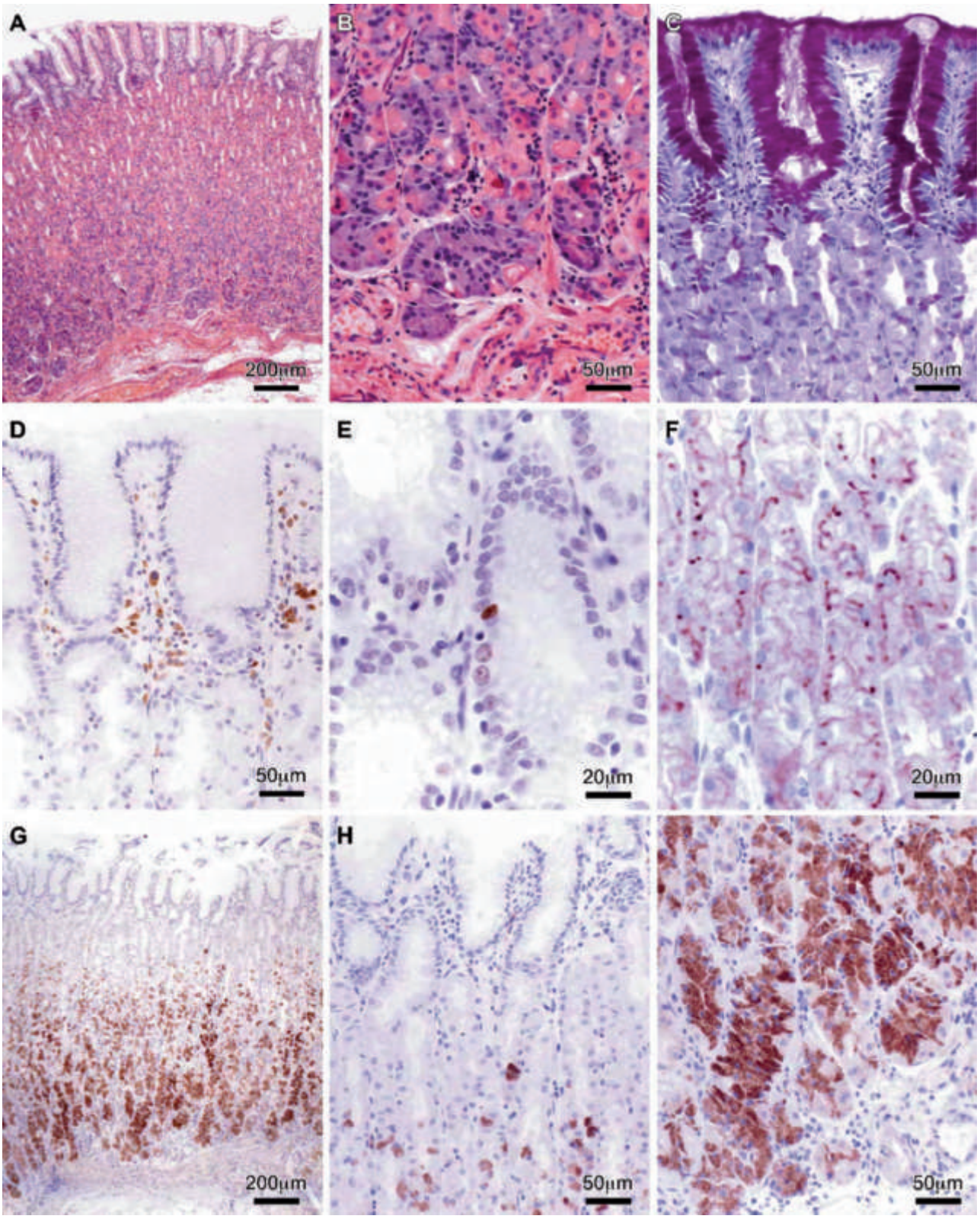

Figure 1. A: Gastric mucosa 4x. HE. B: Gastric mucosa 40x. HE. C: Gastric mucosa 20x. PAS. D: Bcl-2 inmunostaining 20x. E: p53 inmunostaining 40x. F: COX-2 inmunostaining 40x. G, H, I: C-erb-B2 inmunostaining 4x, 20x superficial glands, 20x deep glands, respectively.

Table 1. Characteristics of the antibodies used for immunohistochemical analysis.

Antigen	*Antibody*	*Laboratory*	*Ig type*	*Antigen retrival*	*Incubation*	*Dilution*
P53	DO-7 (mouse)	Novocastra	IgG2b	Stove	Ab1: 30' Ab2: 30'	1/100
Bcl-2	Clon124 (mouse)	Dako	IgG1	Stove	Ab1: 1h Ab2: 30'	1/50
COX-2	Monoclonal	Caiman		Cooker	Ab1: 1h Ab2: 30'	1/250
c-erbB-2	Polyclonal (rabbit)	Dako	IgG	Stove	Ab1: 1h Ab2: 30'	1/200
EPO-R	Polyclonal	SantaCruz	IgG	Stove	Ab1: 30' Ab2: 30'	1/200
β-catenin	C19220	Transducción	IgG1	Stove	Ab1: 1h Ab2: 30'	1/2000
E-cadherin	Clon36 (mouse)	Transducción	IgG2a	Stove	Ab1: 1h Ab2: 30'	1/3000

Table 2. Intracellular location of immunostaining for antibodies under study.

Tipo celular	*Bcl-2*	*p53*	*COX-2*	*c-erb-B2*	*EPO-R*	*β-catenin*	*E-cadherin*
Neck mucous cells	N	N	cyto	memb	Memb +	Memb	Memb
Parietal cells	N	N	cyto	N	Memb ++	Memb	Memb
Chief cells	N	N	cyto	memb	Memb +++	Memb	Memb

N: Nucleus, Cyto: Cytosol, Memb: Membrane

cal analysis.

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 propia lymphocytes showed intense cytosolic stain.

p53 (Figure 1E). It was negative in the immunohistochemical 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 propia 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 cytoplasma 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 cytosol 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 cytosol 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 commonest malignant gastric tumors and one of the leading causes of death from cancer worldwide. A number of authors have reported a correlation between immunoexpression 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 immunoexpression 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-

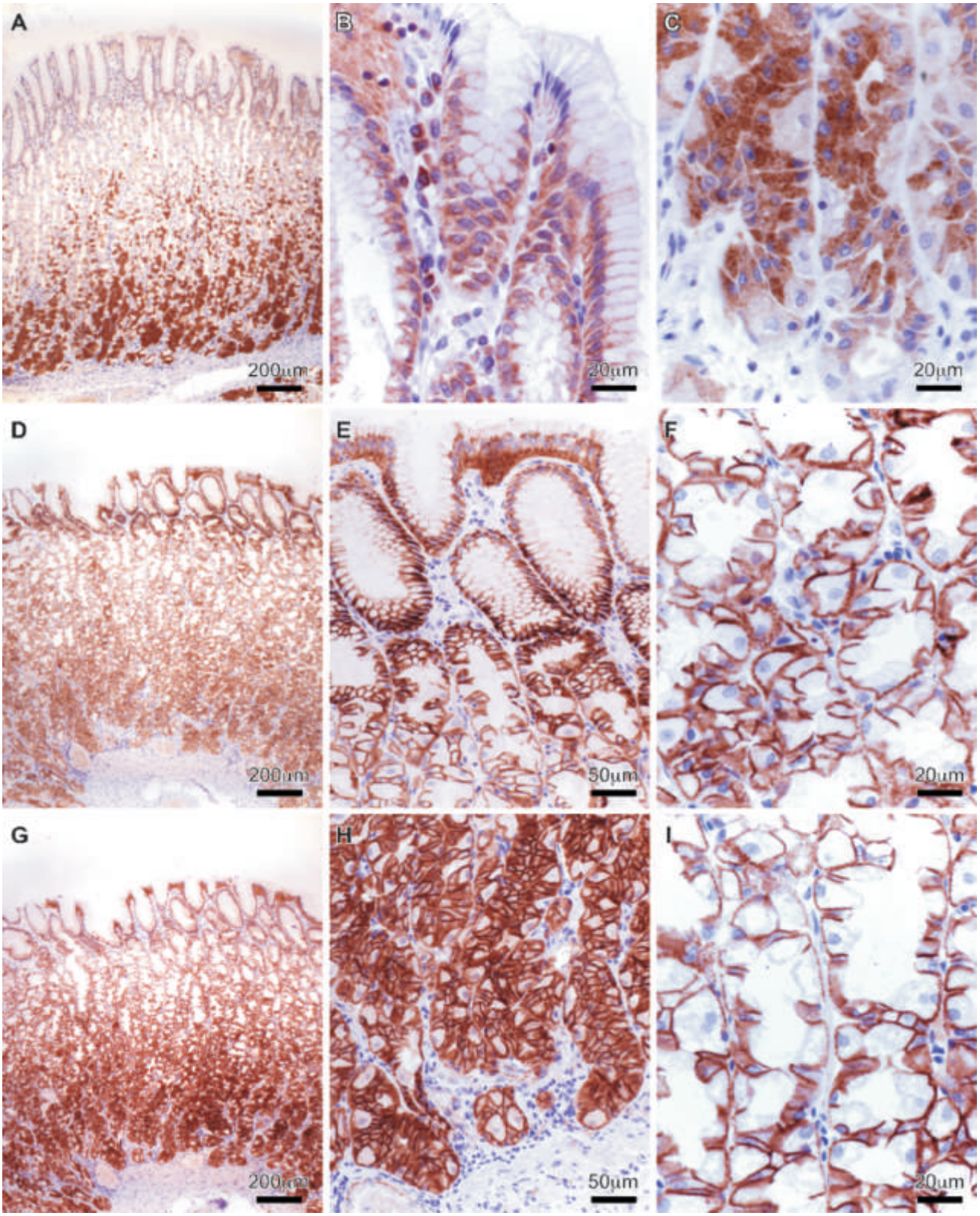

Figure 2. A, B, C: EPO-R inmunostaining 4x, mucosa cells 40x, chief cells 40x, respectively. D, E , F: E-cadherin inmunostaining 4x, 20x and 40x, respectively. G, H, I: β-catenin inmunostaining 4x, 20x and 40x, respectively.

ences in staining intensity between the normal and tumoral gastric mucous membrane but also differences 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 membrane renewal. Bcl-2 is a key protein for cell apoptosis. It exerts antiapoptotic activity, thereby increased Bcl-2 activity would promote uncontrolled 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 presence 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 protein 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 contrast, 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 remodeling (Honjo, 2004; Leung , 2003).

The relationship of COX-2 to gastric carcinogenesis 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-secreting 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 overexpressed. This fact has been observed not only in gastric 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 overexpression 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 (Leung , 2003;Koga, 2004; Honjo, 2004).

In a similar way to that for Bcl-2, the endothelium was stained focally. This phenomenon would reflect the key role played for this protein in physiologic angiogenesis. Several authors have studied the correlation of COX-2 and angiogenesis mechanisms, which would explain our finding (Leung, 2003; Koga, 2004; Honjo, 2004).

One of the proteins studied in the present investigation was HER-2. This protein is included in the family of growth factors with tyrosine-kinase activity, which also includes the epidermal growth factor receptor (EGFR). All mucosal cells in the gastric epithelium showed weak stain of the basal cytoplasm (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 immunoexpression 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 levels correlate to both angiogenesis and tumor progression in patients with gastric cancer, which suggests that EPO administration to these patients may exerts a trophic effect on gastrointestinal tract vasculature (Ribatti, 2003; Koga, 2004).

The expression of the adhesion molecules β-catenin and E-cadherin has been extensively studied in both normal and tumoral gastric mucous membrane. E-cadherin is a protein involving 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 suggests that the immunoexpression of such proteins in the gastric mucous membrane depends on functional factors, which allows further insights in their behavior in the pathologic tumoral or inflammatory mucous membrane. Further investigation is needed in order to better understand pathophysiology of gastric mucous membrane.

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