Differential expression of laminin isoform (α2), integrins (α3β1 and α6β4) and cytokeratin 20 in H. pylori gastritis

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Summary. The expression of laminin-1 chains (β1 and γ1), laminin-2 (merosin), integrin receptors to laminin (α3β1 and α6β4) and cytokeratin (CK20) were studied by immunohistochemical methods in gastric biopsies from antrum of 25 patients. H. pylori gastritis was found in 19 cases and intestinal metaplasia (IM) in four from these 19. Another 13 biopsies, all with IM were immunostained to laminin-2. Laminin-1 chains in normal and gastritis areas without IM were expressed as a strong, linear and continuous deposit in the basement membranes of the superficial and glandular epithelium. In metaplastic glands the reactivity to laminin-1 chains was decreased. Merosin was discontinuous when a moderate to accentuated H. pylori glandular colonization was present. Samples with IM were negative to laminin-2. The α3β1 and α6β4 integrins were negative only in IM gastric biopsies. The CK20 immunoreactivity was strong and homogeneous in the cells at the tip and the strong and homogeneous in the cells at the tip and the parallel both to CK20 expression in H. pylori chronic gastritis.

Key words: Laminins chains, Cytokeratin, Helicobacter pylori gastritis, Integrins

Introduction

The processes of development, cell growth and cell differentiation are closely related with the interactions between cells and the extracellular matrix, which is composed by interstitial components and basement membrane (BM) (Ekblom et al., 1986). BMs are specialized, sheet-like, extracellular matrix structures that separate the connective tissue from epithelia, muscle fibers, blood vessels and nerves (Foeldart et al., 1980; Lissitzky et al., 1986; Edds, 1997). In recent years, the importance of extracellular matrix components, especially those located at the epithelial-mesenchymal interface, has been illustrated in the developing human small intestine (Haffen et al., 1989; Beaulieu et al., 1991). Laminins are the major BM glycoproteins throughout the body, composed by 3 chains (α1-5; β1-3; γ1-2) assembled into heterotrimeric isoforms (Chung et al., 1979; Timpl et al., 1979; Burgeson et al., 1994; Miner et al., 1997). Laminins arise very early during the development, and play an important role in the interactions and in the maintenance of gland and epithelia adult state (Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994; Perreault et al., 1995). In particular, merosin, or laminin α2 chain, is restricted to developing the pit-gland structure of the forming glands during gastric development up 20 weeks of gestation (Tremblay and Ménard, 1996), and in the normal adult state is confined to the gastric and intestinal glandular basement membranes (Simo et al., 1991; Virtanen et al., 1995; Tremblay and Ménard, 1996). However, little is known about the composition and distribution of the different BM components associated with the developing gastric mucosa and mature gastric glands (Tremblay and Ménard, 1995, 1996).

Integrins are heterodimeric cell surface glycoproteins that mediate divalent cation-dependent cell-cell and cell-matrix interactions (Buck and Horwitz, 1987; Hynes, 1987). They are composed of a single α subunit associated with a single β subunit and this association is required for cell surface expression. The interactions mediated by integrins are responsible for certain typical properties of adhesive cells, such as attachment and migration, but these molecules are also recognized to contribute to intracellular signaling processes (Hynes, 1992; Humphries, 1999). Amongst the laminin binding integrins, the α3β1 integrin binds laminin 2, 4 and 5 (Rousselle and Aumailley, 1994) and cannot play a major role in cell adhesion to laminin-1 (Aumailley et al., 1990). The integrin α6β4 has been
studied by immunoelectron microscopy and its distribution has been described at the basal side of several epithelial cell types, suggesting that this integrin plays a role in polarity and adhesion of epithelial cells to the BM (Marchisio et al., 1991). Several works demonstrated that α6β4 polarization is lost upon tumorigenic transformation of epithelial cells (Rabinowitz and Mercurio, 1996).

Keratin is the most complex of intermediate filaments and belongs to a great family of related proteins that vary in molecular weight and are coded by a complex set of genes, expressed in different types of epithelium that contain distinct keratin profiles (Franke et al., 1981). Cytokeratin 20 (CK20), was specifically detected as a component of intestinal and gastric foveolar epithelium (Moll et al., 1990, 1993) and remains unchanged even during intestinal metaplasia or malignant progression (Moll et al., 1993; Schwerer and Baczko, 1996).

H. pylori has been considered as a causal agent of chronic gastritis in humans and may be associated with gastric cancer (Valkonen et al., 1994; Solcia et al., 1996). Several works have emphasized that the adhesion of *H. pylori* to laminin disorganizes the gastric epithelium altering the interactions between laminin and laminin receptor in gastric epithelial cells (Slomiany et al., 1991; Valkonen et al., 1994; Sales et al., 1998). The mucosal BM has been considered to play a role in the high capacity of mucosal repair after injury (Mikami et al., 1994), but the way gastric BM component expression occurs during injury, remains unknown.

We decided to detect, by immunohistochemical methods, the expression of β1, γ1, and α2 laminin chains, their integrin receptors α6β4 and α3β1 and cytokeratin 20 (CK20) in the gastric mucosa with *H. pylori* chronic gastritis. Aim: to investigate whether the inflammatory and metaplastic process caused by *H. pylori* alters the expression of BM extracellular molecules and CK20 intermediate filament.

Materials and methods

**Gastric samples**

Samples of antral gastric mucosa from 25 patients with chronic gastritis were obtained by endoscopic means at the University Hospital of UFRJ, Rio de Janeiro. Patients ranged from 37 to 67 years old, being 18 male and 9 female. The principal symptoms reported were: abdominal pain, epigastric burning and distension, periodic nausea, flatulence and halitosis. The criteria of selection were: a) *H. pylori* negative cases were included based on no previous eradication therapy; b) patients with neoplasia, especially in the gastrointestinal tract, were excluded; c) all the patients had not a specific drug intake, especially non-steroid anti-inflammatory drugs, at least 2 weeks before the endoscopy; d) cases with previous gastric surgery were also excluded. Control fragments were obtained from four patients with focal enanthematous areas in which the histopathology revealed only minimal gastritis; the samples collected from normal endoscopic adjacent areas exhibited normal histology. From each sample area (n = 29), one fragment was immediately frozen in liquid nitrogen and stored at -70 °C, the other being embedded in paraffin. Diagnoses were confirmed from standard hematoxylin-cosin, Alcian-Blue-FAS and Wayssson method for *H. pylori*. Another 13 antral biopsies in which chronic gastritis with intestinal metaplasia without dysplasia was histologically detected, were randomly selected from paraffin-embedded samples of the Labs Patologia Clinica e Investigacao Laboratorial pathology service. Gastritis was evaluated based on the criteria of the Sydney score (Sipponen et al., 1991).

**Antibodies**

The monoclonal antibodies used in this study were against laminin chains: α2 (Mab1922 diluted 1:1000), B1 (Mab1928 diluted 1:500), γ1 (Mab1920 diluted 1:500), from Chemicon International Inc, against integrin receptor to laminins: α6β4 (Mab1982 diluted 1:100) from Chemicon International Inc. and α3β1 (MO608 diluted 1:200) from Dako Corporation. All antibodies were used for immunofluorescence staining on frozen sections and merosin (α2 laminin chain) was also applied in paraffin sections. For the cytoskeleton, the monoclonal antibody anti-cytokeratin 20 (CK 20) from Dako Corporation was used on the paraffin sections.

**Immunohistochemistry**

The frozen tissue specimens from normal and chronic gastritis areas, were sectioned at 5 μm and fixed in acetone precooled to -20 °C. After washing in phosphate buffer saline/serum bovine albumin (PBS/BSA), pH 7.4, the frozen sections were incubated with the primary antibody (anti-α2, B1, γ1 laminin chains, anti-α3β1 or α6β4 integrin) for one hour. The procedure was continued by incubating the sections with a fluorescein-isothiocyanate-conjugated goat anti-mouse, for 30 minutes, followed by three washes in PBS and mounting in buffered glycerol. The specimens were examined in a Zeiss microscope with epi-illumination and the appropriate filter.

For the immunoperoxidase staining, the paraffin-embedded specimens were sectioned at 4 μm on silanized slides to avoid damage to the sections during the procedure. A duet kit (Dako Corporation) was used according to the protocols provided by the distributor. To detect CK20, the sections were dewaxed in xylene and rehydrated with graded alcohol to distilled water. Antigen retrieval was carried out in conjunction with microwave oven heating. Following washing for 16 minutes in PBS at pH 7.4, the endogenous peroxidase activity was blocked with 0.6% H2O2 in methanol for 15 minutes. The sections were then washed in PBS for 10
minutes and incubated with non-immune sheep serum at 1% to reduce the background. After this, the primary antibody was applied (anti-α2 laminin chain or anti-CK 20), diluted with non-immune sheep-serum at 1%, and incubated for one hour. For the evaluation of immunostaining, the primary antibody was replaced by non-immune serum to provide a negative control. This was followed by three washes in PBS and a second incubation with biotinylated goat anti-mouse antibody for 30 minutes. Following this, the sections were incubated with streptavidin-peroxidase complex for 30 minutes. All the incubations were carried out at room temperature. The immunoreactivity was detected by a substrate solution containing H2O2 and 3,3’ diaminobenzidine (DAB) in PBS, pH 7.4. The sections were then counterstained with Mayer’s hematoxylin (Merck) diluted with distilled water 1:2, washed in water, dehydrated in alcohol, cleared in xylene and mounted with Entellan (Merck).

Results

From the 25 samples of chronic antral gastritis, 19 (76%) were positive to H. pylori, having quantities that ranged from minimal (36%) to moderate (36%) and accentuated (4%). In addition, from the total number of

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**Fig. 1.** Immunofluorescence to laminin-1 chains of normal gastric mucosa areas and of H. pylori chronic gastritis with intestinal metaplasia. **A and B.** FITC indirect immunofluorescence with anti-81 chain antibody. **C and D.** FITC indirect immunofluorescence with anti-γ1 chain antibody. **A.** Superficial epithelium (*) basement membrane from normal antrum area showing an intense, linear and continuous reaction to 81 chain (arrow-heads). Basal lamina of capillaries (thin arrows) in the stroma also exhibit intense staining. **B.** Chronic gastritis with intestinal metaplasia. Weak to moderate and irregular reaction to 81 chain in the basement membrane (arrow-heads) underneath the glandular portion with three goblet cells (*). Basal lamina of a capillar (thin arrow) in the stroma exhibit intense staining. **C.** Superficial epithelium (*) basement membrane from normal antrum area showing an intense and continuous reaction to γ1 chain (arrow-heads). **D.** Chronic gastritis with intestinal metaplasia. Irregular and discontinuous reaction to γ1 chain in the basement membrane (arrow-heads) of a intestinalized gland portion. x 860
samples, 4 showed intestinal metaplasia: 2 complete, exhibiting all the cellular components of the intestinal mucosa, and 2 incomplete.

**Expression of β1 and γ1 laminin chains**

Laminin β1 chain was observed on the BM of superficial (Fig. 1A) and glandular epithelium, from normal areas, as a linear and continuous deposit. Those areas, histologically diagnosed as gastritis, showed that, independently of whether or not *H. pylori* was detected, and independently of gastritis activity and the extent of inflammation, the BMs were also stained with a linear and continuous deposit of β1 chain (not shown). The expression of laminin γ1 was similar to β1 chain showing the same pattern of staining (Fig. 1C). However, in the four cases of gastritis with metaplasia, where the inflammation ranged from moderate to accentuated, the reactivity was less intense and staining discontinuity of the BMs was seen both with anti-β1 (Fig. 1B) and γ1 (Fig. 1D) antibodies in these metaplastic cases. Reactivity to basal lamina of capillaries was intense with both antibodies.

**Expression of α2 laminin chain (merosin)**

In the normal areas, the BMs of the glandular epithelium showed a linear and continuous deposit of α2 chain (Fig. 2A). However, in the gastritis areas, the expression of α2 chain was not uniform. The glandular epithelium BMs showed staining patterns varying from linear continuous to discontinuous. In the samples with gastritis where no *H. pylori* was detected, the BMs showed a linear and continuous deposit of α2 chain and discontinuity was seen in those samples exhibiting moderate and accentuated presence of *H. pylori* and

| Table 1. Immunoreactivity of pyloric glands to basement membrane laminin isoforms, to cell membrane integrin receptors, and of superficial epithelium to cytokeratin 20 (CK20) in gastric fragments of patients with chronic gastritis. |
|-----------------|-----------------|-----------------|-----------------|
| **LAMININS**    | **LAMININ α2**  | **INTEGRINS**   | **CK20 SUPERFICIAL** |
| 81 AND γ1       | Intense, continuous and linear | Intense, discontinuous and linear | Moderate to intense baso-lateral deposit |
| Normal antrum and chronic gastritis without *H. pylori* (no inflammatory activity) | Intense, continuous and linear | Intense baso-lateral deposit. Moderate in chronic gastritis | Intense baso-apical cytoplasmatic filaments |
| Chronic gastritis with moderate or accentuated *H. pylori* colonization (moderate and accentuated inflammatory activity) | Intense, discontinuous and linear | α3β1 – moderate to intense basolateral deposit | Intense basal cytoplasmatic filaments |
| Intestinal metaplasia areas of *H. pylori* chronic gastritis (moderate and accentuated inflammatory activity) | Weak to moderate, continuous and linear | Non-reactive | Goëlet cells – non-reactive |
|                  |                  |                  | Absorptive columnar cell – apical pole |

*Fig. 2. Immunostaining to laminin α2 chain of *H. pylori* chronic gastritis with or without intestinal metaplasia. A. FITC indirect immunofluorescence on frozen section. Chronic gastritis without intestinal metaplasia. The glandular basement membranes show an intense, linear and continuous deposit. X 215. B. Immunoperoxidase method on paraffin embedded section. No reaction is seen in the chronic gastritis with intestinal metaplasia. X 860*
inflammatory activity. No reaction was seen in the four cases of intestinalized glands, but in all the 25 samples, strong stromal reactivity was found.

The sections of paraffin-embedded samples were also stained by peroxidase method with anti-merosin antibody. The results were similar to those with the immunofluorescence method (not shown). No reactivity of gastric BM areas with intestinal metaplasia to merosin was confirmed on a further 13 paraffin-embedded samples. The intestinalized glands from complete or incomplete forms were always negative to merosin. In contrast, the normal glands had a linear reaction to α2 (Fig. 2B).

Expression of cytokeratin 20

Paraffin-embedded sections were stained to CK20, in order to ascertain whether any alteration would be seen in the cytoskeleton. Immunoreactivity was restricted to the superficial epithelium, upper foveolae and to a variable number of single CK20-positive epithelial cells in the upper zone of the normal pyloric glands (Fig. 3A). In normal areas, the strong reactivity was basal, with a filamentous pattern with a predominantly baso-apical orientation. Near the lateral and basal membranes, a reinforcement of immunoreactivity was observed (Fig. 3B). The same staining was seen in the cases with gastritis without metaplasia. Intestinal metaplasia areas exhibited a different pattern of staining. The reactivity was irregular and heterogeneous, i.e., some cells showed an intense reaction while others were negative. Goblet cells did not react with the CK20 antibody and or absorptive columnar cells, the reactivity was restricted to the apical cell pole and basal reinforcement was seen, as in the samples without metaplasia (Fig. 3C).

Expression of α3β1 and α5β4 integrins receptors to laminin

In order to investigate the presence of integrin receptors to laminin in chronic gastritis, antibodies against α3β1 and α5β4 were utilized in cryosections stained by indirect immunofluorescence. The α3β1 integrin was detected in all the normal gastric samples as an intense basolateral deposit of the pyloric glands and also in endothelial and interstitial cells (Fig. 4A). In the sections with chronic gastritis and *H. pylori* colonization, the reaction ranged from negative in the metaplastic glands to a moderate to strong reactivity in non-intestinalized epithelium, independently of the inflammatory activity. The absence of reactivity in intestinalized glands was not associated with the type of

Fig. 3. Immunoperoxidase to CK20 on paraffin embedded section. A. Pyloric glands showing few positive CK20 cells (arrows). B. Superficial epithelium of normal area with a regular distribution of CK20. C. Chronic gastritis with intestinal metaplasia exhibiting a irregular aspect of CK20 distribution. Goblet cell did not react with CK20 antibody (*). x 880
intestinal metaplasia. The expression of α6β4, although similar to that seen in α3β1 staining, in some cases showed a lateral reaction and while in others, a basal localization (Fig. 4B). Areas with intestinal metaplasia did not react with the α6β4 antibody (Fig. 4C).

The results were summarized on Table 1.

Discussion

In this report, we analyzed the expression and distribution of α2, β1 and γ1 laminin chains, their α3β1 and α6β4 integrin receptors and the cytokeratin 20 of antral biopsies with gastritis with or without H. pylori cells, and correlated their distribution with morphological changes during this disease.

The patterns of β1 and γ1 expression in normal areas are similar to those reported for the human fetal and adult gastric mucosa (Virtanen et al., 1995; Tremblay and Menard, 1996). Since the basement membranes were also stained with a linear and continuous deposit of β1 and γ1 laminin chains, gastritis with H. pylori and subsequent inflammation not change the expression of these two laminin chains. However, the intestinalization process and the degree of inflammation suggest a modification of the expression of these two laminin chains. In contrast, in areas from gastritis, the merosin reactivity ranged from continuous to discontinuous, and the discontinuity was always H. pylori associated, thus indicating that α2 chain expression can be modulated by H. pylori colonization and by the amount of inflammatory cells present in the lamina propria. The results of this study also showed that when gastric glands lose their typical appearance and acquire an intestinal pattern, the expression of merosin is removed from the BMs. Moreover, even in absence of merosin in the BM intestinalized glands, the stroma stained to it. Then, in gastritis with complete or incomplete intestinal metaplasia, merosin expression may not be regulated by heterologous cell-cell interaction as suggested by Simon-Assman et al. (1994). The interactions with epithelial and mesenchyma-derived cells, on producing merosin, may be dependent on the maintenance of the differentiated state of both cellular types (Parker et al., 1974; Neal and Potten, 1981; Kedingr, 1994). Merosin is not only involved with glandular development, but also with the maintenance of adult glandular structures of all gastrointestinal tract (Simo et al., 1991; Simon-Assman et al., 1994; Perrault et al., 1995; Virtanen et al., 1995; Lohi et al., 1996; Tremblay and Menard, 1996).

Fig. 4. Immunofluorescence to integrins receptors to laminin of normal gastric mucosa areas and of H pylori chronic gastritis with and without intestinal metaplasia. A. α3β1 integrin in glandular epithelium from normal area showing a strong basolateral reaction (arrows). Endothelial and some interstitial cells in the stroma exhibit intense staining. B. α6β4 in chronic gastritis. Moderate reaction is seen in the basolateral portion of the glandular epithelium (arrow-heads). C. α6β4 in chronic gastritis with intestinal metaplasia. No staining in the glandular basement membrane is observed. x 860
Although intestinal metaplasia develops after *H. pylori* infection, it may also appear for other reasons. The fact that a mucosal colonization by *H. pylori* may change the merosin expression (discontinuity), suggests that the binding of *H. pylori* to surface epithelial cells and subsequent inflammatory response play a role in the modulation of this molecule during the process of glandular renewal.

Intestinal metaplasia is a different tissue to the non-metaplastic gastric glands and expresses different antigens to non-metaplastic epithelium. Several works have suggested that the development of gastric adenocarcinoma is related to intestinal metaplasia (Lauren, 1991). Furthermore, intestinal-type dysplasias, adenomas and also adenocarcinomas arise from this region, suggesting that they share a common origin (Hattori and Sugihara, 1996). It seems to us that in the absence of merosin during the intestinalization process, the stem cell loses its pathways of differentiation and this loss may leave the glandular cell in a determined tubule to a preneoplastic state.

The integrins α6β4 and α3β1 have been related to an anchorage process between the extracellular matrix and the actin or keratin-based cytoskeleton (Carter et al., 1991; Niessen et al., 1997). The α6β4 integrin mediates stable anchorage of cells to laminins (Niessen et al., 1997) while the α3β1 integrin plays a role in matrix assembly (DiPersio et al., 1997). In addition, studies relate the loss of polarization (Tennesbaum et al., 1993) or its absence (Downer et al., 1993; Gui et al., 1995) in the tumoral process. In this work α6β4 was detected at basal localization and also at the lateral membranes in glands colonized by *H. pylori*. In intestinalized glands, no reaction to α6β4 and α3β1 integrins was observed, as in the merosin staining. Previously described as a laminin-1 receptor (Lee et al., 1992) it has now been confirmed that α6β4 is a receptor not only for laminin-1, but also for several laminin isoforms (Mercucio, 1995; Spinard et al., 1995). No reactivity of either merosin or α6β4 in the glands displaying intestinal metaplasia, suggests that this integrin is also a merosin receptor. The results of this work are partially different that those found by Tani et al. (1996), in which α6β4 was detected in glands with intestinal metaplasia, although like this study, no merosin was observed in intestinalized glands neither in the complete or incomplete forms.

The results of CK20 showed that in normal areas and in gastritis without metaplasia the immunoreactivity was strong and homogeneous in the cells at the tip and the upper portion of the foveolae. In the lamina propria, pyloric glands, few cells positive to CK20 were exhibited that were probably endocrine cells, as reported by Moll et al. (1993). However, in gastritis with intestinal metaplasia, the reactivity to CK 20 was not homogeneous, showing some cells with intense immunolabeling while others were negative. This difference can be explained by the mosaic-like patterns of positive and negative cells, which is a feature of the distribution of CK20 in normal and transformed tissues (Moll et al., 1990). Schwerer and Baczako (1996) reported an intense and uniform reactivity to CK20 in enterocytes and goblet cells in the same disease. We did not find a positive reaction in goblet cells, probably by the density of intermediate filaments is low in central portions of these cells, due to mucin accumulation, as reported by Specian and Neutra (1984). Moreover, the pattern of expression of CK20 in association with CK7 was recently used to distinguish Barret's esophagus from intestinal metaplasia (Ormsby et al., 1999). In the intestinal metaplasia, cells positive for CK20, alternating with negative cells, probably differ functionally from each other.

Taken together, these findings suggest that a differential expression of laminin isoforms is related to inflammation and subsequent intestinal metaplasia caused by *H. pylori*. In conclusion, the alterations of integrins (α3β1 and α6β4) parallel both modifications in laminin isoform (α2) and CK20 expression in *H. pylori* chronic gastritis.

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