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# Transdifferentiation of mucous neck cells into chief cells in fundic gastric glands shown by GNA lectin histochemistry

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ABSTRACT

The epithelium of the gastric mucosa and its glands in the corpus of rat stomach contains mucous surface cells (MSCs), parietal cells, mucous neck cells (MNCs), zymogenic or chief cells (ZCs), several types of enteroendocrine cells, and intermediate cells with characteristics between MNCs and ZCs also called transitional or prezymogenic cells (pre-ZCs).

The aim of our work was to analyze the expression of Mannose (Man) in the rat gastric glands by means of *Galanthus nivalis* lectin (GNA) histochemistry to identify the differences between MNC, pre-ZCs and ZCs and to establish the relationships between these cells. Most of the cytoplasm of MNCs was negative for GNA histochemistry. Intensity of GNA labeling in the gastric gland showed a graduation from pre-ZCs (weak labeling) to ZCs (moderate labeling). Labeling of ZCs was stronger at the perinuclear and apical cytoplasm.

In the last years, strong evidence has been reported supporting that ZCs differentiate from MNCs. Our work also supports the origin of ZCs from MNCs, because the GNA labeling graduation might be due to oligosaccharides which are not expressed in MNCs, start to express in pre-ZCs and are more abundant in ZCs, indicating that differentiation from MNCs to ZCs is a process in which glycans with Man moieties are synthesized.

## 1. Introduction

The histological organization of the *corpus* of rat stomach is equivalent to that of the *fundus/corpus* region of the human stomach. It contains gastric units, formed by gastric glands connected to short pits of the gastric surface. The epithelium of the gastric mucosa and its glands is an example of self-renewing epithelium. It contains mucous surface cells (MSCs), parietal cells, mucous neck cells (MNCs), zymogenic or chief cells (ZCs) and several types of enteroendocrine cells (Mills and Shivdasani, 2011; Hoffmann, 2013). MNCs are located in the neck region, at the upper region of the gastric gland, next to the pit, whereas ZCs are located at the base, at the bottom of the gland. In addition, cells with characteristics between MNCs and ZCs are located between the neck and the base region, and these intermediate cells are called transitional or prezymogenic cells (pre-ZCs) (Suzuki et al.1983; Yang et al.1997; Madrid et al., 1990, 1998a,b; Karam et al., 2003).

Recent findings have established that all cell types in the gastric epithelium arise from the same somatic stem cell (SSC), not yet identified, located at the isthmus of the gland. This SSC produces the precursor cells of MSCs, MNCs and parietal cells. Most of the evidence shows that MNCs are the precursor cells of ZCs, as has been shown by different approaches, including pulse-chase experiments (Karam and Leblond, 1993; Bredemeyer et al., 2009; Karam, 2010; Mills and Shivdasani, 2011; Willet and Mills, 2016).

During differentiation, cells express new proteins and new oligosaccharides on glycoproteins and other glycosylated compounds. These glycoconjugates can be detected in situ by histochemical procedures by means of lectins, which are carbohydrate-binding proteins which are

*Abbreviations:* AAL, *Aleuria aurantia* lectin; ABC, avidin-biotin-peroxidase complex; BSA, bovine serum albumine; DAB, diaminobencidine; GNA, *Galanthus nivalis* agglutinin; HPA, *Helix pomatia* agglutinin; Man, Mannose; MNC, mucous neck cell; MSC, mucous surface cell; NeuAc, sialid acid; PBS, phosphate buffered saline; PNA, peanut agglutinin; PNGase-F, peptide-*N*-glycosidase F; Pre-ZC, pre-zymogenic cell; SSC, somatic stem cell; TBS, Tris buffered saline; ZC, zymogenic cell

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not enzymes acting on the sugar ligand, carbohydrate-specific antibodies or transport proteins for free mono- to oligosaccharides (Spicer and Schulte, 1992; Kaltner and Gabius, 2012). So, the identification of glycoconjugates by lectin histochemistry could be a useful method to analyze in situ the differentiation of cells in the gastric glands.

The aim of our work was to analyze the expression of Mannose (Man) in the rat gastric glands by means of lectin histochemistry to identify the differences between MNC, pre-ZCs and ZCs and to establish the relationships between these cells.

## 2. Material and methods

### 2.1. Reagents

Biotinylated agglutinin from snowdrop *Galanthus nivalis* (GNA) and biotinylated lectin from orange peel *Aleuria aurantia* (AAL) were supplied by EY (San Mateo, CA).

Peroxidase-labelled agglutinin from snail *Helix pomatia* (HPA), biotinylated agglutinin from peanut *Arachis hypogaea* (PNA), bovine serum albumin (BSA), and 3,3′-diaminobenzidine (DAB) were purchased from Sigma Aldrich (Madrid, Spain).

The enzyme Peptide-*N*-glycosidase F (PNGase-F) from *Flavobacterium meningosepticum* expressed in *Escherichia coli* was obtained from Roche (San Cugat del Vallès, Spain).

Avidin-biotin-peroxidase complex (Vectastain ABC kit peroxidase standard) and avidin-biotin blocking kit were from Vector Laboratories (Burlingame, Ca) and supplied by Atom (Barcelona, Spain).

### 2.2. Tissue samples

Paraffin embedded samples of rat gastric mucosa from our archives were used. These samples were obtained for one of our previous works, as described (Gómez-Santos et al., 2007). In brief, samples were obtained from six adult male Sprague-Dawley rats, weighing 250–300 g. Samples of gall bladder, testis and intestine from our archives, obtained for previous works (Madrid et al., 1994), were also used to carry out the controls of deglycosylation techniques (see below). All applicable international, national and/or institutional guidelines for the care and use of animals were followed at the time when the samples were obtained.

### 2.3. Histochemical procedure

After paraffin removal, sections were hydrated prior to lectin histochemistry. The lectin from *Galanthus nivalis* (GNA), which recognizes mannose (Man) moieties (Shibuya et al., 1988), was employed. As indicated in the control section below, the lectins HPA, AAL and PNA were used as control for some deglycosylation pretreatments.

Histochemistry with peroxidase-labelled HPA was carried out as follows: hydrated sections were immersed in 1% (v/v) $H_2O_2$ in Tris buffered saline (TBS) to block the endogenous peroxidase. After washing in TBS, sections were incubated with 6 µg/ml HPA diluted in TBS. Incubation was done at room temperature in a moist chamber for 1 h 30 min. Then, sections were washed with TBS and the peroxidase was developed with 0.1% (v/v) $H_2O_2$ and 0.25 mg/ml DAB in TBS for 7 min. The sections were then slightly counterstained with hematoxylin.

Histochemistry with biotinylated GNA, PNA and AAL was performed as follows: After blocking the endogenous peroxidase as indicated before, the sections were incubated in a moist chamber at room temperature for 10 min with 1% (w/v) BSA in Phosphate Buffered Saline (PBS) to block unspecific binding. Then, the sections were incubated in a moist chamber with the lectin diluted in 1% (w/v) BSA in PBS for 1 h 30 min at room temperature. The lectins were used in the following dilutions: 60 µg/ml GNA, 50 µg/ml PNA and 10 µg/ml AAL. After incubation with the lectin, sections were washed with PBS and incubated with ABC kit for 1 h. Then, sections were washed with PBS, the peroxidase was developed as indicated before and the sections were then slightly counterstained with hematoxylin.

### 2.4. Deglycosylation pretreatments

The histochemical procedure with GNA lectin was carried out in five ways: 1) without previous treatment; 2) after previous enzymatic deglycosylation with PNGase-F to remove N-linked oligosaccharides; 3) after previous chemical deglycosylation (β-elimination) procedure to remove O-linked oligosaccharides; 4) after acid hydrolysis to remove terminal sialic acid (NeuAc) from oligosaccharides; and 5) after methylation-saponification to remove sulfate groups.

The removal of N-linked oligosaccharides was carried out using the enzyme PNGase-F (Lucoq et al., 1987). The sections were incubated with 40 U/ml PNGase-F in a moist chamber at 37 °C (Martínez-Menárguez et al., 1993).

Beta-elimination is a method to remove O-linked oligosaccharides described by Ono et al. (1983). The technique was carried out by immersing the partially hydrated sections in 0.5N NaOH in 70% ethanol at 4 °C. For every lectin, the technique was performed in different sections for 1 and 5 days, to discriminate both labile and resistant O-glycans (Gómez-Santos et al., 2007).

Both deglycosylation pretreatments, β-elimination and incubation with PNGase-F, enhance background labeling by ABC kit in gastric gland cells (unpublished observations). Thus, this background was blocked using Avidin-biotin blocking kit.

The histochemical procedure was also essayed after removal of terminal NeuAc by acid hydrolysis, immersing the sections in 0.1N HCl at 82–84 °C for 3 h before the histochemical procedure with the lectin (Madrid et al., 1994). This technique was carried out to locate subterminal sugars to terminal NeuAc.

Some carbohydrates could be linked to sulfate groups and then they cannot be labeled by the lectins. To show the presence of sulfated terminal Man moieties in oligosaccharides, the procedure of methylation-saponification was employed. This technique is carried out by immersing the deparaffined sections in pure ethanol for 4 min and then in 1% (v/v) HCl at 60 °C in methanol for 5 h. After rehydration, the sections were immersed in 1.8% (w/v) barium hydroxide at 4 °C for 1 h (Martínez-Menárguez et al., 1992).

### 2.5. Controls

The following controls were used: 1) substitution of the lectin or the enzyme by the buffer alone, 2) preincubation of the GNA lectin with 0.2 M Man (inhibitory sugar for GNA), and 3) staining of sections of other tissues of known altered binding pattern for each of the chemical and enzymatic pretreatment. As specific control of β-elimination, rat testis sections were stained with HPA, the HPA staining removal after β-elimination has been reported for this tissue (Martínez-Menárguez et al., 1993). AAL histochemistry in rat testis was used for PNGase-F control, because this tissue becomes negative to the lectin after removal of *N*-glycans (Martínez-Menárguez et al., 1993). To test the effectiveness of the acid hydrolysis, PNA was essayed in human gall bladder sections to verify the labeling to the secretory granules of principal cells (Madrid et al., 1994). PNA lectin stains the rat intestinal microvilli and globet cells after methylation-saponification (Martínez-Menárguez et al., 1992), thus these lectin and tissues were used as specific control for this desulphation method.

### 2.6. Analysis of results

Three histological sections from each sample were used for the histochemical procedure with the lectin alone and with each deglycosylation procedure. The staining intensity in the gastric cells was evaluated by three independent observers. It was classified into six preestablished categories: no labeling (0), very weak (1), weak (2),

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