Cell/Tissue Injury and Cytoprotection/Organoprotection in the Gastrointestinal Tract

Mechanisms, Prevention and Treatment

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Physiopathology of Esophageal Inflammation, Ulcerogenesis and Repair by Studying the Profile of Glycoconjugate

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Abstract

Esophageal inflammation is the highly complex protective response to cellular/tissue injury, one of the fundamental features in gastro-esophageal reflux disease (GERD) that represents a wide group of acid-related disorders, where a majority of patients appear to have nonerosive reflux disease (NERD) with minimal change esophagitis and no endoscopic abnormalities. Despite recent advances and better understanding of the physiopathogenesis of GERD and NERD, the molecular events of inflammation leading to erosive esophagitis (EE) and nonerosive esophagitis (NEE) development, their delay in healing and recurrence remains unclear. Membrane and integral glycoconjugate transformation of different cells in the esophageal barrier (EB) is associated with the reprogramming of pathways that control inflammation, survival and proliferation. The focus of this review is to summarize our data on the bidirectional relationship between the glycoconjugates – variable mediators for structural, modality roles and inflammatory settings in esophageal disorders. We designed and carried out experimental studies that induced esophageal damage, mimicking the esophageal injury of human GERD and NERD. We examined it using functional, morphologic and molecular biologic tests, and which and if pathways of inflammation precede changes in EB and development of mucosal lesions during the early stages of NEE or EE. We showed that glycoconjugates operate as tags for esophageal mucosal inflammation, ulceration and control communication between cell populations in repair. Detailed characterization of dialglycans and their dynamics offers insights into functional interplay in the esophageal cellular community, whereas they switch the pro-inflammatory events involved in early nonepithelial cell activation and mucosal restitution. Esophageal mucosal repair and ulcer healing through different responses cannot be separated from changes in glycoconjugates upon modulation activity of limited and localized inflammation, cytotoxic nitrogen effects, and oxidative injury of epithelial and endothelial cells, cell proliferation, and migration. This new approach to glycobiology will provide an innovative view on inflammation in the physiopathogenesis of NERD and GERD.

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Esophageal barrier (EB) integrity, function and viability are critically dependent on the cross-talk of epithelial and stromal determinants implicated in counteraction to
the chemical, physical, ischemic and infection factors involved in inflammation that are complex protective responses of the organism. Initial studies on the genesis of gastro-esophageal reflux disease (GERD) focused on nonspecific markers of inflammation, while recent investigations revealed adaptive immunity. Although many different bioregulators of inflammation in esophageal disorders have been identified, there is still much to be discovered, particularly in regard to the evaluation of biomarkers with carbohydrate recognition domains which are highly sensitive and specific tools for the determination of modified and potentially pro-inflammatory contents, structural reorganization and functional change induced by leukocytes trafficking and infiltration. This review focuses on novel knowledge of the physiopathogenesis of GERD and the role of glycoconjugates such as pro-resolution messengers for inflammatory settings in esophageal disorders.

Recent studies have demonstrated that acid-related diseases are common and widespread disorders with increasing prevalence of GERD which is being diagnosed with increasing frequency both in children and adults. The majority of patients appear to have nonerosive reflux disease (NERD) with minimal change esophagitis (MCE) and no endoscopic abnormalities (MCE–). These facts served as a background for the development of a new approach to the classification of different forms of GERD, especially including NERD. In 2009, the international workgroup finished studies focused on NERD. In a symposium held in Vevey, Switzerland, the new diagnostic classification was proposed – extension of the Los Angeles classification of grading erosive esophagitis (EE) –, adding two new categories: (1) grade M – minimal endoscopic lesions such as MCE, edema, hyperemia and friability, and (2) grade N – MCE– [1]. The modern interpretation of esophageal physiopathology includes a chain of events beginning with transient spontaneous relaxation of the lower esophageal sphincter, which increases in quantity, results in nonerosive lesions or EE, and then develops into Barrett’s esophagus. The pathogenesis of GERD is diverse and still under controversial discussion, but current data suggest that the disease is multifactorial. In addition to the epithelial, secretory and motor dysfunctions, natural age-related changes (presbyesophagus), visceral hypersensitivity, and alteration of the gut’s microbial composition and functions – appear to be important in the induction of GERD. On the other hand, gender, general functional state of the organism (pregnancy, 1-year-old children), various neurological and psychological impairments (depression, Parkinson’s disease), and different metabolic dysfunctions also play a key role in GERD.

Current studies revealed that the major and common mechanism in the physiopathogenesis of different forms of esophageal disorders is inflammation. Inflammation in GERD is complex and numerous profiles of the cellular, subcellular and molecular biomarkers may explain the different outcomes: MCE, nonerosive esophagitis (NEE), EE, Barrett’s esophagus. These diseases may potentially cause chronic inflammation and its pro-inflammatory agents contribute to the progression of esophageal carcinogenesis [2, 3]. The WHO names BE as premalignant to esophageal adenocarcinoma,
one of the most dangerous gastrointestinal (GI) cancers. Thus, we decided that it is more important to investigate esophageal epithelial and stromal cells by specific molecular tools of initial inflammation events as proposed candidates for the diagnostic and therapeutic targets for MCE, NEE and EE. Assessing the role of glycoconjugates as new molecular biomarkers contributing to inflammation will provide the new insights into their role as peculiar messengers, i.e. the link between inflammation and GERD for better diagnosis and treatment efficacy in the future.

A Historical Sketch

Most of the research on the esophageal physiopathology was focused on the most fundamental problems, faced by both structural and functional points of view. History of the esophageal physiopathology started in the 2nd century, beginning with the great Galen, who first described the suggestive symptoms of this GI disease. In 1859, Rokitansky was the first to define peptic ulcer of the lower esophagus. After 20 years, Quincke described esophagitis as an acute inflammation of the mucous membrane, which gave extreme odynophagia and often aphagia. Canon was first to give detailed description of the episodic spontaneous occurrence of the reflux recorded by newly developed X-ray imaging with barium contrast during study of digestive process [4]. A turning point in GERD physiopathogenesis came in 1934 when Winkelstein and Hamperl, independently from each other, were the first to correlate the symptoms of heartburn and regurgitation with reflux-induced esophageal inflammation, introducing ‘peptic’ esophagitis, i.e. an esophagitis resulting from the irritant action on the mucosa of free hydrochloric acid and pepsin with the relationship to the acid peptic reflux, esophageal ulceration and reflux symptomatology [5]. A big step forward in the understanding of esophageal pathology was made in 1950 by N. Barrett, who was first to report the columnar lined metaplasia in the lower third of esophagus. Development of the new endoscopic methods, wireless pH capsule and multichannel intraluminal impedance techniques provided an opportunity to identify weak acidic reflux and extra-esophageal manifestation of GERD. Over the last decade, considerable progress has been made in understanding the role of central and peripheral neural, and humoral and cellular mechanisms in BE functions, identifying cross talk in multifactorial interaction of endogenic signal pathways, by advances in cell and molecular biology and pharmacology.

A modern integrated method of probe-based endomicroscopy provides real-time in vivo histology for confirmation, characterization and recognition of cellular and subcellular details during ongoing microscopy and opened new perspectives for molecular imaging. In the postgenomic (postproteomic) era there is a growing demand for the innovations in the field of glycomics since glycans, carbohydrate-binding compounds, are sensitive to modulation of the comprehensive range of biological processes and progression of many diseases, including neoplastic processes [4]. In spite
of their early discovery in 1876 by J.L.W. Thudicum, little progress in understanding the major role of carbohydrates in molecular, cellular biology and physiology was made before the 20th century by Nobel Laureate Hermann Emil Fischer (Chemistry, 1902) in his revolutionary effort ‘in recognition of the extraordinary services he has rendered by his work on sugar and purine syntheses’. Among different modern methods of recognizing glycoconjugates, lectin (from the Latin legere, meaning ‘to select’) histochemistry, which was first introduced by P.H. Stillmark in 1888, is the screening tool for quantitative/qualitative differences during post-translation modification [4]. When apoptosis was discovered in the 1970s, a set of apoptosis-specific biochemical events was detected by the glycoconjugate recognition system through interaction with lectin labeling. In the mid-1980s, it was investigated that glycoconjugates also occurred on the internal (luminal) surface of intracellular organelles, and on secreted molecules, and it became apparent and known now that some discrete types of glycoconjugates are present in the cytosol and nucleus [7]. It is well known that membrane and intracellular glycoconjugates play a vital structural and modality role in the wide-ranging diversity of functions between cells and the surrounding matrix in processes that maintain cellular homeostasis at different times of development (organism-intrinsic functions) or in different environmental contexts (organism-extrinsic functions) [8]. Such changes of carbohydrates provide different recognition signals that are involved in intercellular and intracellular activities via lectin-like molecules that were proposed to mediate this interaction [6]. Specific glycan-protein interactions in vivo are responsible for the recruitment of neutrophils (and other leukocytes) into sites of inflammation or ischemia/reperfusion injury [10]. Also, they are involved in apoptosis, autoimmune cell response as well as intracellular regulatory on/off switching effects and host-pathogen (parasite/symbiont) interactions. The role of glycoconjugates in the progression of many diseases including cancer and probably in other areas are not yet fully investigated [3, 6]. Moreover, data obtained in vitro and in vivo about the synthesis of de novo glycoconjugates (glycoproteins, proteoglycans, glycolipids) presents an effective information coding system about the time sequence of cell behavior and has the ability to modify the cellular microenvironment, such as secretion of glycoprotein mucins that are critical for protection against damaging factors [11]. Reducing N-glycan branching in inhibition, tightening and stabilizing cell-cell junctions and cell migration are important attributes of normal epithelial cells, or inherent paracellular permeability, or cell-cell adhesion or overexpression of changed patterns in glycoproteins of the plasma membrane during apoptosis [8]. These properties are essential to providing dynamic cellular turnover and maintaining resistance of the barrier function of the GI tract in which the mitosis activity of some cells, especially epitheliocytes, in the upper GI is very high.

These facts were the basis for our focus on the glycomic approach to the identification of glycoconjugates of EB as hallmarks of different outcomes of inflammation in multifunctional substrate mucosal and submucosal parts of EB. Our approach has always been to (1) search for a light microscopy equivalent of EB cells in inflammation

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via changes de novo synthesized glycoconjugates in situ, (2) to test their diagnostic value in different models of esophagitis, and (3) to look for a possible role of modified glycoconjugates in the genesis of inflammation.

**Screening Glycoconjugates in Esophageal Mucosa**

The sialic acid network (NEU5AC) is essential for the initiation and regulation of inflammation and an effective tool of representing injury-cell-associated immunogenicity in vivo. We hypothesized that NEU5AC interacted at a defense/receptor level in the same fashion as pro-inflammatory cytokine in the regulation of esophageal barrier function. In our previous work, we discovered that the expression of sialyted glycoproteins and glycoconjugates, which contains NEU5AC, correlated with the cytoaggressive influence under topical and nontopical ulcerogenic agents, and found that they were potent biomarkers of gastric ulcerogenesis and healing [12]. Our laboratory was the first to investigate the relationship between glycoconjugates and inflammation during esophageal injury and healing. In these studies, we used lectin histochemistry analysis for sections of the lower third of esophagus in experimental EE and NEE. The lectins used in our study and their carbohydrate specificities are shown in table 1.

**Inflammation as a Common Mechanism of Esophageal Ulcerogenesis**

Modern data revealed that the functioning of EB depends on the neural control, as the central (e.g. esophago-salivary and esophago-bronchial reflexes) and peripheral (substance P, calcitonin gene-related peptide, CGRP) mechanisms, as well as the non-neural mechanisms, which are realized by humoral and paracrine cellular and intracellular pathways, control submucosal and mucosal homeostasis [13]. However, the usefulness of existing biomarkers or histological diagnosis of GERD, especially for MCE, is limited by poor specificity, sensitivity or high cost of available diagnostic tools. Indeed, only ultrastructural morphometry showed esophageal space dilation to be a reliable sign of reflux disease, but it is still rarely used in routine practice [14]. Latest data state that in damage to the esophageal mucosa, its quality of repair is associated with inflammation, which is the key defense mechanism of esophageal cells/tissue to different injury agents. Signals for inflammation originate from the esophageal epithelial and stromal elements in EB and, in addition, to the cascade reaction – a series of sequential steps related to the recruitment of leukocytes from the bloodstream into the surrounding tissue [10]. It was found that the onset of esophageal lesions is likely to be multifactorial and the esophageal mucosa response to topical or non-topical-induced damage was associated with a failure in several mechanisms that included hypersecretion of pro-inflammatory cytokines, growth factors, adhesion molecules and heat shock protein, upregulation of transient receptor potential channel vanilloid subfamily
<table>
<thead>
<tr>
<th>No.</th>
<th>Name of plant</th>
<th>Source</th>
<th>Name</th>
<th>Carbohydrate specificity of top 3 ligands according to Consortium of Functional Glycomics (source: <a href="http://www.functionalglycomics.org">http://www.functionalglycomics.org</a>)</th>
<th>Molecular weight, kDa</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td><em>Arachis hypogaea</em>, peanuts</td>
<td>Seeds</td>
<td>PNA agglutinin</td>
<td>β0-galactosamin; N-acetyl-galactosamin (β0Gal-H → 3DGal NAcDGal)</td>
<td>110</td>
</tr>
<tr>
<td>22</td>
<td><em>Helix pomatia</em>, edible snail</td>
<td>Extract</td>
<td>HPA</td>
<td>N-acetyl-α-galactosamin (αNAcDGal)</td>
<td>79</td>
</tr>
<tr>
<td>33</td>
<td><em>Sambucus nigra</em>, black elder</td>
<td>bark</td>
<td>SNA</td>
<td>N-acetyleneuraminic acid (Neu5Ac/2→6Gal)</td>
<td>140</td>
</tr>
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member-1 (TRPV1) receptors, and proteinase-activated receptors-2 (PAR-2). These pro-inflammatory and neuro-inflammatory effects are able to alter permeability, transepithelial resistance of EB, sensory nerves and endogenous NO release, and an increase in apoptosis [2, 15]. In addition, advanced data postulates that chemokines and the cytokine network that regulates the leukocyte recruitment cascade play a critically significant role in esophageal epithelial-stromal interactions in esophageal ulcerogenesis but also in maintaining or enhancing inflammatory response and repair.

Furthermore, inflammation caused dysmotility, including impairment of esophageal peristalsis of the lower esophageal and pyloric sphincters to intensify their dysfunction, which also seems to play an important role in the function of ‘anti-reflux barriers’ and the luminal clearance of the esophagus from various irritants [2].

**Detection of Glucoconjugates in Esophageal Injuries**

Most of the experimental physiopathogenic research about GERD has been performed by investigating esophageal injury against acidic pepsin with or without bile

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<th>Molecular weight, kDa</th>
</tr>
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<tbody>
<tr>
<td>44</td>
<td><em>Triticum vulgare</em>, wheat germ</td>
<td>Germ</td>
<td>WGA</td>
<td>N-acetyl-α-glucosamin, sialic acid (βNAcDGlc&gt;NAcNeu)</td>
<td>36</td>
</tr>
</tbody>
</table>

Lectins were purchased from Lectino-test Laboratory (Lviv, Ukraine, http://www.meduniv.lviv.ua/depart/lectinotest.html).
acid-trypsin influences [15]. From the beginning, we focused on the idea of how to re-create an experimental model with the most relevant functional and morphological criteria for different forms of esophageal disorders that existed in humans. Inducing esophageal injury in the current animal models includes using surgical procedures that divert the gastroduodenal contents into the esophagus. The major limitation in these models is the significant postoperative inflammation and the failure to control the amount, concentration and composition of the refluxate components entering the esophagus as well as morbidity. Therefore, revising the role of inflammation in the genesis of EE, we designed a novel noninvasive alternative, less-complicated and well-controlled model of esophageal injury by perfusion of the esophagus in anesthetized rats without surgical intervention [16, 17].

**Erosive Esophageal Lesions**

We investigated EB resistance in two models of erosive acidic and alkaline esophagitis caused by the exogenous administration of acidic (with hydrochloric acid with pepsin) as well as alkaline (with deoxycholic acid with trypsin) solutions with the addition of bile. The proposed combinations of esophageal perfusion and time of exposure were sufficient for induction of the esophageal lesions and their pathology signs are similar to the changes observed in humans (fig. 1). Our study showed that ablation of the capsaicin-sensitive sensory neurons increased the aggressive effects of acid-pepsin-induced damage, partly due to impairment of the local microcirculation in esophageal mucosa that may affect trophic processes and augment mucosal destruction [18]. Also, the obtained findings indicate that nitric oxide (NO) produced by endothelial synthase NO (eNOS) dilates mucosal blood vessels and prevents leukocyte aggregation, and is therefore essential for the maintenance of esophageal mucosal blood flow. We provided evidence that pretreatment with L-NNA, a nonspecific inhibitor of NOS, is accompanied by a decrease in plasma NO levels and that a reduction in esophageal blood flow greatly augments the macroscopic damage score. Therefore, it is reasonable to conclude that the NOS/NO-mediated response is an important component of esophageal cytoprotection. Recently, it was reported that selective NO delivery by NO-donating compounds may offer a new therapeutic approach in the treatment of esophageal dysfunction and the prevention of mucosal injury [19].

Clinical observations revealed new areas of GERD physiopathology, including the close relationship between the prevalence of GERD, Barrett esophagus, esophageal adenocarcinoma in populations with sleep disorders or conditions related to desynchronization of the periodical circadian cycle (e.g. shift work, dysadaptation), and also with overweight and obesity. This fact was the reason we began to investigate the effect of melatonin pretreatment on acidic and alkaline plus bile-induced esophageal injury [17, 18]. So, as long as the stress may contribute to the development and progression of nonerosive GI injuries [20], we hypothesized that stress-associated lesions could be induced in the esophageal mucosa.
For testing the role of inflammation in the development of NEE and MCE, we first had to examine the effect of water and immersion-restraint-induced stress (WRS) on EB by using the 1964 Takagi model. Since the expression of inflammatory mediators also results in mucosal restitution, we studied the time sequence of EB repair after WRS, then after 24 and 48 h [21–24]. Stress-associated NEE results in local hemodynamic disorders with signs of irregular hyperemia, stasis, restricted perivascular diapedesis with hemorrhage and focal edema in the underlying stroma (fig. 1). We also found a significantly elevated content of IL-1β and TNF-α vs. control, but a reduced content

**Nonerosive Esophageal Lesions**

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**Fig. 1.** Macroscopic (a), microscopic by HE staining (b) and lectin histochemical labeling of WGA, SNA, PNA changes of erosive (middle) and nonerosive (right) lesions of lower third of the rat esophagus, as compared to control group esophagus (c). Arrows indicate ulcer (middle b) in erosive esophagitis (EE) and destruction of superficial epithelial layer, subepithelial edema, extravasation and microthrombi in subepithelial capillaries, leukocyte infiltration (right b) while spinous and basal cell layers and were still intact in nonerosive esophagitis (NEE). Three labeling patterns of lectins: WGA is strongly positive on surface on most luminal side of corneal layer in EE vs. NEE and in other layers – weakly reacted in EE and no reactions in NES; SNA is strongly positive with intensity gradually increased towards the submucosa in EE and NEE; PNA shows mosaic expression in EE and faintly or negative staining in submucosa, basal layer with increased intensity toward the luminal surface in NEE.
according to data obtained in the EE group. Also, IL-1β and TNF-α have shown pro-migratory effects to leukocytes, which are intensively infiltrated in the esophageal mucosa. Then, they secreted other pro-inflammatory cytokines that may contribute to the restoration of EB [2, 26]. Pretreatment of melatonin significantly reduced IL-1β and TNF-α. Simultaneously, we were the first to investigated the changes of glycoconjugate patterns in EB during EE and NEE (fig. 1, 2). We reported that epithelial and stromal cells in the esophageal mucosa are exclusively expressed by βDGal-H→3DGal NAcDGal, Neu5Ac/2→6Gal glycoconjugates with intensity gradually increasing toward the luminal surface of corneal layers. Higher-intensity NEU5AC labeling was observed in epithelial cells in EE vs. NEE, where sialic acid-associated changes were more intensive in the subepithelial cells (fig. 2). These observations implicated that altered glycoprotein profiles are able to monitor cytoarchitectural changes in EB and established signs of inflammation of the epithelial and stromal cells during EE and NEE due to impaired cell-cell and tight junctions in epithelial and vascular structures induced by hypoxia and delivery pro-inflammatory mediators. These reactions could trigger the adaptation or elimination of damaged cells [27, 28]. No detectable changes of mapping by WGA and HPA labeling were found in the epithelial layers and stroma, except for the ability of WGA to mimick different specific receptors on the superficial layers. Intensive expression of PNA binding on the corneal layer might be the result of improved defense reactions due to essential changes in the mucus/mucosal maintenance of EB. In accordance with current data, the consequences of these changes may be repeated not only for cell-surface glycoconjugates but also for intracellular glycoconjugates [20, 21].

Nonerosive Esophagitis Associated to Glucose Dysregulation

In accordance with recent data results, the molecular underpinning of the metabolic disorders related to glucose dysregulation, hypothyroidism, hypergastrinemia and obesity show implications for inflammation. Knowing that an increased incidence of metabolic diseases is a global trend and that GI-related metabolic disorders are common chronic and often poorly diagnosed problems mainly due to 'silent' clinical features, we also initiated the discovery of inflammation in esophageal injuries based on glucose dysregulation in streptozotocin-mediated hyperglycemia [29]. The results showed NEE and excess of activity iNOS, which influences the vascular tone of local microvessels, increased leukocyte infiltration and modification of NEU5AC expression in EB that might reflect inflammation. Another important mechanism of the decline in EB functions is strongly associated with changes in the composition and function of the microbiota, which is often also associated with obesity. Therefore, by investigating the modification of microbiota on the improvement EB function, we found that the expression of sialic acid-containing glycoproteins βDGal→3DGalNAcDGal, DGalaNAc, Neu5Ac/2→6Gal domains helps recognize that Fas-FasL-mediated apoptosis contributes to epithelial turnover [7, 30].
Fig. 2. Photomicrographs of esophageal mucosa showing changes of expression glycoconjugates by SNA, PNA, HPA, WGA lectin labeling in stress-associated injury (a) and during repair after stress (WRS) and healing, after 24 and 48 h after WRS and melatonin (20 mg/kg) pretreatment (b). ×600. HPA shows no reactions in basal and spinous layers, but a weak reaction on the corneal layer 24 h after WRS. An increase of PNA staining was detected in the corneal and spinous layers of esophageal mucosa and intensive decrease of expression of SNA binding on apical surface of epithelium during treatment of melatonin are signs of desialization.
Conclusion

In this review, we summarize data on inflammation in esophageal injuries and our newly obtained data with detailed characterization of sialoglycans and their dynamics of expression in esophageal erosive, nonerosive, stress-associated and metabolic-related lesions that operate as tags for mucosal and stromal inflammation, ulceration and communication between cell populations in repair. Moreover, NEU5AC expression plays an important role in esophageal cell survival; however, obtaining details for the interpretation of these activities is an important area for future study. Thus, the glycobiological approach may be an option to achieve an innovative view on the expression of inflammation in NERD and MCE.

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