Abnormal glycosylation and altered Golgi structure in colorectal cancer: dependence on intra-Golgi pH

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Abstract Abnormal glycosylation of cellular glycoconjugates is a common phenotypic change in many human tumors. Here, we explore the possibility that an altered Golgi pH may also be responsible for these cancer-associated glycosylation abnormalities. We show that a mere dissipation of the acidic Golgi pH results both in increased expression of some cancer-associated carbohydrate antigens and in structural disorganization of the Golgi apparatus in otherwise normally glycosylating cells. pH dependence of these alterations was confirmed by showing that an acidification-defective breast cancer cell line (MCF-7) also displayed a fragmented Golgi apparatus, whereas the Golgi apparatus was structurally normal in its acidification-competent subline (MCF-7/AdrR). Acidification competence was also found to rescue normal glycosylation potential in MCF-7/AdrR cells. Finally, we show that abnormal glycosylation is also accompanied by similar structural disorganization and fragmentation of the Golgi apparatus in colorectal cancer cells in vitro and in vivo. These results suggest that an inappropriate Golgi pH may indeed be responsible for the abnormal Golgi structure and lowered glycosylation potential of the Golgi apparatus in malignant cells.  © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Neoplasia; Colorectal cancer; Glycosylation; Golgi apparatus; Acid–base balance

1. Introduction

Genetic analyses of human tumors have revealed a variety of chromosomal abnormalities, such as rearrangements and deletions, epigenetic changes and instability of microsatellite DNA, as well as mutations in oncogenes, tumor suppressor genes, DNA mismatch repair enzymes and signal transduction proteins [1–3]. In addition, about 500 differentially expressed genes have been detected in gastrointestinal cancer cells using SAGE analysis [4]. Yet, no single consistent chromosomal alteration or gene mutation has been detected that is inherently linked to malignant transformation.

Much less is known about the many phenotypic changes that accompany carcinogenesis. One of these is the production and secretion of aberrantly glycosylated proteins and lipids. In colorectal cancer, these glycosylation abnormalities are found in most cancer patients [5–7]. They likely impair many basic cellular functions, since terminal oligosaccharide units serve as highly specific biological recognition molecules implicated in processes such as intracellular protein sorting, signal transduction and cell–cell or cell–extracellular matrix interactions [8]. Proliferation, differentiation and metastatic properties of cancer cells also depend on specific carbohydrate structures expressed at the cell surface [9–11]. The clinical importance of oligosaccharide structures has also been realized recently through identification of a number of genetic defects that cause clinical syndromes by impairing normal glycosylation processes [12].

Glycosylation abnormalities concern both N-linked and O-linked carbohydrate chains on glycoproteins, and glycolipids. In general, the processing of terminal sugars is mostly affected. The overall biosynthesis in malignant cells thus results in the synthesis of shorter and less branched carbohydrate chains, as well as in increased sialylation and reduced sulfation [10,13,14]. Because the final structure of the oligosaccharide chains is mainly dictated by the activity or the expression levels of the relevant glycosyltransferases [10,15,16], and changes in these have been detected in human tumors, they have been considered the primary cause for the observed glycosylation abnormalities in malignant cells. However, no simple correlation between these enzymatic changes and the final structure of the produced oligosaccharide units has been shown to exist [17,18]. These observations, therefore, suggest that additional possibilities may exist. In this report, we have compared the structural and functional state of the Golgi apparatus in different experimental systems, in an attempt to assess whether an inappropriate Golgi pH could also be responsible for these cancer-associated glycosylation abnormalities. Our results show for the first time that abnormal glycosylation in malignant cells is also accompanied by a marked disorganization of the Golgi apparatus, and that both of these structural and functional alterations in the Golgi apparatus can be mimicked by perturbing the existing pH in the Golgi lumen.

2. Material and methods

2.1. Reagents

Anti-β-tubulin monoclonal antibody, rhodamine-conjugated phalloidin, diaminobenzidine (DAB), ammonium chloride, chloroquine, bafilomycin A1, the Hoechst dye (33258), and lectin conjugates (peroxidase- and fluorescein-conjugated peanut agglutinin (PNA), peroxidase-conjugated jacalin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-giantin monoclonal antibody was a kind gift from Dr. H.-P. Hauri (Basel, Switzerland). Polyclonal β-COP

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antiserum was from Affinity Bioreagents (Golden, CO, USA). Anti-
bodies to Golgi-specific ankyrin isoforms Ank119 and Ank195 were
generous gifts from Drs. J. Morrow (Yale University, New Haven,
CT, USA) and K. Beck (University of California at Davis, Davis, CA,
USA), respectively. Cancer-associated carbohydrate markers were vi-
alyzed using monoclonal antibodies against the Tn and the sialosyl
Tn antigens (Calbiochem-Novabiochem, La Jolla, CA, USA), CEA (anti-
carcinoembryonic antigen, Zymed, San Francisco, CA, USA) and CA19-9 (anti-sialyl Lewisα; Novocastra, Newcastle upon Tyne,
UK). Alexa-488- and Alexa-594-conjugated goat anti-mouse and goat
anti-rabbit secondary antibodies were from Molecular Probes (Eugene,
OR, USA).

2. Patients
Fresh specimens of normal colorectal mucosa and carcinoma lesion
were obtained during operative surgery of patients with colorectal
carcinoma, and were processed without delay for further analyses (see below). Samples of normal mucosa consisted of resection surfaces
at a distance of at least 10 cm from the adjacent carcinoma. Histol-
opathological diagnosis of colorectal carcinomas and the histological
grade of the tumors were confirmed using hematoxylin-eosin-stained
tissue sections. Written consent to use tissue lesions for research pur-
poses was obtained from each patient and accepted by the local eth-
ical committee (license 252/08/91).

2.3. Cell culture
Normal rat kidney cells (NRK) and African green monkey kidney
cells (COS-7) were grown at 37℃, 95% air/5% CO2, in Dulbecco’s
modified Eagle’s medium (Gibco-BRL, Grand Island, NY, USA) sup-
plemented with 10% fetal calf serum and antibiotics (penicillin-strep-
tomycin). MCF-7 breast cancer cells (ATCC, Manassas, VA, USA)
and multidrug-resistant MCF-7/AdR cells (kindly provided to us by
Dr. Myles Cabot, Santa Monica, CA, USA) were grown in RPMI
1640 with the same supplements. Colorectal cancer cell lines (Caco-2,
HT-29, SW-48, T84) were cultivated on plastic dishes or glass covers-
slips in a medium suggested by ATCC. Treatment of cells with the pH
gradient dissipating drugs (chloroquine 40 μM; ammonium chloride
10–30 mM; bafilomycin A1 100 nM) was started 1 day after plating
by adding 100× concentrated stock solutions (in H2O; bafilomycin
A1 in dimethyl sulfoxide) directly into the fresh culture medium. After
6 or 20 h incubation, cells were washed, fixed with 2% paraformalde-
hyde, and stained with the lectins or with appropriate primary and
Alexa-conjugated secondary antibodies. All staining procedures were
done using freshly prepared 0.1% bovine serum albumin (BSA)/0.1%
saponin/phosphate-buffered saline (PBS). Hoechst dye (33258, stock
solution 1 mg/ml in 50% ethanol) was diluted 1:1000 with PBS and
applied to the fixed cells for 2–5 min just before the final washing
steps and embedding in Immu-Mount™ (Shandon, Pittsburgh, PA,
USA).

2.4. Immunohistochemistry
A small portion of each specimen was embedded in Tissue-Tek™
(Shandon) and snap-frozen in liquid nitrogen. Frozen sections of 4 μm
were cut and mounted on superfrost slides. The sections were post-
fixed briefly with 2% paraformaldehyde (in PBS) and, after quenching,
subjected to immunostaining with the anti-giantin monoclonal anti-
body (in PBS containing 3% BSA and 0.02 M glycine). Avidin-biotin
detection, using Histostain™-Plus Broad Spectrum Kit (Zymed Labo-
ratories, San Francisco, CA, USA) was then applied. DAB was used
as the chromogen and the sections were lightly counterstained with
the ECL substrates (Amersham).

2.5. Transmission electron microscopy
Tissue or cell specimens were fixed in 2.5% glutaraldehyde (in 0.1 M
Na-phosphate buffer, pH 7.4). The specimens were post-fixed in 1% os-
mycin A1 (100 nM), for 6–20 h prior to staining with the lectins and antibodies. Indirect immunofluorescence showed only faint staining in untreated NRK cells (Fig. 1) and COS-7 cells (data not shown) with either PNA or jacalin. In contrast, drug-treated NRK cells showed a pronounced expression of the TF antigen in the Golgi region (Fig. 1), even after 6 h treatment. After 20 h treatment (Fig. 1), the TF antigen was still concentrated in the Golgi region, but it was also present in small intracellular vesicles that likely represent post-Golgi transport vesicles. The plasma membrane in drug-treated cells was also clearly stained in a substantial portion of the cells. Similar results were obtained with jacalin (data not shown). The expression of the other markers (the Tn, sialosyl Tn and CEA antigens) was also slightly increased in drug-treated cells, whereas no change was observed in the expression of the CA19-9 antigen (data not shown). Thus, Golgi alkalization indeed results in increased expression of at least some cancer-associated carbohydrate antigens in normally glycosylating cells.

The known effects of monensin (a sodium proton ionophore) on Golgi structure [21] prompted us to test whether these functional changes are also accompanied by the structural disorganization of the Golgi apparatus in drug-treated cells. We stained untreated and drug-treated NRK cells with the anti-giantin antibody. Giantin is one of the recently characterized Golgi matrix proteins [22]. We found that untreated cells possessed a perinuclear and well organized Golgi apparatus (Fig. 2, top), whereas in drug-treated cells, the Golgi apparatus was structurally abnormal, and consisted of fragmented and separate Golgi elements that most often were scattered in close proximity to the nuclei (Fig. 2). Identical results were also observed with COS-7 cells. Electron microscopy revealed that the cisternae of the scattered Golgi stacks were severely dilated in both NH4Cl- and chloroquine-treated cells when compared to control cells (Fig. 2, bottom).

Light microscopy and counterstaining of drug-treated NRK cells with the anti-β-tubulin antibody also revealed that microtubules appeared normal and were reminiscent of those seen in interphase cells (data not shown). Therefore, the observed Golgi fragmentation and scattering observed in drug-treated cells did not result from a mere accumulation of mitotic cells with fragmented Golgi membranes. Furthermore, the fragmented Golgi elements also co-stained with antibodies against β-COP, Ank119 and Ank195 (data not shown). Golgi fragmentation, therefore, does not seem to involve marked changes in the association of β-COP with the Golgi membranes, or disorganization of the spectrin-based Golgi membrane skeleton.

The pH dependence of these alterations in Golgi structure and function was confirmed by utilizing a breast cancer cell line, MCF-7, that is known to be unable to acidify its intracellular organelles, including the Golgi apparatus [23,24]. We found (Fig. 3) that the Golgi apparatus is heavily fragmented and scattered in these acidification-defective breast cancer cells. Counterstaining of MCF-7 cells with PNA showed that these cells express the TF antigen at high levels both intracellularly and at the plasma membrane (Fig. 3). This was also confirmed by staining of MCF-7 cells without permeabilization (data not shown). Electron microscopy showed further that the individual Golgi cisternae were also similarly dilated to those seen in drug-treated NRK cells (Fig. 2), and that only few transport vesicles were detected in close proximity to the Golgi stacks (data not shown). In contrast to these observations with MCF-7 cells, an acidification-competent subline (MCF-7/AdrR) possessed a compact, structurally normal juxtanuclear Golgi apparatus (Fig. 3). These drug-resistant cells were also found to express the TF antigen only in the Golgi region (Fig. 3), similarly to NRK and COS-7 cells.

Other cancer-associated carbohydrate markers were also ex-
pressed at higher levels in MCF-7 cells than in MCF-7/AdrR cells, as assessed first by immunofluorescence. The staining intensities of all markers tested (Fig. 4A) were clearly much more prominent in acidification-defective MCF-7 cells than in acidification-competent MCF-7/AdrR cells. Similar results were also obtained by Western blotting experiments (Fig. 4B). Although all markers recognized several bands, some of which appeared to be non-specific (i.e. they were detected using the secondary antibody alone (marked by asterisks)), the overall intensity of the bands was clearly higher in MCF-7 cells. Furthermore, there were several bands, including the CEA antigen (180 kDa band in Fig. 4B), that appeared to be expressed only in acidification-defective MCF-7 cells. These observations indicate that Golgi structure and expression of cancer-associated carbohydrate structures correlate well not only with each other, but also with the known ability or inability of these breast cancer cells to acidify their intracellular organelles. Acidification competence, therefore, appears to rescue ‘normal’ glycosylation potential in MCF-7/AdrR cells.

The validity of these observations to colorectal cancer both in vitro and in vivo was evaluated next by utilizing both established colorectal cancer cell lines and colorectal tissue specimens. Indirect immunofluorescence experiments with the anti-giantin antibody showed (Fig. 5) that the Golgi apparatus is morphologically similar to that of acidification-defective MCF-7 cells (Fig. 3), and to the drug-treated NRK cells (Fig. 1). All these colorectal cancer cell lines possessed a fragmented Golgi apparatus, characterized by the presence of predominantly perinuclear (Caco-2, HT-29) and cytoplasmic Golgi elements (SW-48, T-84). All cell lines also expressed cancer-associated glycosylation changes, as evidenced by their staining with fluorescein-conjugated PNA (data not shown).

The TF antigen was also expressed at high levels in colorectal cancer cells in vivo, as assessed by staining of several cancer tissue specimens with peroxidase-conjugated PNA (Fig. 6). The staining was evident along the whole crypt. Higher magnification (Fig. 6, top right) showed that most
tumor cells were PNA-positive and that the PNA binding glycoconjugates were present both intracellularly and in the luminal secretions. However, not all cells within the tumor were stained by the lectin, in agreement with the view that tumors consist of a heterogeneous population of malignant cells. Expression of the TF antigen in normal colorectal epithelium was always faint and restricted only to the sub-apical Golgi region (Fig. 6), as expected.

The anti-giantin antibody, which worked best on frozen tissue sections, was used for visualization of the Golgi membranes in the same PNA-positive sections. The antibody stained a well-organized and 'horseshoe'-shaped Golgi apparatus in normal colon epithelial cells (Fig. 6, bottom left). In cancerous epithelium, however, the Golgi apparatus was structurally different, and consisted mainly of small punctate structures that often were scattered around, or close to, the nuclei (Fig. 6). Although the morphology in frozen tissue sections is not optimal for visualization of subcellular organelles, the overall microscopic appearance of the stained Golgi elements was thus clearly reminiscent of that of the fragmented Golgi elements seen in acidification-defective MCF-7 cells, in all colorectal cancer cell lines and in the drug-treated NRK cells. All four specimens that represented rectal carcinomas (TNM classification: three T2N0M0, grade II or III, one T3N0M0, grade II) gave identical results.

More detailed structural analyses by transmission electron microscopy revealed that in normal epithelial cells, the Golgi apparatus consisted of several distinct Golgi stacks with flattened cisternae, which were closely associated with each other, usually in a curvilinear fashion (Fig. 7A). We also observed numerous transport vesicles within the trans-Golgi area, indicative of efficient vesicular transport into post-Golgi destinations. In contrast, the 'fragmented' Golgi elements in cancer cells consisted most often of clustered Golgi stacks (Fig.
The stacks themselves did not show any association with the other stacks within the same cluster. Individual Golgi cisternae were often also dilated, showing marked swellings, either throughout or at their rims (Fig. 7B). Other intracellular organelles, however, did not exhibit such a dilatation, and e.g. the endoplasmic reticulum and mitochondria appeared normal (Fig. 7C). In addition, very few transport vesicles were detected in close proximity to the clustered and structurally abnormal Golgi stacks in cancer cells. Altogether, 100 different Golgi areas were analyzed with similar results. Jointly, Golgi fragmentation and scattering, as well as dilatation of the Golgi cisternae and the presence of only few transport vesicles, all appear to be characteristic features of the Golgi apparatus in colorectal cancer cells in vivo.

4. Discussion

In this report, we followed the structural and functional state of the Golgi apparatus in different experimental systems that included colorectal cancer cells both in vivo and in vitro, acidification-defective and -competent breast cancer cell lines, and non-malignant cells that were treated (or not) with pH gradient dissipating drugs. Our results indicate that, in all of these experimental systems, Golgi structure and glycosylation potential are causally related and dependent on the existing pH gradient in the Golgi lumen. If this gradient is disturbed, either by pH dissipating drugs or by an endogenous failure to regulate it normally, morphologically and functionally similar changes to those seen in colorectal cancer cells in vivo take place. Our observations thus provide experimental evidence to suggest that an altered Golgi pH may indeed be an additional cause for the observed glycosylation abnormalities in malignant cells.

Although direct proof for this possibility awaits the direct in vivo measurement of the Golgi pH in malignant cells, this possibility is intriguing, as it provides a unifying theme for a number of seemingly unrelated observations made previously in both malignant and non-malignant cells. For example, Golgi alkalization has been repeatedly reported to interfere with the normal glycoprotein processing and efficient vesicular trafficking through the Golgi [25–30], in agreement with our lectin staining experiments and the morphological data. Golgi alkalization has also been shown to impair proper targeting of newly synthesized proteins in the Golgi either into regulated secretory vesicles [31] or to the apical plasma membrane in polarized epithelial cells [32,33]. Altered Golgi pH in malignant cells may therefore have direct contacts with the observed dedifferentiation and the loss of normal cellular polarity of tumor cells in vivo. Similarly, anomalous secretion of lysosomal enzymes observed in many cancer cell types [34–36] could simply result from missorting of these enzymes in the Golgi due to an inappropriate Golgi pH.
Direct pH measurements have also shown that alterations in intracellular and extracellular pH homeostasis are common in tumor cells [37,38]. Previous studies on multidrug resistance have also shown that the major difference between drug-sensitive and -resistant cells is their ability or inability, respectively, to acidify their intracellular organelles [23,24], and because most tumors in vivo can be successfully treated with chemotherapeutic drugs, such alterations in the pH homeostasis appear to be common in intracellular organelles as well. Such alterations may even be intimately associated with malignant transformation itself, since transformation of mammalian cells with the yeast plasma membrane H^+ -ATPase or with certain viral oncopogenes has been shown to correlate with alkalization of either the cytoplasm [39-41], the Golgi lumen [42], or the lysosomes [43]. In addition, acidic extracellular pH has been shown to alter the gene expression profile [44], and to increase the mutation rate by impairing DNA repair [45].

Exactly how an altered Golgi pH may interfere with the normal glycosylation processes is not known. One possibility could be that Golgi alkalization may simply reduce the activity of the relevant glycosyltransferases, since late-acting Golgi glycosyltransferases are known to have their pH optima at a slightly acidic pH range ([15] and references therein). However, it is not clear how such a reduction in the enzymatic activity alone would result simultaneously in the structural disorganization of the Golgi in tumor cells (Figs. 3, 5-7). Therefore, additional factors are likely to be involved, and one such factor could be an altered Golgi pH which, by impairing balanced membrane flow through the Golgi, would interfere not only with the structural organization of the Golgi, but also with the observed recycling of glycosyltransferases between the endoplasmic reticulum and the Golgi, thereby causing their mislocalization and inability to modify their substrates correctly. Consistent with this scenario, a 130 kDa cis-Golgi resident protein has been shown to be mislocalized in colorectal cancer cells in vivo [46], and in cultured cells, targeted mislocalization of a late-acting Golgi glycosyltransferase to the cis-Golgi has been shown to inhibit the synthesis of branched O-glycans [47]. Very recently, Golgi alkalization has indeed been shown to result in mistargeting of middle- or trans-Golgi glycosyltransferases into endosomes and the plasma membrane [48].

The results of this study raise several important questions that concern not only the in vivo measurement of the Golgi pH in malignant cells, but also the mechanistic details and the identity of the ion transporter(s) that may be responsible for the suggested pH alterations in the Golgi lumen. Because Golgi pH is known to be finely regulated, any perturbations in it may involve changes in either the rate of proton pumping, proton leakage, counterion (Cl^-) transport, or even import or export of base equivalents across the Golgi membranes [49-51]. In this context, it should be emphasized that the Golgi pH in cancer cells could also be more acidic than the pH in non-malignant cells. This possibility, however, was not addressed here. Since the existing pH in the Golgi lumen may also depend on cytoplasmic and extracellular pH gradients, potential ion transporter(s) that may be defective (or remain constantly active) in malignant cells are numerous. The most likely candidates, in addition to the vacuolar H^+ -ATPase [52], are the Golgi-associated chloride channel [53], the Na^+ /H^+ antiporter [54], and the sodium-dependent and -independent chloride/bicarbonate exchangers, such as the DRA (down-regulated in adenoma gene product [55]) and the Golgi-associated Cl^- /HCO_3^- exchanger recently identified by us [56]. The major challenge in the future studies, therefore, is to assess whether any one of these is defective in malignant cells.

5. Note

During the preparation of this article, we became aware of a recent article in which the authors [Campbell, B.J., Rowe, G.E., Leiper, K. and Rhodes, J.M. (2001) Glycobio. 11, 385-393] show that neutralization of intra-Golgi pH with bafilomycin in LS174T cells increases TF antigen expression and reduces mucin sulfation. The results are fully compatible with the lectin binding data presented in this article.

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References
