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Overloading of differentiated Caco-2 cells during lipid transcytosis induces glycosylation mistakes in the Golgi complex

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Abstract: Overloading the intestine enterocytes with lipids induced alteration of the Golgi complex (GC; Sesorova *et al.*, 2020) and could cause glycosylation errors. Here, using differentiated Caco-2 cells with the established 0[I] blood group phenotype (no expression of the blood antigens A and B [AgA, AgB] under normal conditions) as a model of human enterocytes we examined whether the overloading of these cells with lipids could cause errors in the Golgi-dependent glycosylation. We demonstrated that under these conditions, there were alterations of the GC and the appearance of lipid droplets in the cytoplasm. Rare cells produced AgA and AgB. This suggested that after overloading of enterocytes with lipids, AgA were mistakenly synthesized in individual enterocytes by the Golgi glycosyltransferases. These mistakes could explain why in the absence of AgA and AgB antibodies against them exist in the blood.

Introduction

The mechanism responsible for the formation of the antibodies against the human blood group polysaccharide (PS) antigens is unknown. The blood group is determined by the presence of antigens A and/or B (AgA and AgB) on the surface of erythrocytes and on the basolateral plasma membrane of other cells (Ravn and Dabelsteen, 2000; de Mattos, 2016). People with group 0(I) do not synthesize AgA and AgB but have antibodies against AgA (AbA) and AgB (AbB). Persons with the blood group A produce AgA and AbB. Individuals with the blood group B synthesize AgB and AbA. Finally, people with the blood group AB synthesize AgA and AgB but have no AbA and AbB. AgA and AgB are the final parts of PS attached to proteins or lipids (Hakomori, 1999; de Mattos, 2016). AgA and AgB are synthesized by glycosyltransferases A and B (GTA, GTB) (Ravn and Dabelsteen, 2000). GTA and GTB are transmembrane proteins of Type II containing C-terminal catalytic domains in the lumen of the medial Golgi cisternae Antibodies against AgA and AgB belong mostly to the IgM class. They are generated against antigens, which do not exist in the organism (White *et al.*, 1990; Sheffield *et al.*, 2005; Milland and Sandrin, 2006; Branch, 2015).

GTA catalyses the transfer of a monosaccharide residue from UDP-GalNAc (UDP-N-acetylgalactosamine) to Fuc alpha1-2Gal beta-R (H)-terminating acceptors, whereas GTB catalyses the transfer of a monosaccharide residue from UDP-Gal (UDP-galactose) to Fuc alpha1-2Gal beta-R (H)-terminating acceptors (Yamamoto, 2004; Milland and Sandrin, 2006). AgA and AgB are localized at the end of the long oligosaccharide chains, which are attached to the heavily glycosylated proteins such as Band-3 or sphingolipids (glycolipids are formed) (Hakomori, 1999; de Mattos, 2016). It seems that other Golgi glycosylation enzymes could synthesize antigens AgA and AgB although with significant difficulties (Varki, 1998; Varki *et al.*, 1999). In newborn children, their titre of AbA and AbB is very low (Wuttke *et al.*, 1997), it increases rapidly in 3 months after the birth and becomes maximal at 18–20 years of age, and then, their titre is reduced.

Several hypotheses were proposed in order to explain this discrepancy. The first hypothesis suggests that the appearance of AgA and AgB in the blood and subsequent generation of antibody poses that epitopes similar to AgA and AgB are delivered to the blood from the intestine after the digestion

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of food. For instance, many bacteria express similar polysaccharide chains on their cell surfaces. However, AgA and AgB *per se* are polysaccharides, whereas, in adults, human enterocytes cannot transcytose the polysaccharide molecules (Sesorova *et al.*, 2020). Humans lost the ability to absorb PS from the lumen of the intestine soon after their birth. Only immediately after birth and only a small amount of proteins and PS could be transported. Most of these proteins represent antibodies of the IgG class due to the presence of anti-Fc receptors (He *et al.*, 2008).

Currently, there are two models for the formation of antibodies against non-existing antigens A and B. (1) These antibodies could originate from antibodies produced against Gram-negative bacteria, such as *Escherichia coli*, cross-reacting with the α-D-galactose on the B glycoprotein (van Oss, 2004). (2) These antibodies could be caused by viral infection because most viruses contain membranes filled with proteins passing through the host GC (Glingston *et al.*, 2019) where enzymes producing AgA or AgB could be. Appearance of the membranes composed of proteins and lipids containing PS with AgA or AgB could induce an immune reaction.

Here, we tested these hypotheses and also our one, which is based on the assumption that Golgi glycosylation could make mistake especially when the intra-Golgi transport is overloaded. Indeed, after overloading the Golgi with cargo, the synthesis of sugar chains occurs with low preciseness inducing a change in glycosylation (Marra *et al.*, 2007). Caco-2 cells express H Type 1 blood group antigen on the basolateral plasma membrane and chylomicrons (ChMs; Amano and Oshima, 1999). We loaded the differentiated Caco-2 cells with a high concentration of lipids. This led to the alterations of the GC and the appearance of lipid droplets suggesting that enterocytes were overloaded with lipids (Sesorova *et al.*, 2020). Under these conditions, some of these cells produced AgA suggesting that non-existing polysaccharide antigens could be synthesized under some conditions by the human own Golgi glycosidases.

It is impossible to perform these experiments on laboratory animals because they do not have the human blood group antigens. One possibility is to use biopsies of the human small intestine, but it is technically and ethically very difficult to do. Finally, we could use Caco-2 cells able to become enterocytes. On the other hand, Caco-2 cells were from a person with the blood group 0(I) and they express H Type 1 blood group antigen on the basolateral PM and on ChMs (Amano and Oshima, 1999) and due to the absence of both GTA and GTB these cells do not synthesise AgA and AgB. Caco-2 cells derive from human colorectal adenocarcinomas and can spontaneously polarize *in vitro* when cultured in a tight monolayer for 3 weeks. They form a mature brush border, express small intestine-specific enzymes, and use trafficking routes specific for polarized cells, including direct biosynthetic trafficking and transcytosis (Fleet *et al.*, 2003; Schneeberger *et al.*, 2018). Caco-2 cells synthesize both ApoB-100 and ApoB-48 and are able to take up lipids. The ApoB-48/ApoB-100 ratio is maximal in proliferating Caco-2 cells (Amano and Oshima, 1999; Santos *et al.*, 2016; Schneeberger *et al.*, 2018). In non-differentiated Caco-2 cells, apical endocytosis exists. After differentiation, these cells behave as human enterocytes and transcytose lipids (Santos *et al.*, 2016). Several leukocyte markers, namely, CD10, CD13, CD14, CD18, CD21, CD25, CD26, CD28, CD31, CD35, CD47, CD59, CD61, and CD63 are present on both human enterocytes and Caco-2 cells. In contrast to enterocytes in Caco-2 cells, HLA-class 11 molecules are not found, synthesis of fat via the mono-acyl-glycerol pathway is much lower, the mono-acyl-glycerol pathway is inactive, and despite abundant production of ApoB, the secretion of newly synthesized triglyceride-rich lipoproteins is restricted (Trotter and Storch, 1993; Levy *et al.*, 1995). Caco-2 cells express lower levels of intestinal fatty acid-binding protein than enterocytes and cannot induce proliferation of allogeneic lymphocytes (Darimont *et al.*, 1998; Rodriguez-Juan *et al.*, 2001; Hiebl *et al.*, 2020). However, since our primary interest is to utilize the ability of Caco-2 cells to assemble and secrete chylomicrons from a source of fatty acids, it is a useful model cell line for this study. In other mammals, the monosaccharide's very short branches (forks) similar to human AgA or AgB are not found. Therefore, here we used Caco-2 cells.

Materials and Methods

Unless otherwise stated, all chemicals and reagents were obtained from the previously indicated sources (Beznoussenko *et al.*, 2014, Beznoussenko *et al.*, 2016; Sesorova *et al.*, 2020). Cholic (bile) acid (Catalogue number: №C1129) and cholesterol (Catalogue number: №C8667) were from Sigma-Aldrich (Milan, Italy). Protein kinase inhibitor H-89 was from MedChemExpress (Catalogue number: №HY-15979). PKI-1422 (Subramanian *et al.*, 2019) was from EMD Millipore/Calbiochem (Catalogue number: №476485100). Blood Group A Antigen Monoclonal Antibody (HE-193. Invitrogen. Catalogue number: №MA1-19693) and Blood Group B Antigen Monoclonal Antibody (HEB-29, Catalogue № MA1-19691) was from ThermoFisher Scientific. The blood-group antibodies were used at a dilution of 1:100 for 1 h at room temperature, as was described by Gehrie *et al.* (2014). Treatment of cells with NEM was performed exactly as described by Kweon *et al.* (2004). Brefeldin A used at concentration 1 μm/mL (Mironov *et al.*, 2004).

Caco-2 cells were cultivated as it was described (Townley *et al.*, 2012) with the addition of recommendations by Wu *et al.* (2013). Briefly: Cells were seeded on MatTek Petri dishes at a density of 2×10^5 cells per insert and cultured for 3 weeks with media changes every other day. The cell viability was always higher than 95%, evaluated with Trypan Blue solution. We used 12% BSA or adult serum as the replacements for embryonic serum.

In order to test whether the transcytosis through the Caco2 differentiated cells occurred, we used the mixture of fatty acids proposed by Townley *et al.* (2012) and Santos *et al.* (2016) with the addition of 1% of bile acid and 0.5% of cholesterol. The mixture was heated and intermixed. Then it was diluted 5-fold and used in the dilution condition and without dilution. In one MatTek, we added 150 μL of this pseudochyme over the central cavity of the MatTek Petri dish where Caco-2 cells were grown, and cells were incubated for 20 min.

The applied concentration of H89 was equal to 50 μM, whereas the concentration of PKI1422 was equal to 150 μM (Subramanian *et al.*, 2018). In the experiments with COPII, BeFx complexes were prepared according to Antonny *et al.* (2001); namely, BeFx solution contained 10 mM KF, and 250 mM $BeCl_2$ was used for the preparation of the pseudo-chyme. 1 μM NEM was added to the pseudo-chyme in 5 min and 20 min after the beginning of the incubation according to the protocol described by Kweon *et al.* (2004).

We took isolated washed erythrocytes obtained from the person with the blood group AB (department of blood transfusion of S. Petersburg). These cells were fixed with 0.05% glutaraldehyde in PBS (pH 7.4) or 10 min and then and these cells were incubated with endoglycosidase H for 4 h. Erythrocytes were supplemented with 1 U/μL endoglycosidase H (NEB) and were incubated for 2 h at 37°C. Then samples were centrifuged and heated at 90°C for 5 min to induce denaturation of endo H. Next this supernatant was used for the dilution of the monoclonal anti-AgA and anti-AgB antibodies. We found that heating for 5 min at 85°C was sufficient for the denaturation of endoglycosidase-H. In control experiments, where the epitope was already glued to the most-sticky part of the antibody, there was no labelling in the Golgi area and in post-Golgi carriers operating between the Golgi apparatus and basolateral plasma membrane.

Preparation of reagents, immune fluorescent microscopy, conventional electron microscopy (EM), correlative video-light EM, nano-gold immune EM labelling, ultra-thin cryo-sectioning, counting of labelling density, and analyses by electron microscopic tomography were all carried out as previously described (Beznoussenko *et al.*, 2015). Sections were examined using electron microscopes Tecnai-12 and 20 (ThermoFisher, Eindhoven, The Netherlands) as described previously (Beznoussenko *et al.*, 2016).

Results

After 21 days of cultivation, Caco-2 formed a brush border (Figs. 1A–1D) and interdigitating contacts (Fig. 1E). The most complete differentiation of Caco-2 cells into enterocytes was found when the pH of the medium was equal to 7.5: The percentage of cells with microvilli reached 91 ± 7% (Fig. 1D). We also found the cisternae of the SER attached to the basolateral PM. This feature was similar to that observed in enterocytes in the intestine (Sesorova *et al.*, 2020). The Golgi complex (GC) was situated above the nucleus. On optical sections passed perpendicularly to the long axis of a Caco-2 cell GC appeared as dashed rings (Fig. 1F).

When the diluted artificial chyme (pseudo-chyme) was added, these Caco-2 cells exhibited similar phenotypes to those described earlier (Sesorova *et al.*, 2020). After the addition of the diluted pseudo-chyme, we observed the appearance of ChMs in the distensions of Golgi cisternae (Fig. 2A). Lipid droplets were not formed. The IF analysis did not reveal a significant number of spots positive for AgA and AgB (this parameter was at the level of background (Figs. 1G: left, 1I: left).

In order to overload Caco2 cells with lipids, we added the non-diluted pseudo–chyme. The overloading phenotype appeared (Sesorova *et al.*, 2020). The main evidence that we reached the overloading of the Caco2 cells with lipids was the appearance of lipid droplets in the cytosol and accumulation of many ChMs at the trans-side of the GC (Figs. 2B–2D).

Several cells acquired spots positive for anti-AgA and Anti-AgB with the Golgi area (Figs. 1G–1J, Figs. 2E–2G, 2I and 2J). In order to control the specificity of antibodies, we used AgA and AgB obtained from isolated erythrocytes from the AB blood group. The addition of AgA and AgB blocked staining with antibodies (Figs. 1H and 1J). Thus, overloading of enterocytes with lipids could induce rare errors of the protein glycosylation.

Role of SNAREs, COPII and COPI

We tried to identify at what level there could be glycosylation errors. To this end, we tested the role of the most important molecular machines operating at the ER-GC interface COPII, COPI, and SNARE. To inhibit COPI we used BFA, the inhibitor of the COPI assembly (Klausner *et al.*, 1992). In order to inhibit COPII BeFx, H89 and PKI (permeable protein kinase A inhibitor) 1422, inhibitors of COPII assembly (Aridor and Balch, 2000; Omari *et al.*, 2018; Subramanian *et al.*, 2019) were applied. Finally, we treated cells with NEM for 5 min on ice immediately after the moment when ChMs appeared in the smooth ER (5 min and after their appearance in the GC (20 min). Then cells were washed and observed for 5 additional min.

Our analysis revealed that BeFx, 50 μM H89, and 100 μM PKI1422 did not interfere with the ER-Golgi transport of pre-ChMs (Figs. 3H–3J). In all these cases, ChMs were visible inside the GC. In 20 min after the addition of the pseudo-chyme. In contrast, 1 mg/mL BFA blocked the delivery of pre-ChMs from the ER at the Golgi and formed giant ChMs and the tubulated GC (Figs. 3K and 3L). Thus, the errors in the synthesis of AgA were not dependent on COPII but could be dependent on COPI.

In order to evaluate the role of membrane fusion for different steps of intracellular transcytosis, we added the undiluted pseudo-chyme to differentiated Caco-2 cells and, in 5 min, when ChMs were already visible within the SER, we treated cells with 1 mM NEM on ice for 15 min and, after its washout, we incubated cells for additional 5 min (the time when the effect of NEM on mitochondria is minimal, see Kweon *et al.*, 2004) and examined whether ChMs are detectable within the GC. Although in control experiments we found ChMs within the GC, after such treatment with NEM the GC almost did not contain ChMs (Fig. 3M). Also, we treated cells with NEM in 15 min after the pseudo-chyme addition and then incubated additionally for 5 min. In control cells, ChMs were already visible within the space between the basolateral PM (BLPM) of interdigitating contacts (IDCs), whereas after treatment of cells with NEM CHMs were observed less frequently there (Fig. 3N). However, the size of ChMs was not changed (our unpublished observations). Thus, our experiments with NEM indicated that the ER-Golgi and the post-Golgi transport routes were SNARE dependent. Altogether our analysis suggests that the incorrect glycosylation was caused by alterations of the GC.

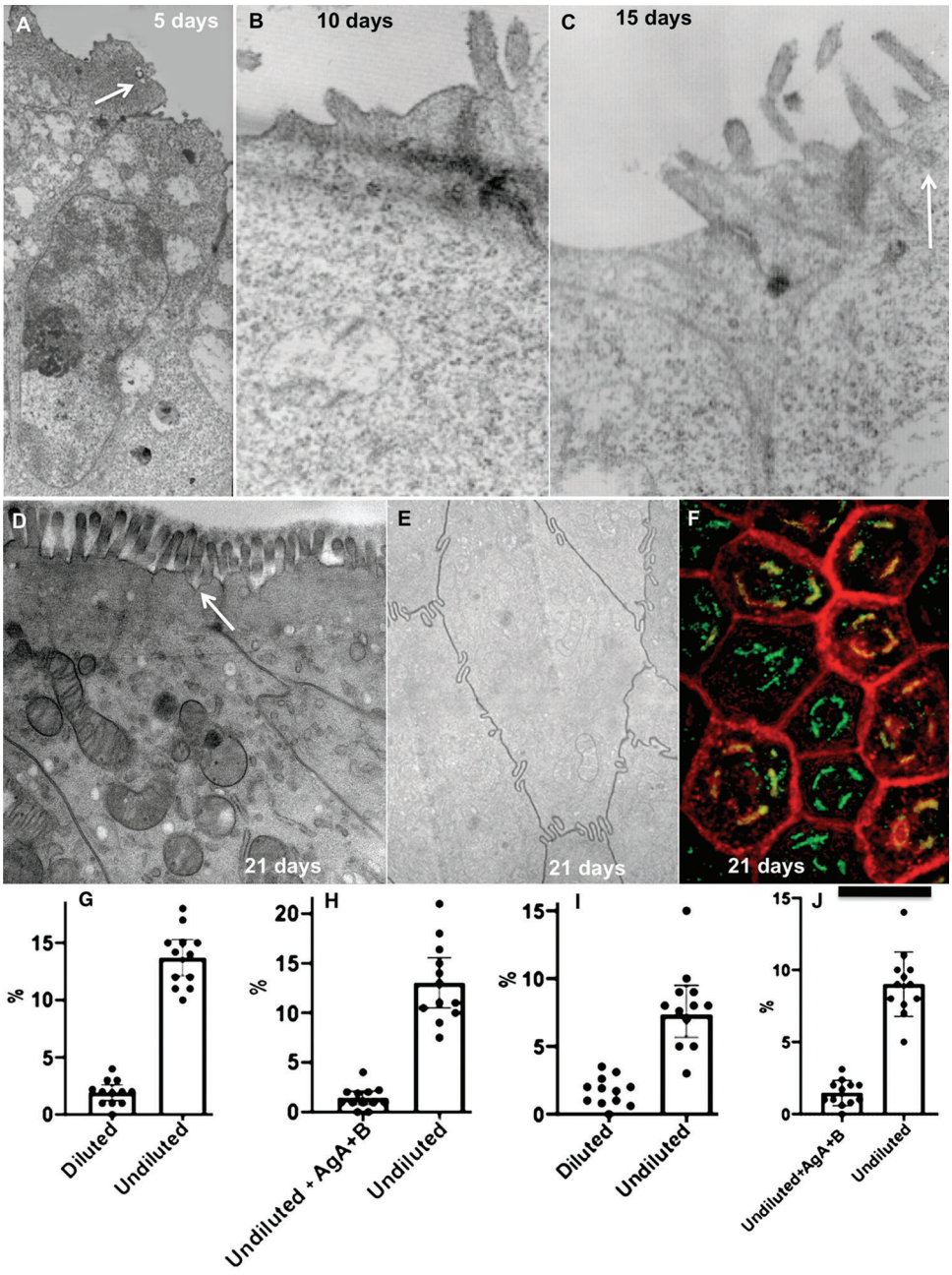

FIGURE 1. Dynamics of the Caco-2 cell differentiation.
Consecutive stages of the formation of epithelial layers. (A–E) Electron microscopy images. (A–D) formation of microvilli on the apical PM. (E) Formation of interdigitating contacts. (F) E-cadherin is coloured in red, GalT is coloured in green. Immune fluorescence. (G–J) Percentages of cells containing spots positive for AgA or AgB (G, H) after addition of the diluted (left bars) or undiluted (right bars) pseudo-chyme (G, I) or after incubation of cells with undiluted pseudo-chyme in the presence (control of antibodies) or absence antigens A + B (H, J). In all graphs, the difference between the two bars is significant ($P < 0.05$). Scales bars: 1.7 μm (A); 400 nm (B, C); 850 nm (E); 350 nm (E); 5 μm (F).

Discussion

Although morphology and functional characteristics of Cavo-2 cells are rather similar to enterocytes, Caco-2 cells slightly differ from standard enterocytes in the organism. For instance, even after differentiation, these cells express lower levels of intestinal fatty acid-binding protein (I-FABP) than enterocytes suggesting that I-FABP is not necessary for Caco-2 cells to absorb and metabolize long-chain FFAs (Darimont *et al.*, 1998). After 7 days of cultivation of filter, the relative apical and basolateral membrane surface areas of the Caco-2 cells was found to be only 1:3 (Trotter and Storch, 1993). Application of an adult serum and BSA accelerated differentiation of Caco-2 cells. Of interest, in undifferentiated Caco-2 cells, the apical endocytosis is visible after differentiation it became less evident as in enterocytes from adult animals (our unpublished observations; see Sesorova *et al.*, 2020).

However, iCaco-2 cells represent a useful model for this study because our primary interest was to utilize the ability of Caco-2 cells to assemble and secrete ChMs (Townley *et al.*, 2012; Santos *et al.*, 2016). Not only enterocytes but also other cells are very sensitive to lipid overloading. Indeed, monocytes from mice incubated with LDL taken from normal animals did not form foam cells. However, when these monocytes were incubated with LDL taken from humans, they are transformed into foam cells (Rekhter *et al.*, 1993).

The appearance of lipid droplets and alter and overloading of the GC suggests that the addition of a lot of pseudo-chyme could induce over-loading of Caco-2 cells with ChMs. Earlier, we demonstrated that when enterocytes were overloaded with lipids containing bile acids and fatty acids, lipid droplets appeared in the cytoplasm, the structure of the GC changes and the transcytosis of ChMs was altered (Sesorova *et al.*, 2020). Experiments with overloading of the differentiated Caco-2 cells with the pseudo-chyme allowed us

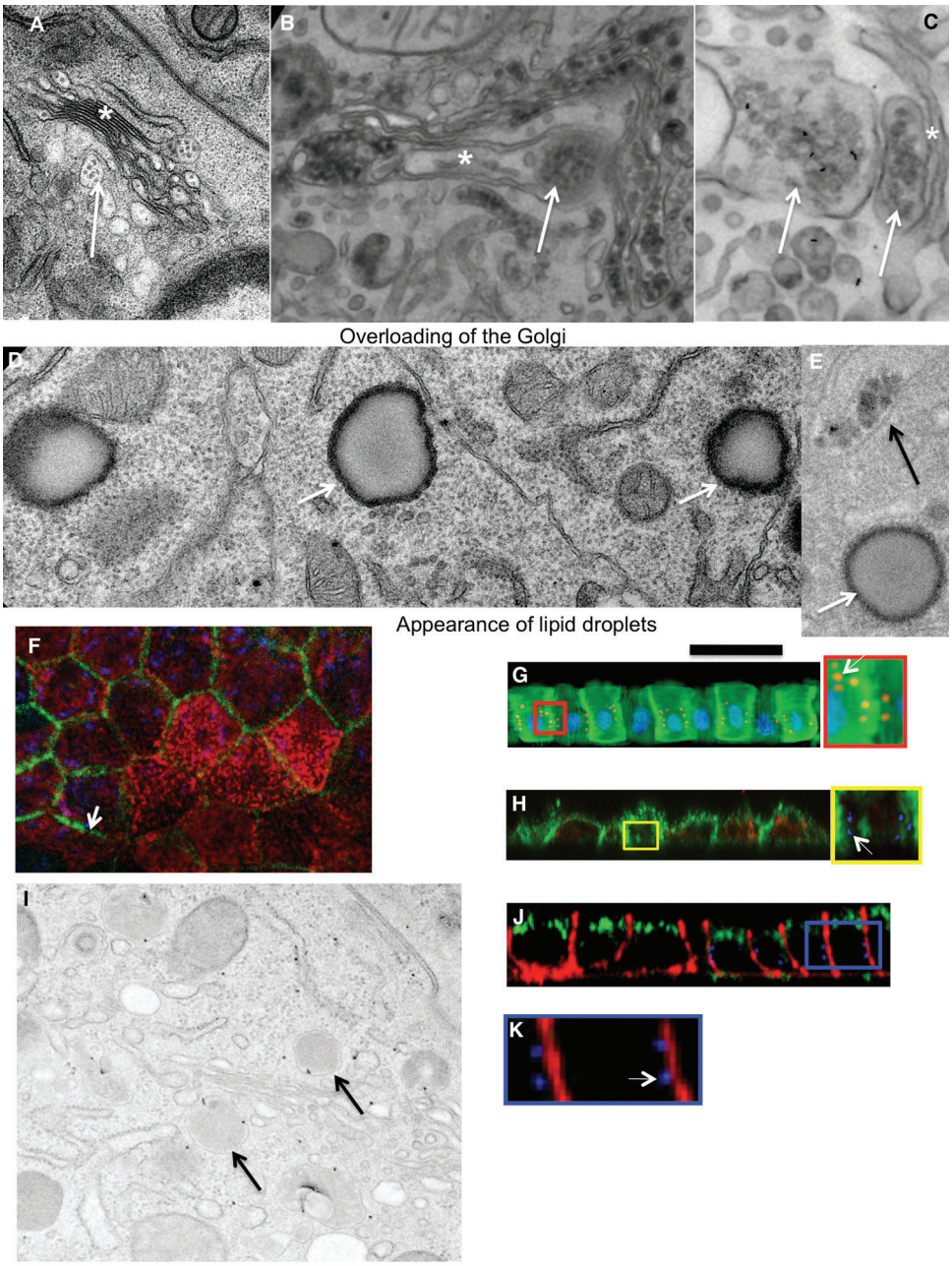

FIGURE 2. Loading of Caco-2 cells with lipid.
(A) Chylomicrons (ChMs) are transported across the GC. ChMs are in the cisternal distensions of the GC. (B, C, E) Appearance of lipid droplets in the cytosol after the overloading. (D) accumulation of ChMs in the GC (asterisk) after its overloading with lipids. (F–H) Appearance of AgA-positive spots in some Caco-2 cells after their overloading with lipids. (I, J) Labeling for anti-A antigen (gold particles). (F) Appearance of anti-A-positive dots in the matured Caco-2 cells after overloading of cells with lipids. Tangential optical section closed to the APM. The red color indicates villin (the marker of the APM). The green color is e-cadherin. Blue color indicates AgA positive dots. (I) Nano-gold (black dots) labeling of ChMs for Antigen A. (G, H, J, K) Representative vertical sections after reconstructions from optical tangential sections demonstrate red and blue dots (white arrows in boxes) positive for AgA. (I) The immune EM nano-gold-enhancement (black dots) labeling of ChMs for Antigen A. Scale bars: 520 nm (A); 260 nm (B-E, I); 4.5 μm (F); 15 μm (G, H–J).

to find the errors in glycosylation of proteins that are present inside ChMs. This was similar to the situation that occurs during the transport of massive amounts of membrane proteins through the GC and leads to a change in standard glycosylation processes (Marra *et al.*, 2007). Our observation is not unique. The altered terminal glycosylation is a common feature of cancer cells conferring new phenotypic properties to the cells (Groux-Degroote *et al.*, 2018).

Glycosyltransferases acting on the histo-blood group carbohydrate biosynthesis have redundancy and degeneration. Redundancy is observed when two separate enzymes synthesize the same antigen (Groux-Degroote *et al.*, 2018). Degeneration occurs when the same enzyme synthesizes different carbohydrate structures. For instance, FUT3 gene-defined fucosyltransferase is capable of synthesizing at least four different blood groups of carbohydrates (de Mattos, 2016). Also after Gal-knockout (KO), pigs were produced in several institutes by knocking out the α(1,3)galactosyl transferase (GGTA1), another transferase, and the GGTA2 remains in pigs (Keusch *et al.*, 2000). Several researchers reported this possibility in Gal (GGTA1)-KO pigs (Taylor *et al.*, 2003; Milland *et al.*, 2006; Sandrin, 2007; Kiernan *et al.*, 2008). ß-D-mannoside, ß 1,4-N-acetylglucosaminyltransferase III (GnT-III) catalyses the branching of N-linked oligosaccharides, producing a bisecting N-acetylglucosamine (GlcNAc) residue. Once a bisecting GlcNAc residue is added to the core mannose by GnT-III, the action of other competitive enzymes such as GnT-IV and GnT-V is prevented from introducing any additional structures into the Golgi stack. As a result, it is likely that all levels of N-linked sugar, including Gal and non-Gal antigens, are decreased. As a strong point of this strategy, overexpression of GnT-III clearly works on non-Gal antigens relate to N-linked sugar (Miyagawa *et al.*, 2001, 2012).

We think that other Golgi enzymes are involved in the synthesis of AgB because GTA and GTB do not exist in Caco-2 cells. Indeed, there is a significant overlapping in

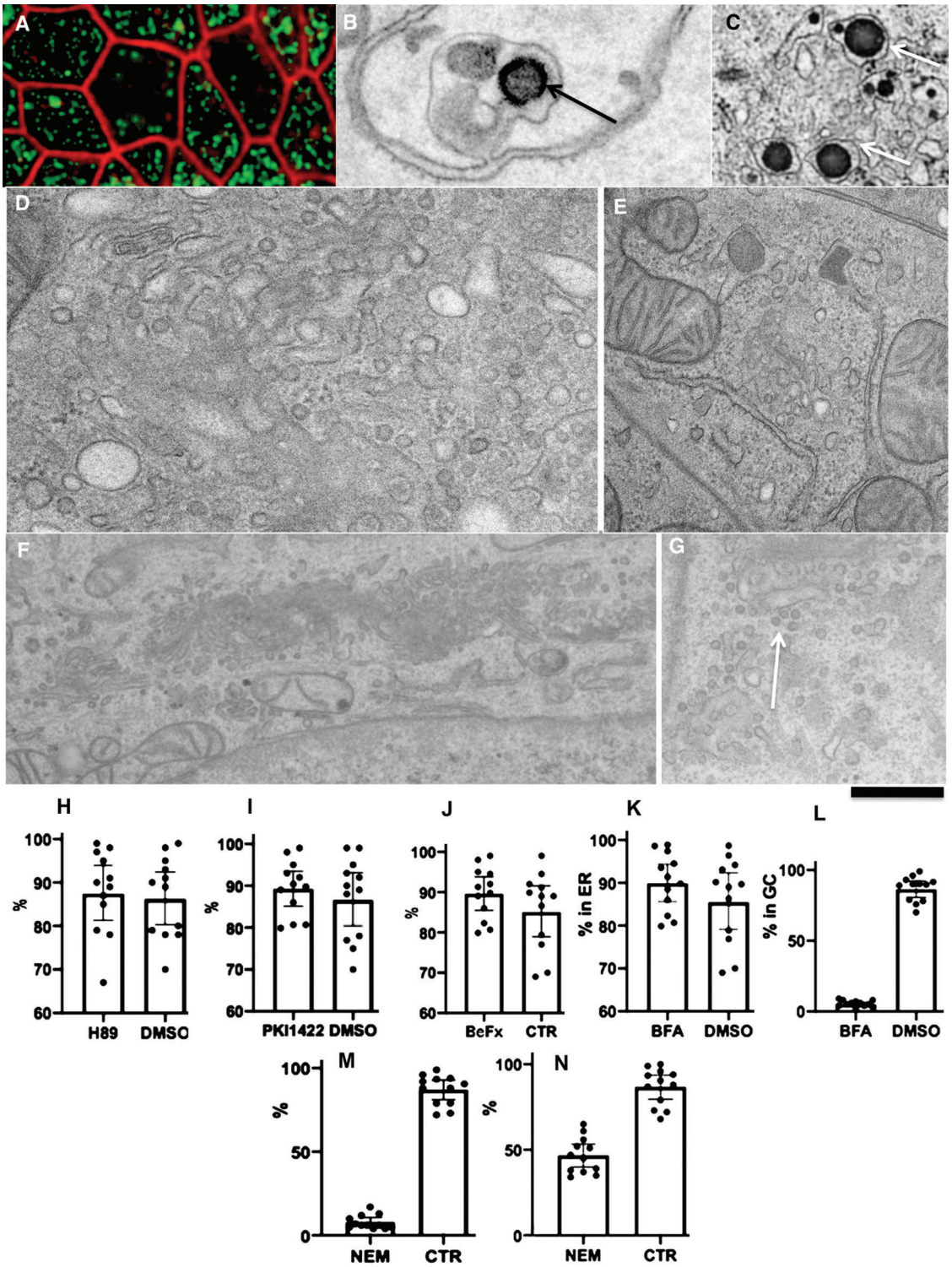

FIGURE 3. Effect of the treatment of Caco-2 cells with different drugs affecting COPII, COPI and SNAREs.
(A) Treatment with brefeldin A. Reduction of the Golgi size is visible. (B, C) Large ChMs (arrows) in the ER after the overloading of Caco-2 cells with lipids. (D, E) Tubulation of the GC under the action of BFA. (F) Treatment of Caco-2 cells with AlF4 induce the Golgi tubulation and vesiculation. (G) Treatment of Caco-2 cells with NEM induced vesiculation (arrow) of the GC. (H–J) Percentage of cells exhibiting ChMs in the GC (H–J, M) after incubation of cells with undiluted pseudo-chyme in the presence (left bar) of absence (right bar) of H89 (H), PKI1422 (I), BeFx (J). (K, L) Percentage of cells exhibiting ChMs in the ER (K) or in the GC (L, M) or in the extracellular space (N) After their incubation with undiluted pseudo-chyme in the presence of the indicated drugs. The difference between the left and the right bars is not significant (H–K; $P > 0.05$) or significant (L–N; $P < 0.05$). Scale bars: 5 μm (A); 330 nm (B); 500 nm (C); 270 nm (D): 450 nm (E); 670 nm (F); 450 nm (G).

functional characteristics among different Golgi enzymes (Diswall *et al.*, 2007, 2010, 2011). Importantly, the altered terminal glycosylation is a common feature of cancer cells conferring new phenotypic properties to the cells (Groux-Degroote *et al.*, 2018). In the same individual various tissues express AgB and AgA in a different way (Oriol *et al.*, 1992). Sialylation increased and fucosylation decreased with age (Lityńska and Przybyło, 1998). This

explains why with age the titre of AbA and AbB decreased. In any case, this question deserves additional analysis.

On the other hand, in differentiated Caco-2 cells, we did not find ER exit sites, COPII-coated buds and mega-buds, and apical clathrin-coated buds or ChM-containing vesicles near the APM before their loading and overloading with lipids. Also, in rat adult enterocytes, we did not find COPII-coated buds and mega-buds. Also, no mega-vesicles ferrying ChMs were observed (Sesorova *et al.*, 2020).

Therefore in order to check whether COPII is necessary for the ER-Golgi transport of ChMs, we treated cells with BeFx, H89 or PKI-1422. These substances impaired the function of COPII. However, this did not block ER-Golgi transport of ChMs, whereas brefeldin A inhibited this delivery leading to augmentation of ChM size and Golgi tubulation. Nevertheless, several authors claim that COPII-coated vesicles exist. However, careful analysis of these papers revealed that after initiation of synchronized transcytosis through enterocytes generated from Caco-2 cells labelling for TANGO1 were never surrounded ApoB-containing BODIPI-positive ChM (Fig. 6C in Santos *et al.*, 2016).

After treatment of the differentiated Caco-2 cells with 1 μm/mL brefeldin A, we found partial fragmentation of the GC at the level of light microscopy but did not observe ChM immune EM labelling for AgA and AgB within the GC. Similarly, Santos *et al.* (2016) demonstrated that loading of differentiated Caco-2 cells with lipids in the presence of brefeldin A, induced the formation of giant ChMs with their diameter of up to 2.3 μm. Thus, in Caco-2 cells, the ER-GC transport of ChMs is dependent on COPI but independent on COPII.

The first hypothesis of the AbA and AbB formation poses that epitopes on gut flora or plant materials similar to AgA and AgB are delivered to the blood from food (van Oss, 2004). Chickens kept in a germ-free environment would produce anti-B but not anti-A when fed bacteria expressing high levels of a B-like antigen and lower levels of an A-like antigen (Springer *et al.*, 1959, 1961; Springer GF).

However, there are several problems with this hypothesis. Indeed, bacterial lipo-PS (LPS) are rather toxic, being able to alter the jejunal absorption of amino acids (Abad *et al.*, 2001) and ascorbic acids (Subramanian *et al.*, 2018), and serotonin (Mendoza *et al.*, 2009). After prolonged exposure of the intestine to LPS, there is a loss of epithelial integrity and could induce detachment of epithelial cells (Wells *et al.*, 1993).

Antibodies against AgA and AgB could be found even in neonates where consumption of bacterial PS is not possible. These antibodies are not of maternal origin (De Biasi, 1923; Thomaidis *et al.*, 1967; Chattoraj *et al.*, 1968; Godzisz, 1979; Mencarini *et al.*, 1982; Wuttke *et al.*, 1997; Merbl *et al.*, 2007). Only in newborn mammals, enterocytes can transcytose macromolecules and polysaccharides (PS) from the intestine lumen (Gossrau, 1975a, 1975b). Moreover, only a small amount of proteins are transcytosed, and most of these proteins represent antibodies of the IgG class due to the presence of anti-Fc receptors (He *et al.*, 2008). Moreover, soon after birth, this capacity disappears and enterocyte form a strong tissue-blood barrier, which does not allow proteins and oligosaccharides to be transcytosed through enterocytes. For instance, in rats, mice and pigs already in 7 days after the birth, such transcytosis was not found (Ekström *et al.*, 1988, Ekström and Weström, 1992). In humans, the level of the maternally transferred IgG1-4 declined over the first week of life (Bennike *et al.*, 2020). In adults, PS is hydrolysed by pancreatic α-amylase (Asanuma-Date *et al.*, 2012).

Large PS molecules could be absorbed by enterocytes from the gut only through apical endocytosis because PS cannot jump through the lipid bilayer and tight junctions between enterocytes are impermeable for PS. In the intestine, carbohydrates undergo digestion and newly formed monosaccharides are absorbed through the APM (Bu *et al.*, 2010; Asanuma-Date *et al.*, 2012; Goto *et al.*, 2012; Howe *et al.*, 2014). However, we did not find clathrin-coated buds on the APM of the rat adult enterocytes (Sesorova *et al.*, 2020). In spite of this inhibition of endocytosis of PS (which occurs very soon after birth) concentration of anti-A and anti-B antibodies increases until 18 years (Springer *et al.*, 1959, 1961; Springer and Horton, 1969). Finally, linear PS's have low antigenic properties. Specific extremely short double-edged fork (branches where each branch from two is composed of only one monosaccharide) at the tips of the bacterial PS and LPS are not found.

The cross-reaction hypothesis poses that these antibodies originate from the immune response towards the influenza virus, whose epitopes are similar enough to the α-D-N-galactosamine on the A glycoprotein could elicit a cross-reaction (Hakomori, 1999; Christen *et al.*, 2010; Arend, 2013; Mujahid and Dickert, 2015). Finally, viruses could transfer AgA and AgB formed within their membrane deliver antigens A and B carry from one person to another. Viruses form the external membrane of their viral envelope from the portions of the plasma membrane. After infection, the virus delivers the antigen into the PM of human cells. Therefore, a person begins to synthesize antibodies against AgA introduced by the virus. However, at the beginning of life, a child often has a very small number of contacts with other people. Also,a the amount of antigen delivered by a virus is too small to stimulate the relevant lymphocyte clones. Moreover, antigen A and B are synthesized on the basolateral plasma membrane and not on the apical plasma membrane. Therefore, viruses bearing these antibodies have no direct access to the external environment, and their infection occurs through the blood. Constant synthesis of AgA or AgB in person with Groups B and A correspondingly could induce immune conflict. Of interest, the altered terminal glycosylation is a common feature of cancer cells conferring new phenotypic properties to the cells (Groux-Degroote *et al.*, 2018). Similarly, alterations of glycosylation of chylomicrons could be one of the main atherogenesis mechanisms (Mironov *et al.*, 2019).

Outlook

Thus, overloading of enterocytes with lipids induces rare glycosylation errors in the structure of PS and the formation of PS antigens within the sugar chain. This could explain the mechanism of the formation of blood group antibodies. Our hypothesis could be tested using the predictions, which it

generates. For instance, it predicts that (1) The titre of AgA and AgB would be higher in those who eat a lot of fatty foods. A similar study could be performed using biopsies from the intestine of humans. Biopsies of jejunum could be obtained during operation for cancer. These samples could be taken from the tumour containing part of the intestine just near the border. Then, the samples could be placed into a culture medium and delivered at 37°C to the laboratory. Next, the samples could be placed epithelial back on the large droplet of chyme from rats and incubated for 1 h. Further, samples could be washed and immune labelled with an antibody against AgA. In turn, the knowledge about mechanisms of formation of AgA and AgB in an organism where these antigens should not be important for organ transplantation especially of kidneys (Irving *et al.*, 2012) because waitlist mortality continues to be a limiting factor for all solid-organ transplant programs (Grasemann *et al.*, 2012). This observation could be useful for the explanation of atherogenesis. The role of glycosylation mistakes in the development of atherosclerosis is explained in our review (Mironov *et al.*, 2020). For instance, alteration of glycosylation, especially sialylation, could induce aggregation of ChMs, ILDLs and LDLs and lead to the formation of antibodies against these apo-lipoproteins.

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