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Alpha (1,2) Fucosylated Glycoepitopes from Invertebrates to Humans

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Review Article

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ABSTRACT

Glycans participate in bio-communication, such as cell to cell recognition, sperm-egg recognition and embryonic development because they have the property of storing biological signals in forms that can be identified by other biological systems, such as anti-glycan antibodies and glycan binding proteins or lectins. Aberrant arise at the cell surface in many diseases, including cancer, and altered carbohydrate moieties are indicated as mediators of tumorigenicity and invasiveness. Alpha-L-fucopyranosyl residues are immunodeterminant, playing an important role in the glyco-language; this is especially true of residues alpha(1,2)-linked to a galactose, that frequently exist as terminal modifications of N-glycans, O-glycans or glycolipids. The H antigen is the product of the enzyme that adds fucose to galactose residues by an alpha(1,2)-linkage. We report work in which we demonstrated the presence and role of alpha(1,2)fucose-containing glycoepitopes in biocommunication between egg and sperm in two invertebrate species, the tunicate *Ciona intestinalis* and the mollusk *Unio elongatulus*. In the mollusk the structure of the fucosylated epitopes involved in sperm recognition in the context of the blood group H-antigen was confirmed by MS/MS data. Using an antibody raised against the bio-communicating glycoprotein of the *Unio* oocyte, the epitope of which was demonstrated to contain the alpha(1,2)fucosylated O-glycans, we detected the same or a very similar epitope in nucleolin, a protein expressed in highly proliferating and cancer cells in humans, known to act as a shuttle between the cell surface and nucleus. We suggest that the glycosylation machinery used to build up the alpha(1,2)fucosyl-containing glycoepitope of the *Unio* egg, is re-stored in cancer cells.

Every free living cell and every cell type in a multicellular organism is decorated externally with a thick layer of carbohydrates, known as *glycocalyx*. Previously considered no more than a protective coat, the glycocalyx actually contains a precise glycan framework, representing a frontline identity card of the cell for contact with the outside world. Glycans participate directly in processes such as cell to cell recognition and communication, pathogen recognition, sperm-egg recognition and fertilization, directing embryonic development and differentiation, as well as the distribution of various cells and proteins throughout the body. Glycans can also shift from normal to a pathological status in many diseases including cancer [1], and altered carbohydrate moieties have been indicated as mediators of tumorigenicity, invasiveness and metastatic potential [2,3]. Glycans participate in bio-communication because they have the property of storing biological signals in forms that can be identified by other biological systems. The main molecules capable of reading the glycan language are anti-glycan antibodies and glycan binding proteins or lectins [4]; the carbohydrate moiety recognized by these molecules is referred to as *glycoepitope* [5]. Glycoepitopes are therefore the words of the glycan language. Unfortunately, the glycan language is very complex and difficult to read. Glycan complexity depends on the fact that carbohydrates can generate a large range of structural diversity. Unlike proteins, which are connected solely by a peptide bond, carbohydrates exploit many possible glycosidic linkages. Two amino acid residues can only produce one dipeptide, whereas two sugar molecules have the potential to generate 11 different disaccharides. A trimer of any of the nine common sugar residues of the human body can theoretically give rise to more than 100,000 different structural isomers. By comparison, 20 different amino acid residues can give rise at most to 8000 tripeptides. The field of research concerned with glycan structures that display glycoepitopes was recently enhanced by the development of advanced profiling and structural characterization strategies, including high-resolution chromatography coupled with exoglycosidase digestion, modern mass spectrometry and nuclear magnetic resonance spectroscopy analysis. However, difficulties in this field persist because the same sugar chain may generate different glycoepitopes when the sugar moiety is presented in different cluster configurations.

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Fucosylated Glycoepitopes

Fucosylation involving attachment of α-L-fucopyranosyl residues to N-glycans, O-glycans or glycolipids is a glycan modification reported to regulate many biological functions of adhesion molecules and to act in cancer progression [6]. Two structural features distinguish fucose from other six-carbon sugars present in mammals. These include the lack of a hydroxyl group on the carbon in position 6 (C-6) and the L-configuration **(Figure 1).** Fucose frequently exists as a terminal modification of glycan structures, thus becoming an immunological determinant. ABO blood group antigens are the best known fucosylated glycoepitopes. The H antigen is the product of an alpha(1,2)fucosyltransferase (FUT1 gene product) that adds fucose to terminal galactose residues on the oligosaccharide precursors **(Figure 1)** that decorate several glycoproteins and glycolipids. In individuals of blood groups A, B or AB, the H antigen is further modified by ABO locus-encoded glycosyltransferase that adds a galactose and an N-acetyl-glucosamine to form the A and B antigens; the unmodified H antigen is expressed on the cell surface of type O individuals [7]. Interestingly, expression of A and B blood group antigens, where galactose and N-acetyl-glucosamine are the determinant sugars, is lost in many tumors with concomitant increases in H expression, where alpha(1,2)linked fucose is determinant. Such changes are correlated with poor clinical prognosis [8-11]. A glycoepitope containing fucose alpha(1-2)linked to galactose has also only been reported in the plasma membrane of Morris hepatoma 7777 but is not expressed in liver, kidney cortex or serum [12].

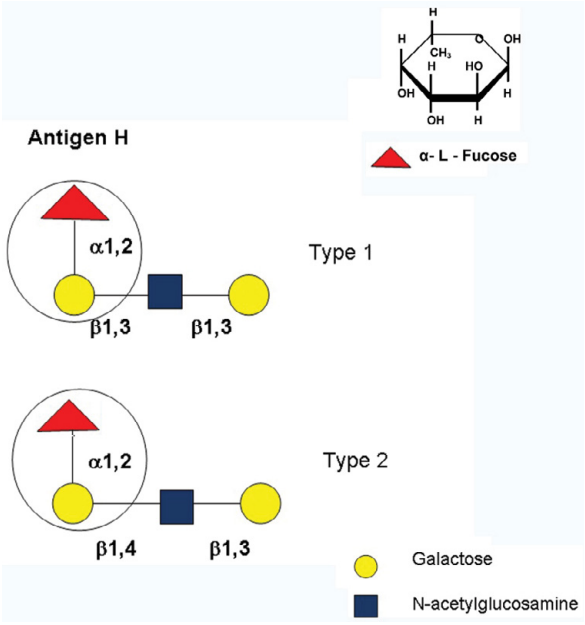

Figure 1. Structure of fucose and H antigen of type 1 and type 2. Circles indicate the immunodominant portion of the antigen.

The main lectins capable of recognizing fucosyl-containing glycoepitopes include *Lotus tetragonolobus* [13] and *Ulex europaeus* [14] lectins from plants, *Anguilla* lectin from eel [15], *Aleuria aurantia* lectin (AAL) from mushroom [16], *Rhizopus stolonifer* lectin [17] and *Aspergillus oryziae* [18] from fungi and *Ralstonia solanacearum* lectin from bacteria [19]. Among them, *Ulex europaeus* and *Lotus tetragonolobus* lectins are reported to prefer α (1,2)linked fucose residues [13,20], whereas AAL, *R stolonifer* and *A oryziae* lectins preferentially bind to α (1,6)fucosylated oligosaccharides [18]. However, as previously mentioned, the spatial organization of glycoepitopes and their cluster configuration may be determinant for recognition by the lectin [19]. Indeed lectins accommodate glycoepitopes larger than mono- and disaccharides and there is also evidence that they prefer certain cluster configurations. For example, the lectin from *Ulex* has been reported to react with fucose in blood antigen oligosaccharides with chain 1 and 2 **(Figure 1),** whereas the lectin from Lotus only reacts with chain 2 [20] **(Figure 1).** Furthermore the lectin from *Ulex* is reported to agglutinate red cells, whereas that from *Lotus* only does so after neuroaminidase treatment, thus suggesting different exposures of the alpha(1,2)fucosylated glycan clusters recognized by the two lectins. *Ulex* lectin has been also reported to require higher concentrations of saccharides to bring about complete inhibition of hemagglutination than did the *Lotus* lectin [21].

A lectin characterization may therefore include but is not limited to:

a) the spectrum of carbohydrate structures that are reactive with it;

b) the conformational properties of the glycoepitopes selectively recognized by it;

c) the cluster effect in lectin-carbohydrate interactions.

Fucosylated Glycoepitopes in Sperm Egg Interaction

In every invertebrate and vertebrate species, and under natural conditions also in man, fertilization of eggs and therefore development of a new individual of the species begins with recognition and binding of one of the million sperm present around the outer coat of the egg. This initiates a series of steps leading the male gamete to fuse with the oocyte. The first evidence that

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sperm-egg binding is mediated by carbohydrates was acquired many years ago, using lectins in mammals [22] and carbohydrates in tunicates [23] as competitors of the process *in vitro*. L-fucose was found to be the preminent sugar for bio-communication with sperm in the tunicate *Ciona intestinalis* **(Figure 2),** since it was the only sugar that interfered with binding; the other sugars tested, including D-fucose, proved completely ineffective [23]. The ability of monosaccharides to effectively block sperm binding has since been demonstrated in a number of invertebrate and vertebrate species, including man. In *Ciona* the role of fucose residues as the major component of the oocyte coat glycoepitope for sperm recognition and binding was confirmed by injecting L-[3H]fucose into female gonads and following its incorporation by means of autoradiography and sodium dodecylsulfate/polyacrylamide gel electrophoresis [24]. Fucose was found to progressively accumulate in the egg coat, especially in three glycoproteins [25]. In this species we were unable to identify which of them was responsible for interacting with sperm, but we know with certainty that it contains an alpha(1,2)fucosylated glycoepitope, since all three proved positive to the lectin from *Lotus*. In our successive experimental animal, the mollusc bivalve *Unio elongatulus,* (C. Pfeiffer) **(Figure 3A)** we identified the egg molecule responsible for recognizing and binding sperm and we demonstrated that alpha(1,2)fucosylated glycoepitopes are an essential part of the recognition event [26]. In *Unio,* unlike other mollusks, the eggs remain in the female and sperm encounter them in the chamber of the gills **(Figure 3B).** The egg of *Unio* is peculiar: unlike other invertebrates in which sperm binding can occur anywhere on the egg coat surface with nothing to aid identification of an animal and a vegetal pole, in *Unio,* sperm only interact with a region of the egg coat located at the vegetal pole [27]. Here the egg coat protrudes to form a truncated cone surrounded by a wrinkled area **(Figure 3B),** where sperm binding occurs. In *Unio* only two glycoproteins were found to account for 90% of the material dissolved from the egg coat [28]. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry determined masses of 273 kD and 180 kD and the two glycoproteins were therefore named gp273 and gp180 [29]. The molecule involved in sperm recognition and binding was identified as gp273 [30], since when the proteins were purified only it was shown to bind sperm; when antibodies were raised against both, only anti-gp273 was positive in the crater region where recognition and binding occur [30]. Interestingly, this region also proved to be the only one positive to the lectin from *Lotus*. Gp273 was found to contain N- and O-linked glycans. Total carbohydrate content was calculated to be about 3.5% by mass and to consist of fucose, mannose, galactose, glucose, N-acetyl-galactosamine and N-acetylglucosamine [31]. The N-linked glycans released by PNGase F and analyzed by a combination of HPLC, ^{1}H NMR spectroscopy and mass spectrometry revealed oligomannose glycans that constituted the major part of carbohydrate content [31]. Fucose, galactose and N-acetyl-galactosamine were presumed to be O-glycan components, indeed carbohydrate analysis of gp273 after removal of N-glycans confirmed their presence in the remaining O-glycoprotein. By fragmentation of the O-glycoprotein, we also obtained an O-glycopeptide that conserved sperm recognition ability, and significantly for our subsequent work, conserved the capacity to interact with anti-gp273 [32]. We then confirmed the importance and determined the structure of the fucosyl-glycoepitope that communicated with sperm. When released and tested for their ability to interfere with sperm binding to the egg, only gp273-derived O-linked glycans proved able to inhibit sperm binding, whereas N-linked glycans had no effect at all. Information on the nature of the O-glycome was then acquired exploiting high sensitivity MALDI-TOF/TOF and the GlycoWorkBench informatics tool [33]. This research revealed that gp273 carries a diverse repertoire of O-glycans, many of which are rich in fucose. Significantly, the MS/MS data showed unequivocally that the fucose residues mostly occurred in the context of the blood group H-antigen and that additional fucose could be linked to the glycan chain [33]. It was therefore definitively confirmed that the coat glycoepitope, with fucose residues alpha(1,2)-linked to a galactose, was responsible for the interaction with sperm in *Unio* and that the lectin from *Lotus* and the anti 273 IgG recognize this epitope. In the species studied by us, the alpha(1,2)fucosylated glycan similar to H antigen seems to be the only component that could communicate with sperm. It is therefore tempting to suggest that the glycan is a syllable of the glycocode that can form a word simply by clustering in a specific way on a protein. As we discuss later, we found a similar glycoword in cancer cells.

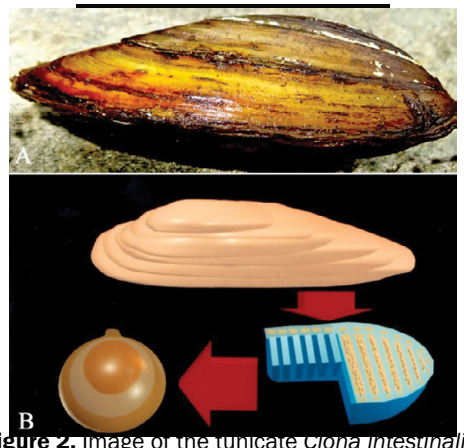

Figure 2. Image of the tunicate *Ciona intestinalis*.

Figure 3. Image of the mollusc *Unio elongatulus* in A and in B graphic representation of *Unio*, the suprabranchial chambers of the gills where the eggs encounter sperm and lastly an egg.

The molecule on the sperm plasma membrane that interacts with the alpha(1,2)fucosylated glycoepitope in the egg coat was suggested to be the enzyme alpha-L-fucosidase, both in *Ciona* [34] and in *Unio* [35]. It has also been suggested that sperm

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alpha-L-fucosidase works as a fucose-binding protein and that the complex formed at the moment of the binding is rather stable, because the glycosidase has an acidic pH optimum, and the rate of hydrolysis in alkaline water is therefore expected to be low. L-fucosyl residues on the vitelline coat and an alpha-L-fucosidase on sperm have been reported as mediators of sperm-egg binding in another tunicate, *Halocinthia* roretzi [36]. Alpha-L-fucosidases on the sperm plasma membrane were more recently suggested to be involved in reproduction in other groups, including insects [37] and mammals [38] including man [39]. However, in none of these cases has the corresponding fucosyl-glycoepitope in the oocyte been analyzed. One reason is that since the success of intra-cytoplasmic sperm injection, the field of research concerned with sperm binding to the oocyte coat has been almost completely abandoned.

The presence and importance of fucose and/or alpha-L-fucosidase in sperm-egg interaction in different and evolutionarily distant species has obviously raised the question of how the same sugar and the same glycosidase can ensure the species specificity necessary for fertilization.

A suggested general answer is that selective pressure probably operates at higher levels of sperm-egg binding, making the encounter of heterospecific gametes highly improbable. At molecular level, an explanation may be that high complexity and specificity evolved through multiple sets of similar or different carbohydrate chains interacting with similar or different carbohydrate binding proteins.

Alpha(1,2)fucosylated Glycoepitopes in Human Diseases

Analysis of cell surface glycans on diseased cells dates back to the 1960s and includes assays of binding between cancer cells and plant-derived lectins [40,41]. Later on, monoclonal antibodies raised against human tumor cell lines were used. These studies revealed that glycan expression by neoplastic cells was radically different from that occurring in the corresponding normal cells [42]. Aberrant glycans were reported in cell lines derived from many adenocarcinomas, certain melanomas and some leukemias and lymphomas. To stress how general the relationship between aberrant glycans and cancer was, in 1984 Hakomori coined the term “tumor-associated carbohydrate antigen” or TACA [42]. In the last three decades, clear evidence has emerged that carbohydrates are essential for manifestation of the malignant and metastatic properties of human cancer cells, as well as for their functional behavior [43,44]. Among the carbohydrates involved in neoplasia, alpha-L-fucose is reported to be one of the most critically important sugars. Unlike fucosylated glycoepitopes functional in sperm-egg interaction in invertebrates, where fucose has always been reported to be associated with O-glycans, in vertebrates including man, fucose can be associated with O-glycans or N-glycans, and particularly with highly branched complex N-glycans which are almost completely absent in invertebrates. Among fucosylated glycans, fucose linked at alpha 1,2 has often been reported in relation to cancer. Tumor progression in the distal colon and rectum is described as associated with expression of the blood group determinants Lewis b, H-type 2, and Lewis y, which share the Fucα1,2Galβ-R motif [45-47]. An increase in α(1,2)fucosyltransferase (FT) activity has been found in colon cancer [48-50] and the H gene is reported to be overexpressed in colon cancer tissue [49]. Goupille et al. [51] transfected a weakly tumorigenic clone derived from a rat colon carcinoma cell line with cDNA encoding for the human H FT and found that expression of α(1,2) fucosylated structures on a variant of CD44 adhesion molecules could be responsible for the aggressiveness of colon carcinoma cells. Beta(1,3)galactosyltransferase (GalT) and alpha(1,2)fucosyltransferase (FT) are reported to be involved in the biosynthesis of type-1-chain carbohydrate antigens in human colon adenocarcinoma cell lines [52]. Globo H is another structure containing Fucα1,2Galβ-R that has been found at the cancer cell surface as a glycolipid or glycoprotein [53-56]. Enhanced expression of MBr1-positive antigens was found on primary and metastatic prostate cancer specimens using the murine monoclonal antibody Mbr1 [53,57,58]. Slovin et al. [59] demonstrated that Globo H is one of several candidate antigens present in prostate cancer cells.

It is therefore legitimate to ask whether any relationship exists between the alpha(1,2)fucosylated glycoepitopes found in invertebrates and those present in human cancer. Our answer to this question is positive, since our IgG anti-gp273 proved to specifically recognize an O-alpha(1,2)fucosylated glycoepitope in nucleolin [60] a protein expressed in highly proliferating and cancer cells in humans, known to act as a shuttle between cell surface and nucleus, and indeed as an extracellular regulator of nuclear activity [61]. The first identification of a relationship between the glycoword of sperm-egg interaction in Unio and that of the shuttle molecule came from electrophoretic analysis of protein extracts of bovine cultured coronary venular endothelial cells (CVEC) and cultured human epidermoid carcinoma A431 cells with anti-gp273 IgG [60]. The only protein that proved immunoreactive to the anti-gp273 IgG was one running at an apparent MW of 110 kDa. When the portion of the gel corresponding to the immunoreactivity was analyzed by mass spectrometry, the protein was found to match nucleolin. The specific immunoreactivity of the anti-gp273 IgG on nucleolin was then confirmed using this antibody at the same time as the commercial anti-nucleolin monoclonal antibody MS-3, known to be directed against the entire peptide sequence of the protein: the antibodies recognized the same molecule [60].

The determinant role of O-glycans in forming the nucleolin epitope recognized by the anti-gp273 IgG was identified first by excluding that gp273 and nucleolin had similar peptide sequences and then by evaluating which glycan chain was involved in forming the epitope by enzymatically removing N-glycans and chemically removing O-glycans from nucleolin and testing the protein with the antibody. Only removal of O-glycans sharply lowered the antigp273 immunoreactivity of nucleolin. As well, the O-glycoepitope of nucleolin was only found to contain fucose linked at alpha 1,2 like that of Unio. In fact, lectin-blot analysis of the protein after removal of N- and O-glycans by lectins from Lotus, Ulex and Aleuria revealed that all three were positive to the entire protein but positivity to Aleuria disappeared after removal of N-glycans and positivity to Lotus and Ulex disappeared after

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removal of O-glycans [61]. We confirmed the presence and importance of the Fucα2-Galβ-R motif in nucleolin by examining whether depletion of the enzymes required for its biosynthesis, namely FUT1 and FUT2, had an effect on expression and activity of the fucosylated nucleolin glycoforms. FUT1 and FUT2 were both found to participate in the fucosylation of O-glycans of nucleolin since expression of fucosylated nucleolin decreased sharply in FUT1/FUT2 depleted cells [62]. Nucleolin was initially reported to be localized mainly in the nucleolus [61,63] but its presence at the cell surface, where it serves as a binding protein for a variety of ligands implicated in tumorigenesis and angiogenesis, has been substantiated by many investigators in different cells [64-66] as has the fact that it carries N-and O-glycans [67]. One of the functions of the protein at the cell surface is to act as receptor to internalize ligands and translocate them into the nucleus [61]. In endothelial cells of angiogenic blood vessels, surface nucleolin is reported to specifically bind endostatin and transport it into the nucleus [68]. Our studies suggest that the protein can be glycosylated in different ways and that fucosylation is important for membrane localization and for its shuttle activity [62]. The presence of antigp273 fucosylated glycoepitopes at the cell surface was subsequently demonstrated in glioblastoma cells and its increasing surface overexpression was correlated with increasing grades of malignancy [69]. This in turn led to demonstration that a nucleolin antagonist triggers autophagic cell death in human glioblastoma primary cells and decreased *in vivo* tumor growth in an orthotopic brain tumor model [70].

CONCLUSIONS

Since the concept of oligosaccharide chains as information-storing coding units is increasingly appreciated, how the sugar words are written and read is a fascinating puzzle. The puzzle is still difficult to solve because of the inherent difficulty of studying these structures. Determining the sequence of an oligosaccharide chain is only the first step. Anomeric positioning and linkage points are other essential aspects needed to define the primary structure of an oligosaccharide chain. The next and most difficult question concerns the third dimension. Depending on conformational flexibility parameters, the same carbohydrate sequence seems able to form more than one three-dimensional shape. Using the classical “lock and key” metaphor, glycans have been envisioned as a “bunch of keys”. We are therefore far from understanding the array of messages with which the versatile glycan assembly endows cells. There is no evolutionary key for glycans that helps us to interpret their language. What is evident is their considerable diversity within and between species. However, our studies indicate that a glycoword used by the oocyte of an invertebrate to communicate with sperm of its own species is closely related or is the same as that used by cancer cells for their physiology in humans. At least in this case, it suggests that the glycosylation machinery that formed the glycoword for biocommunication at fertilization is restored for anomalous biocommunication in the cell shift from normal to malignant status. Obviously we need other data to support this hypothesis, but since research on the molecules at work in sperm egg interaction in invertebrates is no longer “trendy” or economically attractive, it is not currently possible to identify other messages inscribed in this primary interaction mechanism.

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