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Human-specific expression of Siglec-6 in the placenta

Els C M Brinkman-Van der Linden[2,3,5], Nancy Hurtado-Ziola[2,7], Toshiyuki Hayakawa[4,7], Lisa Wiggleton[6], Kurt Benirschke[6], Ajit Varki[1,5], and Nissi Varki[6]

[5]Glycobiology Research and Training Center, Departments of Medicine, Cellular & Molecular Medicine, [6]Department of Pathology, and [7]Biomedical Sciences Graduate Program, University of California at San Diego, La Jolla, CA 92093-0687, USA

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CD33-related-Siglecs are lectins on immune cells that recognize sialic acids via extracellular domains, and deliver negative signals via cytosolic tyrosine-based regulatory motifs. We report that while Siglec-6/OB-BP1 (which can also bind leptin) is expressed on immune cells of both humans and the closely related great apes, placental trophoblast expression is human-specific, with little or no expression in ape placentae. Human-specific transcription factor recognition site changes in the Siglec-6 promoter region can help explain the human-specific expression. Human placenta also expresses natural ligands for Siglec-6 (a mixture of glycoproteins carrying cognate sialylated targets), in areas adjacent to Siglec-6 expression. Ligands were also found in uterine endometrium and on cell lines of trophoblastic or endometrial origin. Thus, Siglec-6 was recruited to placental expression during human evolution, presumably to interact with sialylated ligands for specific negative signaling functions and/or to regulate leptin availability. The control of human labor is poorly understood, but involves multiple cues, including placental signaling. Human birthing is also prolonged in comparison to that in our closest evolutionary relatives, the great apes. We found that Siglec-6 levels are generally low in placentae from elective surgical deliveries without known labor and the highest following completion of labor. We therefore speculate that the negative signaling potential of Siglec-6 was recruited to human-specific placental expression, to slow the tempo of the human birth process. The leptin-binding ability of Siglec-6 is also consistent with this hypothesis, as leptin-deficient mice have increased parturition times.

Keywords: human evolution/parturition/placenta/sialic acids/siglecs

Introduction

The Siglecs are sialic acid (Sia)-recognizing members of the immunoglobulin superfamily (Varki and Angata 2006; Crocker et al. 2007). The CD33-related Siglecs (CD33rSiglecs) are a subgroup of cell surface molecules that are prominently expressed on human immune cells, including neutrophils, myeloid precursors, monocytes, macrophages, eosinophils, and on a subset of B and NK cells (Angata and Brinkman-Van der Linden 2002; Crocker et al. 2007). While this family of receptors appear to be rapidly evolving in many taxa (Crocker et al. 2007), human evolution appears to have been associated with particularly rapid changes, perhaps secondary to the genetic loss of a major hominid sialic acid, *N*-glycolylneuraminic acid (Varki 2007).

Siglec-6 is a CD33rSiglec that was cloned independently by two groups, in one instance from a human placental library (Takei et al. 1997) and in another as a leptin-binding protein (Patel et al. 1999). We confirmed strong human placental trophoblast expression of Siglec-6 and also noted it to be present at variable levels on human B cells (Patel et al. 1999). Here we show that placental trophoblast expression of Siglec-6 is a human-specific phenomenon, which is associated with the presence of Siglec-6 ligands on cells of placental and endometrial (uterine epithelial) origin. We also show a relationship between the tempo of Siglec-6 expression and the onset and progression of labor. Given the presence of inhibitory signaling motifs on the cytosolic tail of Siglec-6 and its leptin-binding ability, we finally speculate about mechanistic connections to the unusually prolonged nature of the human birth process.

Results and discussion

Mouse antihuman Siglec-6 monoclonal antibodies recognize great ape orthologs

Amino acid alignment of the first two extracellular domains of human and chimpanzee Siglec-6 (∼210 amino acid residues) showed a difference of only four residues (data not shown). Two of the amino acid changes were conservative (nonpolar to nonpolar, M206V, or uncharged polar to uncharged polar, S105Y), and only two were nonconservative (basic to uncharged polar, R70Q, or nonpolar to basic, W107R). Thus, the likelihood that antihuman Siglec monoclonal antibodies would crossreact with the chimpanzee CD33rSiglecs is high. An ELISA assay was used to determine crossreactivity between recombinant soluble human, chimpanzee, and baboon Siglec-6-Fc proteins. While both anti-Siglec-6 clones tested (10F10 and E20-1232) recognized recombinant chimpanzee Siglec, there was only moderate to weak recognition of the baboon ortholog (data not shown).

We had previously shown that Siglec-6 is expressed on the surface of circulating human B cells (Patel et al. 1999). We

[1]To whom correspondence should be addressed: Tel: +1-858-534-2214; Fax: +1-858-534-5611; e-mail: a1varki@ucsd.edu
[2]The first two authors contributed equally to this work
[3]Current address: Crucell N.V., Protein Research, Leiden, The Netherlands
[4]Current address: Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

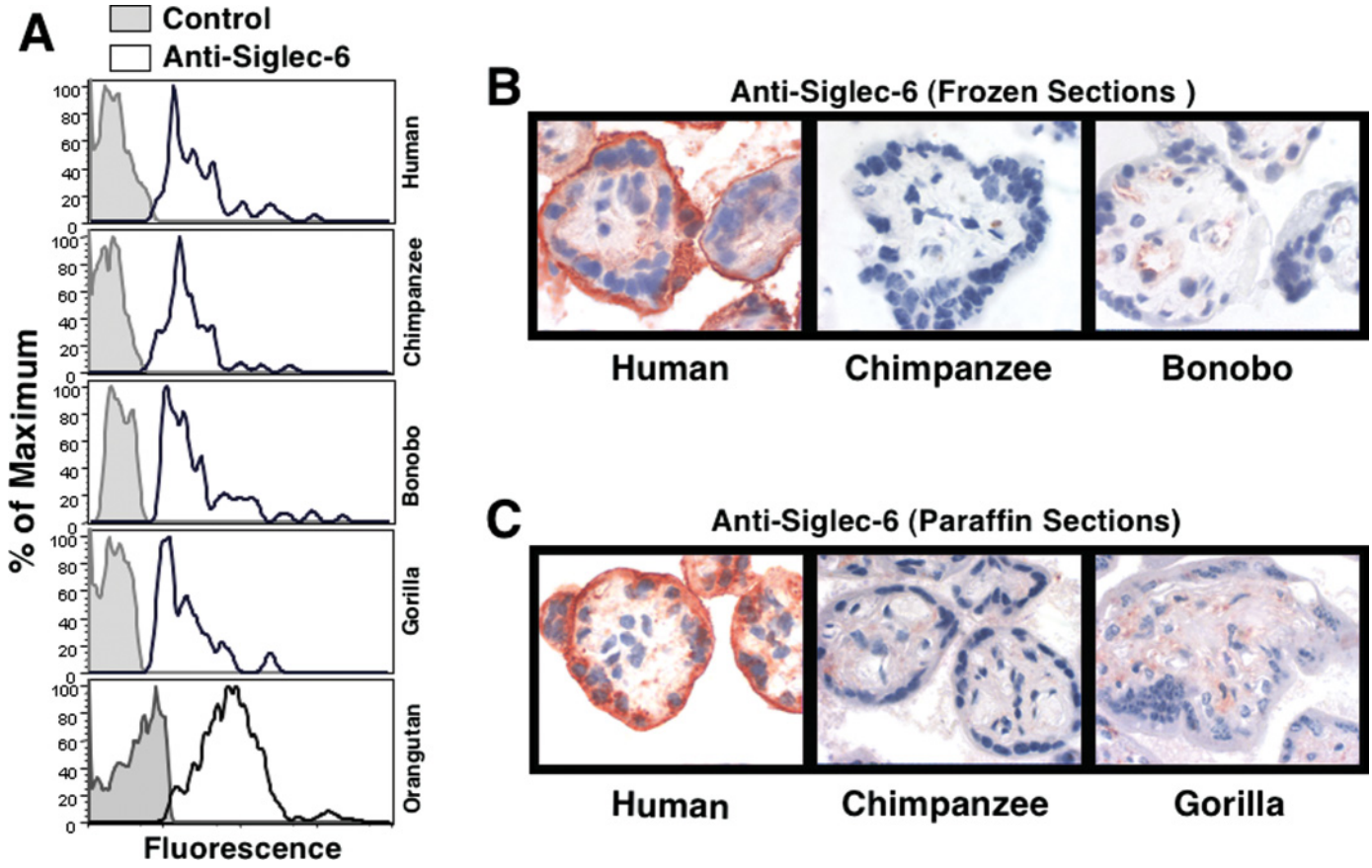

Fig. 1. Siglec-6 is expressed on B cells of great apes, but not in the Placenta. (**A**) Peripheral blood leukocytes (PBLs) were isolated from whole blood by hypotonic erythrocyte lysis, incubated with anti-Siglec-6 clone E20–1232, followed by phycoerythrin-conjugated goat anti-mouse IgG, double stained with either CD3, CD14, CD19 or CD22 antibody and analyzed by flow cytometry on a FACSCalibur. All species had a relatively small population of PBLs that were recognized by anti-Siglec-6. Most but not all of the Siglec-6 positive PBLs were also positive for CD19 or CD22. The sample profiles shown are for CD19-positive cells (human, chimpanzee, bonobo, and gorilla) or CD22-positive cells (orangutan). (**B**) Frozen placental tissue from human, chimpanzee or bonobo was freshly sectioned, incubated in 0.03% H_2O_2 to block endogenous peroxidases, followed sequentially with anti-Siglec-6 Mab in blocking buffer, hydrogen peroxidase-conjugated goat anti-mouse IgG, 3-amino-9-ethylcarbazole (AEC), and finally counter-stained with hematoxylin. Only human placental trophoblast stained positive for Siglec-6, as indicated by the reddish-brown stain. Magnification: 1000×. (**C**) Paraffin-embedded placental tissue for human, chimpanzee, and gorilla, were prepared as described in "Experimental Procedures". Again, only human placental trophoblast cells were positive for Siglec-6 expression. Magnification: 1000×.

now compared Siglec-6 expression of human peripheral blood leukocytes with those of the *great apes*: chimpanzees, bonobos, gorillas and orangutans (the term great apes is used in the colloquial sense, as genomic information no longer supports this species grouping see Goodman, 1999 – technically, these species are now grouped with humans in the family *Hominidae*). Leukocytes from the whole blood of humans or great apes were examined for Siglec-6 expression by flow cytometry using antibody E20-1232. As with humans, Siglec-6 expression was found to be isolated to a subpopulation of great ape lymphocytes. Further investigation showed that Siglec-6 expression is primarily on the $CD19^+$ or $CD22^+$ B lymphocytes of all great apes examined (data not shown). The flow cytometry profiles of Siglec-6 positive lymphocytes shown in Figure 1A are representative of multiple experiments. We concluded that the monoclonal antihuman Siglec-6 antibody E20-1232 can be used to compare Siglec-6 expression in chimpanzee, bonobo, gorilla, and orangutan tissues, and that the pattern of expression in peripheral blood cells is generally similar amongst these species.

Siglec-6 is expressed in human but not in chimpanzee or other great ape placentae

We previously noted that Siglec-6 was expressed on human placental trophoblast using mouse antihuman Siglec-6 clone 10F10 (Patel et al. 1999). Later, we found that the anti-Siglec-6 clone E20-1232 reacted equally well with lymphocytes in both frozen or paraffin-embedded human spleen sections (data not shown). Using the latter antibody, we now confirmed our prior finding of Siglec-6 expression in the trophoblast from placentae obtained after normal labor in humans (see example in Figure 1B).

Unlike the case with humans, it is difficult to obtain properly preserved great ape placental samples. Most great ape births take place at night (Lefebvre and Carli 1985) and the mothers will often consume the placenta immediately after the delivery. Two chimpanzee placenta samples were obtained at emergency cesarean section for fetal distress following a period of labor (courtesy of Dr. Todd Preuss, New Iberia Primate Center in Louisiana). A bonobo placenta was obtained immediately after

labor and delivery (courtesy of Pascal Gagneux, the Zoological Society of San Diego). These samples were in excellent condition and could be compared with corresponding human samples using frozen sections. In striking contrast to humans, we found little or no Siglec-6 expression in the chimpanzee and bonobo placenta sections (see examples in Figure 1B).

As such, elective cesarean sections or collection of placental samples immediately following birth are rare even in captivity, all other great ape placentas we could obtain were paraffin-embedded samples collected after labor associated with normal delivery by zoo-keepers, and archived by one of us (K.B.). Fixed and paraffin-embedded placental samples for chimpanzee, gorilla, orangutan, and baboon were available, and were sufficiently well preserved, as determined by the vimentin and/or cytokeratin positive control (data not shown). These samples had a somewhat higher background with the secondary antibody alone, with an irrelevant primary, likely because most of the great ape samples were preserved in Bouin's fixative, which leaves proteins in a denatured state, producing a higher background in immunohistochemistry (Ananthanarayanan et al. 2005). Regardless, we found little or no staining above background in any of the great ape samples (see Figure 1C for representative examples), indicating again that this is the ancestral state. We conclude that expression of Siglec-6 on placental trophoblast is a human-specific evolutionary change.

Human-specific expression of Siglec-6 in the placenta may be explained by mutations in the 5′ untranslated region (5′ UTR) of the gene

The genomic region encoding chimpanzee and baboon Siglec-6 had been previously sequenced (Angata et al. 2004). The 5′ UTR candidate promoter sites for the human, chimpanzee, and baboon Siglec-6 genes were examined using the TRANSFAC database, looking for human-specific promoter regulatory elements. As shown in Figure 2, two regulatory sites, which could potentially act as a composite enhancer for placental expression, (Oct-1 and E-box) (Wang and Melmed 1998; Kalbe et al. 2000) were detected in the human promoter regions but not in those of the baboon or rhesus monkey – and this region is not found in the chimpanzee genome. A putative GATA-binding site, which is reported as an enhancer element for placental expression (Steger et al. 1994; Shi et al. 1997; Wang and Melmed 1998) is found in nonhuman primate genomes, and appears to have been altered in the human genome to the point of no longer having the consensus sequence of a functional promoter (Figure 2). These promoter sequence changes in humans, i.e., the loss of one element (GATA-binding site) and the gain of two elements (Oct-1 and E-box), indicate that the organization of putative enhancer elements have changed significantly in the human lineage. This is consistent with the induction of selective expression in human placenta (Steger et al. 1994; Wang and Melmed 1998). On a related note, the insertion of a MER11 repetitive element is reported to explain the human placental expression of leptin. However, no MER11 element was found in 16-kb upstream region of Siglec-6 (Bi et al. 1997).

Further studies of the proposed enhanced promoter function of human Siglec-6 need to be done in a Siglec-6 expressing cell line of human trophoblastic origin. Unfortunately, such studies are not currently feasible, because all existing trophoblastic (choriocarcinoma) cell lines that we examined do not express Siglec-6 (data not shown), presumably because of their malignant origin (though the lines do express detectable Siglec-6 ligands, see below).

Ligands for Siglec-6 are present in the placenta and uterine epithelium

Using synthetic binding targets, we previously showed that one preferred sialylated ligand for Siglec-6 was a SialylTn-like structure Siaα2-6GalNAcα- (Patel et al. 1999). However, little is known about the natural ligands of Siglec-6. We therefore probed tissue sections with recombinant soluble human Siglec-6-Fc fusion protein as a pseudo-antibody, and found that Siglec-6 ligands are present in human placenta (Figure 3A) and on human uterine epithelia (Figure 3A). The expression of the Siglec-6 ligands was especially high on invasive trophoblastic X cells (the "extravillous trophoblast") of the placenta (Figure 3A). As a control, we showed that human brain tissue does not express Siglec-6 (Patel et al. 1999), nor the cognate ligand (Figure 3A).

While Siglec-6 was most prominent on the fetal trophoblast, the ligands were most prominent on the maternal side, with significant overlap in ligand expression at the maternal-fetal junction, as seen in serial sections (Figure 3B). Notably, the binding of Siglec-Fc was reduced by sialidase pretreatment of the sections, indicating that the binding was at least partly dependent upon Sia recognition. In keeping with this, an R122A Siglec-Fc construct (mutating the critical Arg residue in the V-set domain required for optimal sialic acid recognition) also showed reduced binding (Figure 3A). Expression of Siglec-6 and the adjacent sialylated ligands was further confirmed using double immunofluorescence studies (Figure 3C). Interestingly, similar Siglec-6-Fc ligands were also noted in chimpanzee placentae, even in the absence of Siglec-6 (data not shown). Thus the sialylation machinery capable of generating potential Siglec-6 ligands was already present in the ancestral ape placenta, prior to the recruitment of Siglec-6 expression to this organ.

As leptin does not carry sialic acids, it cannot be the dominant ligand being detected with the Siglec-6-Fc probe. This is also likely to be the case because the binding of Siglec-6 to monovalent leptin is not as strong as to the sialylated ligands (the leptin used in our original studies was found to be divalent, data not shown). However, there is a direct relationship of placental Siglec-6 expression pattern to that of endogenous leptin (Figure 3C). Thus, Siglec-6 is in a position to act as an alternate binding site for the sequestration of endogenously produced leptin.

Only cell lines of placental or endometrial origin express Siglec-6 ligands

To further explore the distribution of Siglec-6 ligands we probed various cell types, including cultured cell lines of hematopoietic, placental, endometrial, prostatic, and colonic origin, as well as CHO cells. Hematopoietic cells expressed ligands for Siglec-1, -2, and -5 (Table I). In contrast, recombinant Siglec-6-Fc interacted strongly only with the cells of placental or endometrial origin, and weakly with prostate or colon cancer cell lines (Table I). Thus, only cell lines of trophoblastic or endometrial origin were found to be strongly positive for Siglec-6 ligands.

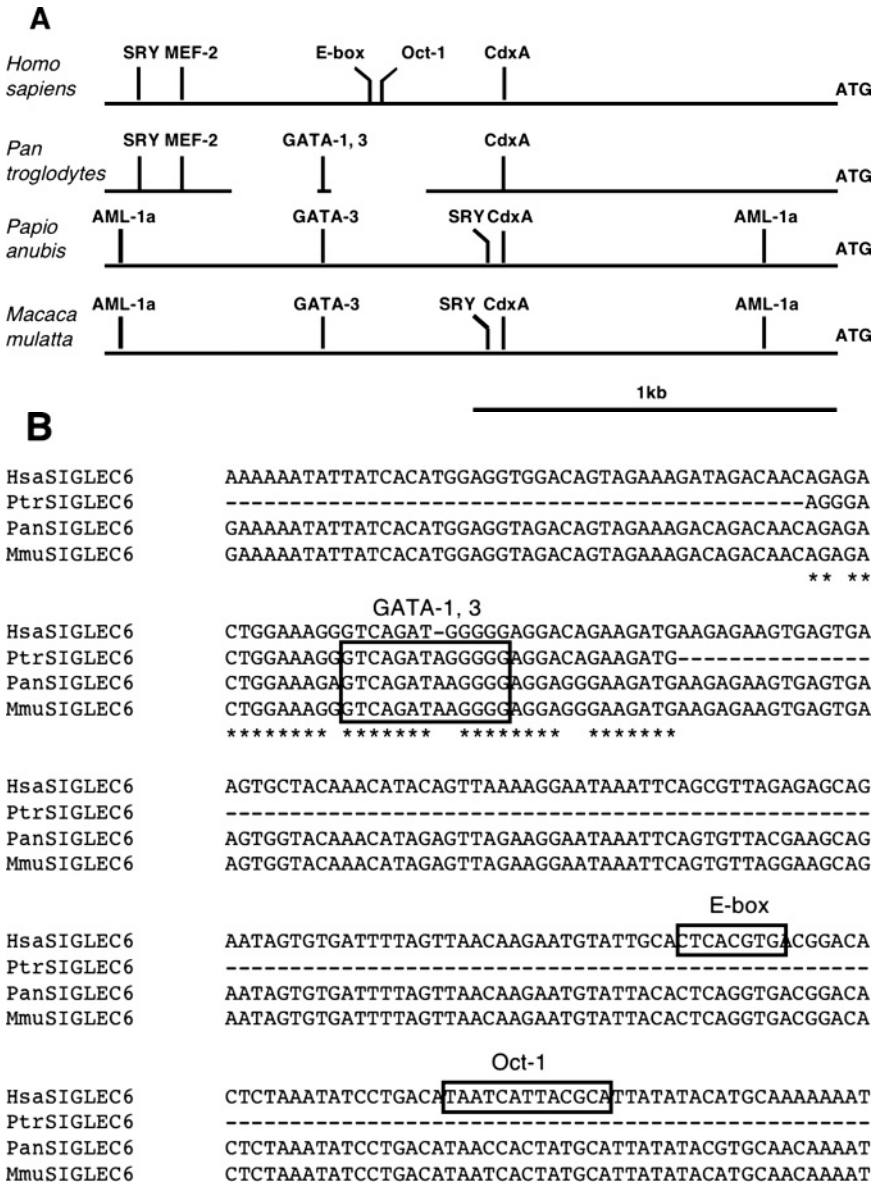

Fig. 2. Human-specific expression of Siglec-6 in the placenta might be explained by mutations in the 5′ Untranslated Region of the Siglec-6 gene. The 5′ UTR candidate promoter site for the human, chimpanzee and baboon Siglec-6 genes was examined using the TRANSFAC database, looking for putative upstream regulatory elements. (**A**) Two new regulatory sites (Oct-1 and E-box) were detected in the upstream regions of human but not in that of the chimpanzee or baboon of the 5′ UTR. A GATA-binding site has also been eliminated in humans. (**B**) Sequence alignments of the putative promoter region that contains the human-specific element changes. Boxes represent the putative transcription factor binding sites.

Natural ligands for Siglec-6 are a mixture of sialylated cell surface glycoproteins

We used JAR and BeWo cell lines of choriocarcinoma origin, as well as placental tissue sections to determine if the natural Siglec-6 ligands are glycoproteins or glycolipids. The R122A Siglec-Fc chimera was used as an additional control, as this mutation reduces sialic acid-dependent binding of the Siglec-Fc. We released adherent cells from culture plates with EDTA or trypsin and studied the binding of Siglec-6-Fc by flow cytometry. Binding of the R122A mutated Siglec-6 was much lower than that observed with the wild type version (Figure 4, note that a log scale is used to indicate fluorescence intensity). Trypsinization of BeWo and Jar cells eliminated binding, indicating that the ligands are predominantly glycoproteins (Figure 4). Consistent with this, methanol fixation, which would eliminate glycolipids, did not destroy Siglec-6-Fc ligand binding on placenta tissue sections (data not shown).

Pretreatment of positive cells with sialidase had no obvious effect on binding (data not shown). Similar results were obtained when probing Western blots of extracts from the cells or placentas with the Siglec-6-Fc protein (data not shown). From these experiments we conclude that Siglec-6 ligands are a family of glycoproteins bearing the cognate ligands, which are relatively resistant to conventional sialidase treatment. This is not too surprising, as the sialidase treatment used (*A. ureafaciens*) did not efficiently remove sialic acid from sialyl-Tn probes in our

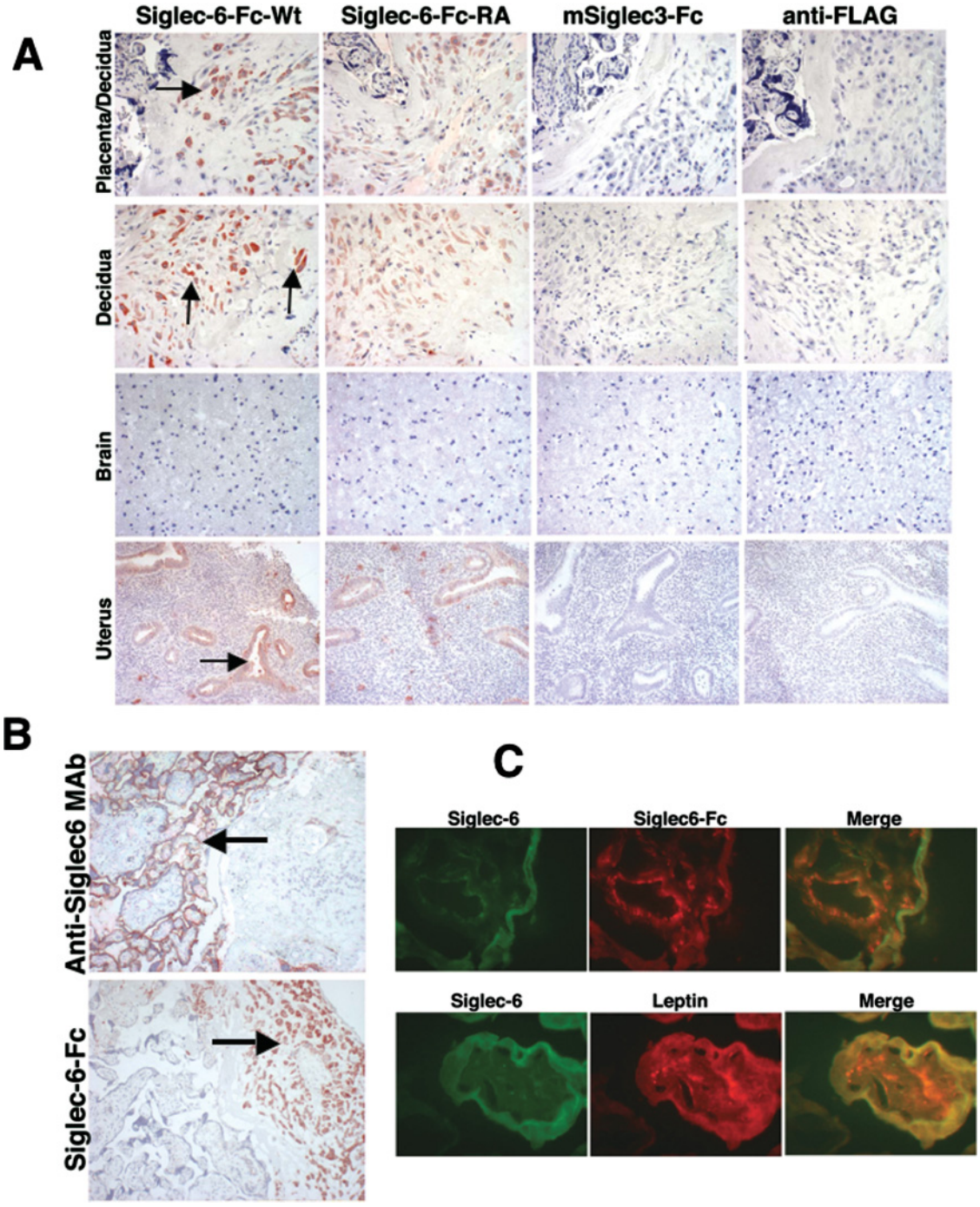

Fig. 3. Detection of Siglec-6 ligands and leptin. (**A**) Siglec-6 ligands are present in human placenta and uterine epithelium. Frozen tissue sections were prepared and probed as in Figure 1, except the *primary antibody* was recombinant Siglec-6-FLAG-Fc (or mouse Siglec-3-Fc as a negative control), followed by detection with mouse anti-FLAG and HRP-conjugated goat anti-mouse IgG, and developed with AEC. An additional negative control was with the anti-FLAG and subsequent steps. Ligands for Siglec-6 were found in human placenta, decidua, and uterine epithelium (see arrows), but not in brain. Magnification: 200×. (**B**) Relationships between expression of Siglec-6 and Siglec-6-Fc-binding ligands in human placenta. At this low power view of serial sections, it can be seen that while Siglec-6 is widely distributed in the placental trophoblast (arrow in upper panel), the ligands are seen prominently in the invasive trophoblast (arrow in upper panel, including "X" cells). Magnification: 100×. (**C**) Relationships between expression of Siglec-6, Siglec-6-Fc-binding ligands, and leptin in human placenta. Frozen sections were studied by double immunofluorescence, as described in *Materials and methods*. Magnification: 1000×.

earlier studies, and we had to use mild acid (Brinkman-Van der Linden and Varki 2000). However, such mild acid treatment would have destroyed the tissues or cells used in the search for natural ligands described here. There are many reasons why sialidase treatment on sections or cell surfaces may not work well such as steric hindrance (Powell et al. 1987) or sialic acid modifications (Varki and Diaz 1984). Thus the diminished binding of the R122A Siglec-6Fc was used to indicate the sialic acid

Table I. Only cells of placental or endometrial origin express natural ligands for Siglec-6

Cell line	Siglec-1-Fc	Siglec-2-Fc	Siglec-5-Fc	Siglec-6-Fc
RBCs	++	+	+	−
Jurkat	nd	++	nd	−
Daudi	++	++	−	−
EBV-tr-B[a]	++	++	++	−
HL60	nd	++	+/−	−
U937	++	++	+	+/−
THP-1	++	++	+	−
HEL	++	++	+/−	−
BeWo (trophoblastic)	++	++	++	++
JAR (trophoblastic)	++	++	++	++
SNG-M[b] (endometrial)	nd	nd	nd	++
PC-3 (prostate)	nd	nd	nd	+/−
LS180 (colon)	nd	nd	nd	+/−
CHO	−	−	−	+/−

Recombinant human Siglec-Fc proteins were precomplexed with phycoerythrin-conjugated goat antihuman IgG. The Siglec-Fc complexes were then incubated with various cells lines and staining examined by flow cytometry. The data is a composite of several similar experiments.
nd = not determined.
[a]Kind gift of Peter Parham (Stanford University).
[b]Kind gift of Michiko Fukuda (Burnham Institute).

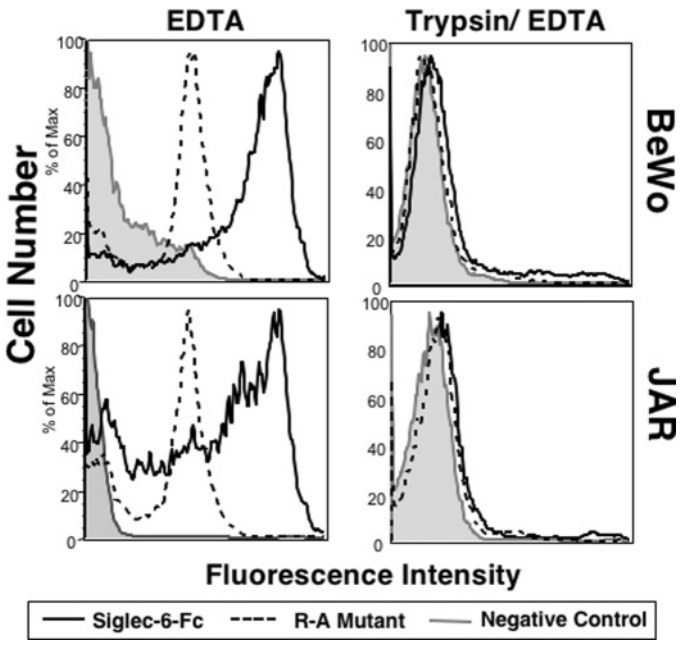

Fig. 4. The ligands for human Siglec-6 are glycoproteins that are at least partially sialic acid-dependent. BeWo and JAR choriocarcinoma cells were released from culture dish with EDTA, incubated with human Siglec-6-Fc precomplexed with phycoerythrin-conjugated goat antihuman IgG, and studied by flow cytometry. The Siglec-6-Fc staining (solid lines) shows that both BeWo and JAR cells have high levels of Siglec-6 ligands. Release of cells from the culture plate by trypsinization (Trypsin/EDTA) completely abrogated Siglec-6-Fc binding, indicating that the Siglec-6 ligands detected are glycoproteins. A single amino acid residue mutation from Arg to Ala (R122A) of the predicted *critical arginine* necessary for sialic acid binding resulted in reduced ligand binding compared to wild type Siglec-6 (dotted lines). A conservative single amino acid substitution of Arg to Lys (R122K) of human Siglec-6 Fc showed Siglec-6 ligand binding that was almost identical to wild type human Siglec-6 Fc (data not shown). These data show that Siglec-6 ligand binding is at least partially dependent on Siglec-sialic acid interactions with glycoproteins.

dependency of these interaction, and to infer that the same binding site in the V-set domain is involved in recognition.

Siglec-6 levels increase during process of human labor

Human pregnancy and parturition are unusual in comparison to that of other mammals, including nonhuman primates (Nathanielsz 1998; Bernal 2001; Smith 2007). Indeed, it has been pointed out that, "human parturition is a uniquely human event" (Smith 2007). It is difficult to predict with accuracy the date of delivery for human infants (normal term gestation has a range of 37–42 weeks) (Liao et al. 2005). In contrast, the gestation period in chimpanzees (our closest evolutionary relatives) shows only a 10–12 day variation (Keeling and Roberts 1972). Human parturition is also a prolonged and arduous process, as a larger fetal head must negotiate a birth canal that was rendered small during the evolutionary emergence of the bipedal posture (Lovejoy 2005). The duration of human labor also tends to be long (mean of ∼10 h for a first pregnancy) (Liao et al. 2005). In contrast, chimpanzees give birth more rapidly, sometimes in less than an hour (Keeling and Roberts 1972). Furthermore, nonhuman primates demonstrate much fewer signs of pain or discomfort during labor (Lefebvre and Carli 1985).

It appears that the feto-placental unit triggers the onset of labor in all viviparous mammalian species, with activation of the fetal hypothalamic-pituitary axis playing a major role (Smith 2007). However, unlike the case in ruminants like sheep (Nathanielsz 1998), the mechanisms determining the onset of labor in humans remain poorly defined (Navitsky et al. 2000, Smith 2007). The placenta may upregulate specific target genes such as corticotropin-releasing hormone, cyclo-oxygenases, and oxytocin, associated with excess production of cortisol from the fetal adrenal and possibly increased placental estriol production (Liao et al. 2005; Smith 2007). Circulating levels of leptin also rise during labor (Nuamah et al. 2004), and leptin-deficient mice show delay in parturition (Mounzih et al. 1998), suggesting a role for this molecule as well. While there is evidence for many such signals leading to human parturition and even possibly a role for inflammation (Yellon et al. 2003; Romero et al. 2006; Smith 2007), the order and importance of events needs further elucidation.

It is also unclear if the prolongation of the birth process in humans is simply due to the mechanical limitations imposed by the disproportionately large head, or if the process is specifically slowed, in order to limit damage to the mother and the fetus. The fact that anencephaly (a congenital anomaly in which the forebrain, and vault of the skull fail to form) does not result in highly shortened labor suggests that mechanical factors are not the primary *cause* of prolonged human labor. Rather, it could be that human labor *needs* to be prolonged in order to limit the damage to the mother and thebaby. Regardless, finding molecular expression differences between humans and great apes may give clues to how the unique birth process of humans evolved.

While performing immunohistochemistry for Siglec-6 localization in additional human placentae we noted a wide range of expression, with the highest expression seeming to be associated with placentae from normal labor and delivery. To pursue this finding, sections from many different human placentae were stained for Siglec-6 as above, and then scored blindly follows: no staining, faint, moderate, and strong staining (see Figure 5

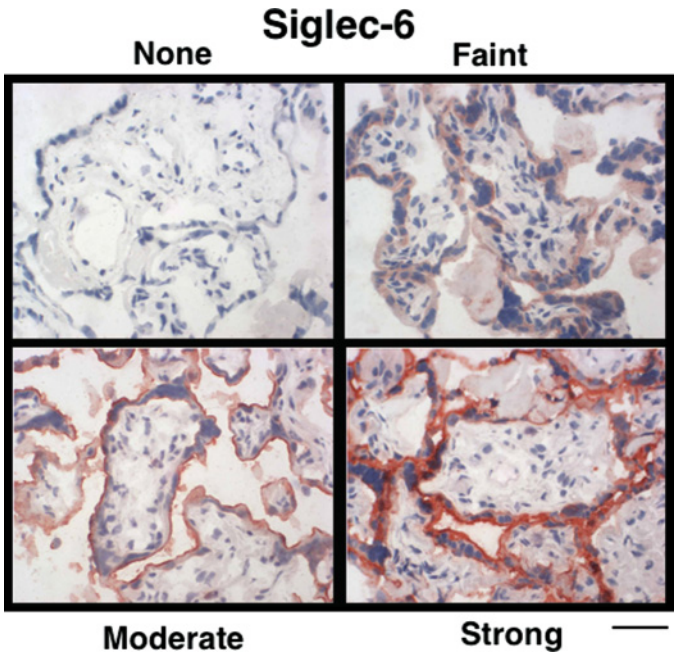

Fig. 5. Examples of Siglec-6 expression increase during the progression of labor. Frozen placental tissues from humans prepared for Figure 1 were used for this panel to demonstrate the levels of Siglec-6 expression as seen by immunohistochemistry. Two individuals blindly scored all micrographs. Examples of no Siglec-6 expression (none), faint, moderate or strong Siglec-6 staining are shown. For complete results, see Table II.

for examples). We found that placentae from women who experienced little or no labor prior to elective cesarean section (either self-reported or as determined by the attending physician) typically only had low levels of Siglec-6 expression with some having no expression at all (Table II). In contrast, most samples from the patients who had experienced labor either prior to normal delivery (or leading up to emergency cesarean section) had stronger Siglec-6 expression. Thus, while 89% of labor samples stained moderately to strongly, 72% of the nonlabor samples had no staining to faint staining for Siglec-6. While the correlation is not perfect, it must be kept in mind that early labor could have begun in some of the women who had elective procedures, and that the duration of labor varied in the other group. We also observed that several placentae of pregnancies that terminated early for various reasons had none to faint staining of Siglec-6 on the trophoblast, with most of these samples having no staining at all (data not shown). Ideally, one would like to correlate the increase in Siglec-6 expression with the duration of labor in each case. However, this type of information is very difficult to determine precisely from the medical record.

Table II. Siglec-6 expression in human placenta appears to increase with onset of labor

	Number of samples with Siglec-6 expression			
	None	Faint	Moderate	Strong
Labor	0	2	9	7
No labor	2	6	2	1

Frozen tissue sections were prepared as in Figure 1, for staining with an anti-Siglec-6 MAb (E20–1232). "Labor" placenta samples were from women who had a normal-term vaginal delivery, or a cesarian section following a period of active labor ($n = 18$). "No labor" samples were from women who had an elective cesarian section without a known onset of labor ($n = 11$). All assignments were made in a blinded fashion by independent observers.

Conclusions and perspectives

We have recently discovered multiple differences in sialic acid biology between humans and great apes (Varki 2007). The most obvious one is that while humans express *N*-acetylneuraminic acid (Neu5Ac) but not *N*-glycolylneuraminic acid (Neu5Gc) on the surface of their cells, great apes express about equal amounts of both sialic acids (Muchmore et al. 1998). This is due to specific genomic mutation that occurred in the lineage leading to humans, after the chimpanzee our common ancestor (Chou et al. 2002). Other examples of human-specific changes in CD33rSiglec expression include T lymphocytes (Nguyen et al. 2006) and brain microglia (Hayakawa et al. 2005). The human-specific loss of cytosolic inhibitory motifs of CD33rSiglecs on T cells probably contributes to the T cell hyperreactivity in humans. We also found that Siglec-11 is selectively expressed on the microglia of the human brain, even though splenic expression is found in both humans and chimpanzees. Here we report another unusual example of human-specific Siglec evolution – expression in the placenta. The finding of these and other human-specific differences in sialic acid biology (Varki 2007) indicate that this system underwent marked changes during human evolution. Overall, our findings are consistent with the hypothesis of King and Wilson, that human uniqueness is at least partly determined by differential expression of shared genes (King and Wilson 1975).

Placental Siglec-6 expression in humans invokes a number of questions and speculations, because of the unusual nature of the human birthing process. Potential cognate ligands for Siglec-6 apparently already existed in placentas of the human – great ape common ancestor. Why should Siglec-6 have been recruited for human-specific placental expression? Why does Siglec-6 expression increase with the onset and progression of labor? Does the expression of trophoblastic Siglec-6 contribute to the prolongation of labor during parturition, helping to limit damage to both mother and fetus? A related question is whether Siglec-6 was recruited because of its tyrosine-based inhibitory signaling capabilities and if so, whether this signaling is mediated through the known pathway for CD33rSiglec signaling in immune cells via the cytosolic ITIM motifs, i.e., recruitment of the tyrosine phosphatases SHP-1 and -2. In this regard, preliminary studies by Western blotting show that Siglec-6 is tyrosine phosphorylated in placental tissue extracts (unpublished observations). These data suggest that phosphorylation may play a role in negative signaling by Siglec-6. However, it is difficult to quantitatively or comparatively study this phenomenon between different placental tissue extracts, because of the complex architecture of the placenta and the inhomogeneous distribution of expression of Siglec-6 itself. Given that leptin-deficient mice show delayed parturition (Mounzih et al. 1998), another (not mutually exclusive) possibility is that Siglec-6 also acts as a low-affinity sink to sequester leptin from its own receptor, and helps slow down the labor process in this manner. Also, is Siglec-6 upregulation related to hypoxia, as has been observed with labor for other molecules? (Hoang et al. 2001). Perhaps, excessively

rapid labor causes more intense hypoxia, and Siglec-6 could serve as a feedback inhibitory loop.

Genetic studies would be needed for conclusive proof that Siglec-6 contributes to the control and tempo of labor and delivery. A human fetus completely deficient in Siglec-6 expression could potentially experience excessively rapid labor and delivery, resulting in more potential damage to both the mother and the newborn. However, such a homozygous null state would occur with only one out of many of babies born to a heterozygous mother. Moreover, if such an individual survived birth, there might be minimal perturbation of immune function because of the overlapping expression of other Siglecs on B cells. For all these reasons, it would be difficult to identify such a human mutant. Meanwhile, the use of a mouse model to study the role of placental Siglec-6 in labor is also not straightforward. Mice do not have a Siglec-6 ortholog (Angata et al. 2004), and the Siglec-6 ligands are likely not present in the mouse. Nevertheless, the study of the human-specific expression of placental Siglec-6 may help us gain knowledge and understanding of normal birth, as well as pathological states of late stage pregnancy such as preeclampsia (McMaster et al. 2004; Levine et al. 2006), which has so far not been reported in great apes (Chez 1976).

Materials and methods

Cell lines

All cell lines were from ATCC unless otherwise stated. Cells were cultured at 37°C, in 5% CO_2, and all media were supplemented with 10% heat-inactivated fetal calf serum (FCS). Daudi, JAR, Jurkat, HEL, HL60, PC-3, THP-1, U937, and human EBV-transformed lymphoblastoid B cells (a gift from Peter Parham of Stanford University), were cultured in RPMI 1640. BeWo cells were cultured in Ham's F12 with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. CHO and LS180 cells (colon carcinoma cells) were incubated in Minimal Essential Medium Alpha (αMEM). Additional cell lines of trophoblastic and endometrial origin (HT-H and SNG-M) were a kind gift from Michiko Fukuda of the Burnham Institute and were cultured in Dulbecco's Modified Eagle Media (D-MEM).

Production of Siglec recombinant proteins

A set of soluble human, chimpanzee, and baboon CD33rSiglec-Fc chimeric proteins was produced for use in antibody crossreactivity studies and ligand binding experiments. These and other Siglec-Fc proteins were produced using a construct that included the two N-terminal Siglec Ig-like domains joined to the human Fc domain and inserted into the pcDNA 3.1(+) vector (Invitrogen, Carlsbad, CA). Some of the constructs included a FLAG tag sequence between the Siglec and Ig Fc portions. Each construct was transiently transfected into Large T antigen-transformed CHO cells (CHO-TAg) with Lipofectamine (Invitrogen). The cells were then cultured in Opti-MEM with 2% low IgG FCS, the supernatant was collected over a 6-day period and pooled for each construct. Recombinant proteins were bound to Protein A sepharose beads (GE Healthcare, Piscataway, NJ), treated on the column with 25 mU of *A. ureafaciens* neuraminidase for 1 h at room temperature with end-over-end mixing, eluted with 0.1 Molar glycine HCl buffer pH 3.0, immediately neutralized with 1 M Tris–HCl, and washed with PBS during concentration in an Amicon Ultra Filtration device (Millipore, Billerica, MA). The final protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, IL) and SDS-PAGE. The functionality of each Siglec-Fc protein was determined by an ELISA-like assay with biotinylated sialic acid polyacrylamide probes (Glycotech, Gaithersburg, MD) followed by alkaline phosphatase-conjugated streptavidin, developed with *p*-nitrophenyl phosphate (*p*NPP, Sigma-Aldrich, St. Louis, MO) and read on a 96-well plate reader at 405 nm (SpectraMax 250, Molecular Devices, Sunnyvale, CA). A point mutation was introduced in human Siglec-6-Fc converting an Arg residue to Ala (R122A) or an Arg to Lys (R122K) using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) and the recombinant protein produced as mentioned above.

Crossreactivity of monoclonal anti-Siglec-6 antibodies with primate Siglecs

The crossreactivity of mouse antihuman Siglec-6 antibodies (10F10 and E20-1232) with chimpanzee and baboon CD33rSiglecs was determined using an ELISA-like method. Incubations were done at 4°C unless otherwise stated and each sample was done in triplicate. Briefly, Protein A (5 μg/mL, Sigma-Aldrich) was immobilized (100 μL/well) overnight in 50 mM carbonate bicarbonate buffer pH 9.5. The wells were blocked with PBS/1% BSA for 1 h. The appropriate Siglec-Fc, including a negative control, was added to each well (100 μL/well of 2 μg/mL in PBS), incubated for 3 h and washed. The wells were then blocked with 1% BSA in PBS for 1 h followed by mouse anti-Siglec antibody for 1 h. Wells were washed and goat anti-mouse IgG conjugated to alkaline phosphatase (1:2000 dilution, Jackson Immuno-Research, West Grove, PA) was added for 1 h and the wells washed once again. Each well was incubated with 10-mM *p*NPP at room temperature and read on a 96-well plate reader. The resulting data were plotted in Excel.

Human placenta samples

Samples of fresh discarded placenta were obtained with Institutional Review Board approval following routine postdelivery examination at the UCSD Medical Center, or were obtained frozen from the NIH-sponsored Cooperative Human Tissue Network. Information was available about whether the placentae were obtained following labor, or following elective cesarean section, without labor. However, all samples were anonymized, and there were no patient identifiers accompanying the analyzed material.

Immunohistochemistry

Immunohistochemistry was done on frozen or paraffin-embedded tissues. Freshly harvested tissue was frozen in OCT (*OCT*, Sakura, Torrance, CA) using an isopentane-dry ice slurry and the tissue blocks kept at −80°C until needed. Frozen tissue slices were air dried onto glass slides and treated with 0.03% hydrogen peroxide and blocked for endogenous biotin before being fixed in 10% buffered formalin or left unfixed. Sections were overlayed with 1% BSA in PBS and then with anti-Siglec-6 antibodies at 1 μg/mL or 1:100 dilution for hybridoma supernatant for 1 h at room temperature in a humid chamber. After PBS washes, bound antibody was detected using the LSAB kit (DAKO Cytomation, Carpenteria, CA), with color development using the HRP substrate 3-amino-9-ethycarbazole kit (AEC, Vector Laboratories, Burlingame, CA). The tissue was then counterstained with Mayer's hematoxylin (Sigma-Aldrich),

washed and mounted with aqueous mounting media and viewed using an Olympus BH2 microscope (Olympus, Center Valley, PA). Images were captured with Olympus Magnafire digital system and formatted using Adobe Photoshop. Paraffin embedded tissues, (fixed in 10% neutral buffered formalin or Bouin's fixative) were deparaffinized, serially rehydrated, subjected to antigen retrieval and biotinyl tyramide enhancement using the CSA kit (Dako Cytomation, Carpenteia, CA) and specific binding revealed with the HRP substrate kit. The isotype antibody P3-X63Ag8 (X63, ATCC) was the negative control, and anti-vimentin or anti-cytokeratin (clone V9 and AE1 respectively, Dako Cytomation) were the positive controls to determine if the tissue blocks were correctly preserved and retained intact proteins.

Flow cytometry

Blood was collected into K_3EDTA Vacutainers (BD Biosciences, Franklin Lakes, NJ) from chimpanzees (Yerkes Primate Research Center, Atlanta, GA, San Diego Zoo, San Diego, CA, or Lincoln Park Zoo, Chicago, IL), bonobos (San Diego Zoo), gorillas (Lincoln Park Zoo, San Diego Zoo), orangutans (San Diego Zoo), or baboons (Southwest Primate Research Center, San Antonio, TX) during routine health checks and shipped overnight on ice packs. Blood was simultaneously collected from human volunteers after obtaining consent following a protocol approved by the UCSD Human Subjects Committee. The human blood was stored overnight on ice packs to mimic the transportation of the ape samples. Leukocytes were isolated by hypotonically lysing erythrocytes with ACK lysis buffer (150 mM NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM EDTA) for 5 min at room temperature for one to three rounds of lysing and then kept in 1% BSA/PBS on ice until needed. In all cases the isolated cells were used within 3 h. Each staining was done with about 1×10^6 cells using an anti-Siglec antibody (1 μg/mL or 1:100 dilution for hybridoma supernatant) or the isotype control X63 for 1 h on ice. The cells were washed, incubated with goat anti-mouse IgG conjugated phycoerythrin (Caltag, Invitrogen) for 30 min and washed once again. The cells were then double-stained with TriColor- or APC-conjugated anti-CD3, -CD14, -CD19, -CD22, or -CD45 (Caltag, Invitrogen) for 30 min and washed. Cells were resuspended in 1% BSA in PBS, interrogated on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR).

Detection of Siglec-6 ligands on tissue sections

Freshly sectioned frozen tissue sections were air-dried, and endogenous peroxidases blocked with glucose/glucose oxidase in 1% BSA in PBS. Residual erythrocytes were lysed with ACK lysis buffer, the sections washed three times, and followed by a 10% neutral buffered formalin fixation. Sections were incubated with Siglec-6-EK-FLAG-Fc (1–5 μg/mL) for 2 h at room temperature, washed and incubated with mouse-anti-FLAG (1:500 dilution). Separate sections were treated with sialidase (from *A. ureafaciens*, Sigma-Aldrich) 100 mU/mL in 50 mM sodium acetate buffer pH 5.6, for 1 h at 37°C degrees followed by washing and then overlaying with Siglec-6-EK-FLAG-Fc. Specific binding was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (1:500 dilution, Jackson ImmunoResearch) and color was developed with AEC or with NovaRed HRP substrate (Vector Laboratories, Burlingame, CA). Nuclei were counter-stained with hematoxylin and slides were mounted with aqueous mounting media. The slides were examined using a Zeiss Axiolab microscope, images captured with a Sony 3CCD camera and the images formatted in Adobe Photoshop.

Flow cytometry detection of Siglec-6 ligands on cultured cells

Recombinant human Siglec-Fc-6 was precomplexed at optimized ratios with phycoerythrin-conjugated goat antihuman IgG, and incubated with various cell lines without a blocking step. The cells were then washed and examined by flow cytometry. In some cases, comparisons were made to the binding of Fc-chimeras of Siglec-1, -2 and -5.

Detection of Siglec-6, Siglec-6 ligands and Leptin using fluorescent markers

Frozen sections of human placenta were immunostained using fluorescent markers so that double labeling methods could be used for detecting colocalizing epitopes. The specific binding of mouse monoclonal anti-Siglec-6 was detected after blocking endogenous biotin binding sites, using a biotinylated anti-mouse (LSAB kit, Dako Cytomation), followed by Alexa-Fluor 488 conjugated Streptavidin (Molecular Probes, Invitrogen). The expression of Siglec-6 ligands was detected using Siglec-6-Fc as a probe, followed by a polyclonal rabbit anti-FLAG (Sigma-Aldrich) and then with a Cy3 conjugated anti-Rabbit antibody (Jackson Immunoresearch). Expression of Leptin was detected using a polyclonal rabbit anti-leptin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by the Cy3 conjugated anti-Rabbit antibody. Digital images were recorded using a Sony CCD camera mounted on a Zeiss Axiophot microscope, using appropriate excitation and barrier filters, captured using Scion and NIH image program and organized using Adobe Photoshop.

Sequence alignment and promoter region analysis

The putative genomic promoter region upstream of the coding region of the *SIGLEC6* gene was compared between human, chimpanzee, baboon, and macaque using the TRANSFAC database (BIOBASE, Biological Databases, GmbH; Matys et al. 2003; Matys et al. 2006).

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Conflict of interest statement

None declared.

Abbreviations

CD33rSiglec, CD33-related Siglec; MAb, monoclonal antibody; Sia, sialic acid; 5′ UTR, 5′ untranslated region of a gene.

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