Ultrastructural localization of glycoprotein IIla (GPIIIa, β3 integrin) on placental syncytiotrophoblast microvilli: implications for platelet alloimmunization during pregnancy

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BACKGROUND: Fetal and neonatal alloimmune thrombocytopenia due to anti-human platelet antigen (HPA)-1a more commonly occurs in first pregnancies, unlike hemolytic disease of the newborn. Anti-D is produced after D+ fetomaternal hemorrhage; this usually occurs at parturition. Anti-HPA-1a could develop during pregnancy if maternal immunization is stimulated by HPA-1a expressed not only on platelets but also on other fetal cells.

STUDY DESIGN AND METHODS: An ultrastructural study of fetal placental chorionic villi was undertaken to determine the localization of glycoprotein (GP)IIla carrying the HPA-1a/1b polymorphism. First trimester and term villi were incubated with a monoclonal antibody (MoAb) to GPIIIa or with positive control MoAbs (anti-placental alkaline phosphatase and ED822 MoAb) to villous syncytiotrophoblast (ST). Binding of MoAbs was detected with a gold-conjugated secondary antibody before processing the tissues and examination of ultrathin sections in an electron microscope.

RESULTS: Gold particles were evident on microvilli on the apical surface of ST when labeled with anti-GPIIIa and the placenta-specific MoAbs but not with an isotype control antibody. Immunolabeling for anti-GPIIIa on first trimester ST was similar to that of term ST.

CONCLUSION: The apical surface of the ST is bathed in maternal blood. During the natural regenerative process of human placenta, senescent parts of the ST are shed into maternal blood during pregnancy. This includes both apoptotic ST nuclei and microparticulate ST debris. The presence of GPIIIa on this circulating ST cellular material could be the source of HPA-1a alloantigen causing primary immunization of susceptible primigravidae early enough for anti-HPA-1a to cause fetal thrombocytopenia during a first pregnancy.

M ost cases of fetal and neonatal alloimmune thrombocytopenia (FNAIT) are caused by alloantibodies against human platelet antigen (HPA)-1a,1,2 and anti-HPA-1a is responsible for the majority of severely affected babies.3 Currently, there is no routine screening for this disease4 despite encouraging findings of a recent large study6 and no consensus on the optimal antenatal treatment,4,6 although good results are generally achieved with current management utilizing intravenous immunoglobulin G (IgG) as the first-line treatment.5,7 Unlike D hemolytic disease of the fetus and newborn, there is no preventative therapy. To develop rational and effective treatments for FNAIT, the etiology of this disease should be understood.

In contrast to hemolytic disease of the fetus and newborn,8 FNAIT often occurs in first pregnancies.1 The D antigen is restricted to erythroid cells and immunization of D− women occurs after fetomaternal hemorrhage (FMH) of fetal D+ red blood cells (RBCs). During pregnancy, small FMHs occur only occasionally. In a large

ABBREVIATIONS: CT(s) = cytotrophoblast(s); FMH(s) = fetomaternal hemorrhage(s); FNAIT = fetal and neonatal alloimmune thrombocytopenia; HPA = human platelet antigen; PLAP = placental alkaline phosphatase; ST = syncytiotrophoblast.

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study, 8 percent (9/114) of samples taken from women during the first 6 months of pregnancy had detectable FMH (fetal RBC); all FMHs were less than 0.1 mL. In the third trimester, the incidence of FMH increased to 15 percent (38/259) with 4 percent (10/259) exceeding 0.1 mL FMH. In contrast, more than half of the women (375/692) had detectable FMH after delivery, with the incidence of FMH over 0.1 mL being 25 percent.\(^9\) Comparable data were recorded in a subsequent small study.\(^10\) Thus D immunization generally occurs only postnatally, because larger fetal bleeds are more frequent at parturition. It is unlikely, therefore, that the amount of fetal platelets (PLTs) present in antenatal FMH could be sufficient to cause immunization of HPA-1a-negative primigravidae, especially because PLTs themselves rarely stimulate anti-HPA responses after allogeneic transfusion.\(^11\) This is despite the fact that pregnant women can make robust alloimmune responses, whereas many patients receiving transfusion are to some extent immunosuppressed. We therefore hypothesized that there might be another source of fetal HPA-1a antigen that is accessible to the maternal immune system during pregnancy.

The HPA-1 antigen is located on PLT glycoprotein (GP)IIa with the HPA-1a/1b phenotypes resulting from a leucine\(^{13}\)/proline\(^{33}\) substitution.\(^12\) On PLTs, GP\(\text{IIa}\) is in association with GP\(\text{IIb}\) forming a heterodimer, GP\(\text{IIbIIa}\) (integrin \(\alpha\)IIb\(\beta\)3, CD41/61), the fibrinogen receptor. The \(\alpha\)Ib integrin subunit pairs only with \(\beta\); expression of \(\alpha\)Ib\(\beta\)3 is restricted to PLTs and megakaryocytes. However, \(\beta\)3 can also bind \(\alpha\)V forming the \(\alpha\)V\(\beta\)3 heterodimer (the vitronectin receptor), which is expressed on several cell types.\(^13\) Therefore, as well as PLTs, other fetal cells may express the \(\beta\)3 integrin subunit (GP\(\text{IIa}\)) encoding the HPA-1 polymorphism.

Fetal trophoblast cells form the interface between fetal and maternal tissues and blood. The placental chori- onic villous syncytiotrophoblast (ST) is in direct contact with maternal blood. The \(\alpha\)V\(\beta\)3 integrin was identified on brush border ST membranes of human term placentas by immunocytochemical and biochemical analysis.\(^14\)-\(^16\) By flow cytometry and immunocytochemistry, we found GP\(\text{IIa}(\beta\)3) and \(\alpha\)V \(\alpha\) but not GP\(\text{IIb}(\alpha\)Ib\) localized on placent ST of first trimester (10 weeks’ gestational age) and term placentas.\(^17\) Reactivity of three anti-GP\(\text{IIa}\) monoclonal antibodies (MoAbs) varied.\(^17\) In this study, we investigated whether GP\(\text{IIa}\) could be identified on first trimester and term placental ST by electron microscopy. We describe the anatomy and biology of placenta and the ways in which fetal material can enter the maternal circulation during normal and pathologic pregnancies. We relate our findings regarding the ultrastructural localization of GP\(\text{IIa}\) on ST microvilli to the natural deportation of ST debris into maternal blood during pregnancy and to the possibility that this could stimulate the development of primary HPA-1a immune responses during pregnancy.

### MATERIALS AND METHODS

#### Antibodies

Primary antibodies used were anti-CD61 (GP\(\text{IIa}, \beta\)3 integrin, clone SAP; Chemicon Europe, Chandler's Ford, UK), anti-fibrin (clone E8; Beckman Coulter, High Wycombe, UK), anti-placental alkaline phosphatase (PLAP, clone 8B6; Sigma, Poole, UK), ED822, a MoAb with specificity for ST,\(^18\) and IgG1 myeloma isotype control (MOPC 21, Sigma). Anti-PLAP and ED822 were positive controls and IgG1 was a negative control. The antibodies were used at 10 \(\mu\)g per mL in phosphate-buffered saline (PBS) for staining tissues. The secondary antibody was 18-nm colloidal gold–conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Strathecly Scientific, Soham, UK).

#### Placentas

Placentas were obtained after elective termination of pregnancy at 8 to 11 weeks’ gestational age (n = 5) or after normal term (37-41 weeks’ gestational age) delivery of healthy infants (n = 2). Ethical permission was granted for the work by the Research Ethics Committees of the United Bristol Healthcare NHS Trust and North Bristol NHS Trust, and written consent was obtained from the patients.

#### Electron microscopy

Fragments of placental chorionic villi were cut from fresh tissues, trimmed under a dissecting microscope to approximately 1 mm\(^3\), fixed in 2 percent glutaraldehyde (Agar Scientific Ltd, Stanstead, UK) in 0.1 mol per L phosphoric acid, and processed through to embedding in epoxy resin (Araldite CY212, Agar Scientific Ltd). Ultrathin sections of 100 nm thickness were cut from the blocks, mounted on 300-mesh EM copper grids (Agar Scientific Ltd), and stained with saturated methanolic uranyl acetate\(^19\) and Reynolds lead citrate solution.\(^20\) The grids were viewed in an electron microscope (CM10, Phillips, Eindhoven, the Netherlands) and digital electron micrographs captured by image analysis software (ITEM, Olympus Soft Imaging Solutions GmbH, Münster, Germany).

#### Immunoelectron microscopy

Fragments of villi were prepared and trimmed as above, incubated with primary antibodies for 60 minutes at 4°C, washed three times in PBS, incubated with the secondary antibody for 60 minutes at 4°C, and then washed three times. The tissues were then fixed and processed as above. Villi from five first trimester placentas and one term placenta were labeled with anti-GP\(\text{IIa}\). Three first trimester villous samples were incubated with ED822 MoAb and
anti-PLAP was used to stain one term sample. IgG1 was incubated with villi from two first trimester placentas and one term placenta.

Immunocytochemistry
Cryostat sections of term placentas were stained with antifibrin followed by detection of the primary MoAb with reagent (VIP, Vector Laboratories, Inc., Peterborough, UK) and then with hematoxylin nuclear counterstain.

RESULTS
Binding of MoAbs to the ST was detected with a gold-conjugated secondary antibody. Gold particles were present on and adjacent to microvilli on the apical surface of the ST of all six placentas incubated with anti-GPIIIa. Examples of first trimester and term specimens are shown (Figs. 1A-1D). With the positive control MoAbs, microvilli of all the first trimester placentas incubated with ED822 MoAb exhibited gold particles (Fig. 1E), and the term placentas bound anti-PLAP (Fig. 1F). Gold particles were often in groups of up to seven after labeling tissues with anti-GPIIIa and anti-PLAP, but were distributed individually or in pairs with ED822 MoAb. Not all microvilli bound gold particles and areas without microvilli were also unlabeled. The patchy distribution of gold particles was more evident with anti-GPIIIa than with the positive control MoAbs, indicating variation of expression of membrane proteins over areas of the ST membrane. No gold particles were seen with any specimens stained with the negative control IgG1 antibody (Figs. 1G and 1H). Approximately 100 fields were examined for each sample.

A description of placental structure is given below to illustrate the fetal and maternal circulations, the villous tree, and localization of the microvilli (a brush border) on the villous ST.

The majority of the placenta is composed of highly branched fetal chorionic villi extending from the chorion to the decidua (Fig. 2A). The villi contact maternal blood that flows from uterine spiral arteries into the intervillous spaces. Here, at the apical surface of the villi, nutrient and gas exchange takes place between maternal and fetal blood. The maternal blood then drains back through the endometrium via the uterine veins (Fig. 2A). The fetal circulation in the placenta is confined within blood vessels of the villi (Fig. 2B). Capillaries extend into all the villous sprouts. The villi consist of mesenchymal stroma surrounding and supporting the blood vessels with an outer continuous epithelial cell layer, the ST. The ST nuclei are closely packed around the periphery of the villi (Fig. 2C). Areas of villi denuded of ST are covered by a layer of fibrin (Fig. 2C). There are frequent aggregations of ST nuclei (syncytiotrophoblast), apparent by light microscopy as darkly stained patches protruding from the villi (Fig. 2C).

By electron microscopy, details of the structure of villi are much more apparent (Fig. 3). Fetal capillaries are surrounded by a thin layer of endothelium (Figs. 3A-3C). The fetal RBCs are electron-dense, often irregularly shaped. Electron-lucent mesenchymal stroma separates endothelial cells from the ST. The ST is highly vacuolated, with a few electron-dense granules. Microvilli (the brush border) cover virtually the entire apical surface of the ST at term, greatly increasing the surface area in direct contact with maternal blood. The ST nuclei are not separated by cell membranes. Those ST nuclei that are aggregated in syncytial knots are unlike those of the endothelial or mesenchymal cells, being highly irregular in shape and with perinuclear patches of electron-dense condensed chromatin (Figs. 3A and 3B). They are in groups of approximately 5 to 15 nuclei. Two main differences between first trimester and term villi are evident. The mononucleated cytotrophoblasts (CTs) that are situated between the ST and the stroma are sparse at term (Fig. 3C) but are more frequent and regularly spaced underlying the ST during the first trimester (Fig. 3D). At term, villi are small with many fetal capillaries abutting the ST (Fig. 3C), whereas no fetal blood vessels are visible in the image of a first trimester villus (Fig. 3D).

DISCUSSION
This ultrastructural study of the immunolocalization of GPIIIa on placental chorionic villi showed that GPIIIa was expressed on ST microvilli of both first trimester and term placentas (Figs. 1A-1D). The MoAb chosen to detect GPIIIa (clone SAP) was earlier found to be reactive on first trimester and term placental ST by immunocytochemistry, unlike clone Y2/51 that did not bind ST by this technique and PM6/13 that was weakly reactive. All MoAbs were strongly reactive with PLTs. Epitopes for some MoAbs raised against PLT GPIIIa (of the αβ3 integrin heterodimer) may be masked by conformational changes of β3 integrin when associated with αV integrin. MoAbs to αVβ3 integrin (23C6 and LM609) also gave discrepant results on placenta, either reacting with or not binding to extravillous trophoblast. Thus, MoAbs to integrins vary in their tissue reactivity.

The positive control antibodies, ED822 MoAb and anti-PLAP, were chosen from earlier data. By immunocytochemistry, they bound to ST of term placentas and ED822 MoAb also reacted with first trimester ST. The enzyme activity of PLAP was found to be localized to ST microvilli and to increase during pregnancy. By cryoimmunoelectron microscopy, with the use of ultrathin sections of term villi labeled with anti-PLAP (clone 8B6), gold particles were frequently visible on the microvilli of the ST. Thus, our findings are consistent with earlier reports of the subcellular localization of PLAP and also show that
GPIIIa is present on the ST microvilli of both first trimester and term placentas.

The relevance of this observation lies in the anatomy and physiology of the human placenta. Although the placenta and amniotic sac form a physical barrier between the semiallogeneic fetus and the mother, three types of fetal material enter the maternal circulation. Subcellular ST microparticulate matter, DNA derived from ST nuclei, and occasionally, fetal blood are all detectable in the peripheral blood of pregnant women. They have relevance for the following aspects of transfusion medicine.

The location of GPIIIa on microvilli of the apical surface of ST suggests that this molecule will be accessible to the maternal immune system. The microvilli are bathed in maternal blood from the first few weeks of pregnancy until parturition. Fetal trophoblastic debris is con-
tinuously deported into maternal blood in all pregnancies, reaching a level of about 3 g per day at term. Some of this particulate ST cellular material will circulate through the maternal spleen, the organ where immune responses to blood-borne antigens are produced, especially under inflammatory conditions. Both apoptotic and necrotic loss of syncytial debris occurs, although the mechanism of shedding is not fully understood. Apoptotic release occurs under controlled homeostatic physiologic processes and is noninflammatory. Necrotic cell death, caused by external damage, is proinflammatory due to liberation of toxic cellular components. Some necrotic shedding of ST in pathologic pregnancies may be caused by hyperoxia, with free radical damage, and some by hypoxia. Those parts of the chorionic villi furthest from the outflow of oxygenated maternal blood from the uterine spiral arteries may be especially prone to hypoxia (Fig. 2A). Therefore, the balance between apoptotic and necrotic shedding of ST, and the resulting pro- or anti-inflammatory environment, may affect the immunogenicity of GPIIIa. For some susceptible pregnant women, however, exposure of the antigenic GP to the maternal immune system early in pregnancy may result in an immune response to HPA-1α developing in time for maternal alloantibodies to cross the placenta and cause fetal thrombocytopenia.

The villous trophoblast is continuously regenerated in a highly regulated, homeostatic mechanism. The ST is formed and maintained by fusion with the underlying proliferative stem cells, CTs. Apoptosis (programmed cell death) is necessary for the renewal of the syncytiotrophoblastic surface of chorionic villi. As areas of the ST age, senescent
Syncytial nuclei cluster into focal aggregations known as syncytial knots (Figs. 3A and 3B) and show characteristic signs of apoptosis. In these bizarrely shaped pyknotic nuclei, chromatin condenses and accumulates in compact patches against the nuclear envelope, leading to nuclear shrinkage, degradation of the nuclear membrane, and nuclear fragmentation. Finally, the syncytial knots are extruded into the intervillous space for clearance by the maternal circulation. Two to four weeks elapse between fusion of CT with the syncytiotrophoblast and exocytosis of the

Fig. 2. Anatomy of the placenta. (A) Diagrammatic structure of the placenta. The fetal blood vessels are within the chorionic villi. These are entirely covered by an epithelial layer of fetal trophoblast that also extends over the areas of contact with maternal decidual tissue. Extravillous trophoblast cells are present in the decidua and in the lumen of the uterine arteries. Maternal arterial blood flows with little resistance (long dark gray arrows) through these dilated and flaccid spiral arteries into the intervillous space and then drains back (pale gray arrows) through uterine veins. (B) Small portion of an isolated villus taken from an area of a term placenta such as that illustrated in the box in A. The villus was washed free of maternal blood, placed in saline, and photographed through a dissecting microscope. The fetal blood vessels can be seen. The villi are highly branched with numerous protuberances containing capillaries in close apposition to the external membrane. (C) Cryostat section of intact term placenta representing an area of tissue such as that illustrated in the box in B, stained with antifibrin (mid-gray) and with hematoxylin nuclear counterstain (dark gray). Numerous villi (V) are evident, most are in transverse section; the villi may be derived from one or more branches of the chorionic tree shown in B as they are entwined. The villi are surrounded by maternal blood, appearing here as unstained spaces. ST nuclei are closely packed forming an almost continuous layer on some surfaces of the villi (arrowheads). On many villi, accumulations of darkly stained nuclei occur, often protruding from the surface; these are syncytial knots (arrows). Where the ST layer has become denuded, fibrin is polymerized from maternal blood to form a thick protective layer. As shown in this section, a fibrin clot has contacted at least seven villi.
senescent ST nuclei. Most of the fetal cells and syncytial knots will be removed by the lungs. More than a century ago, Schmorl (in an article summarized recently) identified thrombi containing multinucleated syncytial giant cells in the small arterial blood vessels of lungs of women who had died of preeclampsia, a maternal systemic inflammatory disorder of placental hypoxia. Trapping of cells occurs here because this is the first capillary bed encountered by uterine venous blood. This explains the paucity of trophoblasts in peripheral blood of pregnant women despite their presence in uterine vein blood. Although most apoptotic ST DNA is probably in these multinuclear cells and is rapidly cleared, some fetal DNA is released in cell-free form into maternal plasma. It is now recognized that the source of cell-free fetal DNA in maternal plasma used for fetal genotyping is ST nuclei. This was recently confirmed by finding normal levels of free fetal DNA in anembryonic pregnancies (comprising a placenta but not a fetus). Free fetal DNA is in small fragments, mostly 145 to 201 bp, consistent with its origin from apoptotic ST nuclei. During apoptosis, activated endonucleases cleave DNA at internucleosomal linker regions to form individual nucleosomes or their multiples. These fundamental repeating units of chromatin comprise 146-bp fragments of double-stranded DNA bound to histone octamers with approximately 50-bp linker DNA. It is because fetal DNA is released from apoptotic ST nuclei and is mainly in short fragments that fetal RHD typing failed when using primers producing amplicons longer than 361 bp but succeeded with amplicons of 82 or 122 bp.

Fig. 3. Electron micrographs. (A, B) Cross-sections of two small terminal villi such as those illustrated in the box in Fig. 2C are shown. Fetal RBCs are in the central capillaries that are surrounded by a thin gray layer of endothelium. The adjacent electron-lucent stroma contains scattered fibroblasts and macrophages. Two syncytial knots are present, with closely packed unevenly shaped ST nuclei containing large perinuclear electron-dense areas of condensed chromatin. There are six ST nuclei in the syncytial knot in A and at least 10 in B, with no cell membrane between them. The ST forms an unbroken layer over the villi. The ST cytoplasm has many vacuoles. Microvilli are present on the entire apical (outer) surface of the ST, where they are in contact with maternal plasma. (C, D) Photomicrographs of the edges of large villi taken from term (C) or 10 weeks' gestational age (D) placentas illustrating the main differences in anatomy at different stages of pregnancy. At term, fetal capillaries are numerous and adjacent to the cytoplasm of the ST (C). Early in pregnancy all the villi are comparatively large with fetal capillaries being usually distant from the ST, as evident here (D) where none are in the field of view. Throughout pregnancy, mononucleated CTs are located at intervals between the ST outer layer and the inner stroma.
Shedding of the ST, to a greater or lesser extent, occurs during normal pregnancies. A mechanism is in place to prevent breaching of the fetomaternal barrier that could lead to FMH. Areas of villi that become denuded of their trophoblast epithelium are protected by fibrin-type fibrinoid deposited from maternal blood onto the trophoblast basal lamina to cover these areas (Fig. 2C) and to provide a scaffold for lateral growth of the villous trophoblasts.41 When FMH does occur, however, it is perhaps most likely in those areas of the villi subject to necrosis. Indeed, FMHs were found greater and more frequent before Week 36 of pregnancy in patients with hypertension due to preeclampsia than normal women42 and the risk of large FMH was increased in preeclamptic patients.43 One of the features of preeclampsia is placental hypoxia due to reduced maternal blood flow through the spiral arteries, probably increasing the likelihood of necrotic shedding of the ST. FMH is more likely later in pregnancy, when 1) the fetal capillaries are close to the ST; 2) the villi are more numerous as the placenta grows, and 3) there are likely to be more hypoxic areas. Evidence for the reverse transfer of maternal cells to fetal blood (maternofetal hemorrhage) suggests that it rarely occurs. Only 4 percent of newborns were found to have maternal D-mismatched RBCs, at low volumes, 0.8, 1.5, and 101 µL.44 Transplacental hemorrhage may occasionally lead to long-term survival of a few alloimmune cells, either from FMH giving rise to fetal microchimerism in the mother or from maternofetal hemorrhage resulting in microchimerism of the offspring.45

In contrast to the almost complete lack of cellular transfer, the placenta actively transports nutrients, waste products, and gases between the mother and fetus. To enhance placental transport, the villi become more numerous and branched while reducing in diameter and minimizing the distance between the fetal capillaries and the ST (Fig. 3C). Estimates46,47 of the maximum villous ST surface area are approximately 10 to 12 m² whereas microvilli greatly increase the apical surface area of the ST in contact with maternal plasma, so that at 36 weeks gestation it is approximately 94 m².48 The ST transports many substances, some in vesicles, including maternal IgG, which gives passive protective immunity to the infant. IgG transfer is mediated by a specialized receptor, FcRn;49 thus only IgG maternal alloantibodies to fetal blood group antigens can cause fetal alloimmune cytopenias.

In conclusion, we identified GPIIa on ST microvilli. Because ST debris is shed during pregnancy, it would be transported in maternal blood to the spleen where cellular components of the immune response (macrophages, dendritic cells, T and B lymphocytes) reside. The ST-derived fetal HPA-1a-positive GPIIa then might initiate sensitization of susceptible HPA-1a-negative women and cause alloimmunization. Antenatal development of anti-HPA-1a could then lead to FNAIT in primigravidae. Future prevention strategies may need to allow for this mechanism.

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