Placental Trophoblast Inclusions in Autism Spectrum Disorder

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Background: *Microscopic examination of placental tissue may provide a route to assessing risk and understanding underlying biology of autism.*

Methods: *Occurrence of a distinctive microscopic placental morphological abnormality, the trophoblast inclusion, was assessed using archived placental tissue. The rate of occurrence of trophoblast inclusion-positive slides observed for 13 individuals with autism spectrum disorder (ASD) was compared to the rate in an anonymous consecutive birth cohort.*

Results: *The occurrence of inclusion positive slides was significantly greater in the ASD group compared to the control group (6/27 slides, 22.2% vs. 12/154, 7.8%; Fisher Exact Test, two-tailed* p = *.033; relative risk 2.85). The proportion of positive cases was also greater in the ASD group (5/13 cases, 38.5% vs. 8/61, 13.1%; Fisher Exact, two-tailed* p = *.044; relative risk 2.93). Behavioral severity scores did not differ across groups of inclusion positive (*N = *4) and negative (*N = *8) ASD individuals.*

Conclusions: *Although probably not functionally detrimental or causative, the greater occurrence of placental trophoblast inclusions observed in ASD individuals may reflect altered early developmental processes. Further research is required to replicate the basic finding, to understand the basis for the trophoblastic abnormality, and to determine the utility of the measure in early detection of ASD.*

Key Words: Autism, early screening, marker, placenta, trophoblast, trophoblast inclusion

Twin and family studies have provided convincing evidence that autism is largely genetically determined (Bailey et al 1995; Bolton et al 1994; Folstein and Rutter 1977). The clinical genetic data and recent genome-wide screening studies indicate that, in most cases, multiple genetic factors are involved, with each likely contributing only a small amount of risk. It also appears that these risk factors can vary substantially across individuals classified as having an autism spectrum disorder (ASD) (Bailey et al 1995; Cook 2001; Folstein and Rosen-Sheidley 2001; Jones and Szatmari 2002; Lauritsen and Ewald 2001; Maestrini et al 2000; Risch et al 1999; Szatmari 1999; Veenstra-Vanderweele et al 2003). This apparent polygenetic and heterogenetic nature of autism, as well as the unclear role of environmental influences on expression, makes an elucidation of the molecular and neurobiological basis of autism extremely difficult. Basic questions about the relative utility of considering autistic behavior from a categorical or dimensional perspective further complicate matters (Adrien et al 2001; Bolte and Poustka 2001; Szatmari et al 2002).

Despite the inherent difficulties in understanding etiological aspects of autism, the identification of early autism-related markers, traits or endophenotypes offers a promising route of investigation (Gottesman and Gould 2003; Gottesman and Hanson 2005; Leboyer et al 1998; McBride et al 1996; Skuse 2001). Early disorder-associated phenotypes could be of great use in focusing neurobiological research, in suggesting candidate genes, and in early screening or risk assessment. Although most measurable biological phenotypes are expected to be multi-determined, they may actually offer advantages in reflecting convergent processes related to neurobiological abnormality. Even if sets of risk alleles can be identified, at best they can be expected to contribute in a probabilistic fashion to autism-related behavior. Thus, a relevant biomarker or endophenotype may be of particular value in light of the apparent genetic complexities.

The placenta may offer a readily available tissue for the detection of developmental abnormality. The placental trophoblasts are among the first fetal cells formed and they are essential to the implantation of the conceptus and the development of the placenta. The cytotrophoblast and the syncytiotrophoblast form a bilayer that surrounds the inner cell mass and serves to separate the fetal and maternal circulations (Boyd and Hamilton 1970; Kliman 1999). Cytotrophoblasts are known to be the proliferative stem cells of the placenta, while the overlying syncytiotrophoblast layer is formed by fusing cytotrophoblasts (Kliman et al 1986). The relative rates of cytotrophoblast proliferation and incorporation into the outer syncytiotrophoblast layer appear to determine the morphology of the finger-like chorionic villi (Huppertz et al 2001; Kliman and Segel 2003; Rejniak et al 2004). When these critical processes are altered, the bilayer can inappropriately bulge inward into the villi, creating invaginations and trophoblast inclusions that can be readily detected upon histological examination of sectioned placental tissue (Figure 1).

Trophoblast inclusions have been associated with a number of frank genetic abnormalities including triploidy, trisomy and Turner's syndrome (Honore et al 1976; Novak et al 1988; Silvestre et al 1996; Szulman et al 1981). The balance between cytotrophoblast proliferation and fusion into the syncytiotrophoblast layer may offer a sensitive early (sentinel) marker for genetic vulnerability to disruptions in the regulation of basic proliferative and cell specification processes (Kliman and Segel 2003). In other words, although trophoblast inclusions probably have no effect on overall placental function, they may be a reflection of genetic diatheses that could have subtle, yet profound, effects in the developing embryo and the forming nervous system. The possibility that just such diatheses or vulnerabilities may influence brain development in children with ASD prompted us to consider whether trophoblast inclusions might not occur at a

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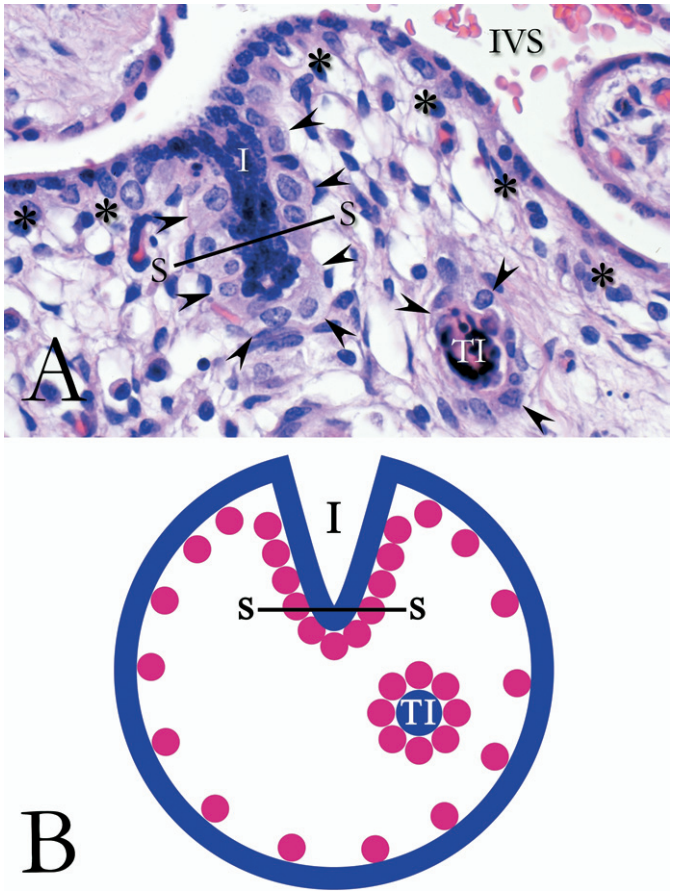

Figure 1. Formation of trophoblast invaginations and inclusions. **(A)** Histologic section of a placental villus which exhibits both a trophoblast invagination (I) and inclusion (TI). Note the increased numbers of cytotrophoblasts (arrow heads) beneath the syncytiotrophoblast layer in the region of the invagination and their paucity away from the invagination (*). When an invagination is sectioned perpendicular to its long axis (S—S) it appears as an inclusion (TI), with dark syncytiotrophoblast nuclei in its center surrounded by cytotrophoblasts (arrow heads). Intervillus space (IVS). **(B)** Diagram of a villus cross-section showing the outer syncytiotrophoblast layer (blue line) and inner cytotrophoblast layer (pink circles) with a trophoblast invagination (I) and inclusion (TI) illustrating the relevant morphology and disposition of cytotrophoblasts in the region of the invagination.

greater rate in the placentas of these children. Using archived placental tissue, we have compared the occurrence rate of trophoblast inclusions in ASD children to the rate seen in controls.

Methods and Materials

Subjects

Cohorts of consecutive patients seen from 1999 to 2005 at the Yale Child Study Center Developmental Disorders Clinic were study-eligible if between 3–13 years-old and meeting DSM-IV diagnostic criteria for autism spectrum disorder [autism, Asperger syndrome or pervasive developmental disorder-not otherwise specified (PDD-NOS)] (American Psychiatric Association 1994; Volkmar and Klin 2005). All patients had undergone a full behavioral, cognitive, and psychiatric assessment within the past 5 years and diagnoses were based on all available information (including psychological and communication assessments, diagnostic screens and assessment, and clinical examination). Parents of potential subjects ($N = 112$) were contacted by mail and asked to participate by granting written permission to access paraffin-embedded placental tissue blocks archived at the child's hospital of birth. Written informed consent was obtained for 76 of the eligible subjects and slides were obtained for 13 of the 76 enrolled subjects. The low slide availability was due mainly to many of the hospitals not routinely archiving placental tissue.

Mean (± SD) age of the final ($N = 13$) ASD group at testing was 4.6 ±1.4 years-old; 11 were Caucasian and two African-American. Patients were assigned consensus clinical diagnosis of autism ($N = 8$) and Pervasive Developmental Disorder—Not Otherwise Specified (PDD-NOS, $N = 5$) based on review of medical and developmental history, and the results of the Autism Diagnostic Observation Schedule—Generic (ADOS-G) (Lord et al 2000) and Autism Diagnostic Interview-Revised (Lord et al 1994). Cognitive level of functioning was measured using the Mullen Scales of Early Learning ($N = 11$) (Mullen 1995) or Wechsler Intelligence Scales ($N = 2$), and standard T scores were obtained (normative values 50 ± 10). Group mean verbal and nonverbal cognitive functioning T scores were 36 ± 20 and 34 ± 14, respectively. The average gestational age in the sample was 38 ± 3.9 weeks (range, 27–42 weeks), with a mean birth weight of 6.9 ± 1.8 lbs (range, 2.3–9.0 lbs). By parental report, 7 of 13 infants were delivered by Caesarian section, 4 experienced perinatal problems [oxygen or respirator required ($n = 3$), hypotonia ($n = 1$)], and 6 of the pregnancies had associated problems [bleeding ($n = 4$), preeclampsia ($n = 1$), and preterm labor ($n = 1$)]. Cytogenetic results were normal for the 7 subjects who had karyotyping performed. Maternal age at birth of the child ranged from 17 to 38 years (mean, 30.8 ± 6.6 years). The study was approved by the Human Investigation Committee of the Yale University School of Medicine (HIC protocol #26018).

Tissue Specimens

The hospitals of birth of enrolled ASD subjects were contacted and asked to provide hematoxylin and eosin (H&E) recut slides of available archived paraffin-embedded placental tissue specimens that were collected at the time of each birth. Anonymous H&E placental control slides ($N = 154$) of a consecutive birth cohort (1999–2000) were obtained from the Department of Pathology, Hospital of Saint Raphael, a community hospital located in New Haven, CT, which at the time of these births submitted all delivered placentas to their pathology department. This aspect of the study was approved by the hospital's IRB (protocol #SR1214). The case and control slides were read by an experienced placental pathologist (HJK). The typical placental slide has a cross section of 2 cm^2 and contains approximately 10,000 chorionic villus profiles. All slides were masked with tape in order to cover completely any distinguishing characteristics. Although H&E staining characteristics (color, intensity) varied somewhat depending upon hospital of origin, staining in control slides was also variable and, thus, differences in staining did not appear to affect the blindness of the reads. The slides were systematically scanned in an attempt to examine all the chorionic villi on each slide and note the occurrence of five specific microscopic abnormalities: well-formed trophoblast inclusions (TIs), questionable TIs, calcified TIs, questionable calcified TIs, and invaginations. The intra-rater test-retest reliability of well-formed TI identification was examined in a validation set of 71 slides enriched (in a rater-blind manner) in slides previously identified as containing TIs. The observed percent agreement across all 71 slides was 83% (59/71), the percent specific

agreement (agreement among slides deemed positive on either read) was 57.1% (16/28), and a kappa of .65 was obtained indicating moderate-to-good reliability.

Statistical Analysis

The primary hypothesis of an increased occurrence of well-formed trophoblast inclusions (TIs) in the chorionic villi of archived placental tissue of ASD children was first examined on a slide-wise basis, using all 27 slides obtained from the 13 subjects with an ASD. The ASD case slides were mixed in with 154 anonymous control slides of the consecutive birth cohort obtained from the Hospital of Saint Raphael. All 181 slides were read, re-labeled and a second read then performed. A positive slide was defined, a priori, as a slide with at least one TI noted on both reads. The proportions of positives slides in the two groups were compared using Fisher's Exact Test (two-tailed statistic reported for all comparisons). The hypothesis was also examined on a case-wise basis, with a positive case defined as a subject with at least one positive slide (as defined above). For this case-wise analysis, 11 ASD subjects each had two slides, one subject had 1 slide and one had 4 slides; 61 anonymous subjects were included, all with two slides each. The inclusion of ASD subjects with one and four slides each was inadvertent, but did not favor the hypothesis of the case-wise test as the singleton slide was positive and none of the quadruplicate slides were positive. Subsequent exploratory analyses used ANOVA to compare TI-positive and TI-negative ASD subjects with respect to ADOS-G and ADI-R domain and total scores, as well as other descriptive and behavioral measures. Additional exploratory analyses compared the occurrence of the other noted microscopic abnormalities in the ASD and control groups using Fisher's Exact test.

Results

When the ASD and control groups were compared on a slide-wise basis (using the a priori TI positive slide definition of at least one TI noted on both reads of a slide), 6 of the 27 ASD slides were inclusion (TI) positive versus 12 of the 154 control slides. The proportion of positive slides in the ASD group (6/27, 22.2%) was significantly higher than that seen in the control group (12/154, 7.8%; two-tail Fisher's Exact test, $p = .033$). The presence of TIs had a sensitivity for ASD of 22.2% and a specificity of 92.2%. A risk ratio of 2.85 (confidence interval-CI: 1.17–6.95) and an odds ratio of 3.38 (CI: 1.15–9.97) were calculated. Exploratory slide-wise analyses were also performed using several post hoc definitions for positive slides. If a positive slide was defined as one having one or more TIs noted on either read, the ASD and control proportions were 12/27 and 29/154, respectively (Fisher's Exact $p = .0059$); using a positive slide definition of 3 or more TIs on either read, the respective proportions were 6/27 and 6/154 (Fisher's Exact $p = .0032$).

The ASD and control groups were also compared on a case-wise basis, with a positive case defined as a subject having at least one positive slide (using the a priori positive slide definition given above). Five of the 13 ASD subjects were TI positive, while 8 of 61 control subjects were TI positive [Fisher's Exact $p = .044$; sensitivity 38.5%, specificity 86.8%, risk ratio 2.93 (CI: 1.14–7.53), odds ratio 4.14 (CI: .76–22.5)].

The TI-positive and TI-negative cases had similar scores in the Communication, Play and Imagination, and Social Reciprocal Interactions domains, although Stereotyped Behaviors and Restricted Interests scores may have been marginally elevated in the TI-positive group ($F_{(1,\ 10)} = 4.31$, $p = .068$). Similar analyses using ADI-R, verbal and nonverbal cognitive scores did not reveal any significant subgroup differences, nor were significant differences seen in the reported ages of onset or other descriptive variables including obstetrical difficulties and perinatal problems. However, pregnancy complications (bleeding, preterm labor, premature rupture, and preeclampsia) were reported more often for the TI positive cases (4 out of 5 versus 2 out of 8 in the TI negative cases).

The intra-rater test-retest reliability for well-formed TI identification across the two reads of the experimental slide set was evaluated using the a priori TI positive slide definition of at least one TI noted on both reads of a slide. A percent agreement of 87.3% (158/181), a percent specific agreement of 43.9% (18/41), and a kappa of .53 were observed, indicating moderate reliability. The other trophoblastic abnormalities (including calcified inclusions and invaginations) were determined with lower reproducibility and were not seen to differ across groups in exploratory analyses.

Discussion

The major finding of the study was the approximate 3-fold increase in the rate of trophoblast inclusion (TI) positive slides or individuals in the ASD group. The results confirmed the hypotheses of increased TI occurrence in the ASD group and raise a number of issues for consideration. It is clear that replication of the basic observation is needed in a larger case-control study. Although the control sample was a sample of convenience, it is presumed to be reasonably representative of the general population. It is possible that the control group included individuals with chromosomal abnormalities and other conditions associated with TIs. The observed control rate of inclusions might, therefore, be somewhat greater than that seen in an assessed healthy normal control group. Improved detection of TIs and optimization of the definition of positive slides and cases may lead to greater group discrimination. As performed, the kappas observed in validation and experimental sets (.65 and .53, respectively) were intermediate to those previously reported for detection of trophoblast inclusions (Genest et al 1995; van Lijnschoten et al 1994) and indicated moderate test-retest reliability.

It is useful to consider the TI findings in the context of research on perinatal risk factors for autism. A number of studies have found that autism is associated with an increased frequency of pregnancy complications and perinatal problems (Bolton et al 1997; Burd et al 1999; Glasson et al 2004; Hultman et al 2002; Juul-Dam et al 2001; Larsson et al 2005; Matsuishi et al 1999; Tsai 1983; Wilkerson et al 2002; Zwaigenbaum et al 2002). Based on family history (genetic loading) data, it is generally thought that the complications and problems are a consequence, rather than a cause of autism. Parenthetically, it is quite possible that the complications and problems, while secondary in nature, can exacerbate the severity of neurobiological abnormalities and behavioral deficits. Most of the obstetrical difficulties, pregnancy complications, and perinatal problems found to be significantly associated with autism have had Odds Ratios of less than 2.0, somewhat lower than the Odds Ratios observed in the present study (3.4 and 4.1). Exploratory analysis suggested that there might have been a greater likelihood of pregnancy complication in the TI positive cases. In future studies, it will be of interest to examine the relationship between the various pregnancy complications/perinatal problems and the occurrence of TIs.

The determination of TI occurrence may eventually prove to be a useful component of a potential multi-marker panel for

autism risk assessment. Closer at hand it might be possible to incorporate TI screening into clinical practice. In cases with obvious dysmorphology or major karyotypic abnormalities, TI screening would not be necessary. However, the presence of inclusions in otherwise apparently normal infants might warrant careful assessment for more subtle dysmorphology and cytogenetic abnormalities. In addition, a careful follow-up for autism-related behavioral abnormality might be recommended in order to determine the suitability of early therapeutic intervention. It should be noted that further improvements in the reliability of detection of inclusions would substantially increase the likelihood of the practical clinical application of TI screening for autism risk assessment.

Finally, presuming replication, consideration of trophoblast physiology and developmental biology may provide insight into the neurodevelopmental abnormalities of autism. Given reported alterations of the neurohormone/neurotransmitter serotonin (5-Hydroxytryptamine, 5-HT) in autism (Anderson 2002), we are particularly interested in 5-HT's role in placental biology and in trophoblast growth and differentiation. Demonstrations of high concentrations of 5-HT in placental tissue (Huang et al 1998) and of the presence of the 5-HT transporter and the 5-HTIA receptor in the human placenta are intriguing (Carrasco et al 2000; Huang et al 1998; Nguyen et al 1999; Padbury et al 1997). Especially relevant to the proposed research are reports of a potent mitogenic effect of 5-HT on the bovine trophoblast (Fecteau and Eiler 2001), since trophoblast inclusions have been suggested to result from a disproportionately increased number of cytotrophoblasts compared to syncytiotrophoblasts (Kliman and Segel 2003; Rejniak et al 2004). It is well established that 5-HT has early growth factor-like effects in the developing nervous system and it appears that 5-HT is present and exerts powerful effects at various stages of embryonic development (Buznikov et al 2001; Janusonis et al 2004; Whitaker-Azmitia 2001). While the role of 5-HT in placental and fetal development remains to be clarified, the available data suggest that its effects are critical.

In summary, the finding of increased occurrence of trophoblast inclusions in ASD requires replication and further characterization. Quite speculatively, it can be suggested that the abnormality may reflect alterations in early developmental processes relevant to autism. Further research is needed in order to understand the basis for the trophoblastic abnormality, to relate the observation to the neurobiology of autism, and to determine the utility of the measure in early detection of autism and related disorders.

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