Denudations as paracellular routes for alphafetoprotein and creatinine across the human syncytiotrophoblast

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We tested two hypotheses: 1) that fibrin-containing fibrinoid-filled denudations of the syncytiotrophoblast may provide a route for paracellular diffusion and 2) that placentas from women who had elevated maternal serum alphafetoprotein (MSAFP) in midgestation had raised permeability to AFP and greater denudation than in normal pregnancy. We measured AFP and creatinine clearance across term placental cotyledons from the above groups and used light microscope morphometric analysis to determine the volume density of fibrin-containing fibrinoid deposits. There was no significant difference between the two groups in terms of AFP and creatinine clearance or volume density of fibrin-containing fibrinoid deposits. The combined data showed a significant (P < 0.05) positive correlation between creatinine clearance, but not AFP clearance, and volume density of fibrin-containing fibrinoid. We conclude that syncytiotrophoblast denudations, with associated fibrinoid, do provide a route for diffusion of small hydrophilic solutes, but that other anatomic features of the placenta are rate limiting for transfer of AFP and similarly sized molecules.

maternal serum alphafetoprotein; placenta; fibrin-containing fibrinoid

HYDROPHILIC SOLUTES, of sizes up to that of circulating proteins such as alphafetoprotein (AFP; molecular mass 60 kDa), may diffuse across the placenta by way of paracellular routes (1, 2, 11, 28, 30). This paracellular diffusion makes a quantitatively important contribution to unidirectional transfer across the human placenta (8, 25).

The exchange barrier of the human placenta in the last two-thirds of pregnancy has two cell layers, the syncytiotrophoblast and the fetal capillary endothelium. The latter is a typical continuous endothelium; the cells are separated by lateral intercellular spaces through which hydrophilic solutes may diffuse, although for molecules of the size of AFP such diffusion is likely to be restricted (10). By contrast, the syncytiotrophoblast is a true syncytium, without obvious lateral intercellular spaces. This has led to controversy regarding the anatomic nature of the paracellular routes across the syncytiotrophoblast. However, we have previously provided evidence that one such route could, potentially, be formed by areas of syncytial denudation, where fibrin-type fibrinoid is deposited (5, 11).

AFP is the major circulating protein of the fetus, with concentrations in fetal plasma up to 10^4 times those in maternal plasma (27). Maternal serum AFP (MSAFP) is measured clinically as a screen for fetal anomalies such as open neural tube defect, where the MSAFP is elevated above normal. However, a small number of pregnancies have elevated midtrimester MSAFP levels in the absence of fetal malformation (21, 27). These women have an increased risk of poor pregnancy outcomes such as low birth weight (27). The elevated MSAFP levels are postulated to arise from an increase in the permeability of the placenta to AFP (3, 21).

In this study, we measured the clearance of AFP and creatinine (a small, 113-Da hydrophilic solute) across the in vitro-perfused human placental cotyledon and then quantified syncytiotrophoblast-associated fibrinoid deposits by morphometric analysis of the same cotyledons. The aim was to evaluate the relationship between maternal/fetal transfer of these solutes and the number of syncytial denudations containing fibrin-type fibrinoid. We tested three hypotheses: 1) the permeability to hydrophilic solutes (AFP and creatinine) of cotyledons from women who had elevated midtrimester AFP would be higher than those from women with normal midtrimester AFP levels, 2) the number of fibrin-containing fibrinoid deposits in the cotyledons from the former group would be higher than that in the cotyledons from the latter group, and 3) the permeability of the cotyledons to AFP and creatinine would be correlated with the number of fibrin-containing fibrinoid deposits.

MATERIALS AND METHODS

This study was approved by the Central Manchester Healthcare Trust Ethical Committee. Patients in the raised AFP group were identified at between 14 and 19 wk gestation

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and recruited for collection of their placentas at the time of delivery. Placentas were collected at term delivery from two groups of women: one group had normal MSAFP (<2.0 multiples of the median (MOM)) and the other group were those who had raised MSAFP (>2.0 MOM) when measured in midtrimester.

Perfusion. Placental cotyledons were selected for perfusion on the basis of the integrity of the decidual plate, which increased the probability that fetal venous perfusate recovery would be high. The method of perfusion was that described by Schneider et al. (22) as adapted in our laboratory (5, 11). Perfusion pumps were set to deliver perfusate to the maternal intervillous space and fetal-side circulation at rates of 14.0 and 6.0 ml/min, respectively. The initial perfusate in each circulation was Earle's bicarbonate buffer containing (in mM) 5.6 glucose, 0.5 dextran 70 (average molecular mass 60–90 kDa; Sigma Aldrich Chemical, Poole, UK), and 0.017 mM 5.6 glucose, 0.5 dextran 70 (average molecular mass 60–90 kDa; Sigma Aldrich Chemical, Poole, UK), and 0.017

mM) 5.6 glucose, 0.5 dextran 70 (average molecular mass 60–90 kDa; Sigma Aldrich Chemical, Poole, UK), and 0.017 bovine serum albumin (Sigma Aldrich Chemical), equilibrated with 95% O2-5% CO2, pH of 7.4. After equilibration, the fetalseide circulation was replaced with perfusate without albumin and dextran, but containing 50 mg/dl creatinine (Sigma Aldrich Chemical) and 20% (vol/vol) pooled fetal plasma as a source of AFP, as described previously (5). Our acceptance criterion of fetal-side venous return flow being >95% of the input flow was enforced throughout the entire perfusion. Perfusion continued for 1 h, with the fetal-side circulation in closed circuit and the maternal-side circulation in open circuit. Samples of fetal reservoir and maternal-side outflow perfusate samples were taken at 10- and 5-min intervals, respectively.

Finally, cotyledons were perfusion fixed for 30 min on the fetal side without a break in flow with a modified Karnovsky fixative (14), excised, and weighed. Subsequent tissue processing and chemical fixation was as previously described (5), with a vertical cross section of the entire cotyledon obtained for paraffin embedding.

Analysis of perfusate samples. The AFP concentrations in the perfusate samples were measured by the RIA technique used for clinical samples at St. Mary's Hospital (16). This RIA uses a well-characterized antibody that is unreactive with other serum proteins; the assay has a sensitivity of 2 ng/ml and a coefficient of variation of <10% at the analyte ranges used. Because blood was incompletely removed from the maternal circulation of the tissue, there was a release of AFP endogenous to the tissue. In a separate study, endogenous AFP output was measured in six placentas over a time course of 40 min. A linear correlation was found to exist between the AFP concentration and the differential in AFP output as a percent of total AFP in the perfusate samples (23). A correction to the raw data was then made by deducting this endogenous value from the measured AFP concentration for each cotyledon. This correction assumed that in the first minute of experimental time, there was negligible transfer of endogenous AFP from the fetal to the maternal circulation. There were no significant differences between the two groups of placentas in terms of absolute calculated endogenous AFP output or in endogenous output as a percent of total AFP in the perfusate samples (data not shown). Creatinine concentrations were measured using a diagnostic kit (Sigma Aldrich Chemical). Transfer of AFP and creatinine was calculated in terms of unidirectional fetomaternal clearance (23).

\[
K_{f-m} = \frac{\text{([maternal side]·Q)/([fetal side]·W)}}{\mu l \cdot min^{-1} \cdot g \text{ placenta}^{-1}}
\]

where Q is the measured flow rate in the maternal circulation and W is the wet weight of the perfused cotyledon corrected to the prefixation value (11).

Tissue processing and morphometry. Tissue specimens in plastic cassettes were stored in buffer and sent by express mail to St. Louis, where they were embedded in paraffin. Five-micrometer sections were processed for hematoxylin and eosin staining and for peroxidase cytochemistry to localize fibrin, as previously described (17). The tissue was sampled as an entire cross-section in the vertical plane. A marking pen outlined a 10% perimeter around each section to avoid the chorionic and basal plates and to exclude the artifactual edge staining that sometimes occurs with cytochemistry. The tissue section was further divided into six equal units, and four micrographs were obtained randomly from each unit (24 micrographs total per section) by an observer blinded to the clinical history and using an Olympus BH2 bright field microscope (Olympus, New Hyde Park, NY), a x x objective, and Kodacolor Gold 100 film (Eastman Kodak, Rochester, NY). A calibration grid was photographed with each micrograph set to verify final print magnifications (x 265) among micrographs from different placentas.

Figure 2 outlines the sampling cascade used in our analysis. The principles of the morphometric techniques used were as described by Elias and Hyde (12). Surface and volume density estimations were carried out in a manner similar to that described in previous placental studies (6) and involved the superimposition of a grid of fine, regular, horizontal straight lines onto the micrograph. Lines were 1 cm in length, equivalent to 16 µm at full scale, with 1 cm spacing. This provided 70 systematic sampling intersects and 140 points per micrograph for analysis. All morphometric features were recorded manually. Variability of measurement was minimized by use of systematic scoring, which satisfied the requirement for random sampling, and all classified objects had an equal opportunity of being represented in the micrograph, as defined by the Nyquist criteria (29). The grid intersects occurred at a density that did not exceed twice the density of the smallest classified feature.

Statistical analysis. Data are presented as means ± SE or median with range, depending on whether they were normally distributed. Comparisons were made between groups using the unpaired Student's t-test or the Mann-Whitney U test, taking P < 0.05 as denoting a significant difference. Correlations between variables were made by linear regression analysis. To estimate the accuracy of our representation of fibrin volume densities within cotyledons from women who
had normal and raised MSAFP, a statistical analysis was performed to monitor how closely sample size approached an acceptable variance, by calculating variance from a running mean (9). This statistical approach considered how incremental increases in the number of observations during the course of the study affected the percentage variance from the previous summed average of the studied morphological feature in the group. An acceptable number of observations was achieved when subsequent increments in n consistently produced a variability that fell within 5% of the previous mean. A comparison of variance in fibrin volume density was made with volume density of trophoblast as a reference feature.

RESULTS

General features of studied pregnancies and perfused placentas. Nine placentas were studied in each of the normal and raised AFP groups. Clinical details of the women who delivered the placentas are shown in Table 1. Other than midtrimester MSAFP levels, there were no significant differences between the two groups.

The perfused cotyledons weighed 38.0 ± 9.9 and 38.6 ± 6.3 g (normal and raised MSAFP groups, respectively, means ± SE, n = 9 for both groups). Actual perfusion rates through the intervillous space and fetal-side circulation were 14.1 ± 0.1 and 6.1 ± 0.1 ml/min (means ± SE), respectively, in the normal MSAFP group and 14.1 ± 0.1 and 6.0 ± 0.1 ml/min (means ± SE), respectively, in the raised MSAFP group. Fetal-side arterial hydrostatic perfusion pressures were 37 ± 5 and 36 ± 7 mmHg (means ± SE) for the normal and raised MSAFP groups, respectively, and were not significantly different from each other (P = 0.84). Fetal-side perfusate recovery rates ranged from 95 to 100% and were not different between the normal and raised MSAFP groups.

AFP and creatinine clearance. Figure 3A shows the steady-state AFP clearance for each studied cotyledon. There was no significant difference in AFP clearance between the normal and raised MSAFP groups; the median (95% confidence interval) for the two groups was 0.60 (0.14, 1.04) and 0.82 (0.18, 3.92) µl·min⁻¹·g⁻¹ placenta, respectively. However, two of the cotyledons in the raised group had AFP clearance values that were 9 and 13 standard deviations higher than the combined

Table 1. Clinical features of studied pregnancies

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<thead>
<tr>
<th>Normal MSAFP Group</th>
<th>Raised MSAFP Group</th>
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<tr>
<td>Number of deliveries</td>
<td>n = 9 (term)</td>
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<tr>
<td>MSAFP MOM values</td>
<td>&lt;2.0 (range: 0.64–1.61)</td>
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<tr>
<td>Gestation at MSAFP measurement</td>
<td>15–19 wk, 5 days</td>
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<tr>
<td>Ethnicity</td>
<td></td>
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<tr>
<td>Caucasian</td>
<td>n = 5</td>
</tr>
<tr>
<td>Asian</td>
<td>n = 3</td>
</tr>
<tr>
<td>Afro-Caribbean</td>
<td>n = 1</td>
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<tr>
<td>Delivery method</td>
<td></td>
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<tr>
<td>Vaginal</td>
<td>n = 7</td>
</tr>
<tr>
<td>Cesarean section</td>
<td>n = 2</td>
</tr>
<tr>
<td>Birth weight, kg (mean ± SE)</td>
<td>3.453 ± 0.173*</td>
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<tr>
<td>Birth weight percentiles</td>
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<tr>
<td>0 to 3rd</td>
<td>n = 2</td>
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<tr>
<td>3rd to 10th</td>
<td>n = 2</td>
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<tr>
<td>10th to 90th</td>
<td>n = 8</td>
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<tr>
<td>90th to 100th</td>
<td>n = 1</td>
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Term is gestational age >38 wk. Derivation of maternal serum alphafetoprotein multiples of the median (MSAFP MOM) values involved a correction for maternal age, gestational age, and maternal weight (20); there were no diabetic patients in either group. In normal group, 1 Cesarean section was because of a breech fetus and 1 because of failure to progress with late fetal decelerations. In raised group, Cesarean section was because of previous obstetric history. *Not significantly different from each other (means ± SE, P = 0.085). Birth weight percentile charts used gave appropriate values for race, sex, and gestational age at time of delivery (4, 13).
mean of all others in both groups. There was no significant correlation between the midtrimester MSAFP values and the AFP clearances of the placentas from the same pregnancies. The cotyledons that gave the highest AFP clearances at term had midtrimester MSAFP MOM values of 2.29 and 3.29, well within the range of values encountered within this raised group (range was 2.04–5.39). No such outliers were observed in the normal group. Figure 3 shows that creatinine clearances in the two groups were also not significantly different: median (95% confidence interval) was 29.9 (17.7, 64.6) and 29.1 (19.5, 38.2) μl·min⁻¹·g⁻¹ placenta for the normal and raised MSAFP groups, respectively. Notably there were no cotyledons for which clearance of creatinine was more than two standard deviations greater than the mean.

Morphometric data. The morphometric data are shown in Table 2. The most striking result was the observation that 7% of the surface density of the villi in each group consists of fibrin-containing fibrinoid deposits. However, there was no significant difference in any of the morphometric variables between the raised MSAFP and normal MSAFP groups for either surface or volume densities, and all values were within two standard deviations of the mean.

The analysis of variance of the running mean for syncytiotrophoblast-associated fibrin is shown in Fig. 4A. Only at n = 8 did both variances begin to consis-

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<th>Table 2. Morphometric variables</th>
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<td><strong>Measurement</strong></td>
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</tr>
<tr>
<td>Surface density, %</td>
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<td>Volume density, %</td>
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Data are shown as means ± SE (n = 9 in both groups). There are no significant differences.
tently fall within acceptable margins of 5% of the previous mean for fibrin. Figure 4B shows variance in running mean for trophoblast volume density, and at n = 6 it can be seen that the mean began to stabilize within acceptable limits (5% variance from the previous mean) and the variance was generally less at greater n numbers. The n of nine in each of the normal and raised MSAFP groups was therefore adequate to minimize variance.

We took volume density of trophoblast-associated fibrin as the most appropriate marker of zones of syncytial denudation available for diffusion of hydrophilic solutes and so correlated this with creatinine and AFP clearance. As shown in Fig. 5A, there was no significant correlation between AFP clearance and the volume density of the deposits in either group or with both groups combined. This remained the case when the data from the two placentas with very high AFP clearances were excluded from the analysis (data not shown). However, as shown in Fig. 5B, there was a positive correlation, significant at the 10% level, between creatinine clearance and volume density of fibrin deposits in the normal MSAFP group, although not in the raised MSAFP group. There was a positive correlation significant at the 5% level when both groups of data were combined.

**DISCUSSION**

In this study, we directly addressed three issues: whether term placentas from women who had raised midtrimester AFP levels have a greater permeability to hydrophilic solutes than do those from women with normal midtrimester MSAFP levels, whether there would be more fibrin-containing fibrinoid deposits (as a marker of syncytial denudations) in the former compared with the latter group, and, finally, whether there was any correlation between the clearance of the hydrophilic solutes and the number of deposits.

The perfused human placental cotyledon seemed a good experimental system with which to investigate these issues. There is a risk that artificial perfusion might increase placental permeability to hydrophilic solutes. However, the clearances of such molecules across cotyledons perfused in this laboratory are very similar to those found for human placentas in vivo; this comparison has been reported previously (11), and the current data are consistent with it.

We found that there was no significant difference between the two groups in AFP or creatinine clearance. However, closer inspection of the data suggests that the situation may be more complex than this. Two of the cotyledons from the raised group had AFP clearance values that were markedly higher than that seen in the rest of this study or in our previous study (5). With the exception of one of the pregnancies resulting in a small neonate, below the third percentile for gestational age, race, and sex, nothing else was remarkable about these two cases, either in experimental outcome from perfusion or in clinical features of the pregnancies and babies. It is noteworthy that creatinine clearances for these two cotyledons were well within the range of values for the other cotyledons. The two placentas with extraordinary AFP clearances might have arisen in the raised MSAFP group by chance; permeability of the normal human placenta to hydrophilic solutes has been shown previously to have a wide range (30). Alternatively, there may be a small subgroup of women with raised MSAFP who do have placentas with elevated permeability to the protein. A third possibility (bearing in mind that each placenta is made up of many cotyledons) is that just a proportion (2 of 9 or 20% based on our data) of cotyledons from the raised group have abnormally high AFP permeabilities. This might well be sufficient to result in significantly increased total placental AFP transfer and the observed increase in MSAFP. Discriminating between these possible explanations for the high AFP clearances in two cotyledons would be difficult, because of the considerable number of placentas that would need to be perfused from both...
groups and the technical difficulty of perfusing more than one cotyledon from each placenta. We conclude from our present data that, overall, there is no increase in the permeability to hydrophilic solutes of placentas of women who have raised AFP levels in midtrimester or that such an increase is not sustained to term.

When expressed in a comparable fashion, our morphometric values compare well with previous estimates in studies where the perfusion fixation method was employed (6, 7). Our fibrin volume densities are slightly higher than those reported by others (19, 26). However, this is likely to be explained by our use of peroxidase immunocytochemistry to optimize localization of fibrin deposition.

It is clear that variability in fibrin density between cotyledons in both groups was high, compared with the reference variance of trophoblast density. We established that the number of placentas sampled in each group to estimate the volume density of fibrin in this study was sufficiently large to accurately represent the mean population of fibrin within each group.

The proportion of syncytiotrophoblast associated with fibrin-containing fibrinoid deposits as found here is remarkable. The number of such deposits is actually an underestimate of the number of denudations in the syncytiotrophoblast, as not all of these become plugged with fibrin. Therefore, our data imply that >7% of the villous surface does not normally have the typical syncytiotrophoblast layer, at least at term. As this layer is the transporting epithelium of the placenta, it is likely that the formation and reepithelialization of fibrin-containing fibrinoid deposits could have important effects on maternal-fetal exchange. There was, however, no significant difference in the density of these deposits between the raised and normal MSAFP groups. Thus we accept the null hypothesis and conclude that syncytiotrophoblast denudations in term placentas cannot explain the midtrimester AFP elevations observed in some pregnancies.

We reported previously that fetomaternal transfer of AFP probably includes a bulk flow as well as a diffusional component (5). Although such bulk flow might be an artifact of the perfusion system, there is certainly evidence that it could occur in vivo (see Ref. 5). Indeed, a raised fetoplacental vascular tone might be an alternative explanation (to that of raised permeability) for raised MSAFP, if this leads to increased fetal capillary hydrostatic pressure and bulk flow from fetal to maternal circulations.

The significant positive correlation between creatinine clearance and the volume density of fibrin-containing fibrinoid deposits in the combined group provides further direct evidence that syncytial denudations do form a paracellular route across the placenta, independent of whether they are a route for higher AFP transfer. The fact that there was no significant correlation between AFP clearance and the volume density of deposits suggests that the syncytial denudations per se are not rate limiting for transfer of such large molecules. A difference in the rate-limiting site for transfer of small solutes versus that for large solutes is consistent with the data showing that creatinine clearances were in the normal range in the two cotyledons with abnormally high AFP clearances.

Stulc (24) reported previously a theoretical analysis of the in vivo clearance of hydrophilic solutes across the human placenta, consistent with the implication from the data reported here that the paracellular pathways are heterogenous and the different layers of the exchange barrier act in series to provide the measured clearance data. There are a number of sites at which AFP transfer might be limited. First, the fibrin-containing fibrinoid forms a matrix that could itself restrict transfer, i.e., the syncytial denudations containing these deposits may not be freely permeable to AFP and similarly sized molecules. Second, the transtrophoblast channels described previously in the human syncytiotrophoblast (15) might be the rate-limiting route of protein transfer across this layer. The basement membrane underlying the syncytiotrophoblast could restrict transfer of proteins, both on a size and charge basis; such restriction by the basement membrane of the renal glomerulus is well documented (18). Finally, it is clear that the lateral intercellular spaces between the fetal capillary endothelial cells would restrict the transfer of AFP (10) and this may therefore be the rate-limiting barrier for diffusion of such large molecules. Further investigation, including new methodologies, is required to dissect the contribution of each of these structures to the paracellular permeability of the human placenta to hydrophilic solutes across a range of molecular sizes.

Perspectives

The permeability of the human placenta to hydrophilic solutes is greater than that of any other species (11). Over 70% of the unidirectional maternofetal transfer of, for example, Ca\(^{2+}\) and Cl\(^{-}\), across the perfused human placental cotyledon takes place via paracellular diffusion (8, 25). Knowledge concerning the nature of the paracellular route and its regulation is therefore essential to a full understanding of the mechanisms of maternofetal exchange. The work here demonstrates that denudations in the syncytiotrophoblast contribute to the paracellular pathway but are probably not the only anatomic feature that must be considered as of importance in this regard.

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