Published in final edited form as:

J Control Release. 2011 March 30; 150(3): 326–338. doi:10.1016/j.jconrel.2010.11.023.

Transfer of PAMAM Dendrimers across Human Placenta: Prospects of Its Use as Drug Carrier During Pregnancy

Anupa R. Menjoge^{1,2}, Amber Rinderknecht³, Raghavendra S. Navath^{1,2}, Masoud Faridnia³, Chong J. Kim², Roberto Romero², Richard K. Miller³, and Rangaramanujam M. Kannan^{1,2,*}

¹Department of Chemical Engineering and Material Science, and Biomedical Engineering, Wayne State University, Detroit, Michigan 48202

²Perinatology Research Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, and Department of Health and Human Services, Detroit, MI 48201

³Department of Obstetrics and Gynecology, of Environmental Medicine and of Pathology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642-8668, USA

Abstract

Dendrimers offer significant potential as nanocarriers for targeted delivery of drugs and imaging agents. The objectives of this study were to evaluate the transplacental transport, kinetics and biodistribution of PAMAM dendrimers *ex-vivo* across the human placenta in comparison with antipyrine, a freely diffusible molecule, using dually perfused re-circulating term human placental lobules. The purpose of this study is to determine if dendrimers as drug carriers can be used to design drug delivery systems directed at selectively treating either the mother or the fetus. The transplacental transfers of fluorescently (Alexa 488) tagged PAMAM dendrimer (16 kDa) and antipyrine (188 Da) from maternal to fetal circulation were measured using HPLC/dual UV and fluorescent detector (sensitivity of 10 ng / mL for dendrimer and 100 ng /mL for antipyrine respectively). C_{max} for the dendrimer-Alexa (DA) in maternal perfusate ($T_{max} = 15min$) was 18 times higher than in the fetal perfusate and never equilibrated with the maternal perfusate during 5.5 hours of perfusion ($n=4$). DA exhibited a significant but low transplacental transport of $\sim 2.26 \pm 0.12 \mu g / mL$ during 5.5 hours, where the mean transplacental transfer was $0.84 \pm 0.11 \%$ of the total maternal concentration and the feto-maternal ratio as percent was $0.073\% \pm 0.02$. The biochemical and physiological analysis of the placentae perfused with DA demonstrated normal function throughout the perfusion. The immunofluorescence histochemistry confirmed that the biodistribution of DA in perfused placenta was sparsely dispersed, and when noted was principally seen in the inter-villous spaces and outer rim of the villous branches. In a few cases, DA was found internalized and localized in nuclei and cytoplasm of syncytiotrophoblast and inside the villous core; however, DA was mostly absent from the villous capillaries. In conclusion, the PAMAM dendrimers exhibited a low rate of transfer from maternal to the fetal side across the perfused human placenta, which is similar to other investigations of large macromolecules, eg., IgG. These overall findings suggest that entry of drugs conjugated to polymers, i.e., dendrimers, would be limited in their transfer across the human placenta when compared to smaller drug

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*Corresponding author: Rangaramanujam M. Kannan, Department of Chemical Engineering and Material Science, Wayne State University, Detroit, Michigan 48202; rkannan.wsu@gmail.com.

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molecules alone, suggesting novel methods for selectively delivering therapeutics to the pregnant woman without significant transfer to the fetus, especially since the half life of the dendrimer in blood is relatively short.

Keywords

Biodistribution; transport; pharmacokinetic; drug delivery; Human Placenta; paracellular; endocytosis

Introduction

Dendrimers have emerged as promising nanoscale delivery vehicles for targeted delivery of drugs and imaging agents [1]. Dendrimer-drug conjugates have been investigated for oral[2], parenteral [3], ocular[4], transdermal[5], colon[6] and topical routes of administration [1]. The dendrimer-drug conjugates are designed to carry therapeutic agents to specific tissues to reduce systemic effects and increase efficacy at the targeted sites. If dendrimer-drug conjugates can be designed to be confined to either maternal or fetal compartments, there will be significant therapeutic benefits. The success of this strategy depends on the stability and specificity of the conjugate in the body as it reaches the target site (or, target tissue) with subsequent release of drugs before the conjugate is eventually cleared from the body [7]. The release of drugs from the dendrimer conjugate is often slow and is largely governed by the nature of linking chemistry [1,7]. The release of drugs from dendrimer conjugates can be modulated by choice of appropriate spacer or linker to avoid release in physiological conditions such as blood or plasma and to trigger release at diseased or target site [8]. Interestingly, this criterion is not essential in all instances, and some dendrimer conjugates exhibit efficacy in their conjugated forms [8]. The dendrimers biodistribute rapidly and are localized in major organs within minutes of administration [9]. The inability to control passage of small drug molecules across human placenta has been accepted[10]; however, progress towards achieving safe and selective drug therapy during pregnancy by proper drug design and development has not been undertaken [11]. The ability to design biocompatible dendrimer-drug conjugates that rapidly biodistribute, are stable during retention in circulation, and release the drug only at a targeted site suggests a possible mechanism to avoid the undesirable transfer of certain small drug molecules to fetus, which could be toxic to the conceptus. The benefits and safety of drugs conjugated to the dendrimers and administered to the pregnant woman and unborn fetuses are yet to be studied. The present study describes the *ex-vivo* transport and biodistribution of dendrimer conjugates in the dually perfused human placental lobule. The flexibility to modulate the drug release from dendrimers using stable linking chemistry, while retaining the efficacy of drugs in conjugated forms provides innovative ways of selectively treating the pregnant woman. This study also develops the potential for use of polymer-drug conjugates as delivery vectors to selectively treat the mother without affecting the fetus.

Small molecules (less than 600Da) are known to transit the human placenta and reach the fetus by passive diffusion while the passage of substances of molecular weight > 1000 Da may be restricted depending upon whether there are specific receptors on the surface of the syncytiotrophoblast to facilitate the movement of these large molecules, e.g., Immunoglobulin G or Transcobalamin II –B₁₂ [12-19]. Importance of the drugs transported from the mother to the fetus and its implications for the fetus were recognized by the teratogenic defects induced by thalidomide [20]. The *ex-vivo* perfusion of human placental lobule was introduced by first by Panigel et al (1962) and modified by Schneider et al. (1972) and Miller et al (1985) [21]. Placental perfusions have been extensively used to investigate transport of xenobiotics from pregnant woman to fetus [15,22-24]. Nanoscale

medicines continue to be a powerful factor in the advancement of clinical therapy. Recently, the need to evaluate the transport of nanoparticles from mother to the fetus has been expressed. [25] Liposomes in the size range 70 nm to 300 nm were transported across the human placenta to the fetal side by endocytosis and were largely retained within the placental tissues [26]. PEGylated gold nanoparticles 10-30 nm were internalized in the placental cells (trophoblast cells), but were not measurable on the fetal side after 6 h of an open or recirculating perfusion [27], while IgG coated gold nanoparticles (5nM) were detected in the fetal circulation, and human serum albumin gold nanoparticles were not [28]. However, polystyrene nanoparticles up to 240 nm were shown to cross the human placenta *ex-vivo* within 3 hours of perfusion [29]. The macromolecules such as IgG antibody (~150 kDa) and vitamin B12 (1.3 kDa) are known to cross the placenta by carrier mediated pathways [15]. Interestingly, the macromolecules IgG-Fab fragment (abciximab) (~145.6 kDa) [23], erythropoietin [30] thyrotropin stimulating hormone (TSH) (28 kDa), heparin (12-15 kDa) [31,32] and horseradish peroxidase [33] show negligible transplacental transport. These agents demonstrate that there are specific transport mechanisms relative to size and type of biological compounds, including nanomaterials that could limit or facilitate transplacental transfer.

A major challenge in drug therapy is to develop safe and selective targeting strategies during pregnancy [16]. Use of any drug during pregnancy is complicated by concerns of adverse effects on the pregnant woman and the fetus [34]. Drugs administered orally or parenterally to the mother reach the systemic circulation and have a potential to pass to the fetus [20]. In spite of the risk, there is a continuing need to receive medicines for genital infections (bacterial, yeast, herpes, etc), epilepsy, diabetes, asthma and variety of other conditions during pregnancy [11,20]. Dendrimers are used as biocompatible nanocarriers for the delivery of many different types of therapeutic agents to intracellular target sites for pain management, inflammation, cancer and HIV, anti-malarials, antihypertensive drugs, ocular delivery, gene transfer and as carriers for antibodies [1]. These dendrimers could be used to selectively identify active sites for medicine delivery during pregnancy. However, the role of dendrimers as macromolecular carriers for drugs and imaging agents to be administered during pregnancy remains unexplored. In light of the recent developments and extensive research directed towards evaluating the efficacy of different dendrimer-drug conjugates, it is prudent to establish their transport and biodistribution through the human placenta both from efficacy and fetal toxicology perspectives. To the best of our knowledge, this is the first report describing the trans-placental transport and biodistribution of dendrimers across the perfused human placenta. The present study introduces the concept of polymer-drug conjugates as drug delivery systems in maternal-fetal medicine and toxicology.

2. Materials and methods

2.1.1. Synthesis of G₄PAMAM-Alexa (1) (DA)

DA was synthesized as shown in Scheme 1 (see supporting information Fig. S1, S2, S3, S4 and S5 for details).

2.1.2. HPLC analysis

HPLC characterization of conjugates and perfusates was carried out with a Waters HPLC instrument equipped with one pump, an auto sampler and dual UV and fluorescent detector interfaced to Millennium software. The mobile phase used was acetonitrile / water (both 0.14% TFA by v/v) and water phase had a pH of 2.25. Supelco discovery BIOwide pore C5 HPLC column (5 μ m particle size, 25 cm $\times$ 4.6 mm length $\times$ I.D.) equipped with two C5 supelguard cartridges (5 μ m particle size, 2 cm $\times$ 4.0 mm length $\times$ I.D.) was used for characterization of the conjugates. (For details see supporting information).

2.1.4. Dynamic light scattering measurements

Dynamic light scattering (DLS) analyses was performed using a Malvern Instruments Zetasizer Nano ZEN3600 instrument (Westborough, MA) with reproducibility being verified by collection and comparison of sequential measurements. DA sample was prepared using PBS pH = 7.4. DLS measurements were performed at a 90° scattering angle at 37 °C. Z-average sizes of three sequential measurements were collected and analyzed.

2.1.5. Immunofluorescence Histochemistry

Double immunofluorescent staining was performed on 20 µm thick, paraffin sections of tissues placed on silanized slides. The different regions in the placental villous structure were identified based on the presence of syncytiotrophoblast as documented by staining with cytokeratin and the presence of the endothelial cell lining the villous capillaries, as documented by staining with CD-31, PECAM-1. The immunofluorescent staining was performed using Ventana Discovery autostainer for controlled and optimised reaction environment using the automation-optimized reagents from Ventana Medical Systems Inc. (For details see supporting information). Images were captured from Leica TCS SP5 Laser Scanning Confocal Microscope (Leica Microsystems GmbH, Wetzlar, Germany). All study specimens were analyzed by a pathologist blinded to the clinical information.

2.2. Human Placental Perfusions

2.2.1. Patient Selection—Human placentae from normal term deliveries were obtained within 5 minutes of delivery and examined for tears and gross lesions. Within 25 minutes post delivery, a fetal artery and vein on the surface of the chorionic plate and the maternal spiral arteries on the decidual side of the selected lobule were cannulated (Fr 5 umbilical catheters) and perfusion initiated. Details of the placental perfusion technique have been previously published [21,23,35]. These investigations were reviewed and approved by the University of Rochester Human Subjects Review Board.

2.2.2. Preparation of Perfusates—The maternal and fetal perfusates consisted of M199 media without phenol red (Gibco) and modified with dextran (35-45kDa; 7.5 mg/mL maternal, 30 mg/mL fetal), D-Glucose (2mg/mL), sulfamethoxazole (80 µg/mL), trimethoprim (16 µg/mL) gentamicin (52 µg/mL) and heparin (20 USP IU/mL).

2.2.3. Perfusion conditions and study design—The maternal perfusate was gassed with 50% O₂/45% N₂/5% CO₂ and the fetal perfusate was gassed with 95% N₂/5% CO₂. The volume of maternal and fetal perfusate in reservoir was 250 mL each, and the circulating tubings have an additional volume of 125 mL. An initial 2-hour control perfusion (1st hour open circuit; 2nd hour closed, re-circulating circuit) was conducted prior to adding the DA to ensure proper function of the placental lobule. During this interval, perfusion flow rates (maternal and fetal: 15 and 3 ml/min), fetal vessel pressure (<60 mmHg) and net fetal oxygen transfer (>50 mm Hg O₂ difference between the fetal vein and fetal artery) were assessed and criteria met in order to proceed with the 5.5 hour experimental perfusion period (closed circuit perfusion). At this point, maternal and fetal perfusate reservoirs were exchanged, with DA (0.10 mg/mL) and antipyrine (0.10 mg/mL) added to the maternal perfusate in the form of homogeneous solutions of these solutes (test compounds). The transfer of antipyrine and DA was studied from maternal to fetal side. To measure the initial transfer kinetics of dendrimer and antipyrine from the maternal to the fetal circuits, perfusate samples (2.5 mL) were drawn every 5 minutes from the maternal artery and vein, as well as from the fetal vein and the volume loss was calculated for each time point. Thereafter, perfusate samples were drawn every 15 minutes (2.5 mL for each time point) to measure dendrimer and antipyrine transfer into fetal perfusate and human chorionic gonadotropin

(hCG) production. Perfusate samples were stored at -80°C until time of analysis. Perfusate pH, fetal venous pressure and flow rate (2.9 – 3.1 ml/min), and net oxygen transfer were continuously monitored to ensure functional integrity of the placenta lobule. At the termination of the perfusion, perfusates were collected and the placenta was weighed and examined to determine distribution of perfusates reflecting highly perfused, modestly perfused and unperfused (i.e., white, pink and red regions, respectively). Tissue samples were collected for DA content and histology (placed in buffered formalin) and biochemical assessments (frozen in liquid nitrogen).

2.2.4. Biochemical Analysis—A Roche COBAS E411 Analyzer (West Sussex, UK) was used to measure the hCG (β -subunit) levels in both maternal and fetal perfusates (sensitivity: 1.0 mIU/ml). Perfusate gases and pH levels were determined using an Instrumentation Laboratory GEM Premier 3000 (Lexington, MA).

2.2.5. Transplacental transfer percentages—The concentration of DA and antipyrine were measured using HPLC analysis as described in section 2.1.6. The peak concentration and its corresponding sampling time in any of the arteries or veins were defined as the C_{max} and the T_{max} for that maternal artery or fetal vein, respectively. Area under the plasma concentration versus time curve (AUC) was estimated via trapezoidal method.

2.2.6. Estimation of DA retained in the placental tissues—At the end of each perfusion experiment, placental tissue was collected from different regions of the perfused lobule, such as from between the two maternal catheters (highly perfused region) and from the edge of the perfused lobule (modestly perfused region). Tissue samples to be used as control, were collected prior to the perfusion experiment and from an area of the placenta adjacent to the tissue that was loaded into the perfusion chamber. Approximately 200 mg were collected in duplicate from each site, and were rinsed twice in fresh maternal perfusate to remove excess DA unassociated with the tissue. Samples were then homogenized in 1.0 ml of phosphate buffered saline (PBS pH =7.4) and spun at 50,000 rpm for 20 min. The supernatant was further centrifuged at 100,000 rpm for 30 minutes and used for analyzing DA levels according to the above described HPLC method. The results are expressed as ng DA/g tissue. The assay was validated by adding known quantities of DA to fresh placenta, followed by the homogenization and centrifugation process, as described above. The recovery of DA ranged from 90-96%. PAMAM dendrimers have high aqueous solubility and hence their extraction into the phosphate buffer yielded higher recovery.

3. Results and Discussion

Molecules are known to transit the human placenta and reach the fetus. Such drug therapy during pregnancy can be a potential concern for adverse actions in the conceptus [11,20,34]. The purpose of the present study is to determine whether (a) G₄PAMAM dendrimer (and its conjugates) can cross the human placenta from the maternal circulation and can enter the fetal circulation in significant concentrations and (b) could the drug-macromolecule conjugates be used for selective treatment of the pregnant woman without crossing the fetal membranes and affecting the fetus or vice versa. PAMAM dendrimers are nanosized macromolecules and the alexa labeled G₄PAMAM dendrimer (**1**), synthesized in the present study, has a size of 5.6 nm as seen from our particle size analysis by DLS (see supporting information of details). It is known that entry of nanomaterials in the body can be controlled by the epithelial membranes, which act as general barriers. Further, the paracellular transport of nanomaterials could be limited by the presence of tight junctions and adherens which have a small gap < 2nm [25]. Fluorescein has been established as a marker for transplacental transfer [24,25], hence we chose to use the highly stable fluorophore (Alexa 488) tagged

G₄PAMAM dendrimers (DA) for the present study. This is the first study to demonstrate the transfer of a polymer-conjugate from maternal to fetal circulations in the human placenta.

3.3. HPLC analysis of Antipyrine and DA in Perfusates

The dendrimer and its conjugates are effectively resolved using the Supelco discovery BIOwide pore C5 column. The mobile phase acetonitrile / water both containing 0.14% v/v TFA making its pH = 2.25 resolved the DA in the fluorescent channel with retention time of 15.5 min. The maternal and fetal perfusates are composed of several amino acids, which interfere with the detection of antipyrine. Change in the chromatographic conditions (solvent gradient) largely affected the resolution and retention times of antipyrine, without considerably influencing the DA resolution. Under the described gradient method starting with water-acetonitrile (100:0) to water-acetonitrile (30:70) in 35 minutes followed by returning to initial conditions in 25 minutes, antipyrine is efficiently resolved and does not interfere with the endogenous substances in the perfusates. The chromatographic conditions described exhibit a clear resolution of antipyrine and endogeneous substances in the perfusates by UV at 210 nm and by fluorescent detector for DA at λ_{ex} = 495 nm and λ_{em} = 519 nm as seen in Fig. S6 and S7 (Supporting information). The calibration curve for antipyrine and DA in perfusates is linear over the concentration range 0.01 to 0.0001 mg/mL and 1 μ g/mL to 0.05 μ g/mL with a correlation coefficient of $r^2=0.9973$ and $r^2=0.999$ respectively (n=3). The inter-day (n = 3) and intra-day (n=3) studies have an acceptable precision and accuracy of the method. The results of within and between day variability are presented in Table S1 and S2 (Supporting information). These data demonstrate that the method is reproducible within day and between days. The stability of the DA in maternal and fetal perfusates has been confirmed by incubating the DA in the perfusates (2 hr equilibrated) for 72 h and analyzing the samples by HPLC. The chromatograms show a single peak corresponding to DA in the fluorescent channel and the absence of other peaks confirms that Alexa is not cleaved from the DA conjugate (data not shown). DLS studies in PBS showed no change in size of DA as a function of time, over a period of a week, suggesting that there is no aggregation. The proteins, dextran, heparin and other molecules present in the perfusate have similar size to that of the dendrimer and hence it limits the DLS studies to determine aggregation of DA in perfusate. The DLS plot of the maternal perfusate shows a size distribution in the range of 2-8 nm (Fig. S8 Supporting information). The DLS plot of dendrimer-alexa dissolved in maternal perfusate shows a size distribution in the range of 2-8 nm (Fig. S9 Supporting information). The zeta potential for the maternal perfusate was found to be -8.12 and the zeta potential of dendrimer-alexa dissolved in maternal perfusate was found to be -8.23. If there were appreciable aggregation of this neutral dendrimer in the perfusate, we would expect objects larger than 8 nm, with changes in the zeta potential. These results suggest that there is no agglomeration in the maternal perfusate after the addition of dendrimer-alexa. The aggregation of DA in perfusates was therefore evaluated in terms of changes in retention times using HPLC equipped with size exclusion column. We observed no changes in the elution time from samples collected at various time points from the placental perfusion.

3.4. Biochemical and physiological evaluation

For the four placental perfusions, the average mass of the placenta in the perfusion chamber was 80 ± 19 g and 25 ± 13 g for the perfused lobule. All monitored biochemical and physiological data were within specifications for human placental perfusions. Results from the experimental period were compared to those of the last hour of the initial control recirculation period (Table S3). Thus, each placental perfusion becomes its own control, which reduces the large variance due to interplacental differences. Maternal oxygen delivery, net fetal oxygen transfer from the maternal to fetal perfusate, and the production rate for human chorionic gonadotropin into the maternal circuit remained stable pre- and

post-dendrimer perfusion. Further, throughout the control and experimental periods, little hCG was released into the fetal circuit (all values < 0.5 mIU/mL/min/kg) as would be anticipated. The human term placental lobules based upon previously established physiological and biochemical parameters (see Table 1) demonstrated that the placenta functioned normally during the control period and that the dendrimer did not produce adverse effects on the endpoints monitored, as might occur with overtly toxic agents, e.g., cadmium [38]. The cytotoxicity of PAMAM dendrimers is influenced by its generation, surface charge and functionality [1]. The PAMAM dendrimers used in the present study have neutral surface hydroxyl groups which are reported to be non toxic and hence no adverse effects were expected and our findings support this.

3.5. Transplacental Transport of Antipyrine and DA

Antipyrine and DA were added to the maternal perfusate after the 2h equilibration period at exchange transfusion and the transfer from maternal to fetal side was evaluated. The initial concentration of antipyrine and DA in maternal perfusate reservoir was $100 \mu\text{g/mL}$ each. After 10 min of perfusion, the samples collected near the maternal arterial sampling port had concentrations of $66.7 \mu\text{g/mL}$ for DA and antipyrine, and this dilution is attributed to the perfusate volume in the maternal circuit contributing to a decrease in the reservoir concentrations of DA and antipyrine. The freely diffusible marker antipyrine crossed the placenta rapidly, as expected (Fig. 1A). Equilibrium was attained for antipyrine after 45 min. This result is consistent with those reported previously [27]. The peak antipyrine concentration on the maternal and fetal side was achieved within the first 15 min. The decline in the antipyrine concentration on the maternal side was biphasic characterized by a rapid fall in maximum concentration attained in 15 min over 2 h time frames (Fig. 1A, B). The antipyrine values remained steady in the fetal perfusates from 1 h until the end of perfusions (5.5 h). The mean DA concentrations in the 4 to 5.5 h perfusions on the maternal and fetal side following its addition after 2 h equilibration period are shown in Fig. 1C. The critical observation for the DA is the continuing appearance of DA in the fetal circulation resulting in approximately 4.1% of the DA in the fetal circuit compared with the maternal circuit at 5 hours. The C_{max} values for the DA in maternal perfusate ($54.4 \pm 7.51 \mu\text{g/mL}$) are 18 times higher than in the fetal perfusates at the termination of the experiment. The C_{max} value is comparable to the initial concentration of the DA used in the study ($66.7 \mu\text{g/mL}$). The pharmacokinetic parameters determined from these data are summarized in Table 2. The maximum DA concentrations on the maternal side were reached in 15 min (T_{max}). DA was first detected on the fetal side after 15 min of perfusion. At the end of 2 h about $1.45 \pm 0.20 \mu\text{g/mL}$ of DA was transported across to the fetal side, and $\sim 2.26 \pm 0.12 \mu\text{g/mL}$ by 5.5 h. The fetomaternal ratio at end of the perfusion was 0.07 ± 0.02 (Fig. 1D). Equilibrium was not achieved between the fetal and maternal sides as seen from the AUC ratio 0.014. The DA values demonstrated a slow increase in the fetal circuit during the 5.5 h re-circulating perfusions. The DA on the maternal side slowly declined through the study period (Fig. 1D). Thus, DA did cross from maternal to fetal side but at significantly lower amounts as compared to the antipyrine (Table 3). This is quite consistent with other molecules that slowly passage through the placenta, e.g., IgG and Transcobalamin II – B₁₂ [15,17,23].

The mass balance of the DA in the maternal and fetal perfusate was calculated at the end of the experiment taking into account the DA removed for sampling on the maternal side. The HPLC analysis showed that DA was stable in the perfusates and no metabolites were detected during the 72 h incubation period *in vitro*. The initial maximum concentration achieved by DA in maternal perfusates was $54.4 \pm 7.5 \mu\text{g/mL}$ ($n = 4$) in 15 min and at the end of the experiment (5.5 h) about $44.0 \pm 9.9 \mu\text{g/mL}$ was found in the maternal perfusate. The observed difference can be attributed to the amount of DA lost from the maternal

perfusates due to sampling during the entire 5.5 h perfusion period or due to retention in the tissue. Approximately 7-9 $\mu\text{g} / \text{mL}$ of DA was lost during the 5.5 h due to the sampling based upon calculated loss. Retention of DA in the placental tissues was measured by quantifying the amounts of DA in the various regions of the perfused placental lobule collected at the termination of the experiment. Placental tissue collected from the central perfusion area and peripheries have very low and comparable levels of DA retained in the tissue (Table 3). The amount of DA retained in the different regions of placenta is comparable. The overall results suggest that PAMAM dendrimer conjugate was not retained to appreciable levels in the human placental tissues, compared to other nanomaterials or nanoparticles [27,36]. Polystyrene nanoparticles up to 240nm were taken up by the placenta and transported across into the fetal circulation [29]. This suggests that dendrimers may be partitioning less into the placental membrane, compared to other types of nanoparticles.

Throughout gestation, the placenta provides a conduit or barrier for the transfer from the mother to the developing fetus for most xenobiotic materials [24,25,10]. At term, the terminal villi are responsible for maximal materno-fetal exchange. The maternal and fetal blood is separated by the placental membrane cell layers which become thin towards the term [39]. In another recent study by this team, the transport of G4-PAMAM dendrimer-FITC across human chorioamnion membrane was found to be $< 3\%$ in 5 h [10]. The major route of transport was by passive diffusion and to a smaller extent by transcellular pathways. The transport of 70 nm liposomes was found to be 4 % in 2 h while the larger liposomes 300 nm exhibited 1.5 % transfer in 4 h [36]. The macromolecules IgG Fab Fragment (abciximab) (~145.6 kDa) [23], heparin (12-15 kDa) [31,32], erythropoietin [30], dextran (50-70 kDa) and horseradish peroxidase (40 kDa) [33] show negligible transplacental transport. Our finding for the transplacental transport of DA (**1**) is in agreement with those molecules that are transferred but at a low rate as identified with molecules like immunoglobulin G.

The two possible pathways for the xenobiotics to cross the fetal barriers are (a) transcellular route and (b) water filled trans-trophoblastic channels [33]. The solute membrane partition coefficient is known to affect transport across biomembranes. The Log P values for the G₄PAMAM dendrimers are negative, indicative of its hydrophilic nature [40]. For the xenobiotics to cross from the maternal circulation into the fetal circulation they have to pass across two layers; (a) the syncytiotrophoblast and the cytotrophoblast cell layer and (b) fetal villous endothelial lining [41]. The human placenta is known to offer resistance for the transport of substances with molecular weight > 1000 Da [12,13]. The membrane acts physicochemically as a porous and partially semipermeable membrane and its cell junctions which are made of desmosomes, gap junctions and occasional tight junctions offer resistance for paracellular transport. This membrane behaves as a sieve with large water filled extracellular channels and also the intercellular spaces. The paracellular transfer across the placental interface is dependant on the different pore sizes. The trophoblast cells have a limited number of dilated branching wide openings with a diameter of 15-25 nm which regulate the overall permeability [42,43]. While the non dilated channels provide transport for the smaller substances having an effective molecular radius under 2 nm [44]. The molecules arriving from the syncytiotrophoblast have to cross the villous endothelial cell layer to enter into the fetal circulation [63]. The fetal capillary endothelium has few clefts at the intercellular junctions which have a mean width of 15.6nm and contain up to four tight regions of 4.1 nm in diameter restricting the passage of large molecules [27,43,45]. The placental endothelium possesses tight and adherens junctions, an extensive glycocalyx and a continuous basement membrane offering resistance to intercellular transport [43]. The size of DA (**1**) was found to be 5.6 nm, and hence its paracellular passage could occur through the limited dilated openings, a possible reason for the observed low transport of DA across the human placenta. There are reports for the transcellular transport of IgG antibody (~150 kDa) and vitamin B12 (1.3 kDa) across the fetal endothelium [15]. The villous endothelial

cells have abundant intracellular transport structures called caveolae. These caveolae have a flask shaped structure when associated with cell membrane surface and have a size of 50-100nm [41]. It is postulated that the small amount of DA that passes across the syncytiotrophoblast layer, could be transported across the fetal endothelial cells by endocytosis. The histological evaluation was performed to further evaluate the mechanism of transport and biodistribution discussed in subsequent sections.

3.6. Biodistribution of G4PAMAM dendrimer in Human Placenta

Confocal microscopy was used to visualize biodistribution of DA within the perfused placenta. Villous tissues from each placenta were studied before and after perfusion. The general morphology and structural orientation of the human placental villous tissue, i.e., the inter-villous space (IVS) representing the maternal blood and the fetal villous structure was compared between perfused and non-perfused placental specimens (Fig. 2). The syncytiotrophoblast (SCT) forms a continuous layer over the surfaces of the villous trees (Fig. 2C). The villous core has occasional cytotrophoblast cells, basal lamina and the connective tissue core has fetal capillaries, mesenchymal cells and Hofbauer cells. The histological evaluation of the fresh placenta and the perfused placenta does not demonstrate any morphological differences between the two except for the presence of maternal and fetal red blood cells in the unperfused, fresh placenta which is washed during the initial two hour equilibrium period and hence not detected in the perfused placenta (Fig. 2A-B). The histology of the perfused placenta shows no signs of dilation of trans-trophoblastic channels at the end of the experiment. The dilation of trans-trophoblastic channels is known to increase the materno-fetal exchange as these channels act as an extracellular route connecting the stromal space with intervillous space [64].

The transplacental transport of DA from maternal to fetal circulation would involve its transfer across two barrier cell layers viz. the villous syncytiotrophoblast (SCT) and the endothelial cells (EC) lining the fetal capillaries inside the villi [68]. To identify the different cells and regions in the placenta the nuclei for all cells are stained blue (by DAPI), the syncytiotrophoblast and the cytotrophoblast cells separating the maternal and fetal blood regions are stained positive with cytokeratin 8 and the fetal blood cells are stained with CD 31. The Fig. S10A identifies the control placenta (without the treatment with dendrimer (I)) and Fig. S10B shows the perfused placenta with negative control mouse isotype replacing the primary antibodies demonstrating the nuclei stained blue with DAPI and the DA appearing as green fluorescence. The control placenta does not demonstrate any green fluorescent staining, i.e. autofluorescence, confirming that the signal observed in the perfused samples is due to the presence of DA. The fresh placenta stained with cytokeratin 8 and CD 31 is shown in Fig. S10C and D respectively. The staining for the cytokeratin 8 and CD31 is specific and positive as confirmed by its absence in sample with negative control (Fig. S10B).

The Fig. 3 shows the histology of the perfused placenta. The morphology of the perfused placenta was studied in the areas near to the basal plate, farther regions in the terminal villi and midway between them. The syncytiotrophoblast and cytotrophoblast separating the maternal and the fetal blood is stained red (cytokeratin 8 positive). At term, very little cytotrophoblast is present and hence mostly the syncytiotrophoblast is seen marking the rim of villous capillaries. The anti-cytokeratin fluorescence signal for all the syncytiotrophoblast cells lining the terminal, stem and intermediate villous capillaries was comparable. DA signal is stronger near the stem villi and the interstitial trophoblast cells seen in the basal plate while it becomes weaker as one moves interior (Fig 3B-C). The sparse presence of dendrimer in the maternal intervillous space (IVS) is seen as green fluorescence (Fig 3C). The dendrimer appears around the rim of the syncytiotrophoblast in the maternal space. These observations were consistent for all the placentae evaluated (n=4). The biodistribution

pattern in the perfused placenta correlated well with the data for the DA retention in the placental tissue at end of perfusion as seen in Table 3. In very few places the DA appears to be internalized in the outer rim of the syncytiotrophoblast cells (Fig. 4A). This was not a typical observation and was found in only one region. The merged composite image shows the localization of dendrimer in both; the cytoplasm and nuclei of the syncytiotrophoblast (Fig. 4 top right panel). The localization of DA only in the nuclei of the syncytiotrophoblast can be seen from the merged composite image in Fig 4 (bottom panel). The colocalization of G4PAMAM dendrimer in nuclei of cytotrophoblast cells in human chorioamnion was recently reported by our team [10]. In one or two exceptional cases DA was seen beyond the syncytiotrophoblast cells inside the villous core (Fig 4, middle panel). The villous core contains connective tissue and fibers, stroma, basal membrane and the fetal capillaries. The distribution of DA in villous core and the fetal blood vessels was confirmed by staining with CD 31 a positive marker for endothelial cells (Fig 5). Again the sparse presence of DA in the inter-villous space can be seen in Fig. 5A-B. The sparse presence of DA inside the villous core can be seen in Fig 5C and the companion DIC image (Fig. 5D) confirms the presence inside the villous core. In general, the DA was not detected inside the fetal capillaries (VC) marked by endothelial cells, but sparse presence of DA in the connective tissue can be seen from merge composite DIC image under higher magnification (63x) (Fig. 5 bottom panel). None of the sections from the perfused placenta showed presence of DA inside the fetal blood vessels. It must be noted that this immunohistochemistry for DA does not resolve individual dendrimers, since they are only ~ 5nm. Therefore, the presence of green color reflects the fact that dendrimers are present in the region, relative to other regions. It is possible that some low levels of dendrimers are present in the placental tissues and blood vessels as seen from Table 3; however, they are not present in sufficient concentration to be seen in confocal microscopy.

Thus, the transport data suggest that a measurable, but low levels of dendrimer transport across the perfused placental lobule. The fetal DA concentration at the end of 5.5 h perfusion was in agreement with the observed difference between the initial DA concentration in maternal perfusate and that observed at end of the experiment. The experimental and the inferential evidence suggest that DA is not retained to a significant degree in the human placenta. Since a measurable amount of dendrimer is transported across the placenta, with very little quantities retained in the placental cells, our studies suggest that the transfer of PAMAM dendrimer could largely occur by paracellular pathways and to a very small extent by transcellular pathways. Previously, the transfer of horseradish peroxidase molecule (40 kDa) was reported to occur by paracellular route through the discontinuities in the syncytiotrophoblast layer [33] and through the paracellular clefts in placental vessels [43]. The transfer of materials across placenta can be dependent upon gestational age. When term and early human placentae are studied in vitro for uptake of molecules into the placenta, it is noteworthy that the early human placenta appears to concentrate higher levels molecules that are actively transported, e.g., amino acids. In utero, the transfer of inulin into the conceptus is significant in early pregnancy [47]. Even though the molecular weight of PAMAM dendrimers investigated in present study is 3 times larger ($M_w=16\text{kDa}$) than inulin, transfer of DA was observed at term. Thus is it possible there could be a very small transfer of DA across the placenta during first trimester. The transmembrane transfer of TRH (28 kDa) was similar from preterm and term human placenta [31]. Further, there have been reports investigating the bidirectional transfer of xenobiotics from maternal to fetal and from fetal to maternal circulations. It has been observed that the clearance from fetal to maternal circulation is higher than that observed from maternal to fetal side [48,49]. To understand the transfer rates of PAMAM dendrimers from fetal to maternal side and from placenta's in first trimester, extensive studies need to be undertaken and these are beyond the scope of the current manuscript.

The overall results demonstrate that the PAMAM dendrimer conjugate (**1**) in size range 5-6 nm does cross the human placenta ($2.26 \pm 0.12 \mu\text{g} / \text{mL}$ in 5.5 h) in relatively small amounts. This combined with the fact that dendrimers biodistribute relatively rapidly (within ~ 1-2 hours), suggest that dendrimers may be candidates for selective delivery to the mother without rapidly transferring to the fetus. It must be noted that the present *ex-vivo* measurements of transplacental transfer are for re-circulating perfusions of same high concentration DA for 5.5 h and would overestimate the transport, when compared to the *in-vivo* conditions, where the actual large volume of the body fluids and biodistribution would apply. The size dependant barrier capacity of human placenta is known [29]. The recent study on fluorescent quantum dots (QD) showed that smaller QDs transfer at higher rate than larger QDs and their use during pregnancy is therefore limited. The experimental observation in present study and inferential evidence suggests that if the drugs are conjugated to the dendrimers or other polymers of large molecular weights, then their transport across human placenta would be restricted due to the larger size in conjugated form, and these agents may be considered for use as selective treatment of pregnant woman without considerable transfer to the fetus. These results are preliminary and more in-depth studies for understanding the transfer of PAMAM dendrimers are required based upon the half life of PAMAM dendrimers in maternal circulation in-vivo as well as the required therapeutic dosage required for maternal benefit. These studies are underway.

Conclusion

Drug therapy during pregnancy is a challenge. Is the therapy for the pregnant woman, the conceptus or both? Is the therapy perhaps teratogenic or producing fetal toxicity? Can one design a therapy which can treat only the mother or be facilitated in its transfer to the conceptus and specific organs in the fetus? Dendrimers conjugated to known drugs could provide a vehicle for such selective therapies; however, this field of perinatal nanomedicine is only beginning. There is little information concerning the transfer of drugs conjugated to dendrimer and other polymer scaffolds from mother to conceptus. To expand our knowledge, we employed a dually perfused human placental lobule model to examine the kinetics for maternal to fetal transfer of the PAMAM dendrimer as a drug nanocarrier. The dendrimer alexa conjugate was transferred at a slow rate when compared to antipyrine. The rate of transfer was consistent though with other large molecules. The C_{max} for the dendrimer-Alexa in maternal perfusate was attained in 15 min and was 18 times higher than in the fetal perfusate by the termination of the experiments. In 5.5 hours, DA exhibited low transport of $\sim 2.26 \pm 0.12 \mu\text{g} / \text{mL}$ of the total maternal concentration ($66 \mu\text{g} / \text{mL}$). Negligible amounts ($< 33 \mu\text{g/g}$ tissue) of DA were retained in human placental tissue. Thus, depending upon the half life of the dendrimer conjugate in the maternal circulation, the required therapeutic dosage, and the kinetics of placental transfer, entry of drugs conjugated to polymers may be restricted in their transfer from mother to conceptus across the human placenta when compared to small drugs alone. Such results may provide novel mechanisms for the selective delivery of therapeutics to the mother without significant therapeutic transfer to the fetus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS and by NIH R03 HD059027. We thank Dr. Asad Abbas for help with placental tissue sections.

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Appendix. Supplementary Data

Materials and methods (^1H NMR and HPLC analysis) are provided.

Fig.1.

The transport of G4PAMAM dendrimer conjugate and antipyrine across perfused human placenta (at term). (A) Antipyrine concentrations in maternal (blue) and fetal (orange) circulations with 100 $\mu\text{g}/\text{ml}$ of antipyrine, (B) feto-maternal ratio of antipyrine in the same perfusions as in (A), the feto-maternal ratio of antipyrine at end of perfusion was found to be 1.25 ± 0.122 , (C) G4PAMAM dendrimer conjugate (DA) concentrations in maternal (red) and fetal (green) circulations with 100 $\mu\text{g}/\text{ml}$ concentration of DA and (D) the feto-maternal ratio of G4PAMAM dendrimer conjugate (DA) in the same perfusions as in (C). The feto-maternal ratio at end of perfusion was 0.073 ± 0.02 . The results are based on $n=4$ successful perfusions of 5.5 h each. The C_{max} for antipyrine and DA was reached in 30min in maternal perfusate.

Fig. 2. Morphology of human placental villous tissue before (A) and after perfusion (B) (20x). Fresh placenta shows presence of red blood cells (marked by arrow) in the intervillous space (IVS) and inside villous (fetal) capillaries (A). The perfused placenta shows absence of vacuolization in syncytiotrophoblast and red blood cells (B). (C and D) The villous structure shows intact villous capillaries (VC), intervillous space (IVS) and the outer lining of syncytiotrophoblast (SCT) (100x).

Fig.3.

Biodistribution of DA in the human placental tissue. (A) The morphology of the tissue showed little presence of dendrimer in the inter-villous space (IVS) (20×). (B) The dendrimer is seen primarily surrounding the stem villi (40×). (C) Some dendrimer also is noted along the basal plate (40×). (D) Largely, the dendrimer is found surrounding the syncytiotrophoblast and confined to the maternal space (40×). The merge composite image demonstrates that dendrimer is located in the maternal lacunae (outside of the syncytiotrophoblast) (bottom panel) (63×). The syncytiotrophoblast marked by cytokeratin is red, the nuclei for all cells are stained blue and the dendrimer-alexa is seen as green fluorescence. SCT=syncytiotrophoblast, DA =dendrimer alexa conjugate, DAPI= nuclear stain

Fig 4.

Biodistribution of DA in the syncytiotrophoblast cells. (A) The presence of DA in the rim of the syncytiotrophoblast is noted in few villous structures (40×). The merged composite image demonstrates the localization of DA in both the cytoplasm and nuclei of the syncytiotrophoblast (marked by arrows, top right panel, 63×). In an exceptional case, DA was found inside the villous core as seen from the merge composite image (middle panel, 63×). The bottom panel shows the localization of DA only in the nuclei of syncytiotrophoblast cells (63×). SCT=syncytiotrophoblast, DA =dendrimer alexa conjugate, DAPI= nuclear stain

Fig. 5.

Biodistribution of DA inside the human placental villous core. (A) The overall distribution of DA in the placental villi is sparse (20 $\times$). (B) The companion DIC image demonstrates the orientation of the tissue and the presence of DA is largely noted in the inter-villous space. (C) The villous capillaries are stained CD-31 PECAM-1 positive (red) and the nuclei is stained with DAPI (blue) and the dendrimer appears as green fluorescence (40 $\times$). The presence of DA inside the villous core is marked by arrow heads (D) The companion DIC image confirms the presence of DA inside the villous core in a few places (arrow heads) (40 $\times$). The merged composite DIC image in bottom panel demonstrates the presence of DA inside the villous core in connective tissue but not inside the villous capillaries marked by CD-31 (63 $\times$). SCT=syncytiotrophoblast, DA =dendrimer alexa conjugate, DAPI= nuclear stain, VC = villous capillaries, IVS= intervillous space.

Scheme 1.

The schematic representation for the synthesis of fluorescently labeled G₄-PAMAM-dendrimer: DA (1)

Table 1

Biochemical & Physiological Characterization of the Dually Perfused Human Placentae Lobule: Exposure to Dendrimer-alexa conjugate and Antipyrine.

Response	Experimental exposure		
	Control (1-2 h) <i>N</i> =4	Experimental (1-5 h) <i>N</i> =4	Acceptable range
Fetal pressure (mmHg)	43 ± 18	40 ± 17	<70
Fetal venous flow rate (mL/min)	2.99 ± 0.02	2.95 ± 0.04	2.90 – 3.10
	(mL/min/kg)	Per cent of control	
Maternal oxygen delivery	9.9 ± 1.9	104 ± 6.3	>80 - <135
Net fetal oxygen transfer	0.4 ± 0.1	110 ± 14	>76 - <150
	(mIU/mL/min/kg)	Per cent of control	
hCG production (Maternal)	72 ± 47	129 ± 51	>58 - <154

Mean±SD.

N=number of placental perfusions.

Table 2

Kinetic parameters for dendrimer-alexa (DA) in maternal and fetal perfusates

Parameters	Maternal	Fetal
C _{max}	54.4 ± 7.51 µg/ mL	ND
T _{max}	15 min	ND
AUC ^{0-∞}	2976.8 µg hr / mL *	42.76 µg hr / mL *
AUC ratio (fetal / maternal)	0.0143	

* = AUC was calculated using the numerical approximation using trapezoidal rule

ND = Not determined as the values kept slowly increasing over the entire perfusion period

Table 3

Dendrimer-alexa (DA) retained in Human Placental Tissues

Tissue Details	DA level ($\mu\text{g/gm}$)*	SD
Perfused placenta center	33.01	0.019
Perfused placenta center (washed)	25.00	0.004
Perfused placenta periphery	31.88	0.005
Perfused placenta periphery (washed)	18.43	0.007

* Results are in $\mu\text{g/gm}$ tissue and are the mean and standard deviation of triplicate samples from four placentae at the end of a 5.5 hour perfusion.