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***In utero* cell transfer between porcine littermates**

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Abstract

Trafficking of cells between mother and fetus during the course of normal pregnancy is well documented. Similarly, cells are known to travel between twins that share either a placenta (i.e. monozygotic) or associated chorion (i.e. monochorionic). Transferred cells are thought to be channelled via the vessels of the placenta or vascular connections established via the chorion and the long-term presence of these cells (i.e. microchimerism) can have important consequences for immune system function and reparative capacity of the host. Whether cells can be transferred between twins with separate placentas and separate chorions (i.e. no vascular connections between placentas) has not been investigated nor have the biological consequences of such a transfer. In the present study, we tested the possibility of this type of cell transfer by injecting human cord blood-derived cells into a portion of the littermates of swine and probing for human cells in the blood and tissues of unmanipulated littermates. Human cells were detected in the blood of 78% of unmanipulated littermates. Human cells were also detected in various tissues of the unmanipulated littermates, including kidney (56%), spleen (33%), thymus (11%) and heart (22%). Human cells were maintained in the blood until the piglets were sacrificed (8 months after birth), suggesting the establishment of long-term microchimerism. Our findings show that the transfer of cells between fetuses with separate placentas and separate chorions is significant and thus such twins may be subject to the same consequences of microchimerism as monozygotic or monochorionic counterparts.

Additional keywords

cord blood; maternofetal transfer; microchimerism; stem cells; tolerance

Introduction

Microchimerism refers to the presence of a small number of foreign cells (fewer than 1 in 100 cells) within the tissues or circulation of an organism. Microchimerism can occur as a result of iatrogenic interventions, such as transplantation or transfusion, or naturally between fetuses or mother and fetus during gestation. As early as 1893, fetal material was detected in women who had died from eclampsia (Lapaire *et al.* 2007). Decades later, and with advancing technology, cellular exchange between bovine monochorionic twins was suggested by studies of skin graft tolerance (Anderson *et al.* 1951; Billingham *et al.* 1952), detection of fetal leucocytes in the maternal circulation (Walknowska *et al.* 1969; Schroder and De la Chapelle 1972) and detection of maternal melanoma cells in the fetus (Reynolds 1955; Freedman and Mc 1960).

Fetal to maternal microchimerism impacts on maternal immunity and tissue maintenance. The mechanisms by which fetal cells, especially haematopoietic cells, evade the maternal immune system have yet to be determined. However, survival of fetal T-cells, B-cells, macrophages and natural killer cells implies an altered composition of the maternal immune system and so perhaps altered function. That microchimerism impacts on maternal immunity was first suggested by studies indicating that pregnancy ameliorates the autoimmune disease rheumatoid arthritis for most women (Østensen *et al.* 2005). Subsequent reports demonstrated a significant inverse correlation of fetal–maternal chimerism with arthritis activity (Yan *et al.* 2006). Whether fetal cells act centrally (i.e. in the thymus) or peripherally (i.e. at the site of inflammation) to reduce inflammation has yet to be determined. However, similar mechanisms could play a role in the improvement of other autoimmune diseases with pregnancy, including multiple sclerosis and Graves' thyroiditis.

Fetal immune cells in the maternal circulation may also improve immune surveillance. Associative studies have been conducted to determine whether fetal cells improve the ability of the maternal circulation to clear cancer cells. One case-control study, indicated that women with breast cancer were relatively deficient in circulating fetal cells compared with controls (Gadi and Nelson 2007; Gadi *et al.* 2008). For cervical cancer, fetal microchimerism has been augmented to combat the disease. For example, a woman with cervical cancer with pre-existing fetal microchimerism from her daughter received a transfusion of granulocyte colony stimulating factor (G-CSF)-mobilised cells from the same daughter, which resulted in a successful graft-versus-tumour effect (Tokita *et al.* 2001).

Fetal microchimerism may also contribute to tissue repair. Fetal cells with organ-specific phenotypes have been detected in individuals with thyroid and liver damage (Srivatsa *et al.* 2001; Cirello *et al.* 2008). To further probe the associative clinical evidence, animal models have been developed to determine the role of fetal cells in maternal tissue repair. One such rat model administered ethanol and gentamycin to females post partum to induce hepatic and renal injury (Wang *et al.* 2004). Subsequent examination of maternal tissues identified fetal cells as hepatocytes in the maternal liver and renal tubular cells in the maternal kidney. Similar studies have shown evidence of repair via fetal cells in the maternal spleen (Khosrotehrani *et al.* 2007).

Microchimerism between fetuses may facilitate the same outcomes as microchimerism between fetus and mother described above. In addition, microchimerism between fetuses can result in the freemartin syndrome (Lillie 1917) or the masculinisation of the female reproductive tract to varying degrees (Padula 2005). Freemartin syndrome affects female fetuses with male twin(s) and is a consequence of the invasion of male cells in the developing female organism.

The transfer of cells between mother and fetus or between fetuses is thought to occur principally at the maternal–fetal tissue interface within the placenta or through vascular connections that can form between separate placentas of certain species, namely cattle (Lillie 1917; Padula 2005), sheep (Smith *et al.* 2000) and goats (BonDurant *et al.* 1980). Thus, the possibility that dizygotic, dichorionic (i.e. with separate placentas and chorions) fetuses may transfer cells to each other seems remote and so has not been carefully studied. We have recently conducted studies of human T-cell engraftment in pigs after injection of human cord blood-derived cells into 40-day fetuses (Ogle *et al.* 2009). Here we further probed the blood and tissues of pigs (both those injected with human cells in utero and those not injected) to determine whether cells could transfer between porcine littermates. We found that littermates that did not receive the injection still harboured low but detectable levels of human cells in the blood and occasionally in parenchymal tissues. These results indicate that substantial cell transfer can occur between dizygotic, dichorionic litter-mates and suggest an alternative route of transfer and the possibility of considerable contribution of sibling cells to the formation of blood and tissue.

Materials and methods

Cord blood preparation and surgical procedure

Human umbilical cord blood was obtained from healthy human donors in compliance with established institutional guidelines (Mayo Clinic, Rochester, MN, USA) and depleted of T-cells, as described previously (Ogle *et al.* 2004). T-Cells comprised <0.1% of the remaining mononuclear cells. The T-cells were removed because the present studies were initiated for a separate purpose, namely to determine whether human T-cells could develop *de novo* in a surrogate host. It is important to note that because T-cells were removed, the scenario depicted here does not directly reflect the physiological state of microchimerism transfer, which has been shown to include T-cells (Loubière *et al.* 2006). The T-cell-depleted mononuclear cells were transduced using spin inoculation with replication-incompetent retrovirus encoding enhanced green fluorescent protein (pMSCV-IRES2-EGFP) produced in RetroPack PT67 cells (Clontech, Mountain View, CA, USA). Transduction efficiency was $65 \pm 12\%$ based on microscopic examination of green fluorescent protein (GFP)-positive cells 48 h after infection. Treated cells were washed to remove residual retrovirus, followed by a brief incubation with 5 ng mL^{-1} each of recombinant human interleukin (IL)-3 and granulocyte–macrophage colony stimulating factor (GM-CSF; Sigma, St Louis, MO, USA) before injection into the fetus.

Surgical procedure

Pregnant Landrace swine were maintained according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health. At 40–43 days gestation, swine were anaesthetised with i.m. injections of telazol (5 mg kg^{-1}), xylazine (2 mg kg^{-1}) and glycopyrolate (0.06 mg kg^{-1}) and anaesthesia was maintained with inhalational isoflurane (3–5%). A paramedian incision was made along the dorsolateral margin of the mammary glands with the pig in lateral recumbency. One horn of the uterus, containing four to eight fetal swine was then exposed. Guided by ultrasound, 50 million T-cell-depleted umbilical cord blood cells ($5 \times 10^9 \text{ cells kg}^{-1}$) were injected into the peritoneum of three to four fetal swine per litter. After surgery, the pregnant sows were given the antibiotic cefazolin (2 mg kg^{-1} , i.v.). At birth, weekly during the first month and monthly thereafter, blood samples were obtained from each member of the litters.

Polymerase chain reaction

Total DNA was isolated from peripheral blood mononuclear cells (PBMC), bone marrow or tissue (including the heart, lung, liver, thymus, spleen and kidney) of piglets using the

DNeasy Mini Kit (Qiagen, Valencia, CA, USA). Prior to using the DNeasy kit, tissues were homogenised using a hand-held electric homogeniser (Fisher Scientific, Madison, WI, USA). For polymerase chain reaction (PCR), the following were added to a 0.2-mL PCR tube: 100 ng DNA, 12.5 μ L of 2 $\times$ QuantiFast SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 1 μ L sense primer (5 mM), 1 μ L antisense primer (5 mM) and water to make up a total volume of 25 μ L. Primers specific for GFP (sense 5'-CAGCTCGCCGACCACTACC-3'; antisense 5'-GAACTCCAGCAGGACCATGTG-3'; 141 bp) were generated. The PCR products were sequenced to ensure product specificity. Real-time PCR was performed using a LightCycler (Roche, Madison, WI, USA). A standard curve was generated to correlate GFP copy number with PCR cycle using samples with known numbers of copies of GFP ranging from 10 to 10⁶ in 10-fold increments. To ensure DNA quality, all samples were amplified with the housekeeping gene GAPDH, as described previously (Ogle *et al.* 2006).

Tissue studies

Paraffin-embedded tissues were cut at 5 μ m and the paraffin removed. Slides were incubated with purified rabbit antibodies directed towards GFP (14-6774-81; eBioscience, San Diego, CA, USA) or neurofilament (clone 2F11; Dako, Carpinteria, CA, USA). Primary antibodies were detected as described previously (Ogle *et al.* 2004).

Epstein–Barr virus transformation

In cases in which the level of human microchimerism in piglets (as determined by PCR) was very low, human B lymphocytes from the peripheral blood of chimeras were enriched using Epstein–Barr virus (EBV). EBV is an oncogenic herpes virus that can transform human B lymphocytes into indefinitely proliferating lymphoblastoid cell lines (LCLs; Forte and Luftig 2009). EBV does not transform porcine primary B lymphocytes (or any other cell of the porcine haematopoietic system; Ogle *et al.* 2004) and so EBV transformation provides a means to enrich human cells of the chimeras. The PBMC of the chimeras were infected with EBV as reported previously (Davies *et al.* 2010) with minor modifications. Briefly, 2 million cells were exposed to 1 mL filtered B95-8 supernatant and 1 mL RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM L-glutamine, 100 μ g mL⁻¹ penicillin–streptomycin and 0.5 μ g mL⁻¹ cyclosporine A (Sandoz, East Hanover, NJ, USA). Cultures were incubated for 21 days, after which time the media (lacking cyclosporine A) were replaced every 3 days. EBV-infected human B-cell aggregates were apparent after 3–5 weeks.

Statistical analysis

For comparison of levels of microchimerism between injected and non-injected groups, a normal distribution was assumed and one-way ANOVA and Student's *t*-test for unpaired samples were used. Data were analysed with JMP 5.0.1 for Windows (SAS Institute, Carey, NC, USA). A 95% confidence interval ($P < 0.05$) was applied for statistical significance.

Results

Human cell detection in the blood of porcine littermates

To determine whether cells can transfer between porcine lit-termates, human cord blood cells expressing GFP were transplanted into a portion of swine litters at 40–43 days gestation. Members of the litters were studied at various ages after birth. A total of seven injected swine and eight littermates (giving a total of 15 swine) from two separate litters were studied. Blood was drawn at birth, weekly after birth for the first month and monthly thereafter. Human cells were detected by real-time PCR for GFP DNA in the blood of all

injected piglets at birth and until they were sacrificed up to 2 years later (Table 1). By the same measure, human cells were detected in seven of nine unaltered littermates (~78%) at birth and in six of nine unaltered litter-mates (~67%) until they were sacrificed up to 8 months later (Table 1). Generally, human cell engraftment in injected piglets (range 0.1–0.001% of blood mononuclear cells) was approximately 10-fold greater than human cell engraftment in unaltered littermates (range 0.01–0.001% of blood mononuclear cells), although given the high degree of variability, strict statistical differences did not exist between the groups ($P=0.1$).

To further substantiate these findings we generated cytopins of PBMC from injected and unmanipulated littermates. Because it is often difficult to distinguish true GFP expression from autofluorescence of apoptotic cells, cytopins were probed for GFP protein expression via immunohistochemistry and the percentage of cells expressing GFP, indicative of transferred cells, was determined (Fig. 1a–c). Generally speaking, the percentage of cells containing GFP DNA corresponded to the percentage of cells expressing GFP protein. In the two unmanipulated littermates with the lowest fraction of GFP DNA, human cells could not be confirmed via protein analysis for GFP. Thus, we attempted to enrich the peripheral blood cells of chimeras for human cells. This was accomplished by exposing PBMC of the piglets to EBV. EBV is only able to infect human and not pig cells (Ogle *et al.* 2004) and, once infected, human cells proliferate rapidly to form LCLs. The LCLs derived from the peripheral blood of chimeras were probed as above for GFP protein expression. All LCLs were found to contain GFP-positive human cells (Fig. 1d, e). This result supports the presence of transferred human cells in piglets and negates the possibility that GFP-expressing cells recovered from unmanipulated littermates were pig cells transduced by residual retrovirus carried over during the transplant.

Human cell detection in parenchymal tissues of porcine littermates

To determine whether human cells, transferred to unmanipulated piglets, could engraft in parenchymal tissues, we obtained heart, kidney, lung, skin, spleen, thymus, pancreas and brain tissues. DNA was isolated from one fraction of each tissue and another fraction was processed for histological analysis. Human cells were detected by real-time PCR for GFP DNA and by immunohistochemistry for GFP protein. GFP-positive cells were detected in the kidney, heart, spleen, thymus, bone marrow and brain of injected piglets. Unmanipulated piglets harboured human cells in the kidney, spleen, thymus and heart (Table 2). Detection levels for all tissues did not exceed 0.001%. Owing to the low level of microchimerism, the detection of human cells in the tissue via immunohistochemistry was difficult and, when observed, was typically restricted to one or two cells per section. However, a large grouping was detected in the cerebrum of one piglet (S24P4) and several of these cells exhibited the morphology and expressed proteins characteristic of pyramidal neurons (Fig. 2b–d).

Discussion

It is now well documented that, in addition to oxygen and nutrients, intact cells can traverse the maternofetal barrier or vascular bed of the placenta. The transferred cells are thought to be primarily non-adherent types present in the blood and bone marrow, including immune cells and stem cells. Thus, the composition of the immune system of the host can be altered, as can tissues harbouring transferred stem cells or their progeny; such a change can render the individual more or less susceptible to disease. Similarly, twins that share a placenta and/or chorion are susceptible to cell transfer from their sibling. But whether substantial cell transfer can occur between siblings with separate placentas and separate chorions has not been investigated. Here we show that cord blood-derived cells injected into fetal pigs (40 days gestation) can transfer to littermates and are found not only in the blood, but also in

several parenchymal tissues. We confirmed that GFP-positive cells were in fact transplanted human cells and not porcine cells inadvertently infected with the GFP-encoding retrovirus because LCL cultures could be derived from porcine peripheral blood and that such cultures were GFP positive.

It is especially surprising that cells of porcine littermates can be transferred given the anatomical arrangement of fetuses within the porcine uterus. The porcine uterus is a horn structure containing multiple amniotic cavities along its length. Each cavity harbours a fetus and corresponding placenta and intact chorion. The placenta of the pig is epitheliochorial, meaning that the chorion of the fetal placenta is in contact with the epithelium of the endometrium of the uterus (Leiser and Dantzer 1988). In this case, six tissue layers separate the cells of the maternal and fetal circulation (Amoroso 1952; Frandson 1981; Johansson *et al.* 2001). In contrast, the human placenta is haemochorial, meaning the fetal vessels and the fetal chorion of the placenta are invaginated into pools of maternal blood (Krohn *et al.* 1970; Leiser 1985). In this case, only three tissue layers separate the cells of the maternal and fetal circulation (Rocklin *et al.* 1979). Thus, if cells can readily move between siblings in the pig, there is a high probability of the same occurring in humans. In addition, our results support the possibility that cells might be transferred via strict tissue migration and not necessarily via the circulation.

Regardless of the mechanisms of delivery, transferred cells were found in several tissues. Cells detected in the spleen, thymus and bone marrow were not surprising because T- and B-cells had been detected in the blood of littermates. Human cells were detected in the kidneys of nearly all injected piglets and littermates by real-time PCR for GFP. Histological evidence was more difficult to detect; however, we did note human cells in renal tubules of two injected piglets. The kidneys may have a high concentration of cells if transferred cells are trapped during the course of fluid filtration. Perhaps the most surprising observation was the presence of human cells in the brain of one piglet with morphological characteristics consistent with that of neurons. The implications of these findings as they may translate to humans is intriguing; it is tempting to postulate that transfer of neurons, or more likely stem cells that give rise to neurons, may temper genetic neurodegenerative diseases of dizygotic twins. These findings also support the development of innovative (perhaps *in utero*) strategies to treat degenerative diseases.

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Fig. 1.

Detection of green fluorescent protein (GFP) in the peripheral blood of injected piglets and littermates. After birth, weekly during the first month of life and monthly thereafter, blood samples were obtained from the piglets and cytopins were made from mononuclear cells. Cytopins were probed for GFP protein (red label). (a) Negative control. Cytopsin prepared from peripheral blood mononuclear cells (PBMC) of a piglet of a different litter (i.e. not litter S24 or S25). (b) Positive control. Cytopsin prepared from a cell line constitutively expressing GFP. (c) Cytopsin prepared from PBMC of an uninjected littermate (litter =S24, piglet =P7; i.e. S24P7). The arrow indicates the positive cell. (d) Cytopsin prepared from PBMC of an injected piglet (litter =S24, piglet =P9; i.e. S24P9). To further substantiate GFP staining, PBMC were exposed to Epstein–Barr virus, which selectively infects human B-cells. Infected cells rapidly proliferate and ultimately dilute other cells of the piglet to generate a lymphoblastoid cell line (LCL). Live cultures were imaged using epifluorescence; green cells express GFP. (e, f) LCL derived from PBMC of (e) S24P7, an uninjected littermate and (f) S24P9, an injected piglet. Scale bars =10 μ m.

Fig. 2.

Enhanced green fluorescent protein (EGFP) detection in the cerebrum of an injected piglet (S24P4). The brain tissue of piglets was prepared for histological staining and probed for green fluorescent protein (GFP). (a) Negative control. The cerebrum of a piglet from a different litter. (b, c) The cerebrum of the injected piglet S24P4. GFP-positive cells are red and denoted with arrows. (d) The cerebrum of the injected piglet S24P4. GFP-positive cells are red and neurofilament-positive cells are blue. Note two double-positive cells in the centre of the image. Scale bars = 20 μm (a, b); 10 μm (c, d).

Table 1

Detection of human cells in piglet blood

Piglet ID	At birth	At the time of death	%
Non-injected group			
S24P1	+	+	0.001
S24P3	+	+	0.001
S24P5	+	+	0.001
S24P7	+	+	0.001
S25P1			NA
S25P3			NA
S25P5	+	+	0.01
S25P6	+	+	0.001
S25P7	+	+	0.01
Injected group			
S24P4	+	+	0.001
S24P6	+	+	0.1
S24P8	+	+	0.01
S24P9	+	+	0.001
S25P4	+	+	0.001
S25P9	+	+	0.1
S25P10	+	+	0.001

NA, not applicable

Table 2

Detection of human cells in piglet tissues

Piglet ID		Tissues	
Non-injected group			
S24P1	Kidney	Spleen	
S24P3	Kidney	Spleen	Heart
S24P5	Kidney	Heart	
S24P7	Kidney		
S25P1	Kidney		
S25P3	Spleen	Thymus	
S25P5	NA		
S25P6	Kidney		
S25P7	NA		
Injected group			
S24P4	Kidney	Heart	Bone marrow
S24P6	Kidney	Spleen	Bone marrow
S24P8	Kidney		
S24P9	Kidney	Thymus	
S25P4	Kidney	Spleen	Bone marrow
S25P9	NA		
S25P10	Kidney	Spleen	Bone marrow

NA, not applicable