INTESTINAL ABSORPTION OF COLOSTRAL LEUKOCYTES, PERIPHERAL BLOOD MONONUCLEAR CELLS, AND PORCINE UMBILICAL CORD MATRIX STEM CELLS BY NEONATAL PIGS

by

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Abstract

Intestinal absorption of colostral leukocytes (CL), peripheral blood mononuclear cells (PBMC), and porcine umbilical cord matrix stem cells (PUC) was analyzed in neonatal pigs. Maternal CL have previously been demonstrated in pigs, and maternal PBMC have been observed in calves to enter neonatal circulation after ingestion. PUC are primitive stem cells that are easily isolated from Wharton's jelly of the porcine umbilical cord. These cells do not have an immunogenic effect on the host upon initial transplantation. The general characteristics of PUC may allow them to serve as a delivery system to the neonate.

Cellular migration through the duodenum, jejunum, and ileum was assessed using confocal microscopy. In vitro experiments utilized an organ explant culture system to determine the trafficking of labeled cells. Small-intestine tissue was collected from stillborn and sacrificed neonates. All three cell types (CL, PBMC, and PUC) were detected below the luminal surface, after 72 h of culture with media, and regardless of whether explants were from stillborns or live-born pigs.

In vivo trafficking was assessed using neonatal pigs that were fed PBMC isolated from their mother or PUC from an unrelated pig. The effect of prior exposure to 25% acellular colostrum (AC) in medium was evaluated for both cell types. Piglets were euthanized 8 h or 24 h post feeding and sections of the small intestine collected. Both PBMC and PUC were found in all intestinal samples. Exposure to AC had no detected effect on the ability of either cell type to attach and migrate into the tissue. Labeled PUC were detected on the surface of the epithelium and in the lamina propria 8 h post treatment. PBMC were observed on the surface of the

epithelium, in the lamina propria, and superficial submucosa 8 h following ingestion. In neonates sacrificed 24 h post treatment, both PUC and PBMC were observed on the surface of the epithelium, in the lamina propria, superficial submucosa, and deep submucosa of the small intestine. PUC and PBMC were noted at the apex, intermediate between the apex and the base, or at the base of the villus.

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CHAPTER 1 - GENERAL REVIEW OF THE LITERATURE

Introduction

Colostrum plays an integral role in the biological function of many mammalian species. It has an impact on neonatal growth, health, and immune system development. This yellow, viscous, mammary secretion contains maternal antibody that provides the neonate with passive immunity to infectious diseases. In order for passive immunity to be acquired by the neonate, intestinal absorption of maternal leukocytes, cytokines, and antibodies must occur. These immunologically important elements originate from the mucosal system of the mother and are transferred through colostrum into the digestive tract of the newborn animal (Tuboly et al., 1988).

Pig umbilical cord matrix stem (UCMS) cells are a population of cells that have the ability to self renew or differentiate into a diverse range of cell types (Carlin et al., 2006). There is growing interest in UCMS cells as they can be easily isolated from the porcine umbilical cord and from several other species and are showing therapeutic potential in a variety of situations. This review will describe current knowledge about intestinal absorption of colostrum by the neonatal pig and the properties and therapeutic uses of mesenchymal stem cells.

Mammary Development

The immune functions of mammary secretions vary between species. These differences are relevant to differences in placental structure that affect placental transfer of immunoglobulins between the mother and the fetus. The six layers of the epitheliochorial placenta of swine prevents prenatal passage of maternal immunoglobulins to the fetus (Wagstrom et al., 2000).

During pregnancy, the trophoblast of pigs and other ungulates does not invade the maternal tissues and even small peptides in maternal blood are not transferred to the fetus. This leaves mammary secretions as the sole source of maternal antibody for the newborn. Newborns are dependent on colostrum for nutrients, luminal growth factors, and passive immunization through immunoglobulins. Colostrum is a yellow fluid secreted by the mammary gland just before and two to three days after parturition. Uptake of colostrum just after birth is also essential to stimulate normal organ function and maturation. Colostrum contains large amounts of growth factors that may be important for organ growth and development (Jensen et al., 2001). Extensive research has demonstrated the importance of maternal colostral immunoglobulins in colostrum as providers of passive immunity to infectious disease. Colostral immunity is needed for high rates of neonatal survival.

Mammary gland function and development is an important physiological process in the sow. The amount of mammary tissue present is a likely predictor of milking capacity. The external structure of the sow mammary tissue is comprised of approximately seven pairs of glands. These are arranged in two parallel rows, one on each side of the ventral median line. The mammary glands extend from the pectoral to the inguinal region and are suspended by medial and lateral fibrous laminae derived from the abdominal fascia (Sack, 1982). After the onset of puberty, the mammary gland begins allometric growth. This is most likely stimulated by estrogen production from the ovaries. Repeated estrous cycles stimulate duct construction. The ducts begin to branch and the diameter increases due to an increase in estrogen. The terminal sections of each branch begin to form due to the influence of progesterone. During puberty, prolactin and growth hormone increase duct development and cyclic exposure of these hormones continues with repeated estrous cycles. Lobulo-alveolar structures develop during the prenatal

period, about 45 days post conception and constitute about 90% of the cellular mass (Senger, 2005). Final mammary development occurs during the last trimester of pregnancy. In the latter part of gestation, mammary gland growth rate is increased by 3-fold. The composition of this tissue changes from one of high lipid content to one of high protein content. With these changes, mammary gland histology changes from high adipose content to increased development of ductal and lobular structures. Changes in mammary tissue are indicative of mammary gland development. The sow has multiple mammary glands and these glands have different rates of maturation (Ji et al., 2006). Farmer et al., (2001) describe the glandular development as more progressive in the middle region and slower to develop in the anterior or posterior glands during the period after 90 days of gestation. A prolactin spike and an increase in the concentration of serum cortisol occur prior to farrowing, along with an abrupt decline in progesterone concentrations in serum. These hormone changes induce milk production by the gland. The mammary gland then has the ability to secrete milk at the onset of parturition to ensure that the neonate has an adequate supply of nutrients (Senger, 2005).

Mammary secretions

Ductal growth occurs during the first half of gestation while most growth is lobuloalveolar during the second half of gestation. Mammary growth promotes colostrogenesis and then initiates lactation. There is a dynamic shift in mammary tissue function beginning from about one week before farrowing and continuing several days after farrowing (Schnulle and Hurley, 2002).

Lactation describes the secretion of milk by the mammary glands. Lactation ensures that the neonatal mammal does not have to obtain food on its own. The sow will consume all of the necessary nutrients and will transform these into a highly nutritious secretion called milk. In the

sow, there is an increase in lactose concentration in the mammary secretions just prior to parturition. At this time, milk synthesis increases and then peaks shortly after parturition (Senger, 2005).

The newborn piglet must acquire immunoglobulins from ingested colostrum and milk for humoral immune protection until its own immune system has matured to respond to foreign antigens. The neonatal piglet is agammaglobulinemic at birth. Maternal antibodies are first transported into colostrum by Fc-dependent receptors on mammary epithelial cells. Neonates acquire the antibody through consumption of colostrum and will absorb the immune components in the gastrointestinal tract. Sow colostrum differs from sow milk in cell population and composition. This difference is explained by the different functions of colostrum and milk. Milk provides local antibody protection of the intestinal mucosa while colostrum is a source of circulating antibody to the neonate. The total cell count in colostrum is higher than in milk. The lymphocyte population is also higher in colostrum (Meriläinen et al., 1979). The lymphocyte percentage decreases with an increase in days of lactation. Colostrum contains about 14.9-19.8% lymphocytes of the total population of cells, on day 0 of lactation. Percentage of lymphocytes decreases to about 6% by day 14 of lactation. The immunoglobulins in colostrum differ from milk in that IgG is the primary immunoglobulin in colostrum while IgA is preponderate in milk. Colostrum also has buffering properties and protects antibodies and immunoglobulins from denaturization (Wagstrom et al., 2000).

Immunity of the small intestine

Gut associated immunity develops in order for neonates to have their own immune system and the ability to respond appropriately to foreign antigens. The intestine is the largest immune organ in the body (Furness et al., 1999). It must balance the processes involved in the

breakdown of food to prepare them for absorption while preventing excess antigen from passing through the mucosa. The intestine provides a barrier that prevents microorganisms from entering the systemic circulation. If antigens do pass through this mucosal barrier, normal responses must ensure that systemic tolerance or adequate immune responses ensue. In the intestine, the first detectable villi appear at approximately the 10^{th} week of gestation. Columnar epithelium and microvilli form just after the first discernable villi around the 10^{th} to 11^{th} week of gestation. Beginning in the last trimester, the majority of the gut associated lymphoid tissue (GALT) is found in Peyer's patches. These are lymphoid cells of the lamina propria and intraepithelial lymphoid cells (Insoft et al., 1996).

Peyer's patches appear in the distal small intestine primarily in the ileum. Specialized epithelial cells overlay the developing Peyer's patches. These have evolved to allow macromolecules to transverse the gut in a controlled manner. The microfold (M) cells have microvilli and mucus covering their surfaces and allow easier access and penetration of gut macromolecules. M cells have deep invaginations of their basal membrane that allows lymphoid cells to intrude and position themselves close to the intestinal lumen (Insoft et al., 1996). This mechanism is the primary route for non-receptor transport of macromolecules in the intestine. Peyer's patches may also serve as a site of antigen dependent T-cell proliferation (Iwasaki and Kelsall, 1999).

Another component of the small intestine is the lymphoid cells of the lamina propria. This site consists of a mixture of B and T lymphocytes that have migrated to, or differentiated within the lamina propria. Prenatally there is no antigen presentation to the gut. Antigen exposure occurs within one to two weeks of birth and IgA-producing and IgM-producing plasma cells become evident in the lamina propria (Insoft et al., 1996).

The final major component of the neonatal small intestine is intraepithelial lymphoid cells (IEL). These cells appear between intestinal epithelial cells and are situated almost entirely in the basal portion of the epithelial layer (Insoft et al., 1996). The characteristics of these cells are consistent with the hypothesis that at least some of them are T cells, and are capable of both cytotoxic and helper functions (Cerf-Bensussan et al., 1985). These intraepithelial cells lack natural killer cell activity. Porcine IEL cells are similar to those found in mice, rats, and humans but should be characterized further (Insoft et al., 1996).

Passive immunity

Adequate and timely consumption of immunoglobulins by the piglet helps control the effects of exposure to pathogens early in life. In colostrum the total solids and protein are high while fat and lactose are relatively low. During the first 6 h postpartum IgG accounts for most of the total protein in colostrum (Klobasa et al., 1987). IgG comprises about 80% of the immunoglobulins in colostrum and IgA about 14% of colostral immunoglobulins. Ig-containing cells are infrequent or absent in the gut tissue of newborn piglets although surface IgM and IgA are present in Peyer's Patches. Ingestion of colostral immunoglobulins by epithelial cells has been documented immediately after feeding and ceases around 12 h after administration (Butler and Brown, 1994). During the first 24 to 36 h post farrowing the intestinal epithelium of the neonate is highly vacuolated, immature, and has the ability to absorb macromolecules. Absorption of immunoglobulins occurs as the macromolecules move across the intestinal epithelium. The rate of absorption decreases significantly by 3 h and gut closure is related to the amount of colostrum ingested. The immunoglobulins absorbed by the neonate first enter the lymphatic system and then move into neonatal blood circulation (Wagstrom et al., 2000).

In addition to immunoglobulins, the mammary secretions from all species also contain cells. Swine mammary secretions contain T and B lymphocytes, neutrophils, macrophages, and epithelial cells. The cell types present, as well as the quantity of cells, vary between individuals. Ten to 25% of the cells that comprise swine colostrum are lymphocytes, the majority being T cells. Memory T cells dominate among the T cell population. B lymphocytes comprise about 30% of the lymphocyte population and are selectively transported across the mammary epithelium (Wagstrom et al., 2000). Neutrophils and macrophages are phagocytic cells found in the colostrum of swine. The functional ability of these cells remains in question and it has been suggested they have lower functional capacity (Lee et al., 1983). When compared to neutrophils found in circulating blood, mammary neutrophils display decreased phagocytosis, motility, respiratory burst, and polymorphonuclear-mediated killing (Eglinton et al., 1994). Capabilities of macrophages in mammary secretions have only been documented in vitro. The phagocytic activity of macrophages in sow milk may be inferior to that of autologous alveolar macrophages (Wagstrom et al., 2000). Lee et al., (1983) observed that both neutrophils and macrophages in sow mammary secretions exhibit the capacity to engulf lipid and cellular debris. This may lead to decreased functionality. Sow colostrum also contains epithelial cells. About 20% of the cellular component of colostrum is small epithelial cells. It is believed these small cells function as antigen presenting cells and may produce cytokines (Wagstrom et al., 2000). About 60% of the cells in colostrum are large epithelial cells. These cells are morphologically similar to alveolar epithelial cells but the protective function they provide has not been discerned (Le Jan, 1996).

Colostral Immunoglobulins

Immunoglobulins are proteins used by the immune system to identify and remove foreign entities from the body. Immunoglobulins have several isotypes or classes. Each isotype is distinguished by unique amino acid sequences in the heavy-chain constant region. Variations within this sequence result in structural and functional differences among different isotypes (Kuby, 1994).

Colostral immunoglobulins are mainly IgG and IgA. Both isotypes have four heavy chain domains and a hinge region. IgG is a monomer consisting of two heavy γ chains and two λ light chains. Secretory IgA consists of a dimer, a J-chain polypeptide, and a secretory component (Kuby, 1994). Immunoglobulins are absorbed in the neonate by endocytosis and transcellular movement of the macromolecules across the intestinal epithelium (Burton and Smith, 1977). Immunoglobulins are absorbed through the neonatal intestinal epithelium for only a few hours post parturition in most species. Absorbed immunoglobulins enter the intestinal lymphatic system and then enter the neonatal blood circulation. Absorbed IgA is secreted onto mucosal surfaces of the neonate while IgG remains in circulation (Wagstrom et al., 2000).

Mucosal immunity

Control of mammary secretions

The components of colostrum provide the newborn with passive immunity to disease and the amount of colostrum available can be affected by suckling intensity. The suckling demand imposed by the number and size of piglets in a litter can influence milk yield. The typical suckling interval varies from 30 to 70 minutes for individual sows during the first week of lactation. A sow suckling one litter of six piglets has lower suckling demands than when cross-suckling groups of six piglets at increased frequency. The cross-suckled sows have higher

suckling demands and this causes an increase in milk yield (Auldist et al., 2000). According to Marshall et al. (2006), a decrease in suckling frequency caused by increasing the intersuckling interval results in increased milk intake per suckling but decreased daily milk production by the sow. This is also supported by a study in which fostered piglets that were either age-matched with the day of lactation of the gilt or were seven days older than the day of lactation. The gilts suckling older piglets had a rapid increase in milk yield in early lactation and there were greater weight gains by the older suckling piglets. Several sow-litter interactions affect milk yield ultimately increasing or stimulating piglet growth. Interactions such as suckling frequency, age and size of piglets, and litter size affect milk production by the sow.

Once the constituents of the mammary secretions reach the gland cistern they are transferred through the colostrum into the digestive tract of the newborn. In newborn piglets it has been suggested that colostral cells are absorbed from the digestive tract and reach the mesenteric lymph nodes within 8 h of ingestion (Tuboly et al., 1988). In piglets that received colostral cells from another sow, absorption of the cells occurred and the labeled cells were found in the epithelium of duodenal and jejunal samples and were absent from lymph nodes. In contrast, lymphoid cells isolated from the blood of an animal's mother were not absorbed. These data indicate the process is selective (Tuboly et al., 1988). Intestinal absorption of FITC-labeled colostral leukocytes occurs in colostrum-deprived piglets and these cells begin to appear in the blood about 2 h post feeding and peak at 5 to 7 h. Absorption of FITC-labeled colostral leukocytes is a specialized function. When the same procedure was repeated with FITC-peripheral blood mononuclear leukocytes, the cells were not found in the epithelial lining of the duodenal, jejunal, or ileal explant cultures (Williams, 1993). Williams (1993), provides in vivo and in vitro data that indicate the absorption of colostral leukocytes takes place through duodenal

and jejunal intercellular spaces of the epithelium. Colostral leukocytes must migrate into submucosal spaces for absorption to occur. The data implies that the lymphocytes, as antigen presenting cells, can transfer positive and negative signals to the lymphoid system of the neonate. The presence of maternal colostral lymphocytes may regulate T and B cell activities and this may be a feedback mechanism operating in many enzyme systems (Williams, 1993).

A similar mechanism occurs in neonatal calves (Reber et al., 2006; Stott et al., 1979). Maternal cells can traffic from the gut of the neonatal calf into circulation. It has been demonstrated that exposure to 25% acellular colostrum induces changes in homing and trafficking markers on leukocytes in culture (Reber et al., 2006). Colostrum was collected from Holstein cows, diluted to reduce viscosity, and pooled. The colostrum was washed, centrifuged, and filtered through a series of sterile syringe filters. The defatted acellular colostrum (AC) was applied in vitro to maternal peripheral blood mononuclear cells (PBMC). The PBMC were fluorescently labeled and fed to neonatal calves within 6 h of parturition. The fluorescent cells were monitored by flow cytometric analysis of leukocytes collected from circulating blood samples in the calf. Blood was collected at several intervals for 48 h post feeding. The cells treated with AC had significant changes in the expression of CD11a, CD11c, CD43, and CD62L when compared to PBMC incubated in media. There was an increase in the number of cells expressing CD11a, CD11c, and CD43 while there was a decrease in the number of cells expressing CD62L. Detection of fluorescent cells began to increase at 8 h and a peak in fluorescence was found in the jugular samples at 24 h post feeding. Collectively these data imply culture in AC enhanced the trafficking of maternal leukocytes into the circulation of the neonatal calves. Important cellular receptors for transendothelial migration include CD11a and CD43. When cells are exposed to 25% acellular colostrum there is an increase in the number of

cells expressing CD43 and an increase in the density of that protein on the cell surface. This work infers colostrum exposure affects the absorption of maternal cells. Additionally, peripheral blood mononuclear cells (PBMC) were only capable of trafficking into neonatal peripheral blood if they were cultured in 25% AC. Cells that were not exposed to acellular colostrum did not traffic into neonatal circulation (Reber et al., 2006). Liebler et al., (2002), note that intestinal absorption of FITC labeled material and cells from colostrum could be found in calves in the intestinal lumen 1 to 2 h post treatment. FITC-labeled cells were observed exclusively in the Peyer's patches. These secondary lymphoid organs are usually found in the lower portions of the small intestine, the jejunum and the ileum. Peyer's patches establish an immune surveillance of the intestinal lumen and facilitate the generation of an immune response in the mucosa (Gullberg and Söderholm, 2006). The translocation of cells through Peyer's patches is the most effective route to pass the intestinal barrier even though paracellular uptake of material may occur along the entire intestinal tract through breaks in the epithelial barrier. The researchers noted an increased uptake of labeled material and cells in the Peyer's patches of the ileum as compared to the jejunum. This is most likely due to differences in the cellular composition of the follicle-associated epithelium and the more exposed domes in the jejunum (Liebler et al., 2002).

FITC-labeled leukocytes have also been used for tracing the circulatory pathways in crossbred sheep. Maternal leukocytes are capable of penetrating the epithelial lining of the gastrointestinal tract of the neonatal lamb. This is not however, a specialized function limited to colostral leukocytes in the sheep according to Sheldrake and Husband (1985), as peripheral blood leukocytes are also capable of crossing the intestine . Leukocytes labeled with FITC appeared in significant numbers 6 to 9 h post administration. Labeled colostral leukocytes from unrelated ewes are able to penetrate the intestinal wall, suggesting this process is not restricted

by maternal-neonatal recognition factors (Schnorr and Pearson, 1984). Sheldrake and Husband (1985), note that intact lymphocytes of maternal origin are able to enter the neonatal intestine in lambs, and consequently appear in the lymphoid tissue.

Umbilical cord matrix stem cells

The term stem cell is used to describe populations of cells that retain the ability to self renew through mitotic cell division and can also differentiate into a diverse range of cell types. Pluripotent stem cells have the ability to indefinitely self renew and the ability to differentiate into any specialized cell in the body. Multipotent stem cells also have the ability to self renew but these cells can only differentiate into a limited number of specialized cell types.

The umbilical cord is initially apparent as the umbilical ring is formed. The human embryo undergoes flexion, around day 17, and is discernible by day 28 (Karahuseyinoglu, 2006). The human umbilical cord is embryologically derived around day 26 of gestation (Sarugaser, 2005). The structure continues to grow forming a 30 to 50 cm long helical organ at birth. The human and pig umbilical cords contain two arteries and one vein (Kadner, 2004; Sarugaser, 2005; Wang, 2004). These components are surrounded by a connective tissue termed Wharton's jelly as first described by Thomas Wharton in 1656 (Romanov et al., 2003). Wharton's jelly is composed of myofibroblasts-like stromal cells, collagen fibers, and proteoglycans (Mitchell et al., 2003). This gelatinous connective tissue also contains mesenchymal cells that possess stem cell properties. Umbilical cord mesenchymal stem (UCMS) cells were first described for pigs (Mitchell et al., 2003). They have since been identified in several species. Some investigators have identified cells are in the subendothelial layer of the human umbilical cord vein with similar characteristics (Romanov et al., 2003).

Mesenchymal stem cells (MSCs) were initially isolated from bone marrow (Horwitz et al., 2002) and have since been studied by other investigators. These cells have the potential to differentiate into many cell types including; muscle cells, adipocytes, osteocytes, and chondrocytes in controlled culture environments (Pittenger et al., 1999). MSCs also express receptors and integrin markers. Using flow-cytometric analysis, Wang et al., (2004) found cells isolated from the human umbilical cord (HUC) to express matrix receptors CD44 and CD105 as well as integrin markers CD29 and CD51. These cells also express MSC markers SH2 and SH3. Recent advances suggest umbilical cord derived stem cells can be differentiated into osteogenic and adipogenic cells in vitro. These cells were found positive for markers CD13, CD29, CD90, CD105 and CD166 (Wang et al., 2004). This phenotype is similar to the MSCs isolated from bone marrow. HUC cells have also been induced to differentiate into a population containing dopamine neurons. This may lead to potential therapy for Parkinson's disease. Collectively this data suggests umbilical cord derived stem cells are a group of undifferentiated stem cells but are unlike hematopoietic stem cells (Wu et al., 2007).

Many cell markers are used to characterize MSC but the intrinsic ability of these cells to adhere to uncoated plastic substrates is the most consistent property regardless of their origin (Minguell et al., 2001). HUC cells possess the capacity to proliferate and differentiate in vitro. This is complemented by the cells ability to freeze and thaw, yielding a significant number of cells. Characterization of HUC exposes two distinct cell populations, type I and type II. Each type expresses differential vimentin and cytokeratin filaments. In culture, HUC retain their original phenotype without spontaneous differentiation and also contain multipotent stem cell characteristics (Karahuseyinoglu et al., 2006).

When comparing HUC cells to MSC derived from bone marrow, many characteristics are similar. Lu-Lu et al., (2006) isolated cells from human full term umbilical cords and characterized them. Fibroblastic morphology, immunophenotypic markers, cell cycle status, adiopogenic and osteogenic differentiation capacity, cytokine spectrum, as well as hematopoiesis-supportive function are all shared characteristics of HUC and bone marrow derived MSC. A difference between the two populations is that a faster population doubling time was observed for HUC. Bone marrow derived cells have a slower population doubling time which increases after passage six. These data suggests HUC have a higher proliferation capacity. HUC also have a lower expression of CD106 than bone marrow derived cells. The stem cell marker CD106 is an important mediator of adhesive and co-stimulatory interactions that occur in immune responses. Low expression of CD106 has also been identified in adipocyte-derived MSC. With the advantages of UCMS cells such as, painless collection procedures, accessibility, and low risk of viral contamination, UCMS cells may provide a promising alternative stem cell source. In addition to these advantages, Cho et al., (2008) describe the immunogenic characteristics of porcine umbilical cord tissue. These researchers found that a single injection of pig UCMS (PUC) cells does not induce a detectable immune response. These data offer promising results for this alternative stem cell source.

UCMS cells as therapeutic cells

Mesenchymal stem cells are being studied to further advance the field of regenerative medicine. Due to the difficulty of harvesting bone marrow derived MSC; the UCMS cells are being studied. Friedmann et al., (2007) reported that HUC are amenable to gene transfection and augmented engraftment using standard transfection protocols. The umbilical cord can be minced, frozen, and later thawed and expanded in culture resulting in large numbers of

mesenchymal stem cells. Through electroporation HUC can then be readily transfected with a plasmid-based vector. These characteristics make umbilical cord mesenchymal stem cells (UCMs) contenders for many cell-based therapies.

HUC (Weiss et al., 2006) and PUC (Medicetty et al., 2005) cells have been tested for therapeutic use in a Parkinson's disease rodent model. Parkinson's disease is a neurodegenerative disorder characterized by a progressive loss of dopaminergic function. Most effective treatments gradually diminish due to the progressive degeneration of dopaminergic terminals. The ultimate goal would be a treatment that would restore the damaged dopaminergic system, possibly via the transplantation of cells that synthesize catecholamines. HUC cells have the ability to differentiate into neuron-like cells (Mitchell et al., 2003). They also have surface markers suggestive of a primitive mesenchymal stem cell population. When flow cytometry is used to characterize this cell population, about 85% of the HUC stain for CD44, a hyaluronic acid receptor. This marker is also found on other stem cell populations. Researchers also suggest umbilical cord matrix cells may be a more primitive population of mesenchymal stem cells because they lack some of the surface markers found on bone marrow derived mesenchymal stem cells (Weiss et al., 2006). HUC have also been transformed into dopaminergic neurons in vitro (Mitchell et al., 2003). These neurons were transplanted into the striatum, the area of the brain involved in motor and cognitive planning, of Parkinsonian rats. The results of this study indicate that lesion-induced rotational behavior in rats can be partially corrected. This occurs through transplantation of HUC and the transplanted cell viability is at least four months (Fu et al., 2006).

In addition to therapeutic use in a Parkinson's disease model, human umbilical cord stem cells have been used to study myocardial infarction. Myocardial infarction, commonly referred

to as a heart attack, is a medical condition that occurs when blood supply to at least part of the heart is interrupted. Wu et al., (2007) provide evidence that transplanted HUC can improve cardiac function in a rat myocardial infarction model. During this study male Sprague-Dawley rats underwent ligation of the left coronary artery to produce myocardial infarction. Two weeks post infarction; the surviving rats were randomized into two groups. The test group received HUC while the control group received an equal volume of phosphate buffered saline (PBS). Cardiac function was assessed on the mice by echocardiography prior to treatment and 2 weeks and 4 weeks after cell transplantation. The results of the study suggest that delivery of HUC into the damaged cardiac tissue improves left ventricular function. It appears that transplanted HUC can improve the status of damaged tissue through paracrine mechanisms; likely through vascular endothelial growth factor (VEGF) secretion. The transplanted stem cells secrete VEGF and this contributes to therapeutic neovascularization. HUC transplantation can preserve myocardial function by promoting the survival of endangered cardiac cells by inhibition of apoptosis (Wu et al., 2007).

Umbilical cord derived cells have been tested for efficiency in preserving photoreceptor integrity and visual function after injection into the subretinal space of the rat. Photoreceptor degeneration is a major neurodegenerative disease and the leading cause of blindness in the developed world (Friedman et al., 2004). For this condition, there are few available treatments. Lund et al., (2007), show that HUC are effective in sustaining visual function for up to several months after administration. In addition to this, a larger area around the injection site was restored in the rats injected with HUC than those injected with human placental-derived cells or human bone marrow mesenchymal stem cells. The authors also note that there were no results associated with the injection. The cells were able to preserve visual function without being

genetically altered, engineered to over express any growth factors, or eliciting an immune response.

HUC stem cells are being investigated for therapeutic potential of ischemic diseases. These diseases are characterized by restriction in blood supply, often resulting in damage or dysfunction of tissue. Wu et al., (2007) induced unilateral hind limb ischemia by femoral artery ligation, in nude mice. Cultured HUC or phosphate-buffered saline (PBS) were injected into the left adductor muscle adjacent to the ligation site, 24 h post induction of ischemia. Quantification of blood flow compared the left hind limb to the right, nonischemic hind limb. If cells are differentiated in culture, the differentiated cells can be transplanted into ischemic hind limbs. The transplant results in marked improvement, increase in blood flow, and ischemic damage is ameliorated after the injection of cells when, compared to PBS treated mice.

Equine derived umbilical cord matrix cells (EUCM) have also been isolated and described. These cells were found to proliferate extensively in vitro and able to maintain morphological characteristics over many passages. EUCM cells are primitive cells that express Oct-4, SSEA-3, SSEA-4, and TRA-1-60; all important markers of primitive ES-like cells. EUCM cells also exhibit properties similar to MSC cells. The markers CD54, CD90, CD105, and CD146 are also expressed in these cells. These are common markers used to define MSC populations. All of the aforementioned characteristics suggest EUCM cells may play an important role for stem cell-based therapies in the horse or EUCM cells may have pluri- or multipotent capabilities (Hoynowski et al., 2007).

Umbilical cord stem cells or, cells derived from umbilical cord mesenchyme, were first collected from the pig. These porcine umbilical cord matrix cells (PUC) can be collected in a non-invasive manner, may be cryogenically stored, thawed, and expanded in vitro (Carlin et al.,

2006). The immunogenicity of porcine derived umbilical cord stem cells has recently been explored. Characterization of the isolated porcine cells confirms that these cells are phenotypically similar to human umbilical cord stem cells. Cho et al., (2008) suggest that a single injection of unactivated PUC across a full MHC barrier does not elicit a detectable adaptive immune response. Animals injected once had no detectable alloantibody production. Reduced immunogenicity may be observed due to the lack of MHCII expression and a low level of MHCI expression. Under altered circumstances, PUC can elicit an immune response. If the cells have been previously exposed to IFN-γ stimulation, the injection of cells occurs near an inflammatory region, or the injection is repeated multiple times, alloantibody production is detected within one week.

Colostrum in cell culture

Cells in culture are most often grown in a nutrient and growth factor supplemented medium. Serum is a common supplement and is derived from blood. After clotting, serum contains a broad spectrum of macromolecules, proteins, attachment and spreading factors, hormones, and growth factors making it beneficial to cells in culture. The most widely used animal supplement is fetal bovine serum. Human and bovine milk also contain growth promoting factors (Belford et al., 1995). Milk can maintain viable cells and has been considered as a replacement for serum in cell cultures.

Klagsbrun (1980) compared the use of milk and colostrum in cell culture, as a supplement in the absence of serum. Bovine milk was obtained from Holstein and Jersey cows and immediately frozen. Day-one colostrum was collected within 8 h of parturition and day-eight milk was obtained 176 h post parturition. The milk and colostrum were sterilized through sequential centrifugation and filtrations. Both were used as a supplement for various epithelial

cells or fibroblast cells in culture. The data reveals that colostrum can replace serum but acts selectively in supporting the growth of cells. The epithelial cells in this study were able to proliferate but human, rat, and bovine early passage fibroblasts did not. Cell-type selectivity may be due to toxic effects but the authors consider it more likely that colostrum contains the growth factors necessary for epithelial growth and lacks those necessary for fibroblast growth. Although colostrum supports epithelial cell growth, milk renders these cells inactive. Milk obtained one week post parturition is completely inactive. Only if supplemented with transferrin will the milk support cell growth and proliferation. Milk may be lacking the nutrients and/or growth factors present in colostrum and this may explain ineffectiveness for replacing serum in cell culture.

Although bovine colostrum does not support long term culture of fibroblasts, it has been shown to enhance the migration of human fibroblasts in type I collagen gels. A fibroblast collagen gel culture is a model used to explore the interaction that occurs between the cells and the extracellular matrix. Collagen gel contraction occurs when the fibroblasts migrate and form a tissue like structure. This phenomenon is similar to the reorganization of the collagen matrix accompanied by wound healing of skin. The contraction can be increased by growth factors (Martin, 1997). These same growth factors can be found in bovine colostrum. When human fibroblasts are suspended in serum-free Dulbecco's modified Eagle's medium and placed on a polymerized gel, collagen gel contraction activity can be monitored. The results of this study indicate that cell migration promoting agents exist in bovine colostrum. As collagen gel contraction is used as an in vitro model of wound healing, specific growth factors in bovine colostrum could be used to promote faster healing (Takayama et al., 2001).

Goat mammary secretions also contain growth factors. Colostrum from goats has been shown to contain material that causes a marked inhibition of the binding of ^{125}I-labelled epidermal growth factor (EGF) to receptors on mouse fibroblasts. In a study by Brown and Blakely (1983), the ability of goat mammary secretions to inhibit EGF binding was found to be closely correlated with their ability to stimulate DNA synthesis in mouse fibroblast cell cultures. More specifically, colostrum from goats and sheep stimulates the proliferation of mouse fibroblasts in culture but inhibits EGF binding to receptors. EGF is a polypeptide growth factor that has been shown to influence the growth and development of mammary tissue in vitro and may also play an important role in neonatal growth and development. EGF is known to be present in mouse and human milk. It should be noted, the role and origin of cell growth-promoting agents in colostrum remains undetermined. It could be that growth factors in mammary secretions are involved in controlling the early growth and maturation of mammary epithelial cells and of the neonatal intestine.

Brown and Blakely (1984) partially purified EGF from goat mammary secretions using acid decaseination, ion-exchange chromatography, and gel filtration. It has been named, colostric basic growth factor (CBGF) and is a basic polypeptide that inhibits the cellular binding of ^{125}I-EGF. Although CBGF differs from mouse and human EGF, it is closely related to the growth factor isolated from human platelets, human platelet-derived growth factor (PDGF). Human PDGF inhibits ^{125}I-EGF binding to human fibroblast cells by a mechanism similar to that found for the inhibition of ^{125}I-EGF binding by colostrum. Incubation of cells with EGF causes a reduction in cell EGF surface receptors. This process includes an indirect, temperature-sensitive mechanism. This indicates that the colostral growth factor does not bind directly to EFG receptors but inhibits ^{125}I-EGF binding. PDGF has been shown active only on connective tissue-

derived cells and not epithelial cells. The strong biochemical similarities between CBGF and PDGF suggest this factor will fail to stimulate epithelial cells. As a result of this, any effect of the colostric factor on the growth of intestinal epithelium would have to occur indirectly. It may be possible that other growth factors present in the colostrum cause this effect and should be considered.

Conclusion

In terms of the capacity of pig umbilical cord matrix stem cells to enter the circulation of neonatal pigs, no research has been conducted. Research with colostral leukocytes and peripheral blood mononuclear cells implies that exposure to the colostral environment may induce changes in the cells that facilitate trafficking into neonatal circulation. The umbilical cord tissue, Wharton's jelly, provides a source of stem cells. PUC cells are suggested to be MHC class I dull and negative for MHC class II. This data indicates that upon initial presentation to the host, a detectable adaptive immune response does not ensue (Cho et al., 2008). Pig umbilical cord matrix stem cells could offer a possible delivery system to neonatal circulation and exposure to a colostral environment may modulate their ability to traffic into the gut. Studies in this thesis are a first evaluation of the ability of PUC cells to traffic to or through the neonatal pig's intestinal tract.

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CHAPTER 2 - INTESTINAL ABSORPTION OF COLOSTRAL LEUKOCYTES, PERIPHERAL BLOOD MONONUCLEAR CELLS, AND PORCINE UMBILICAL CORD MATRIX STEM CELLS BY NEONATAL PIGS

Introduction

Ingestion of colostrum provides a source of maternal antibodies to the alimentary tract and circulation of neonatal pigs (Banks, 1982; Porter, 1969). This provides an important role in protecting the neonate against pathogenic infection. Neonatal immunity may occur passively through the transfer of maternal immunoglobulins offering protection or protection may occur as a result of intestinal absorption of milk soluble factors or maternal cells (Sheldrake and Husband, 1985). The relationship between the colostral environment and the function of leukocytes has not been clearly defined. Previous studies of maternal peripheral blood mononuclear cells (PBMC) and colostral leukocytes (CL) have demonstrated that the cells have the ability enter neonatal circulation after the ingestion of colostrum.

Williams et al., (1985) developed a method for culturing explants of neonatal pig intestine and observed migration of CL into submucosal sites in the explants. The CL were observed to localize as aggregates in the submucosa and to spread throughout the submucosa as independent cells (Williams et al., 1985). In vitro cultivation of intestinal explants with labeled FITC (fluorescein isothiocyanate) -CL provided data to imply that the cells could be taken up by the small intestinal explants. According to Williams et al., (1993) by 24 and 28 h, cells were

observed to migrate into submucosal spaces. In contrast, maternal peripheral blood mononuclear cells (PBMC) were not taken up by the explants. Others have demonstrated that CL enter the bloodstream and mesenteric lymph nodes when ingested by rats, lambs (Sheldrake et al., 1985), and calves (Liebler-Tenorio et al., 2002). The mechanisms for CL trafficking in the neonatal gut are not understood nor are the cell characteristics that allow migrations of the CL known. Here we report results of in vitro and in vivo experiments that evaluate the trafficking of PBMC, pig umbilical cord matrix stem cells (PUC), and CL. We also tested the potential for acellular porcine colostrums (AC) to modulate the trafficking of PUC and PBMC.

Materials and Methods

General Methods

Animals

Pigs used for these experiments were provided by the Swine Teaching and Research Center, Kansas State University. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Preparation of acellular colostrum (AC)

Colostrum was collected from sows within 24 h of parturition. Sows were administered oxytocin in a marginal ear vein (20 USP units, RXVeterinary Products, Westlake, Texas) and colostrum collected by hand milking into sterile 50 ml polypropylene centrifuge tubes. Colostrum was diluted with an equal volume of sterile Roswell Park Memorial Institute medium (RPMI [GIBCO, Grand Island, New York]) to reduce viscosity and centrifuged (1000 x g) for 20 min to remove colostral cells. The lipid layer was discarded and the supernatant was centrifuged (5000 x g) for 60 min. The lipid was discarded again and the remaining supernatant was centrifuged (18,500 x g) for 60 min. All centrifugations were at 7° C. The supernatant was filtered by vacuum through a 0.22 μM cellulose acetate filter (Millipore, Bedford, Massachusetts). This was followed by a series of sterile syringe filtrations through a 0.22 μM (Millipore) cellulose acetate filter in a sterile air hood. The resulting viscous yellow liquid was 50% AC and was stored at -20° C until use.

Isolation of CL

Colostrum was collected from sows at parturition by hand milking into sterile, 50ml conical polypropylene tubes. Casein and free fat were removed by centrifugation (1100 x g) for 15 min. Colostral whey was carefully removed by pipette. The CL pellet was resuspended in 20 ml media (RPMI-1640 with 10% FBS, antibiotic/antimycotic) and washed three times by centrifugation (200 x g for 5 min). Colostral Leukocytes were resuspended in 1 ml Phosphate Buffered Saline, pH 7.2 (PBS, Invitrogen, Chicago, Illinois) and counted by hemocytometer. Cells were centrifuged to remove PBS and resuspended at the desired concentration (10^7) per ml in Recovery Cell Culture Freezing Medium (GIBCO, Grand Island, New York). Vials of CL were frozen to -50°C and then transferred to the liquid phase of a LN freezer for storage. Samples were quick-thawed at 37°C.

Isolation of PBMC

Blood was collected from the jugular vein of pigs using vacutainers (Monoject, Mansfield, Massachusetts) containing heparin (143 USP units). Heparinized blood was diluted 1:4 in phosphate buffered saline (PBS) at room temperature. The diluted blood sample was slowly layered above the Histopaque 1083 (Sigma, St. Louis, Missouri) in a 50 ml conical polypropylene tube using a 20 ml sterile syringe attached to a 1.2 mm X 25.4 mm needle. The tubes were centrifuged (800 x g) for 45 min with the centrifuge brake off. The supernatant was carefully aspirated to within 2-3 mm of the opaque interface that contained the mononuclear cells. The mononuclear cell layer, along with the remaining serum layer, was collected using a sterile pipette taking care not to disturb the red blood cell layer. The mononuclear cell layer was resuspended in PBS and centrifuged for 15 min at 800 x g at with the centrifuge brake on. The

supernatant was removed and the cells were washed three times in equal volumes of PBS containing 0.5 % bovine serum albumin (BSA) and centrifuged for 5 min at 400 x g. All centrifugations were at 6 to 8°C. The PBMC were counted using a hemacytometer and trypan blue was used to assess viability. For in vitro studies the cells were resuspended in Recovery Cell Culture Freezing Medium and cryopreserved in liquid nitrogen. PBMC for in vivo studies were not cryopreserved.

Harvest of PUC

Umbilical cords were collected from newborn pigs from the Kansas State University Swine Research and Teaching Center. Umbilical cords were collected aseptically at birth and placed in sterile PBS plus an antibiotic/antimycotic (Sigma-Aldrich, St. Louis, Missouri) containing 100units Penicillin, 100µg Streptomycin Sulfate and 0.25µg Amphotericin B/ml and refrigerated until processing for culture. Once in the laboratory, the cords were rinsed in fresh PBS with antibiotic/antimycotic and placed in a sterile Petri dish. Umbilical cord segments (1–3 cm) were cut longitudinally to expose the two umbilical arteries and the umbilical vein and the vessels removed and discarded. Wharton's Jelly was removed from cord sections, minced with scissors into 2–5 mm^3 explants, and transferred to 75cm^2 culture flasks (Corning Inc, Corning, New York) containing 12 ml of High-Glucose Dulbecco's Minimum Essential Medium (DMEM, Invitrogen)) supplemented with 20% Fetal Bovine Serum (FBS, Invitrogen), β-Mercaptoethanol (55µM/ml, Sigma-Aldrich) and antibiotic/antimycotic (25µg Gentamicin Sulfate (Sigma), 100µg Normocin™ [Invivogen, San Diego, California]/ml). Culture flasks were incubated at 38.5°C with 5% CO_2 and saturated humidity. Tissue explants were removed from culture following observation of adherent PUC cells. Cells were passaged by dissociation with 0.1% trypsin + 1.0 mM EDTA in PBS (trypsin, Gibco, EDTA, Invitrogen,).

GFP PUC

Umbilical cords from transgenic fetuses expressing green fluorescent protein (GFP) were kindly provided by Dr. Randy Prather, University of Missouri. Cords were collected as fetuses were removed during surgeries at mid-gestation and were shipped by commercial carrier in PBS at ~5º C. Cords were received within 24 h of collection and PUC were isolated as described above and stored frozen until use.

Culture of intestinal explants

Methods for explants cultures were adapted from those described by Williams et al., (1985). Intestinal sections were prepared for explant culture from either stillborn pigs or from pigs sacrificed prior to nursing. Small intestinal sections of the duodenum, jejunum, and ileum were removed aseptically. To protect the mucosal epithelium, intestinal sections were everted with the mucosa positioned to the exterior. This orientation allows the contents of the culture system direct contact with the mucosa and the insertion of a synthetic sponge (described below) provides stability for the tissue when the intestinal sections are sliced. The intestinal explants were sectioned into thin pieces to allow medium infiltration. Explants of approximately 1.0 mm or thicker displayed rapid degeneration and the loss of the ability to remain in association with the central sponge. A hemostat was inserted into the lumen of each intestinal section and clamped to the far end of the section with the aid of another hemostat. The clamped hemostat was gently pulled in the opposite direction to expose the mucosal surface of the tissue. The hemostat was reinserted into the tissue section and slightly opened. A rectangularly-shaped sponge made of polyvinyl alcohol (PVA) (instrument wipe, 2MM, Ultracell, North Stonington, Connecticut) was cut and inserted into the open hemostat and pulled through the lumen of the tissue. The tissue section, including the sponge, was washed by submerging in PBS containing antibiotic/antimycotic (Gentamicin Sulfate (Sigma), Normocin™, Invivogen, /ml) This hydrated the sponge causing it to swell and forming the tissue into a rectangular block. The block of

tissue was placed in a glass syringe fitted with a plunger. The tissue section was flooded with warm agar medium (Difco, Lawrence KS, 25 g/L). The agar was solidified by cooling to 4°C and explants, as thin (about 0.3-0.5 mm thick) rings, were cut from the section. The solidified agar medium was gently pulled away from the mucosa. The explant, with the central sponge, was positioned onto the window area of a Leighton tube (borosilicate glass, 16 x 83mm, 11 x 55 window #0 stopper, Bellco Glass, Vineland, NJ) containing McCoy's 5a medium (Invitrogen) containing 15% FBS, 0.5% lactalbumin hydrolysate (Sigma), and antimicrobial agents, with a pH near neutrality was added (1 ml) and the explant rotated in the gaseous-phase position for 72 h.

Medium was replaced at 24 h intervals by positioning the Leighton tubes upright and allowing the cellular material and intestinal tissue to settle for 15 min. Next media was aspirated from the Leighton tube taking care not to disturb the explant culture and 1.0 ml of McCoy's 5a medium was added.

The explants were washed for 1 min in 1.0 ml of sterile PBS prior to fixation to remove contents adhering to the mucosa. For confocal microscopic observations of the explants, tissue sections were stained with Propidium Iodide (Invitrogen), and fixed in 10% formalin. The explants were removed from the sponge and placed on slides with the mucosal surface up.

Experiment 1, in vitro tests of cell trafficking using intestinal explants from stillborn pigs

These experiments utilized intestinal explants from pigs that were dead at birth. Fully-formed pigs that are dead at birth are considered to have died during parturition (Trujillo-Ortega et al., 2007). Thus, at delivery, they are generally within 1 h of death. For this experiment, stillborn pigs were processed for explant cultures within 2-3 h of birth.

Experiment 2, in vitro tests of cell trafficking using intestinal explants from pigs alive at birth

Sows were observed during farrowing and pigs removed prior to nursing. Pigs were kept warm (21-27°C) and transported to the laboratory where they were anesthetized by inhalation of halothane (Halocarbon Laboratories, River Edge, New Jersey). Blood was collected by cardiac puncture using a 1.2mm X 38.1mm needle attached to a 35 ml syringe and then 0.5 g sodium pentathol (Abbott Laboratories, North Chicago, Illinois) administered intracardially which resulted in death. Pigs were exsanguinated by severing the carotid arteries and intestinal tissues were collected aseptically.

Experiments 3 and 4, in vivo tests of cell trafficking

Procedures for feeding cells to neonatal pigs

PBMCs and PUC were labeled with the lipophilic dye PKH26GL (Sigma-Aldrich). Approximately 1 X 10^7 single cells were placed in conical polypropylene tube and washed once using a serum free medium. The cells were centrifuged (400 x g) for 5 min into a loose pellet. After centrifugation the supernatant was aspirated leaving no more than 25 µl of supernatant on the pellet. The cells were resuspended cells by adding 1 ml of Diluent C (provided by the manufacturer), and pipetting to ensure complete dispersion. Immediately prior to staining, a 4 X 10^{-6} M PKH26 dye was prepared in polypropylene tubes using Diluent C. The 1ml of 2x cells was rapidly added to 1ml of 2x dye. The sample was mixed by pipetting. The cells were incubated at 25°C for 5 min. The staining reaction was stopped by adding an equal volume of serum and incubating for 1 min. The serum-stopped sample was diluted with an equal volume of complete medium. The cells were centrifuged (400 x g) for 10 min at 25°C. The supernatant

was removed and the cells were transferred to a new tube and washed twice by adding RPMI 1640 with 0.5% BSA. Cells were centrifuged and resuspended to approximately 1 X 10^7 cells/ml. The cells were examined using fluorescent microscopy to verify staining.

Sows were observed during farrowing and pigs were not allowed to nurse until the treatments were administered which occurred within 5 min of birth. Feeding of the labeled cells was by a modified 12 French feeding tube (cut to 187 mm in length, Kendall Healthcare, Marietta, Georgia) and the treatment was administered in 3 ml slowly behind the tongue as the pig was observed to swallow. The feeding tube was flushed with saline (1ml) to deliver the residual volume and the pig ear notched for identification and returned to the farrowing crate to suckle *ad libitum* until sacrifice. Neonates received either PBMC from their mother or PUC from an unrelated pig. The cells were incubated with RPMI 1640 containing 0.5% BSA (Gibco) or 25% AC. The experimental designs are shown in Table 1 (Exp. 1) and 2 (Exp. 2). Either 8 h (Exp. 1) or 24 h (Exp. 2) after receiving the labeled cells, the neonates were anaesthetized, cardiac blood collected for flow cytometry, and the pigs were euthanized and intestinal samples were collected.

Lymphocyte/monocyte isolation for flow cytometry

Blood was collected from anesthetized neonatal pigs that had been fed cells by cardiac puncture using a 1.2 mm X 38.1 mm needle and syringe. Blood was transferred to a 10ml heparin tube and mixed well by inversion. The contents were removed from the tube using a Pasteur pipette, being careful to avoid coagulates. Approximately 3 ml of PBS was added (1:1 dilution). In a 15 ml conical tube, blood was gently layered over an equal volume of Ficoll-Paque Plus (GE Healthcare 17-1440-02). The diluted mixture was centrifuged for 30 min at 400 x g at room temperature. After centrifugation, the mononuclear cell layer was removed with a

Pasteur pipette and placed in a 15ml tube and centrifuged for 5 min at 1,000 x g to pellet cells. The supernatant was discarded and the cell pellet was resuspended in expansion medium (RPMI 1640 with10% FBS and penicillin and streptomycin). The cell pellet was fixed in 1% formaldehyde, light protected and stored at 4° C until analysis on flow cytometer within 24h.

In vivo *tissue collection*

Tissue was collected from 15 neonatal pigs after administration of cells. Sections of the duodenum, jejunum, and ileum were removed and fixed in 10% formalin for 1 h, transferred into a 2% solution of glutaraldehyde in PBS, and stored light protected at 4°C.

Evaluation of tissue

Tissue sections were examined using a Zeiss LSM 5 Pascal microscope. Images were collected using Plan-Neoflaur 40X and Plan-Apochromat 63X objectives. The red and green wavelengths were imaged using HFT 543, LP560, and BP 505-530 respectively. The collected images were evaluated using Image J 1.38X and Zeiss LSM Image Browser 4,2,0,121.

RESULTS

Explant culture system

The duodenal, jejunal, and ileal sections were cultured for 72 h in rotating Leighton tubes and after fixation the tissue sections were easily discernible under a confocal microscope. The tissue was intact and cells appeared healthy (Fig. 1). The smooth muscle layers maintained elasticity as noted by the retention of a circular structure on the sponge. The latter has been considered a measure of tissue integrity for this explant system (Williams et al., 1993). In explants sectioned larger than approximately 1 mm in length, degeneration of the epithelium was noted and the villous structures were no longer discernable under confocal microscopic examination. This is similar to a report by Williams et al., (1993) who considered intestinal sections of 0.3 to 0.5 mm thickness preferable.

Experiment 1. Explant culture system of intestinal sections collected from stillborn neonates

Small intestinal tissue was collected from stillborn neonates to evaluate trafficking of cells in vitro. All three cell types, CL, PUC, and PBMC incorporated into the intestinal explants during the 72 h incubation (Fig 1). No differences in cell incorporation due to incubation in AC were observed (Fig 2). The labeled cells did however; appear to be more abundant in sections of the jejunum when compared to the duodenum or the ileum. When analyzed by confocal microscopy, z-stacks revealed cells in the duodenum to depths of 15to 20 μm below the luminal surface. In ileal explants, labeled cells were detected to depths of 12 to15 μm and in jejunal explants, cells were found to depths of 32 to36 μm.

Experiment 2. Explant culture system of intestinal sections collected from sacrificed neonates.

Fluorescent labeled CL, PUC, and PBMC were incubated with tissue of the small intestine collected from sacrificed neonates. After 72 h the explants were fixed and analyzed using confocal microscopy. Results were similar to explants collected from stillborn neonates (Exp. 1). Treatment of PUC and PBMC with AC had no detectable effect on their incorporation into the explants. CL, PUC, and PBMC were present in all duodenal, jejunal, and ileal explants with more cells found in the jejunum. When comparing the sections of the small intestine using z-stack imaging, the jejunum contained cells to depths of 36-38 μm below the luminal surface of the tissue. Both the duodenal and ileal explants contained cells to depths of 15-20 μm below the luminal surface. When explant cultures were incubated with cells treated with or without AC (Fig. 2), PKH-26 labeled cells were observed to distribute similarly in the explants. Control explants without added cells exhibited only background fluorescent staining (not shown).

In vivo Experiments

Exp. 3 and 4 evaluated cell trafficking in vivo and the influence of AC on the transfer of cells through the intestine of the neonate. Intestinal sections of the duodenum, jejunum, and ileum were collected from neonatal pigs 8 and 24 h after feeding PKH-26GL labeled cells. The tissue sections were imaged on a confocal microscope to detect the presence of labeled cells.

Experiment 3. In vivo, 8 h post treatment

In neonates that were fed labeled cells, intestinal sections of the duodenum, jejunum, and ileum contained PKH-26 labeled cells 8 h post treatment (Fig. 3). The piglet that received PBMC from its mother (piglet 2) incubated in AC, had labeled cells in the jejunum and ileum but none were

detected in the duodenum (Table 1). The labeled cells were located in the lamina propria, deep submucosa, superficial submucosa, and around Peyer's patches. The PBMC were found individually as well as in cell clusters. Fluorescent PBMC appeared to migrate deeper into the mucosa than PUC cells.

In piglets that received PUC (piglets 3, 4, 5, 6), absorption into the intestine was observed in the duodenum, jejunum, and ileum (Table 1). The PUC cells were more often present in a cluster of 2 to 3 cells in the epithelium of the villi (Fig 3). Piglets 4 and 5(fed PUC incubated in AC) displayed fluorescent labeled cells in the duodenum, jejunum, and ileum of the small intestine.

Experiment 4. In vivo 24 h post treatment

Labeled PBMC or PUC were found in the intestine of all pigs at 24 h after feeding (Table 2, Fig. 4 and 5). They were located deeper in the submucosa than observed in pigs sacrificed at 8 h (Exp. 3). One specimen (piglet 1, without AC) revealed labeled PBMC in the duodenum around Brunner's glands (Fig. 4 B). Piglet 2 revealed labeled PBMC (with AC) in or around Peyer's patches within the jejunum (Fig 4A). Piglet 3 (AC) exhibited PBMC in or around Peyer's patches in both the jejunum and the ileum. PBMC were observed in the deep submucosa whether or not they were incubated in AC before feeding.

In intestinal sections of piglets that received PUC incubated without AC (piglets 4, 5) absorption of the cells was confined to the epithelium of the villi (Table 2). In contrast to PBMC, the PUC cells were not observed to migrate past the superficial submucosa. Labeled PUC cells were found in all piglets in the duodenum regardless of exposure to AC and one (piglet 6) fed AC-treated PUC, the labeled cells were in the deep lamina propria of the ileum.

Discussion

Labeled colostral cells are capable of penetrating the porcine gastrointestinal tract (Williams, 1993). Williams et al. (1985) devised an organ explant culture system for short term maintenance of piglet intestine. This procedure allows the use of duodenal, jejunal, and ileal explants for testing their ability to associate with specific cell types. The in vitro tissue explants were cultured with FITC labeled CL. The in vitro and in vivo studies indicate CL are taken up by the epithelial lining of the gastrointestinal tract of neonatal pigs and circulate by blood to various tissues. The CL seem to localize as aggregates in the submucosa and spread throughout the submucosa as independent cells. CL migrated into the explants by 24 and 48 h. Williams (1993) collected blood and intestinal tissue from piglets after they ingested FITC-labeled cells. FITC-CL were observed in the blood stream within 2 h and appeared to peak in circulation at 5 h. Mesenteric lymph nodes, spleen, liver, lung, and pulmonary lavage smears demonstrated FITC-CL at 24 h post treatment. Similarly, the duodenum, jejunum, and ileum also contained cells. In contrast, when the neonates ingested PBMC isolated from their mother's blood, the cells were not observed in the blood stream or tissues.

In vivo studies of trafficking PBMC have also been conducted in neonatal calves (Reber et al., 2006). Maternal PBMC were treated with fluorescent tracer PKH26-GL. The cells were fed to neonatal calves within 6 h of birth; once continuous suckling had been initiated, the calves were fed PBMC incubated for 24 h in medium with or without AC (25%). Jugular blood samples were collected from the calves and analyzed by flow cytometry. Labeled PBMC were recovered from the calves receiving cells incubated in AC. The number of cells detected increased from 8 to 24 h, and decreasing 28 h post treatment. Exposure to 25% AC also induced changes in homing and trafficking markers on leukocytes. Reber et al., (2006) demonstrated that

the number of cells expressing CD43 and CD11a are increased when exposed to a AC. These receptors are implicated in cellular trafficking and migration. The number of cells expressing CD62L is also increased and this receptor works in conjunction with CD11a. CD62L induces rolling along the surface of high endothelial venules thus increasing contact time with the endothelial lining and facilitating adhesion. All of the mentioned receptors are critical for leukocyte adhesion to and migration across the endothelial surface. Colostral leukocytes enter neonatal circulation after the ingestion of colostrum in several species. Sheldrake and Husband (1985) demonstrated that labeled colostral leukocytes enter the bloodstream and mesenteric lymph nodes of rats and lambs. Following laparotomy, labeled cells were injected into the lumen of the duodenum and the jejunum of suckling neonatal rats. The neonates were deprived of milk for 4 h and were then sacrificed. Sections of the duodenum and the jejunum were processed for autoradiography. The findings show that CL migrated from the intestinal lumen into the lamina propria in rats. Laparotomies were also preformed on lambs between 6 and 14 h post partum. PBMC were injected into the duodenum. Samples of the duodenum and the mesenteric lymph nodes were collected 2 h following the injection. The cells were transported from the duodenum via the lacteal lymph to the mesenteric lymph nodes in lambs. In lambs, during the period before gut closure, the lymphocytes are able to enter the neonatal intestine.

Several possibilities have been proposed to explain absorption of CL in the neonate. The cells in question may get into the epithelium through the microvilli (Sheldrake and Husband, 1985). This process may also take place through the intercellular spaces of the epithelium (Ogra et al., 1977). Tuboly et al., (1988) demonstrate that lymphoid cells are absorbed through the intercellular spaces of the epithelium unlike immunoglobulins that enter the cytoplasm of intestinal epithelial cells by endocytosis after having pushed apart the microvilli. Additionally,

when cells are exposed to 25% AC there is an increase in the number of cells expressing CD43 and CD11a. Both are cellular receptors that have been implicated in cellular migration across the epithelial cells of the neonatal gut. These receptors are important for cellular transendothelial migration and often these receptors work in conjunction (Reber et al., 2006).

Once CL are absorbed by the neonate, their role in the circulation of the neonate, and their effect on the development of immunity warrant further studies. Tuboly et al., (1988) conclude that the cells absorbed transfer a positive or negative signal to the lymphoid system of the neonate. The CL also have their own immunocompetence or antigenic commitment that should be considered. The presence of maternal CL may also regulate T- and B- cell activities. The peripheral blood leukocytes of the neonatal pig responded to T- and B- cell mitogens, indicating that the PBL were immunologically competent. This could be attributed to nonantigen-specific immunosuppression of the developing immune system (Williams 1993).

Stem cells are a populations of cells that have the ability to self renew and to differentiate into a diverse range of cell types. There is growing interest in PUC stem cells as they can be easily isolated from the porcine umbilical cord. PUC cells can be collected in a non-invasive manner, may be cryogenically stored, thawed, and expanded in vitro (Carlin et al., 2006). UCMS cells have been isolated from several species and may have therapeutic potential. Immunogenicity studies reveal that a single injection of PUC, across a full MHC barrier, does not elicit a detectable adaptive immune response. Animals that were injected once with PUC had no detectable alloantibody production. This is most likely because PUC are MHC class I dull and are negative for MHC class II. PUC may also play a role in modulating immune responses (Cho et al., 2008).

In our experiments, we evaluated the ability of labeled CL, PBMC, and PUC to traffic into the pig intestine. The labeled cells were not found in peripheral blood of the pigs (data not shown) but we observed incorporation into the intestine both in vitro and in vivo. PUC but not PBMC tend to localize as clusters in the submucosa similar to CL.

Reber et al., (2006) demonstrate that exposure to 25% AC induces changes in PBMC, enhancing trafficking and homing abilities. The authors detected migration into circulation using flow cytometric analysis of blood samples. They did not evaluate the potential for cells to traffic into the intestinal tissues. Our results indicate that exposure to 25% AC did not affect cell migration either in vivo or in vitro. In our observations, both PUC and PBMC were able to associate with the duodenum, jejunum, and the ileum regardless of exposure to AC or medium. Tuboly et al., (1988) note that labeled cells were observed in the epithelium of piglets that had received their mother's CL. In piglets that received cells from another sow, the cells were demonstrated in the epithelial wall but not in lymphatic ducts. When we analyzed intestinal epithelium, cells were observed in the intestine similar to Tuboly et al., (1988). If exposure to colostrum is important, it may be contact with ingested colostrums conferred some properties to the PBMC and PUC that were not exposed to AC before feeding. This could have occurred in our experiments because piglets were allowed to suckle *ad libitum*, beginning approximately 3 to 4 min after administration of cells, when they were returned to their mothers. The ingested colostrum may have had an effect on trafficking and migration patterns of cells in the gastrointestinal tract.

Another consideration is the method of tissue analysis. Williams (1993) used fluorescein isothiocyanate (FITC) to label colostral leukocytes. We used PKH26-GL which produces more intense fluorescence. In our studies, sections of the duodenum, jejunum, and ileum were

collected, fixed, thinly sliced, and placed on microscope slides. The tissue was analyzed by confocal microscopy providing visual images of the tissue at every 2 μm. This may be a more sensitive assay when compared to cryostat sections, sliced at approximately 10 μm that were evaluated by Williams (1993).

The amount of time allotted for the cells to migrate through the gastrointestinal tract was important in our experiments. The piglets that were allowed to suckle for 24 h after receiving labeled cells demonstrated cells deeper into the mucosa. Those that were allowed to suckle for 8 h had labeled PBMC mainly on the surface of the epithelium of the gastrointestinal tract. Further studies will be necessary to the time-course for migration and whether PBMC and PUC eventually reach the circulation and other tissues.

Our in vitro studies were designed to determine if the cells were able to migrate into intestinal tissue, warranting the study of live animals. In vitro cultivation of intestinal explants with CL, PBMC, and PUC demonstrated the cells could be taken up and migrate deep into the explants. In previous work by Williams (1993), PBMC were not taken up by any of the intestinal explant cultures. These differences may reflect the labeling and detection procedures described above. Another procedural difference between our in vitro experiments and those of Williams (1985; 1993) was the use of a PVA sponge rather than the cellulose sponge used by Williams (1985). The latter sponges are no longer available. Of importance, in our in vitro studies similar results were obtained with stillborn and sacrificed neonates. This method of organ culture and tissue analysis may provide a method of studying the neonatal gastrointestinal tract and the use of stillborn neonates offers an experimental tissue without euthanizing live subjects. In our in vitro and in vivo models CL, PBMC, and PUC were able to migrate into the neonatal intestinal tract regardless of treatment in AC. More labeled cells were seen in the

explant cultures than in the intestine after oral feeding. This might be explained by the greater number of cells per surface area in vitro or the longer duration of exposure (72 h) in vitro.

PUC are primitive cells that have the ability to differentiate into a diverse range of cell types and are also nonimmunogenic following their initial allogenic transplantation. These characteristics make these cells a potential delivery source to the neonatal gastrointestinal tract and possibly the neonatal immune system. Further studies of cellular interactions within the neonatal intestine will contribute to our knowledge of immunological and other potentials of orally transplanted cells and may lead to development of delivery systems useful for the treatment and prevention of neonatal disease.

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Table 1. Location of PKH-26 labeled cells in the small intestine at 8 h after feeding (Exp. 3).

Cell type	PBMC	PUC			
no. of pigs	1	2		2	
Exposure to AC[1]	+	-		+	
	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5
Intestinal Section:					
Duodenum	IV, DLP[2]	none	AV, SLP	AV,SLP	AV, SLP
	AV,SLP			BV, DLP	IV, DLP (cluster[3])
	SS (6-10)				
	DS				
Jejunum					
	IV, DLP (10-20)	BV,DLP (cluster)	none	AV,SLP	AV, SLP
	SS (>20)			IV, SLP	
	DS			IV, DLP	
Ileum					
	BV, DLP (10-20)	IV, DLP (6-10)	BV, DLP	AV, SLP	BV, DLP (cluster)
	SS (>20)			IV, DLP (cluster)	
				SS (cluster)	

[1] Exposed to acellular colostrum (25%) during 4 h incubation before feeding the cells.

[2]Region of the labeled cells in the villi. Cells were either at the apex (AV) intermediate between the apex and the base (IV), or at the base (BV) of the villus and the cells were in the superficial lamina propria (SLP) or deep in the lamina propria (DLP). Labeled cells below the villi were in the superficial submucosa (SS) or deep in the submucosa (DS). Unless noted in parentheses the number of cells observed was 1-5.

[2]A cluster of cells in which individual cells could not be counted.

Table 2. Location of PKH-26 labeled cells in the small intestine at 24 h after feeding (Exp. 4).

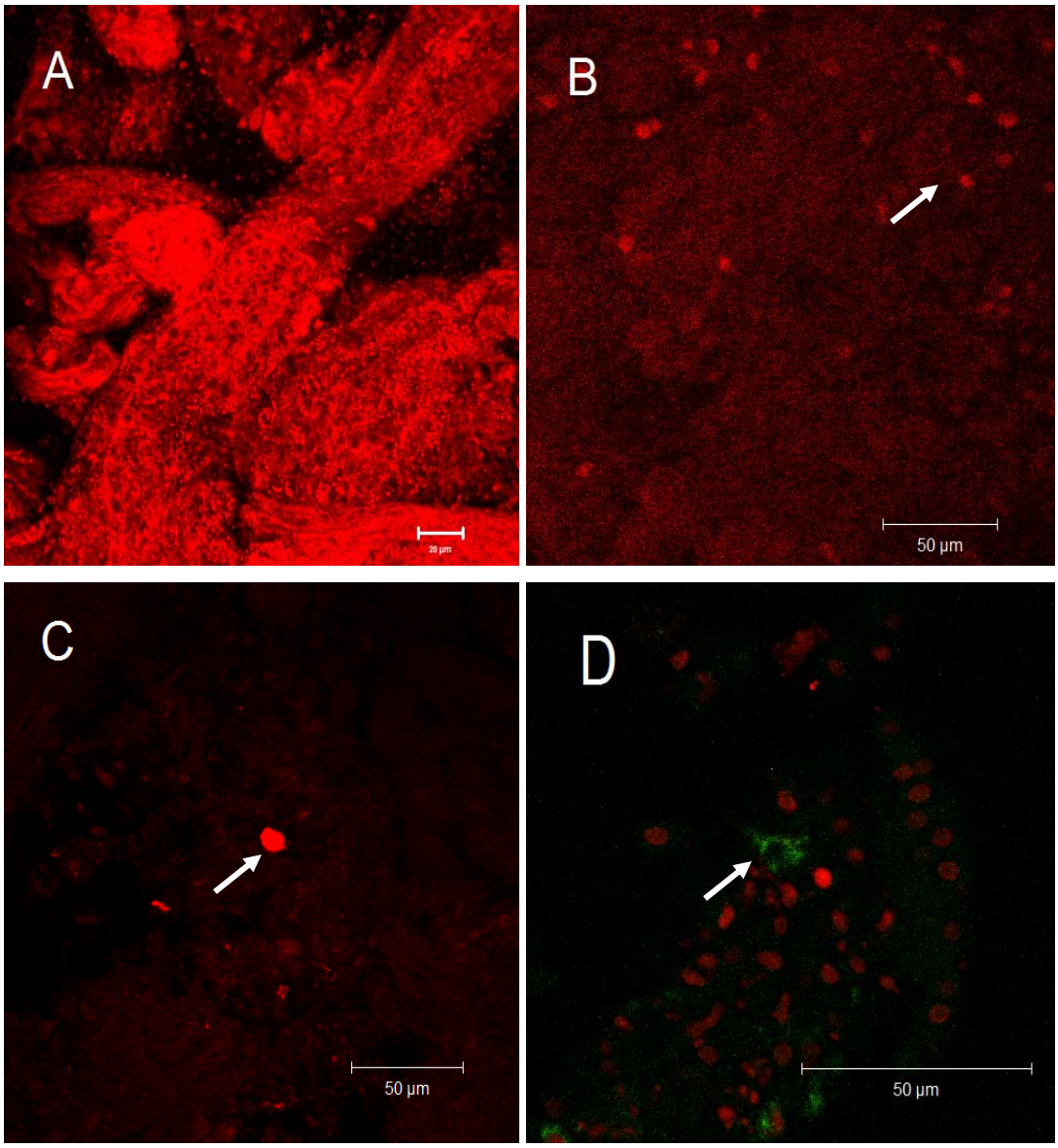
Cell type	PBMC			PUC			
no. of pigs	3			4			
Exposure to AC	-	+	+	-	-	+	+
Pig	1	2	3	4	5	6	7
Intestinal Section:							
Duodenum	IV, DLP[1]	AV, SLP	AV, SLP (cluster[2])	AV, SLP	IV, SLP (cluster)	IV, DLP	IV, DLP
		SS (>20)	IV, DLP	BV, DLP (cluster)		BV, DLP	BV, DLP
		DS (cluster)					
Jejunum							
	IV, DLP	IV, DLP	AV, SLP (cluster)	SS (10-20)	none	none	none
	SS (>20)	SS (>20)					
	DS (6-10)	DS (>20)					
Ileum							
	SS (>20)	IV, DLP	none	AV, SLP	none	AV, SLP	none
	DS (10-20)			IV, DLP		BV, DLP	

[1] Exposed to acellular colostrum (25%) during 21 h incubation before feeding the cells.

[2]Region of the labeled cells in the villi, were either at the apex (AV) intermediate between the apex and the base (IV), or at the base (BV) of the villus and the cells were in the superficial lamina propria (SLP) or deep in the lamina propria (DLP). Labeled cells below the villi were in the superficial submucosa (SS) or deep in the submucosa (DS). Unless noted in parenthesis the number of cells observed was 1-5.

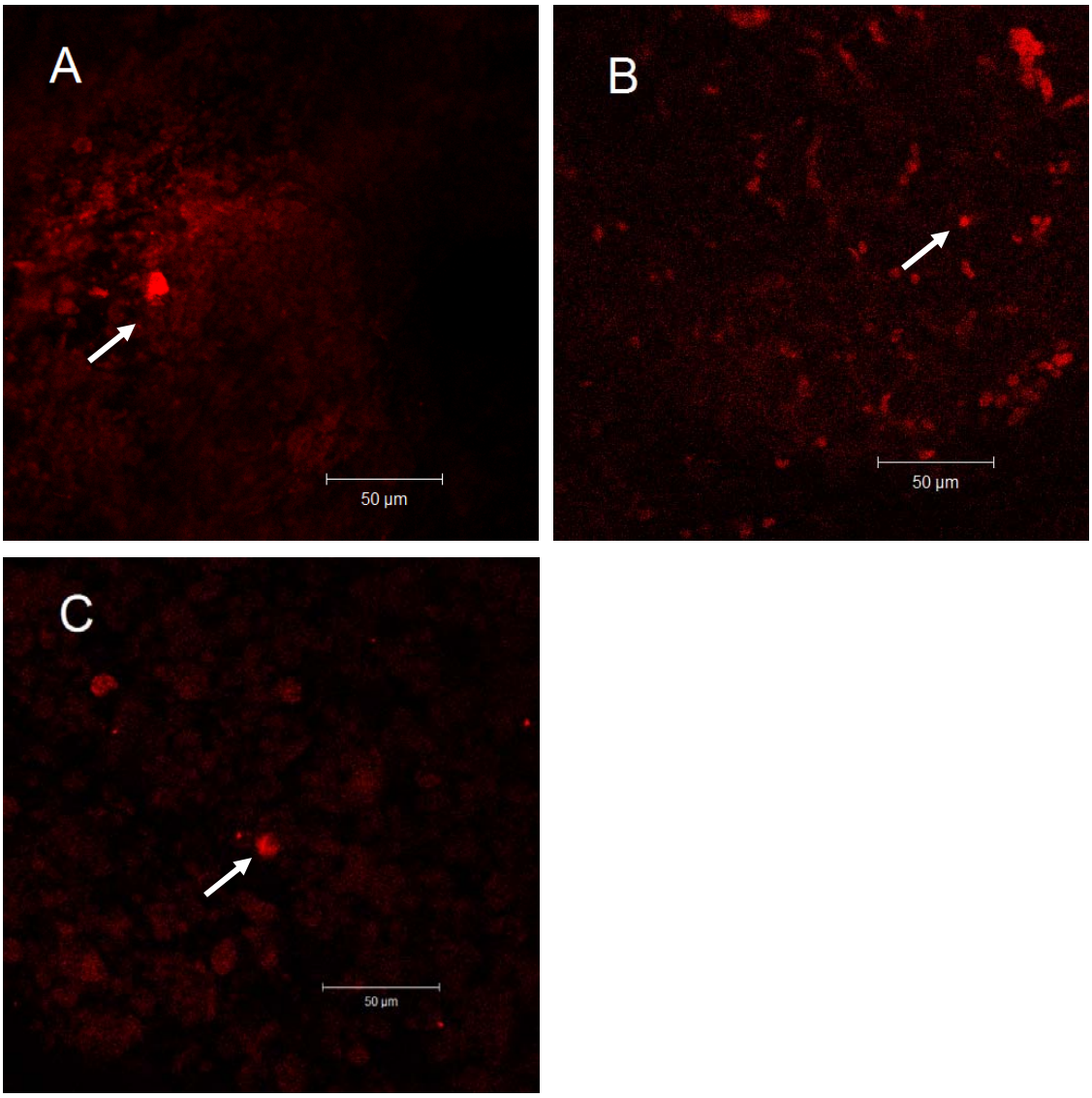
[2]A cluster of cells in which individual cells could not be counted.

Figure 1. Jejunal tissue explanted from stillborn pigs and cultured 72 h (Exp 1).



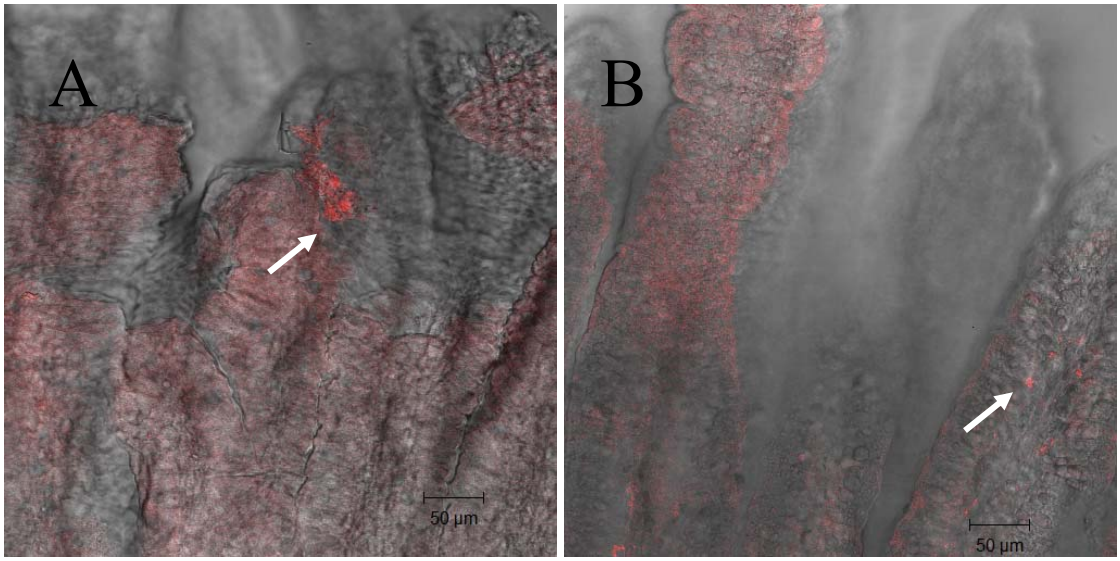
Jejunum, explants in culture containing colostral leukocytes (CL), pig umbilical cord matrix stem cells (PUC), and peripheral blood mononuclear cells (PBMC). Jejunal explant cultures incubated with PKH-26GL labeled cells (arrows indicate PKH labeled cells). **A**. A control culture (no added cells) with identifiable villi (note: background autofluorescence). **B**. Explant culture with PBMC without acellular colostrum (AC) **C**. Explant culture incubated with CL **D**. Explant culture incubated with GFP- PUC without AC.

Figure 2. Tissue explanted from pigs alive at birth after 72 h culture (Exp 2).



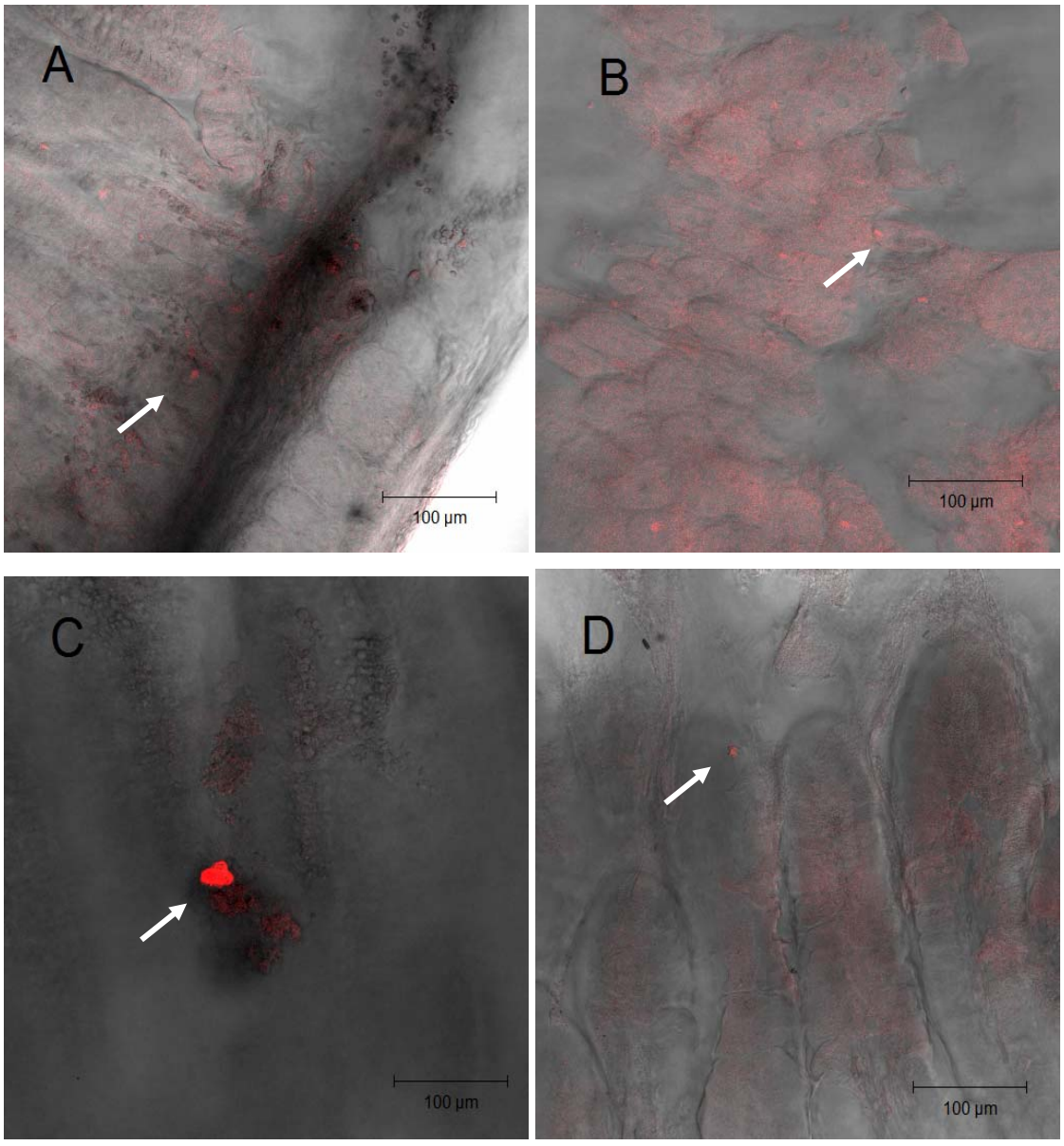
Tissue explants in culture containing PKH-26GL labeled CL, PUC, and PBMC (arrows indicate PKH labeled cells). **A**. Duodenal explant incubated with CL **B**. Ileal explant incubated with PBMC with and AC **C**. Duodenal explant incubated with PUC without AC.

Figure 3. Duodenal tissue from pigs fed labeled cells and collected 8 h post feeding (Exp 3).



Duodenum containing PKH labeled PUC and PBMC (arrows indicate PKH-labeled cells) **A**. Cluster of PUC (no AC treatment) located in deep lamina propria at the apex of a villus, **B**. PBMC (incubated with AC) located in deep lamina propria, intermediate between the apex and the base of the villus.

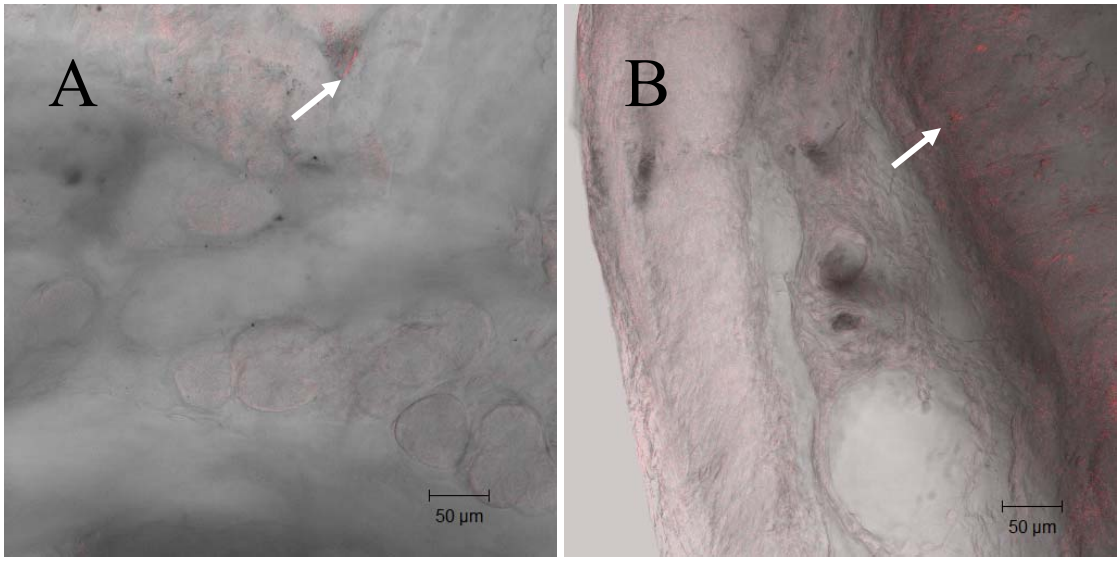
Figure 4. Intestinal tissue from pigs fed labeled cells and sacrificed 24 h post feeding (Exp 4).



Jejunum and Duodenum containing PUC or PBMC (arrows indicate PKH-labeled cells). **A**. Jejunum containing PBMC (AC treated). Labeled cells present in the superficial submucosa, deep submucosa, and in or around Peyer's patches. **B**. Duodenum containing PBMC (AC treated). Cells present in superficial submucosa and in or around Brunner's glands. **C**. Jejunum containing PUC (no AC). A cluster of cells is present at the base of the villus within the deep lamina propria. **D**. Duodenum containing PUC

treated with AC. Cells are present at the apex of the villus and in the superficial lamina propria.

Figure 5. Jejunal tissue from pigs fed labeled cells and sacrificed 24 h post feeding (Exp 4).



Jejunum containing PBMC and PUC (arrows indicate PKH labeled cells). **A**. PUC incubated with AC, a cell is present at the base of the villus. **B**. PBMC incubated without AC. More than 20 cells are present in the deep submucosa.

Appendix A

Table A. 1. Assignment of pigs to Exp. 3 and 4.

Sow	Piglet	Cells[a]	Hours post-feeding
W35	**1**	**N/A Control**	**N/A**
W37	**2**	**PBMC in AC**	**8**
W13	**3**	**PUC in AC**	**8**
W13	**4**	**PUC in RPMI**	**8**
W13	**5**	**PUC in AC**	**8**
W13	**6**	**PUC in RPMI**	**8**
W80	**1**	**PBMC in AC**	**24**
W86	**2**	**PBMC in AC**	**24**
W80	**3**	**PBMC in RPMI**	**24**
W6	**4**	**PUC in RPMI**	**24**
W6	**5**	**PUC in RPMI**	**24**
W6	**6**	**PUC in AC**	**24**
W6	**7**	**PUC in AC**	**24**
W6	**8**	**N/A Control**	**N/A**

[a]Peripheral blood mononuclear cells (PBMC) isolated from the sow and incubated with or without 25% acellular colostrum (AC). Pig umbilical cord matrix stem cells (PUC) incubated with or without AC.