REPORT:

Placenta derived viable tissue matrix leads to robust integration and regeneration of implanted tissue scaffolds

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ABSTRACT

The human placenta has been used to harvest Extracellular Matrix (ECM) and growth factors for over 100 years. Historically, placenta derived products have been shown to reduce pain, infection, and to improve wound healing. In these investigations, an injectable amnion-derived tissue matrix product (BioDFactor™), was analyzed and its cytokine composition compared to native amnion tissue. Additionally, an acellular 3D tissue scaffold was incubated in the amnion derived product and then implanted into a rat model for 5 days to assess initial integration and vascularization. These studies revealed that BioDFactor contains a complex cytokine composition, including osteogenic, angiogenic, neurogenic, and immune related cytokines. In vivo results show the BioDFactor™ infused scaffolds to have a significantly decreased fibrous capsule formation, evidence of neovascularization and increased cell migration compared to control samples.

INTRODUCTION

The human placenta allows the exchange of nutrients between maternal and fetal circulation systems while avoiding the immunological complications that would arise if the two blood supplies came into direct contact. It is also one of the largest harvestable human vascular networks, and contains numerous angiogenesis and growth related cytokines. The unique concentrations and combinations of these molecules make the placenta an excellent source of native human ECM and an array of growth, immune and angiogenic related factors.

Products derived from the placenta range from human umbilical vein vascular grafts to amniotic membrane sourced ocular grafts. Placental derived products have been used in medical applications since the early 1900's. The first reported use of the human amniotic membrane was in 1910 with a review of 550 wounds treated at John Hopkins Hospital. This patient review showed wounds treated with the amniotic membrane resulted in a considerable reduction in pain and infection, as well as increased epithelialization[1]. A later study in 1980 evaluated the effectiveness of using the amniotic membrane on chronic leg ulcers, and suggested that the membrane also has angiogenic capability in regenerative medicine. It was found that the ulcers had developed new blood vessels, thought to be due to angiogenic factors acting on the capillary endothelium at the wound site[2]. Historically, many studies have concluded that amniotic membrane derived products can improve wound healing and reduce pain and infection[3-6].

BioDFactor™ is an injectable tissue matrix derived from the human amnion (BioDLogics™, LLC, Memphis, TN). In these studies, we performed a characterization of BioDFactor™ cytokine profile and compared it to native amniotic membrane cytokine composition. To further define the *in vivo* effects of BioDFactor™, a model (acellular) tissue engineered scaffold derived from a decellularized human umbilical vein (dHUV) was incubated in BioDFactor™ and then implanted into a rat model. After 5 days of implantation, the scaffolds were removed and analyzed to determine effects on tissue remodeling, cell migration, fibrous capsule formation, and vascularization.

METHODS

Cytokine analysis. Relative cytokine levels were determined using a sandwich immunoassay array from RayBiotech, Inc. (Human Cytokine Antibody Array C Series 1000, Inc, GA, USA). Before analysis, all samples were homogenized on ice 3x 15 seconds with 30 seconds of pause between each step to prevent sample heating and maintain the sample at 4º C. Chemilumenescence was detected using a Foto/Analyst Luminaryfx Workstation (Fotodyne Incorporated, WI, USA) and the signal intensities were measured using TotalLab 100 software (Nonlinear Dynamics, Ltd, UK). Intensity

values measured in blank wells were subtracted from all wells, and then data was normalized to a linear curve determined by setting 0% intensity values equal to negative control measurements and 100% intensity values equal to positive control measurements. Final chemokine measurements were given as percent intensity.

Human umbilical vein scaffold preparation and incubation with BioDFactor™. Placentas were collected from Shands Hospital at the University of Florida (Gainesville, FL). HUVs were then dissected using an automated method as previously described[7]. Dissected HUV samples were decellularized in a 1% SDS (Thermo Scientific, Rockford, IL) solution with PBS to obtain a solvent/tissue mass of 20:1 (w:v). Samples were decellularized on an orbital shaker plate at 100 rpm for 24 hours and then rinsed with PBS prior to incubation overnight at 37˚C in a 70 U/mL DNase I solution (Sigma-Aldrich, St. Louis, MO) in PBS. Samples were then terminally sterilized using a 0.2% peracetic acid/ 4% ethanol (Sigma-Aldrich, St. Louis, MO) solution for 2 hours and finally pH balanced (7.4) using multiple PBS washes. Following decellularization, scaffolds were mounted onto 4 mm diameter silicon tubing which was inserted through the lumen, prefrozen to - 85 C, and then lyophilized using a Millrock bench top manifold freeze dryer (Kingston, NY) for 24 hours at -85 C under 10 mT vacuum. Lyophilized and decellularized HUV scaffolds were then soaked (until complete saturation) for 2 hours in BioDFactor™ prior to immediate implantation.

Animal implant study. Male Sprague-Dawley rats (200 g) were purchased from Charles River Laboratories (Wilmington, MA, USA), and all procedures were approved by the University of Florida's IACUC protocol UF#201207728. Upon arrival, 6 month old rats were handled daily by laboratory technicians to reduce contact stress during procedures and allowed to acclimate for a period of 7 days. Scaffold were cut into 1.5 cm x 1.5 cm x 0.075 cm sections and incubated (independently) for 2 hours with either 2 mL BioDFactor™ or 2 mL PBS (control). Animals were anesthetized using isoflurane inhalation with 5% induction followed by 2% maintenance. While under anesthesia, rats were shaved and disinfected with 3 alternating preparations of Betadine (Stamford, CT) and 70% ethanol (for rinsing). Under sterile conditions, a 2 cm skin incision was made down the middle of the spine and a subcutaneous pockets were created by blunt preparation on the medial and lateral sides of the spine. Using forceps, one scaffold was inserted into each of the subcutaneous pockets (total 2 scaffolds per animal), after which the skin was closed with non-absorbable sutures (4-0 sutures; Coviden, Mansfield, MA). After 5 days implantation, animals were euthanized according to approved procedures, and samples removed for analysis.

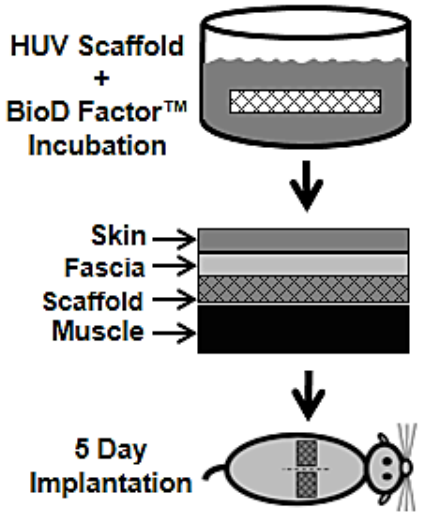

Figure 1: Procedural overview of animal implant study. Scaffolds were saturated in BioDFactor™ and allowed to incubate for 2 hours prior to implantation. After 5 days of implantation, scaffolds were removed from the rats ans analyzed for fibrous capsule formation, vascularization, and cell migration.

Capillary network formation within each scaffold was analyzed immediately after removal from the animal, fibrotic capsules were dissected with a scalpel and the HUV samples were placed onto glass slides. Top-down images of the 750 µm semi-translucent scaffolds were taken using an Imager M2 light microscope (Zeiss, Oberkochen, Germany) with an Axiocam HRm digital camera (Zeiss, Oberkochen, Germany). For histological analysis, tissue samples were embedded in Neg-50 frozen section medium, sectioned to 7 µm (Microm HM550 cryostat, Thermo Scientific, Waltham, MA) and stained using standard hematoxylin and eosin (Richard-Alan Scientific, Kalamazoo, MI) (H&E). Images obtained using the M2 Zeiss scope were used to assess and cell migration and remodeling.

RESULTS

Cytokine Profile Analysis. Chemokine analysis detected 13 cytokines within the composition of BioDFactor™ (Fig 2). Many osteogenesis related cytokines were identified including BMP-4 BMP-6, and CCL 23. Immune related cytokine were also detected including ENA-78 (CXCL 5), and ICAM-2. The most prevalent angiogenesis related cytokines were ACRP 30 and GRO. Neurogenesis related cytokines detected include ciliary neurotrophic factor (CNTF) and brain-derived neurotropic factor (BDNF). Additional detected cytokines include EGF (epidermal growth factor), IGFBP (Insulin-like growth factor binding protein) 1, IGFBP 2, and Oncostatin M. See Appendix I for description of individual functions of each detected cytokine.

A comparison between native tissue and BioDFactor™ reveals no statistical differences in cytokine composition. It is also probable that additional cytokines, not part of this assay, were also present. Additionally, it is possible that some of the cytokines from our assay were not detected due the sensitivity of our assay, but may be present at extremely low concentrations or remain bound to ECM particles within the solution and as such did not bind to the immunoassay.

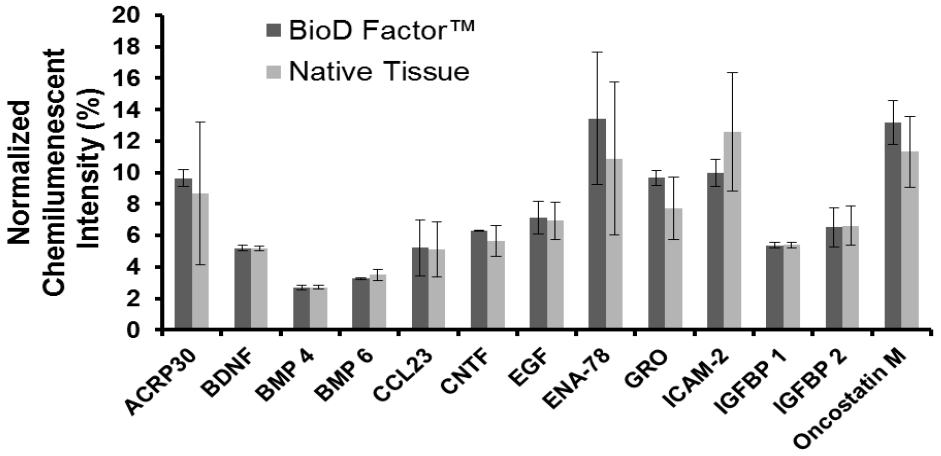

Figure 2: Comparison of the cytokine profiles of BioDFactor™ and Native Placenta Tissue. Cytokines related to angiogenesis, immune regulation, neurogenesis, and osteogenesis were detected. Compared to native amniotic tissue, BioDFactor™ did not have a statistically different cytokine composition. n=3.

In vivo cell migration, immune response and angiogenesis induction. After 5 days implantation (Figure 3a), HUV scaffolds incubated in BioDFactor™ had significantly less fibrosis encapsulation in comparison to control scaffolds (Figure 3b). Upon surgical removal of the scaffolds, BioDFactor™ incubated scaffolds were loosely connected to the surrounding fascia and capillaries, and their removal was simplified by the lack of fibrous encapsulation in contrast to controls that were difficult to remove due to the formation of a thick fibrous capsule. No significant differences in vascularization were observed between control and BioDFactor™ incubated scaffolds, and early stage capillary formation

was observed in both sample groups (Figure 3c, 3d). H&E stained sections show cells to have migrated throughout scaffolds infused with BioDFactor™ compared to control samples (Figure 3e, 3f) with cells limited to the scaffolds periphery.

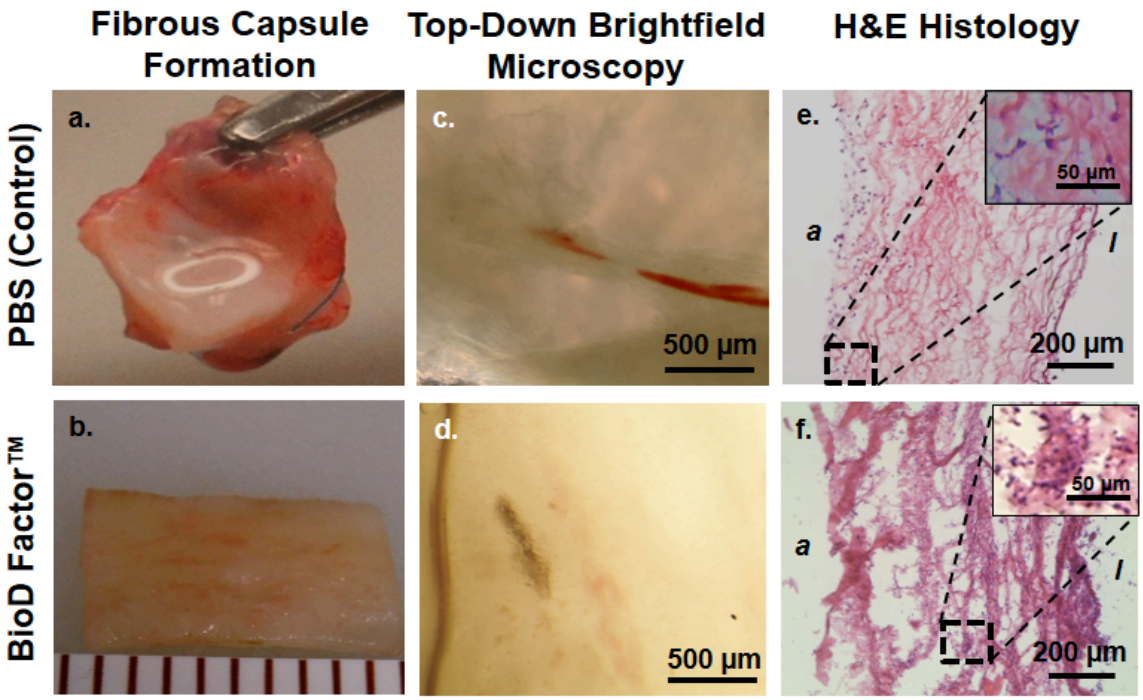

Figure 3: Fibrous Capsule Formation, Bright-Field Microscopy, and Histological Analysis of BioDFactor™ incubated human umbilical vein tissue scaffolds **a**fter 5 days of implantation. Scaffolds incubated in BioDFactor™ had reduced fibrous capsule formation in comparison to controls (**a**,**b**). Although no significant differences were seen in vascularization in comparison to controls (**c**, **d**), BioDFactor™ incubated scaffolds had improved cell migration with cells migrating completely across the scaffold from the ablumen (indicated by '***a***') to the lumen (indicated by '***l***') (**e**, **f**); additionally they had higher cell densities. n=3.

Cellular remodeling of the tissue scaffold structure also appeared to be increased with BioDFactor™ incubated scaffolds having a more defined collagen ECM fibrous structure in zones where cells had migrated into the scaffold. In addition to increased cell migration and scaffold remodeling, BioDFactor™ incubated scaffolds also had higher cell densities through each scaffolds regions of cell migration. Overall, the results from this *in vivo* investigation suggest an important role for placenta derived products in the development of clinically applicable engineered organs because of their unique ability to provide immuno-supressive qualities while increasing the rate of cell-repopulation and ultimately scaffold remodeling.

DISCUSSION AND CONCLUSION

Placenta derived products have immense potential in the fields of wound healing, tissue engineering and regenerative medicine due to their increasingly well known immunoregulatory and healing properties. These studies revealed that incubation of a 3D engineered tissue scaffold in an amnion-derived viable tissue matrix (BioDFactor™) improves *in vivo* integration and reduces the immune reaction when implanted in a rat model. Specifically, BioDFactor™ incubated scaffolds had decreased fibrous capsule formation and increased cell migration and scaffold remodeling in comparison to controls. It is hypothesized that key to this response is the unique composition and combination of physiologically relevant cytokines present within BioDFactor™.

Analysis of BioDFactor™ identified the presence of cytokines associated with osteogenesis (BMP-4, BMP-6, CCL23), angiogenesis (ACRP30, GRO), neurogenesis (CNTF, BDNF) and immune regulation (ENA-78, ICAM-2). The osteogenic cytokines present within BioDFactor™ indicate that it may be useful in bone tissue regeneration applications. BMP-4 is known to induce *in vivo* endochondral osteogenesis[8], and BMP-6 is known to induce numerous osteogenic markers in mesenchymal stem cells[9]. The immunoreductive properties of BioDFactor™ may be strongly related to the presence of ENA-78 and ICAM-2. ENA-78 has been found to reduce sensitivity to pain and is produced following the simulation of cells with inflammatory cytokines IL-1 and TNF-α[10]. I-CAM 2 has been shown to mediate interactions important for the antigen-specific immune response, NK-cell mediated clearance, lymphocyte recirculation, and other cellular interactions important for immune responses[11]. Interestingly, Oncostatin M which has been shown to be a cell growth regulator that inhibits the replication of A375 melanoma and other human tumor cells, but not normal human fibroblasts cells was also detected[12]. This may have important implications with cancer treatment.

The unique combination of cytokines detected within BioD Factor™ is best understood in terms of its derivation from the human amnion. The amnion is derived from 2 germ layers, the medodermal on the outer side and the ectodermal on the inner side[13]. The mesodermal germ layer is the precursor to the formation of bone, cartilage, muscle, the urogenital system, and the blood system, while the ectodermal germ layer is the precursor to the formation of the nervous system, hair, eye lenses, and the skin[13]. A comparison between the cytokine compositions of BioDFactor™ and native human amniotic tissue did not reveal any significant differences, and thus we hypothesize that the presence of osteogenesis, angiogenesis, neurogenesis, and immune related cytokines in this product is the result of its derivation from the mesodermal and ectodermal germ layers.

In summary, these studies highlight the potential of combining placental based products for clinical use in a wide range of applications from wound healing, regenerative medicine, and tissue engineering fields.

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APPENDIX I

DESCRIPTION OF CYTOKINES DETECTED IN BIOD FACTOR™

Table A. Cytokine symbol, name, and function

Cytokine Symbol	Full Name	Function
ACRP30	Adiponectin	Stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells.[14]
BDNF	Brain-derived neurotropic factor	Promotes endothelial cell survival and induces neoangiogenesis. Activity restricted to central arteries, vessels of cardiac and skeletal muscle, and skin. Activates TrkB receptor and recruits bone marrow-derived cells.[15]
BMP-4	Bone morphogenic protein 4	Involved in bone and cartilage development. Plays an important roles in the onset on endochondral bone formation.[8]
BMP-6	Bone morphogenic protein 6	An autocrine stimulator of chondrocyte differentiation that has been shown to enhance chondrogenesis in a human marrow stromal cell line.[9]
CCL23	Chemokine (C-C motif) ligand 23	Chemotactic for human osteoclast precursors and is expressed in bone tissues.[16] It has been shown to induce endothelial cell migration and promotes angiogenesis.[17]
CNTF	Ciliary neurotropic factor	A nerve growth factor that is a potent survival factor for neurons and oligodendrocytes. It may help to reduce tissue destruction during inflammatory attacks.[18]
EGF	Epidermal growth factor	Growth factor that stimulates cell growth, proliferation, survival and differentiation by binding to EGF-receptor.[19]
ENA-78	Also known as C-X-C motif chemokine 5	Activates neutrophils during acute inflamatory responses. May plays a role in reducing sensitivity to pain.[10]
GRO	Chemokine (C-X-C motif) ligand 3	Regulator of angiogenesis activity, but also has been found to be pivotal in thrombin-induced angiogenesis.[20]
ICAM-2	Intracellular adhesion molecule 2	Mediates interactions important for antigen-specific immune response, NK-cell mediated clearance, lymphocyte recirculation, and other cellular interactions important for immune responses.[11]
IGFBP 1	Insulin-like growth factor binding protein 1	Aids in modulation of blood glucose levels.[21]
IGFBP 2	Insulin-like growth factor binding protein 2	Believed to regulate growth and development in the placenta in an autocrine/paracrine manner. [22]
Oncostatin M	Oncostatin M	A cell growth regulator that inhibits the replication of A375 melanoma and other human tumor cells, but not normal human fibroblasts cells.[12]