

A Unique Human Tissue-Based Angiogenesis Assay

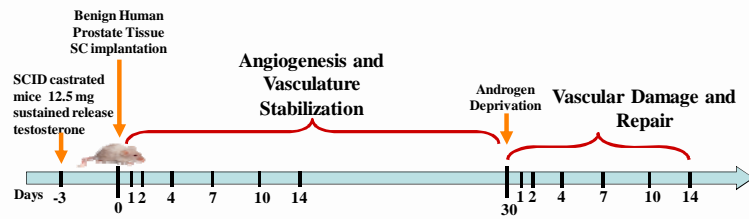
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ABSTRACT

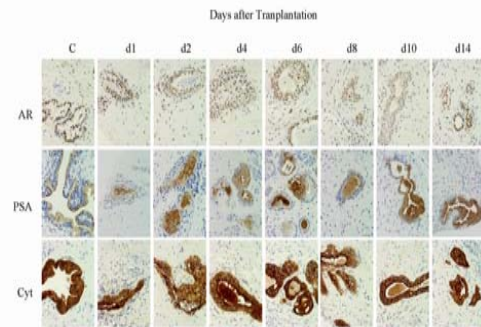
AndroBioSys, Inc. (ABS) has developed a unique pre-clinical model for analysis of anti- and pro-angiogenic activity in human endothelial cells within an intact human tissue microenvironment, termed the *AngioGraft™ Assay*. The model system utilizes primary xenografts of freshly resected human prostate tissue (benign and cancer) that re-vascularizes with micro-vessels of human, not host, origin. The human vasculature of the primary xenografts undergoes a wave of angiogenesis shortly after implantation (Days 6-14), resulting in a 5-10-fold increase in the microvessel density (MVD) of small caliber vessels. Castration results in rapid apoptotic death of the human prostate endothelial cells, followed by complete recovery of MVD by Day 14 after castration. We have demonstrated that human prostate endothelial cells express androgen receptor (AR) protein, AR trans-activates of androgen-regulated gene expression, and that primary cultures of human prostate endothelial cells require exogenous androgen stimulation for survival and growth and undergo apoptosis upon removal of androgens. Each angiogenic response is preceded by a transient wave of expression of VEGF in the xenografts. The 2-tiered *AngioGraft™ Assay* was developed based on the characterization of the human prostate primary xenograft model. The first tier of the *AngioGraft™ Assay* evaluates apparently true angiogenesis by human vessels occurring in an intact human tissue microenvironment during the initial 2 weeks following subcutaneous implantation of the human tissue in immuno-compromised mouse hosts. The second tier evaluates the recovery of a selectively damaged vascular network, potentially modeling the dynamics of the remodeling vasculature of a tumor or damaged tissue. AndroBioSys uses this unique model to provide testing services for clients to study the efficacy of lead agents that promote or inhibit angiogenesis.

METHODS

The *AngioGraft™ Assay* system: Host immuno-compromised mouse hosts are implanted with a slow release testosterone pellet 3-7 days before subcutaneous transplantation with fresh human tissue. Analysis of pro- or anti-angiogenic effects of a lead compound is performed over the initial 14 days after transplantation, during which there is a burst of angiogenesis by the human endothelial cells resulting in a 5-10 fold increase in MVD; MVD stabilizes by 2 weeks after implantation. Host mice are implanted with 8-10 pieces of tissue from 1 or multiple donors. Analysis of anti-endothelial cell effects of a lead compound in a stable vascular network is performed at any time after 3 weeks post-implantation. As a model, androgen deprivation (castration of the host mouse) induces acute endothelial cell involution that can be monitored over 14 days after castration.



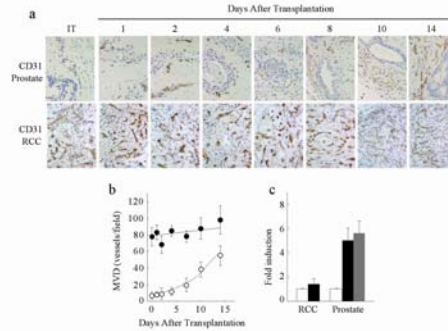
Over the initial 14 days after implantation, the tissue architecture of xenografts of benign prostate or prostate cancer (CaP) is maintained. Furthermore, expression of androgen receptor (AR), prostate-specific antigen (PSA) and epithelial cell specific cytokeratin (Cyt) is maintained in the epithelial cell compartment of xenografts. The success rate of establishment of individual xenografts exceeds 90%, and necrosis is not observed within the xenografts.



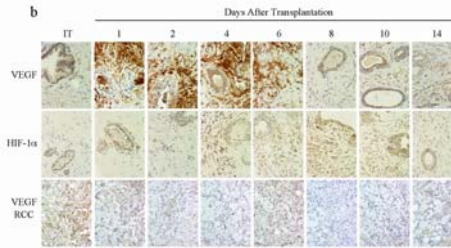
RESULTS

AngioGraft™ Model of Human Angiogenesis in an Intact Human Tissue Microenvironment

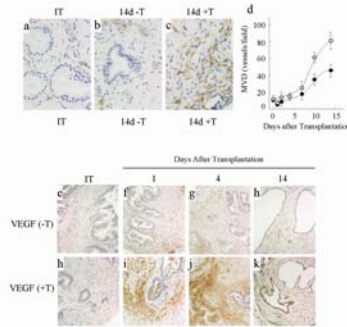
The human vasculature of primary xenografts of human prostate tissue undergoes a dramatic burst of angiogenesis in the 14 days after transplantation (Panel a). In contrast, the human vasculature of primary xenografts of human kidney tissue or renal cell carcinoma (RCC) do not undergo angiogenesis. The MVD in prostate xenografts increases 5-10 fold relative to the tissue before transplantation while the vasculature of RCC does not change (Panel b and c). Panel c demonstrates the increase in MVD in primary xenografts of benign prostate (black bar) or CaP (grey bar).



The increase in MVD begins on Days 5-6 post-transplantation, and has plateaued by Day 14. The wave of angiogenesis marked by increased MVD is preceded by marked up-regulation of expression of vascular endothelial growth factor (VEGF), that peaks on Day 2 post-transplantation. In contrast, expression of HIF-1 α did not peak until Day 8, concurrent with the wave of angiogenesis. VEGF expression was not up-regulated in xenografts of RCC.

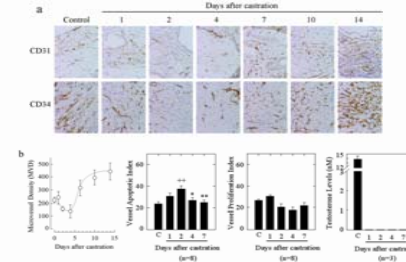


VEGF expression in primary xenografts of human prostate tissue is regulated by the androgen axis. Panels b and c demonstrate the MVD in prostate xenografts transplanted to mouse hosts without (b) and with (c) testosterone pellets. Panel d is the quantitation of MVD in hosts with (open circles) and without (closed circles) testosterone pellets. Panels e-k demonstrate that the wave of VEGF expression occurs in primary xenografts transplanted to hosts implanted with testosterone pellets, but is not apparent in xenografts transplanted to hosts in the absence of testosterone supplementation.

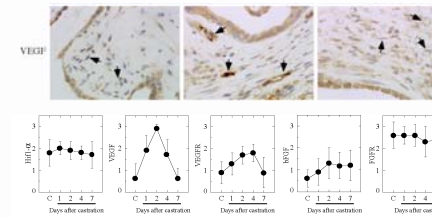


AngioGraft™ Model of Anti-Angiogenic Effects on an Intact Human Vascular Network

Androgen deprivation (castration) provides a model for candidate anti-angiogenic agents since castration induces acute apoptotic death of human prostate endothelial cells, with the apparent MVD reaching a nadir on Days 2-4 post-castration, but recovering to the original MVD by Day 7, and exceeding the original MVD by Day 14 post-castration (Panel b). The nadir of MVD is concurrent with the time of peak apoptotic death, measured by immunostaining for Caspase-3 activation.

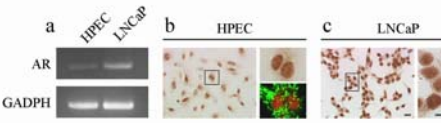


Recovery of MVD in the primary xenografts of human prostate tissue was preceded by up-regulation of expression of potent pro-angiogenic peptides/cytokines in the human endothelial cells. VEGF expression by the endothelial cells peaked on Day 2, and rapidly returned to pre-castration levels by the time the MVD had recovered. Expression of VEGFR and bFGF also were markedly up-regulated in response to castration, with expression peaking before the recovery of MVD.

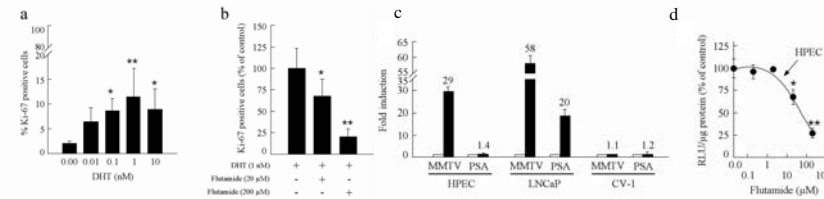


Primary Cultures of Human Prostate Endothelial Cells

Human endothelial cells in clinical specimens of benign prostate and CaP, and primary xenografts of benign prostate and CaP, express the androgen receptor (AR). Primary cultures of human prostate endothelial cells (HPEC) established from fresh surgical specimens express AR at a level of approximately 15% that of LNCaP cells (Panel a). AR is localized to the nucleus when cultured in the presence of testosterone in the media (Panel b); the colored inset demonstrates colocalization of AR (red) and vWF (green) in human prostate endothelial cells.

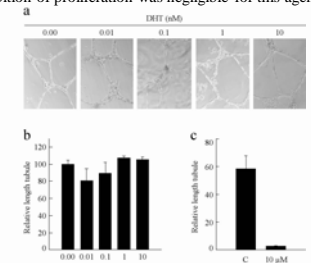


Proliferation of primary cultures of human prostate endothelial cells depends on androgen stimulation, and proliferation is inhibited by the inhibitor of AR function, flutamide (Panel a and b). Transfection of primary cultures with luciferase reporter constructs driven by a promiscuous AR-transactivated promoter (MMTV) or a prostate epithelial cell specific AR-transactivated promoter (PSA) demonstrated AR-mediated gene transcription in HPEC, but not of the PSA-promoter driven reporter (Panel c). The MMTV promoter can be transactivated by glucocorticoids or progesterone. Panel d demonstrates that the transactivation in prostate endothelial cells is inhibited by flutamide, which confirms that the effect is AR-mediated.

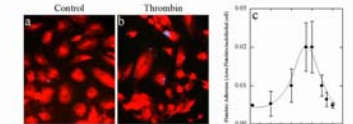


Application of the AngioGraft™ Model for Evaluation of Induced Vascular Damage

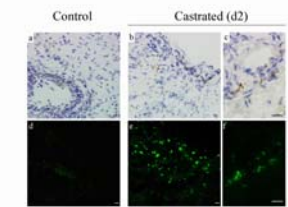
a. Inhibition of endothelial tube formation in Matrigel. Primary cultures of human prostate endothelial cells (HPEC) perform comparably with the standard test cell line, HUVEC (human umbilical vein endothelial cell) in the Matrigel Tube Formation Assay. Endothelial cell tube formation by HPEC was not affected across the dose-range that inhibited proliferation (Panel a and b), suggesting independent mechanisms of regulation. In contrast, a proprietary candidate inhibitor of angiogenesis reduced endothelial tube formation in a dose responsive manner, reducing tube formation by >90% at a 10 μ M exposure level (Panel c). Over the treatment regimen, toxicity and inhibition of proliferation was negligible for this agent.



b. Damage of endothelial cells in stable vascular networks. Damaged endothelial cells represent a natural substrate for the binding of platelets. Platelet adhesion to the apoptotic prostate endothelial cells in primary culture and *in vivo* in primary xenografts of human prostate tissue would validate selective damage to the vascular network. Panels a-c demonstrate the adhesion of fluorescently labeled, freeze-dried human platelets (Entegron, Inc., Chapel Hill, NC) to primary cultures of prostate endothelial cells activated by exposure to thrombin. Maximal endothelial activation measured by platelet adhesion was induced by exposure to 0.5-1.0 U/ml of thrombin.



Fluorescently labeled, freeze-dried human platelets administered by tail vein injection rarely adhered to endothelial surfaces in intact human prostate xenografts (control). However, in xenografts exposed to platelets on Day 2-3 after castration of the mouse host, significant levels of platelet adhesion was observed. The time of maximal platelet adhesion was concurrent with the time of maximal levels of leakage of fibrin/fibrinogen into the interstitial tissue space, suggesting compromise of the endothelial cell vascular barrier.



SUMMARY

- The *AngioGraft™ Model* provides a pre-clinical human endothelial cell-based assay of anti- and pro-angiogenic effects in an intact human tissue microenvironment.
- Pro-/anti-angiogenic effects can be evaluated in primary xenografts of human prostate tissue during the initial 14 days after transplantation during which the MVD increases 5-10 fold.
- Anti-vascular stability/remodeling effects can be evaluated in primary xenografts of human prostate tissue beyond 3 weeks after transplantation when the vascular network has stabilized.
- Androgen deprivation induces acute apoptotic death of the human endothelial cells in primary xenografts of human prostate tissue providing a reproducible control for studies of induced vascular damage.
- Up-regulation of VEGF expression precedes increased MVD in both components of the model, providing a test system to evaluate treatment modalities targeting VEGF/VEGFR.
- Selective platelet binding to the labilized endothelial surface in the human prostate xenografts provides a useful model for testing targeted imaging and therapeutic delivery modalities.