Optimized iron-oxide labeling and MR parameters for endothelial cell tracking in a non-invasive angiogenesis assay

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Introduction

Angiogenesis, the new blood vessel formation through the recruitment of endothelial cells, is a critical factor in providing nutrients and oxygen to a solid tumor, and is required for tumor growth and metastasis. Neovasculature also provides a conduit through which anticancer drugs may be delivered to the tumor. Therefore, the effective evaluation of the role of angiogenesis in cancer, as well as the establishment of suitable antiangiogenic therapy, necessitates a thorough understanding of endothelial cell response to chemoattractants, angiogenic growth/inhibition factors, and antiangiogenic drugs. We have developed an assay to track endothelial cell motility, invasive potential and network response in the presence and absence of cancer cells. The assay comprised MDA-MB-231 breast cancer cells, extracellular matrix (ECM) gel, and Human Vascular Endothelial Cells (HUVECs), to mimic the solid tumor environment. HUVECs were labeled with the superparamagnetic iron oxide T_2 contrast agent, Feridex®, and their motility and network structure was tracked over time using MRI. Our assay observed the response of HUVECs to paracrine factors secreted by MDA-MB-231 cancer cells, concomitantly observing HUVEC motility and network formation.

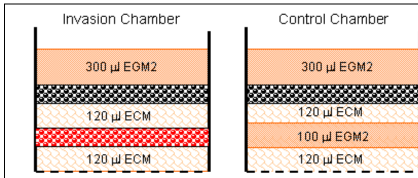

Fig. 1: Schematic of invasion chambers: 1) 120 µl of ECM gel was added, with 30 min incubation for polymerization, 2) 1.5 x 10^5 MDA-MB-231 cells were seeded in 100 µl EGM-2 on the surface of the ECM gel, with 4 h incubation for attachment; 100 µl EGM-2 was added to the control chambers, 3) 120 µl of ECM gel was added, with 30 min incubation for polymerization, and 4) 1.5 x 10^5 labeled HUVECs were seeded, with 24 h incubation for attachment and formation of lumen-like structures.

Methods

Cell culture and maintenance: MDA-MB-231 breast cancer cells were cultured in RPMI 1640 supplemented with 9 % fetal bovine serum, 90 U/ml Penicillin, and 90 µg/ml Streptomycin. HUVECs (Clonetics, USA), and maintained in EGM-2 (Clonetics, USA). ***HUVEC labeling:*** HUVECs were incubated with either 2 µg or 9 µg Feridex® per ml of EGM-2 and 0.028 µg/ml or 0.125 µg/ml Poly-L-lysine, respectively, for 24 h prior to seeding on ECM gel. *Chamber preparation*: Invasion chambers were housed in non-tissue-culture Millipore inserts. The cells were seeded and the ECM gel was added as detailed in Fig. 1. *MRI*: All MR experiments were performed on an 11.7 T Bruker Avance spectrometer with triple-axis gradients. T_2-weighted spin-echo MR images of the chambers were obtained in the axial and the coronal direction, with imaging parameters as detailed in Figs. 2. The chambers were imaged 24 h after HUVEC seeding, and in two 48 h increments thereafter. *Immunohistochemistry:* HUVEC presence and network structure were observed by phase contrast light microscopy, Prussian blue staining (indicating the presence of iron), and by the endothelial specific fluorescence monoclonal antibody, CD31.

Results

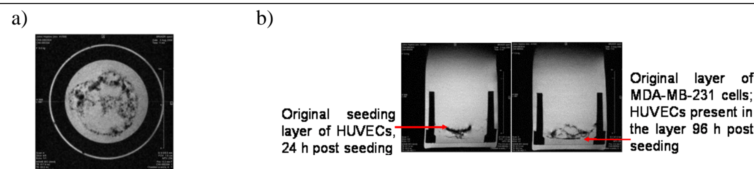

Fig. 2: a) A representative axial image of a chamber containing Feridex®-labeled HUVEC, showing detailed network-like structure 24 h post seeding. The T_2-weighted spin echo MR image was obtained with a single acquisition over a 0.5 mm slice, 1.6 cm field of view, 256 x 256 acquisition matrix, TR = 857 ms and TE = 90 ms. The axial MR images were binarized and the fractional area of HUVECs (areas of hypointensity) was calculated by applying adaptive thresholding b) Coronal images show HUVECs in the upper seed layer 24 h post seeding, whereas an increased presence of HUVECs is observed in the MDA-MB-231 cancer cell layer 96 h post seeding. No invasion was observed in control chambers (not shown).

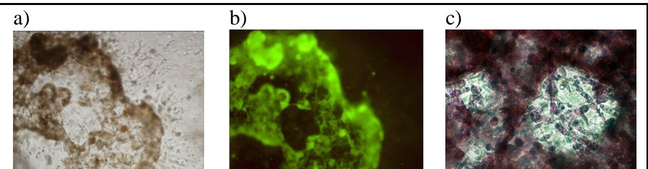

Fig. 3: HUVECs were detected in the MDA-MB-231 cancer cell layer (area of sharp focus) 96 h post seeding, as seen by a) phase contrast micrography, b) fluorescence microscopy with the endothelial cell specific antibody, CD31, and c) Prussian blue staining for the presence of iron.

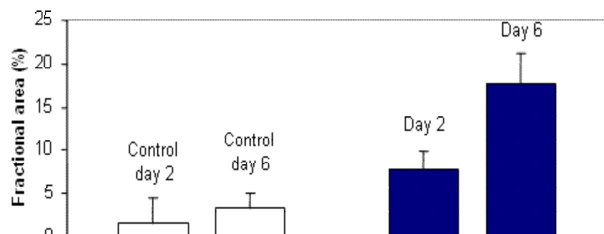

Fig. 4: This graph shows a significantly increasing fractional area of HUVECs in the MDA-MB-231 cancer cell layer, from day 2 to day 4 to day 6 ($p < 0.01$), indicating the invasion and migration of HUVECs through the ECM gel toward the cancer cells (n = 6). No significant invasion or migration was observed in the control chambers (n = 3). The fractional area of HUVECs in the cancer cell layer was significantly higher than in a corresponding layer in control chambers, for both time points ($p < 0.01$).

Discussion and Conclusion

We characterized HUVEC motility, invasive potential, and network formation in the presence and absence of cancer cells. This non-invasive assay can be used to study endothelial cell and network response to various growth and inhibition factors involved in the angiogenic process. The assay can also be used to study endothelial response to synergistic effects of various competing pro- and anti-angiogenic factors.

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