Stroma fibroblasts are recruited systemically to contribute to the tumor angiogenic rim: Cell tracking by MRI and two photon microscopy

D. Granot[1], Y. Addadi[1], V. Kalchenko[2], and M. Neeman[1]

[1]Biological Regulation, Weizmann Institute of Science, Rehovot, Israel, [2]veterinary Resources, Weizmann Institute of Science, Rehovot, Israel

Introduction

The involvement of tumor microenvironment in the biology of tumorigenesis and tumor angiogenesis is widely recognized. However, the precise role of the invading fibroblasts as key modulators in the process is yet to be studied. Interactions that occur between fibroblasts and endothelial cells were demonstrated for *in-vitro* tumor models (1, 2), and the recruitment of bone marrow derived fibroblasts into tumors was shown *in-vivo* in a mouse model (3). We previously reported that myofibroblast infiltration into implanted ovarian carcinoma spheroids marked the tumor exit from dormancy (4) and that these cells contributed to vascular stabilization in ovarian tumors by expression of angiopoietin 1 and 2 (5). However, gaining deeper knowledge regarding their interactions with the endothelium during tumor angiogenesis requires specific *in-vivo* monitoring. We have previously shown that fibroblasts labeled *in-vitro* with biotin-BSA-GdDTPA are detectable *in-vivo* by MRI (6). Additionally, we recently demonstrated the prolonged *in-vivo* MR visibility of these biotin-BSA-GdDTPA labeled fibroblasts and detected their recruitment into subcutaneous ovarian tumor (7). In the work presented here, the pattern by which intraperitonealy administered fibroblasts are recruited into subcutaneous tumors was evaluated. Thus, we used MRI for detection of the migration track of the recruited cells using SPIO labeled fibroblasts and applied two-photon microscopy (2PM) for high resolution analysis of the distribution of the recruited fibroblasts. Three color 2PM was performed on excised tumors using labeled fibroblasts and tumor cells and a fluorescent blood pool marker. MRI studies were performed using fibroblasts pre-labeled with SPIO (Feridex) and biotin-BSA-GdDTPA as a blood pool marker. Here we show that the recruited fibroblasts were primarily distributed at the tumor-host interface, coinciding with tortuous tumor neovasculature. Furthermore, MRI study of SPIO labeled fibroblasts revealed labeled cells at the tumor rim and additionally along the femoral vein suggesting the possible migration track for recruitment of the cells from the peritoneum to the tumor.

Material and Methods: Cell labeling: For MRI CV-1 fibroblasts were labeled *in-vitro* with 0.73 mg/ml Feridex (Advanced Magnetics, Cambridge, MA). Labeling was terminated after 48 hours by 4 washes in serum free medium. For 2PM cells were stained with 7.5 μg /ml CM-DiI (Molecular Probes).

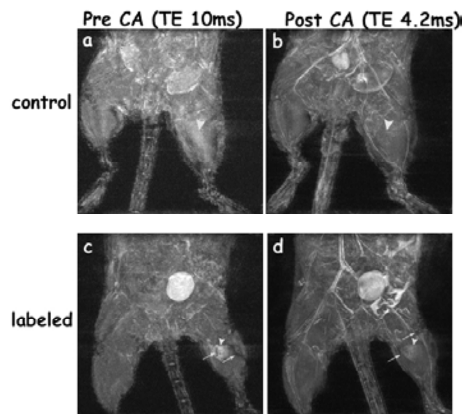
In-vivo model: Subcutaneous ovarian tumors were initiated in the hind limb of CD-1 nude mice by inoculation of human epithelial ovarian carcinoma MLS cells. After a tumor was establised, labeled or unlabeled control fibroblasts were injected into the peritoneum of tumor bearing mice. Mice were studied 2-7 days after fibroblasts administration. For 2PM, MLS tumor cells were pre-stained with 7.5 μg /ml DiD or 5 μM CFSE (Molecular Probes) prior to inoculation.

MRI study was performed on a horizontal 4.7 T Bruker Biospec spectrometer using a whole-body birdcage transmission coil. T2*-weighted 3D gradient-echo (GE) images were acquired with pulse flip angle of 15°; TR 13.69 ms; TE 10 ms; 4 averages; spectral width 50,000 Hz; FOV 4X4X2 cm, 128X128x64 pixels, zero filled to 256X256X256; total acquisition time per frame 448 s. Blood vessels were visualized by intravenous injection of biotin-BSA-GdDTPA into the tail vein via a catheter and contrast enhanced MRI was performed using 3D_GE as described above, with the exception of TE 4.227 ms.

Two-photon microscopy (2PM): Excised tumors were placed in glycerol and imaged by two-photon microscopy, Zeiss LSM 510 META NLO, Germany equipped with Mai Tai One Box Ti:Sapphire Tunable Laser from Spectraphysics, USA, for two photon excitation. Images were acquired from the surface as well as from the inner parts of the tumor. For visualization of blood vessels 550KDa dextran-FITC was injected into the tail vein prior to sacrifice of the mice.

Results: Recruitment and distribution of labeled cells within the tumor was monitored *in-vivo* by T2*-weighted 3D_GE MRI and *ex-vivo* by two-photon microscopy of excised tumors. T2*-weighted images revealed the presence of SPIO labeled fibroblasts within the tumor (figure 1A panels c and d). Recruited cells were mostly detected in slices corresponding to the surface of the tumor, localizing the fibroblasts at the rim of the tumor. The presented maximal intensity projections (MIPs) additionally suggested a migration track of SPIO labeled fibroblasts, that is parallel to blood vessels (figure 1A panel d). 2PM of excised tumors revealed recruited fibroblasts primarily at the tumor- host interface co-localizing with tumor neovasculature (figure 1B, panel A). The inner of the tumor was devoid of recruited fibroblasts (figure 1B, panels A and C insets, D).

A



B

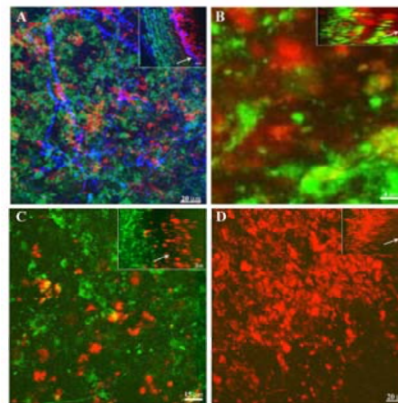


Figure 1. **Distribution of recruited fibroblast in subcutaneous MLS tumors. A)** Maximal intensity projections of 3D_GE images of mice two days after intraperitoneal injection of control or SPIO labeled fibroblasts. Presented are pre- and post contrast MIPs. Arrowheads correspond to tumor sites, arrows indicate SPIO labeled cells / track. **B)** Two photon microscopy of excised subcutaneous fluorescently labeled MLS tumors. MLS cells were labeled with DiD (red; A-D), fibroblasts (green: A-D) were labeled with CM-DiI (A,B) or with CFSE (C,D), blood vessels were visualized by iv injection of dexstran-FITC (blue; A). Insets) ZY projection (arrows point towards the tumor center). Images were acquired from the tumor rim (A, C) and center (B, D). Panels A, C and D: MLS tumors were initiated prior to intraperitoneal injection of labeled fibroblasts, panel B: MLS tumor cells were co-inoculated with labeled fibroblasts.

Conclusions: This study shows that fibroblasts which were recruited from the peritoneum into subcutaneous tumors located at the hind limb, localized at the rim of the tumor, coinciding with tortuous tumor capillaries, thus suggesting a role for fibroblasts in tumor neovascularization. Moreover, *in-vivo* MRI of SPIO labeled fibroblasts, suggested a migration path along the primary blood vessels.

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