Multiphoton Tomography Visualizes Collagen Fibers in the Tumor Microenvironment That Maintain Cancer–Cell Anchorage and Shape

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ABSTRACT

Second harmonic generation (SHG) multiphoton imaging can visualize fibrillar collagen in tissues. SHG has previously shown that fibrillar collagen is altered in various types of cancer. In the present study, in vivo high resolution SHG multi-photon tomography in living mice was used to study the relationship between cancer cells and intratumor collagen fibrils. Using green fluorescent protein (GFP) to visualize cancer cells and SHG to image collagen, we demonstrated that collagen fibrils provide a scaffold for cancer cells to align themselves and acquire optimal shape. These results suggest a new paradigm for a stromal element of tumors: their role in maintaining anchorage and shape of cancer cells that may enable them to proliferate. J. Cell. Biochem. 114: 99–102, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: MULTIPHOTON IMAGING; TOMOGRAPHY; SECOND HARMONIC GENERATION; CANCER CELLS; GFP; COLLAGEN; TUMORS

Two-photon imaging has enabled collagen structures to be visualized in tissue by second harmonic generation (SHG) [So et al., 1998; Brown et al., 2003; König and Riemann, 2003]. For example, Provenzano et al. [2006, 2009ab] and Campagnola [2011] showed, with two-photon imaging and SHG, that increased collagen density of breast tissue is related to the formation of mammary cancer. Friedl et al. [2012] showed in vitro that disseminating breast cancer cells preferentially invade along bundled collagen. Chen et al. [2009] showed high regularity of collagen fibrils/fibers in ovarian cancers, suggesting the assembly of newly-synesized collagen during growth. However, none of these studies investigated the relationship of cancer cells and collagen fibrils in living mice in real time.

In vivo multiphoton tomography, based on nonlinear detection of endogenous fluorophores and fluorescent proteins and SHG imaging of collagen, provide the possibility of monitoring cancer cell behavior within three dimensional extracellular matrixes in live mice in real time. Deep-tissue 3D imaging is achievable due to the low absorption and scattering coefficients of near infrared (NIR)

light in the spectral range of 700–1,200 nm. NIR light is less harmful for the tissue and cells due to the absence of significant one-photon absorbers and due to limited sub-femtoliter fluorescence excitation volume [König, 2008].

In the present study, high-resolution in vivo multiphoton SHG tomography of the tumor environment in live mice implanted with green fluorescent protein (GFP)-expressing colon cancer cells, demonstrated that collagen structures provide for cancer-cell anchorage and shape that may enable them to proliferate.

MATERIALS AND METHODS

MULTIPHOTON TOMOGRAPHY

The MPT*flex*™ multiphoton tomograph (JenLab GmbH, Jena, Germany and MultiPhoton Laser Technologies, Inc., Irvine, CA) was equipped with a tunable 80 MHz titanium:sapphire femtosecond laser (710–920 nm). The optical unit consists of an active optical power attenuator to regulate the in situ power of the laser tissue depth penetration, an active beam-stabilization device, a safety unit

and a flexible articulated mirror-arm with its compact scan head. The scan head consists of a fast galvo-scanning device to generate 2D (XY) scans, a piezodriven z-scanner, and high NA focusing optics (NA 1.3). The optical arm is stabilized with a mechanical arm. The scan head also contains a dual-photon detector unit for the measurement of fluorescence and SHG. The overall field-of-view of the optical system covers $350 \times 350 \, \mu m^2$. The acquisition time for one optical section is typically 7 s. Low picojoule pulse energy was used for multiphoton excitation. The PMT1924 photodetector was used to detect signals from both fluorescence and SHG channels. Filter sets LP409 and BP 395/14 were used for GFP and SHG, respectively [Uchugonova et al., 2011].

CELLS

Mouse CT26-W7-GFP/Colo 26-GFP (mouse colon cancer) were used in this study. The cells were stably transfected with the GFP gene using previously-described techniques [Hoffman, 2005; Hoffman and Yang, 2006abc]. The cells were cultured in RPMI 1640
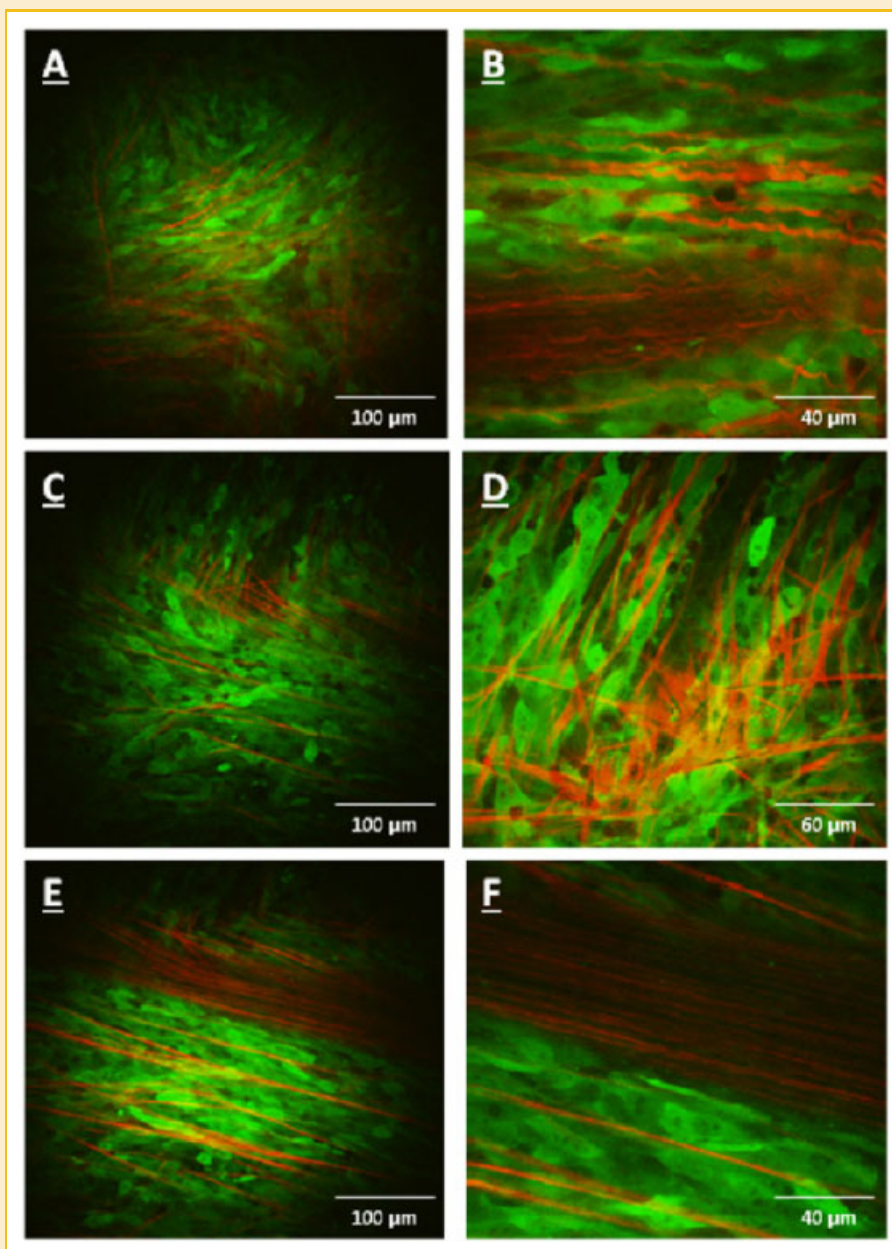

Fig. 1. Various locations of tumors in living mice were investigated with high-resolution multiphoton tomography (A, C, E). Images in panels (B, D, F) were taken under higher magnification to visualize single cells and collagen fibers. Cancer cells express GFP and fluoresce green. Collagen fibrils, visualized by SHG, are red. Cancer cells align parallel to collagen fibrils. Bundles of collagen fibers, varying in diameter up to several micrometers, are detected. The collagen fibrils are organized parallel and perpendicular to each other with a wide interfiber spacing. SHG of collagen and GFP were excited simultaneously at a wavelength of 790 nm. Filter sets LP409 and BP 395/14 were used for GFP and SHG, respectively.

supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator.

MICE

Colo26-GFP cells (2×10^6) were transplanted subcutaneously in 8-week-old non-transgenic nude mice bred at AntiCancer, Inc. (San Diego, CA). All animal studies were conducted in accordance with the principles of and procedures outlined in the NIH guide for the care and use of laboratory animals under assurance number A3873-1.

SKIN–FLAP WINDOWS

Cancer cells in the tumor microenvironment (TME) were visualized by multiphoton tomography after a skin flap was raised over the tumor. The animals were anesthetized with a ketamine-mixture of Ketaset and PromAce (both from Fort Dodge Laboratories, Fort Dodge, IA) and Xylazine HCl (American Animal Health, Wisner, NE). An arc-shaped incision was made in the skin, and s.c. connective tissue was separated to free the skin flap. The skin flap could be opened repeatedly to directly image the cancer cells and simply closed with a 6-0 suture [Yang et al., 2002].

RESULTS AND DISCUSSION

High resolution multiphoton fluorescence and SHG tomographic images of tumors were acquired from living mice with sub-cutaneously-grown Colo 26-GFP tumors. Bundles of collagen fibers, varying in diameter up to several micrometers, were detected. Modified collagen fibrils were observed to be organized parallel and perpendicular to each other with a wide interfiber spacing. Collagen SHG and cancer-cell GFP were excited simultaneously at a wavelength of 790 nm.

As can be seen in Figure 1, the cancer cells, which are readily visualized by GFP expression, are exquisitely aligned along the collagen fibrils, visualized by SHG. The cancer cells appear elongated.

The results suggest that collagen fibrils are essential for cancer cell proliferation. The cancer cells appear stretched as they align along the collagen fibrils. It has been known for a long time that cells can be anchorage dependent [Benecke et al., 1978; Farmer et al., 1978; Ben-Ze'ev et al., 1980; Berezovska et al., 2006; Nelson and Bissell, 2006; Ingber, 2008]. When deprived of their anchoring substrate, anchorage-dependent cells lose the ability to proliferate. The anchorage requirement appears related to the need for cells to acquire a specific shape in order to proliferate [Folkman and Greenspan, 1975; Folkman and Moscona, 1978]. Our results suggest that collagen fibrils provide the scaffolding for cancer cells to anchor and acquire optimal shape in vivo. Very highly malignant cells, such as those which grow as ascites, appear to lose their anchorage dependency.

The results of the present report develop a new paradigm for the collagen-fiber stromal element of tumors: their role in maintaining proper anchorage and shape for cancer cells that enable them to proliferate. Collagen fibers in the TME thus present a new visible target for cancer therapy using powerful two-photon imaging [Uchugonova et al., 2011; Rompolas et al., 2012].

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