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In vivo imaging of extracellular matrix remodeling by tumor-associated fibroblasts

Jean Y Perentes[1,2,3], **Trevor D McKee**[1,2,3], **Carsten D Ley**[1], **Hannah Mathiew**[1], **Michelle Dawson**[1], **Timothy P Padera**[1], **Lance L Munn**[1], **Rakesh K Jain**[1], and **Yves Boucher**[1]

[1] Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, 100 Blossom Street, Boston, Massachusetts 02114, USA

Abstract

Here we integrated multiphoton laser scanning microscopy and the registration of second harmonic generation images of collagen fibers to overcome difficulties in tracking stromal cell-matrix interactions for several days in live mice. We show that the matrix-modifying hormone relaxin increased tumor-associated fibroblast (TAF) interaction with collagen fibers by stimulating β_1-integrin activity, which is necessary for fiber remodeling by matrix metalloproteinases.

Our current understanding of extracellular matrix remodeling derives from *in vitro* experiments[1,2], which are difficult to interpret and relate to *in vivo* physiology. Multiphoton laser scanning microscopy and second harmonic generation (SHG) of fibrillar collagen allow visualization of the matrix of normal and tumor tissues *in vivo*[3]. Using these technologies, it is possible to study the real-time movement of cells through the collagen network on time scales of minutes to hours[4,5]. However, the slow rate of extracellular matrix remodeling makes it difficult to monitor matrix reorganization by stromal cells.

To track stromal cell collagen fiber interactions *in vivo*, we grew tumors (human sarcoma HSTS26T) in transparent dorsal skinfold chambers of immunodeficient mice expressing GFP under the control of the *Vegfa* promoter (VEGF-GFP mice). The transparent tumor chamber facilitated the tracking of the same cells and fibers for several days (Supplementary Methods online). Also, previous data in VEGF-GFP mice[6] and our immunostaining results suggest that peritumor GFP-positive cells are tumor-associated fibroblasts (TAFs) (Supplementary Fig. 1 online).

To induce collagen remodeling in a reliable fashion, we treated tumors with the small hormone relaxin, which causes matrix remodeling and increases tumor invasion and progression[7]. *In vitro*, relaxin enhanced the invasion of fibroblasts through increased collagen I degradation but did not affect the HSTS26T tumor cells (Supplementary Fig. 2 online). These data suggest that

Correspondence should be addressed to Y.B. (yves@steele.mgh.harvard.edu) or R.K.J. (jain@steele.mgh.harvard.edu).
[2]Present addresses: Department of Thoracic and Vascular Surgery, Centre Hospitalier Universitaire Vaudois, rue du Bugnon 46, 1010 Lausanne, Switzerland (J.Y.P.) and Department of Signaling Biology, Ontario Cancer Institute, 610 University Avenue, Toronto M5G 2M9, Canada (T.D.M.).
[3]These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS

J.Y.P. designed and performed experiments, analyzed data and wrote the paper. T.D.M. conceived the project, designed and performed experiments, analyzed data, and wrote the paper. C.D.L. performed imaging and immunostaining experiments. H.M. performed imaging and analyzed data. M.D. performed flow cytometry analysis. T.P.P. analyzed data and wrote the paper. L.L.M. analyzed data and wrote the paper. R.K.J. coordinated the project and wrote the paper. Y.B. coordinated the project, designed experiments, analyzed data and wrote the paper.

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HSTS26T cells implanted in VEGF-GFP transgenic mice could provide a model for studying stromal-specific extracellular matrix remodeling. Because the SHG signal attenuates with tissue depth, we were able to analyze collagen remodeling within 100 μm from the tumor surface (Supplementary Methods).

Temporal changes in tumors and the inability to place mice under the microscope in the same orientation between imaging sessions necessitated the development of a registration method to align image sequences. The SHG signal arising from fibrillar collagen provided a convenient registration landmark because of the relative stability and the wide distribution of collagen fibers in comparison with other potential landmarks, such as blood vessels or exogenous fluorescent beads. Blood vessels within tumors are porous to injected tracer particles, can have time-dependent perfusion fluctuations and are more sparsely distributed than the extracellular matrix, which forms a distinct network distributed throughout the tumor. Likewise, fluorescent beads pose the problem of potentially being unevenly distributed in the region of interest or being unstable because of photobleaching or cellular phagocytosis. We used an intensity-based registration approach (Turboreg)[8] to align subsequent imaged volumes with those at the initial time point (Supplementary Methods).

After image registration, we assessed fibrillar collagen changes. For all images and time points we measured mismatch in SHG signal, calculated by subtracting the maximum intensity projection at a registered time point from its corresponding image at day 1 (Supplementary Fig. 3 online). Mean SHG mismatch remained constant between day 1 and days 2, 3, 4 and 5 (Supplementary Fig. 4a online). The observed variation between time points was related to changes in specimen alignment (imperfect physical registration) and accumulation of biological changes (tissue growth and collagen reorganization). Mean mismatch values remained constant between control phosphate-buffered saline–treated and relaxin-treated tumors, even though it is well established that relaxin modifies fibrillar collagen structures[3] (Supplementary Fig. 4b). The inability to detect relaxin-induced changes may indicate that small modifications in fiber morphology may not affect SHG signal intensity, or that the intrinsic noise of the SHG signal is greater than changes in fiber structure. Thus, SHG-based registration offered two advantages: (i) it relied solely on an intrinsic signal that was widely distributed and therefore did not require the insertion of external landmarks, and (ii) alignment of registered volumes was stable for ~4 d and was not substantially influenced by tumor growth, collagen fiber formation or relaxin treatment.

To track TAF migration and recruitment in HSTS26T tumors, we imaged 4–8 regions per tumor every 24 h for 4 d. In some areas the number of TAFs increased, in others it decreased and in some no change occurred; relaxin did not affect stromal cell recruitment (data not shown). We evaluated the effect of relaxin on TAF migration by tracking selected cells (Supplementary Video 1 online). The fraction of migrating TAFs was significantly lower ($P < 0.05$) in relaxin-treated (37%, $n = 68$) tumors compared to control (57%, $n = 89$) tumors. We defined a migrating cell as one with displacement of more than 10 μm from day 1 to day 4 (Supplementary Methods). Although relaxin reduced the fraction of migrating cells, it significantly increased ($P < 0.01$) the speed of motile cells, from 11 ± 1.5 μm d^{-1} (range, 1–41 μm d^{-1}) in control to 21 ± 2 μm d^{-1} (range, 2–44 μm d^{-1}) in relaxin-treated tumors. Notably, the *in vivo* migration speed of TAFs is approximately one order of magnitude slower than fibroblast movement in collagen gels (5–100 μm h^{-1})[9,10].

A detailed analysis of single fibers indicated that TAFs could drag or push fibers during their migration; after the cells detached, fibers either returned to their initial (‘resting’) position or remained at their new conformation (Supplementary Video 2 online). In control tumors, the collagen network was stable, whereas relaxin induced dramatic and permanent changes in fiber morphology. The direct juxtaposition of TAFs with single fibers was often associated with

fiber buckling or loss of fiber continuity (gap formation). In some cases, TAFs remained close to a fiber for several days before we observed fiber buckling (Fig. 1 and Supplementary Video 3 online). Gap formation occurred in areas of cell-fiber interactions where fiber thinning could initially be observed, followed by loss of fibrillar material 1–2 d later (Supplementary Video 4 online). Notably, continuous TAF-fiber juxtaposition was not required for gap formation, as we observed cells temporarily interacting with fibers and moving on before the gap appeared.

Because bulk mismatch calculations were insensitive to detailed extracellular matrix changes induced by relaxin, we analyzed individual fibers. First, we measured the end-to-end length of randomly selected fibers (selected at day 1). Fiber length between day 1 and days 2, 3 and 4 significantly decreased (paired *t*-test $P < 0.05$) in the relaxin group but not in control tumors (Fig. 2a). Next we assessed fiber buckling and loss of fiber continuity (gap formation; see Supplementary Methods for how we interpreted the data to indicate buckling versus gap formation). In relaxin-treated mice, we observed fiber buckling and gap formation in 53% and 15% of analyzed fibers, respectively. Notably, 15% of fibers in control tumors developed buckles similar to those in relaxin-treated tumors, but fiber length was not affected. Moreover, in some cases, collagen fibers exhibited higher-order reorganizations, with fibers winding through the tissue in parallel groups, with evidence that relaxin caused buckling and condensation remodeling at this larger scale as well (Supplementary Video 5 online).

Because collagen fiber remodeling was associated with close juxtapositions between TAFs and fibers, we determined whether relaxin enhanced TAF-fiber overlap. We measured TAF-fiber overlap as the area of interaction between TAFs and fibers, in pixels, normalized to the length of the fiber, in micrometers. As a function of time, the overlap between cells and fibers increased significantly in relaxin but not in control tumors (Fig. 2b; paired *t*-test $P < 0.05$). We analyzed TAF movement and TAF-fiber overlap in volumes (regions of interest) with constant and changed fiber lengths (Supplementary Methods). Of all TAFs tracked in the relaxin-treated group, 65% of cells in regions with changing fiber lengths and 60% of cells in regions with constant fiber lengths overlapped at least once with fibers, but the mean time of overlap per cell was significantly longer ($P < 0.05$) in ‘changing’ (3 d ± 0.03 d) than in ‘constant’ (2.2 d ± 0.27 d) regions. In ‘changing’ regions, only 6% of TAFs overlapping with fibers were mobile (>10 μm d^{-1}) (Supplementary Table 1 online); in contrast, in ‘constant’ regions, 55% of TAFs overlapping with fibers were mobile. In ‘changing’ regions of control tumors, 29% of TAFs interacting with fibers were mobile. Taken together, these results suggest that relaxin stimulates collagen fiber remodeling by increasing the number of less-motile cells that interact with fibers.

Flow cytometry analysis of GFP-positive cells revealed a potential adhesion pathway involved in cell-fiber interactions: relaxin significantly increased the expression of β_1 integrin (*t*-test; $P < 0.05$) in GFP-positive cells (Fig. 3 and Supplementary Methods). β_1 integrin enhances matrix metalloproteinase-14 expression and activity in fibroblasts plated on collagen I gels[11]. It is also thought that relaxin affects fibrillar collagen remodeling by stimulating the expression and activity of matrix metalloproteinases such as MMP-1, -2 and -14 (refs. [7,12]). To determine the relationship between TAFs, β_1 integrin, matrix metalloproteinases and collagen remodeling, we treated tumors with relaxin combined with either a mouse β_1 integrin–blocking antibody or the pan–matrix metalloproteinase inhibitor GM6001. Both β_1 integrin and pan–matrix metalloproteinase blockade inhibited relaxin-induced fiber remodeling (Fig. 2a) but had differential effects on TAF-fiber interactions. β_1 integrin blockade prevented the relaxin-induced interaction between TAFs and fibers, whereas pan–matrix metalloproteinase inhibition did not (Fig. 2b). Thus, in relaxin-treated tumors, both TAF adhesion to collagen fibers, mediated by β_1 integrin, and matrix metalloproteinase activity were essential for collagen fiber remodeling.

In conclusion, although prior studies have studied cell-matrix interactions *in vitro* or collagen reorganization alone *in vivo*, to our knowledge our study is the first quantitative dissection of stromal cell–extracellular matrix interactions in living tissue. The integration of image registration with multiphoton laser scanning microscopy allowed us to track tissue-remodeling events in which both TAFs and matrix are changing dynamically over multiple consecutive days. Cell interactions with the extracellular matrix are central to fibrosis, wound healing, stem cell recruitment and tissue engineering. Hence, our methodology could be applied to track different cell types in healthy or diseased tissues *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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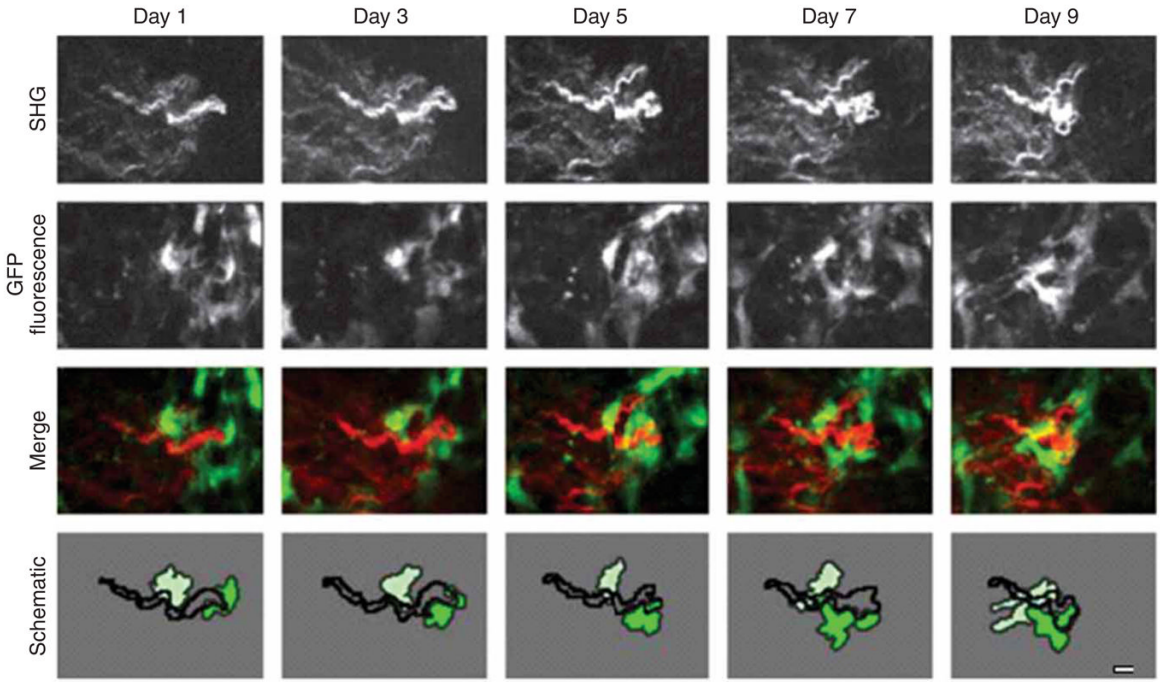

Figure 1.

Buckling of a collagen fiber in a relaxin-treated tumor. For each time point (days 1–9), the SHG, GFP fluorescence, and the merged GFP fluorescence (green) and SHG (red) maximum intensity projections of the acquired volumes are shown. Schematics of the images with the collagen fiber (black) and two overlapping stromal cells (green) are shown at the bottom. Scale bar, 10 μm.

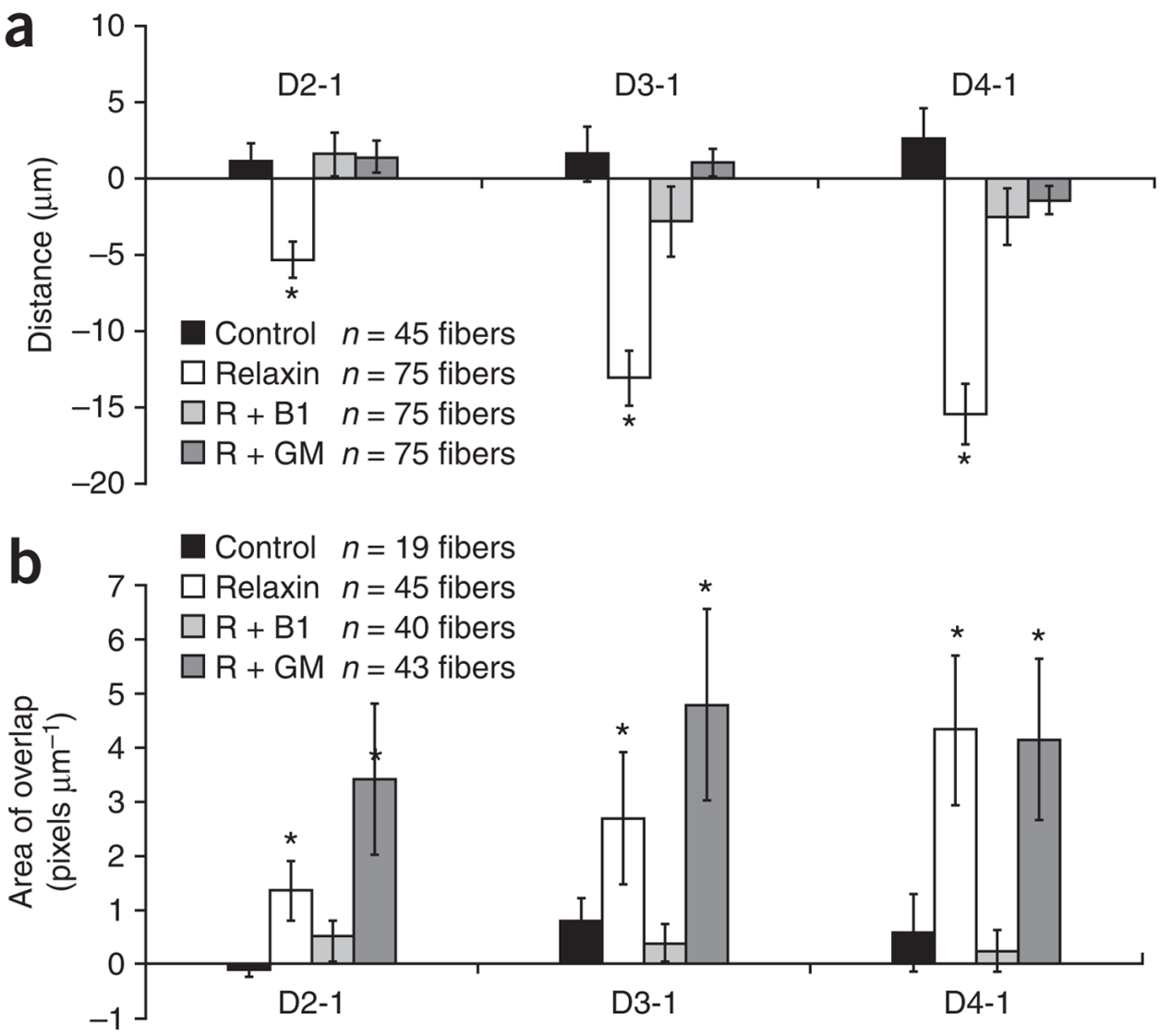

Figure 2.

Quantitative analysis of collagen fiber length and area of colocalization between stromal cells and fibers. (**a**,**b**) The end-to-end fiber length (**a**) and the area of overlap between stromal cells and collagen fibers (**b**) was determined over 4 d in control groups as well as those treated with relaxin, relaxin and β_1 integrin antibody (R + β1) and relaxin and GM6001 (R + GM). Shown are the averaged differences between day 1 and days 2 (D2-1), 3 (D3-1) and 4 (D4-1). A paired *t*-test was performed for fiber length and area of overlap in all groups. Error bars, s.e.m.; **P* < 0.05.

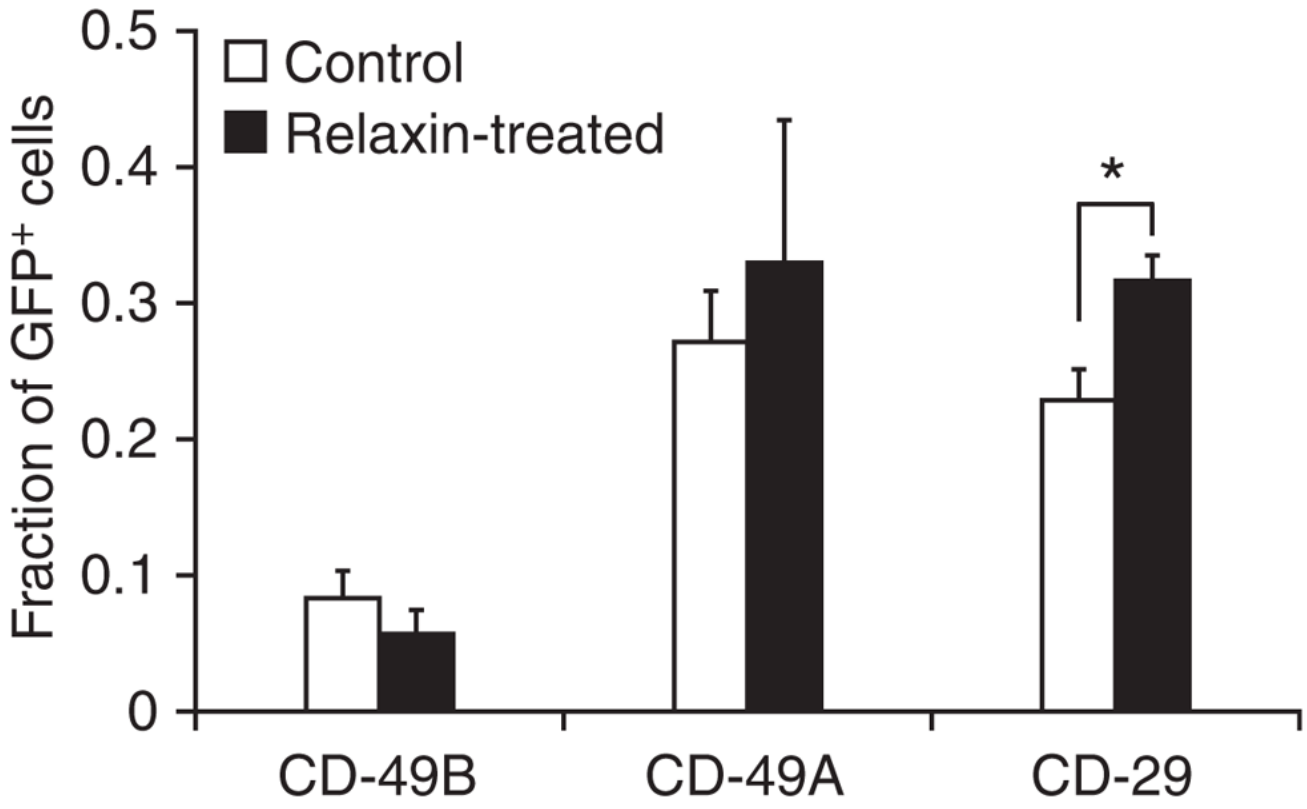

Figure 3.

Flow cytometry analysis of HSTS26T tumor–associated fibroblasts. Flow cytometry analysis for CD-29 (β_1 integrin), CD-49A (α_1 integrin) and CD-49B (α_2 integrin) was performed on GFP-positive cells from HSTS26T tumors growing in VEGF-GFP mice. On average, GFP-positive cells represented 1% of the 1 million events counted for each sample. The fraction of GFP-positive cells expressing the respective integrin is shown for control and relaxin-treated tumors. Error bars, s.e.m. ($n = 3$); $^*P < 0.05$.