Microscopic Diffusion Anisotropy in Formalin Fixed Prostate Tissue: Preliminary Findings

Roger M. Bourne,1* Nyoman Kurniawan,2 Gary Cowin,2 Paul Sved,3 and Geoffrey Watson1

Diffusion tensor microimaging at 16.4 T with 40 μm isotropic voxels was used to investigate anisotropic water diffusion in prostate tissue at spatial resolution approaching the cellular scale. Nine normal glandular tissue samples were collected from the peripheral zone of six formalin fixed radical prostatectomy specimens. Fibromuscular stromal tissue exhibited microscopic diffusion anisotropy (mean fractional anisotropy range 0.47–0.66) significantly higher (P < 0.01, Student’s t-test) than in epithelium-containing voxels (mean fractional anisotropy range 0.31–0.54) in six of the seven normal tissue samples in which both compartments could be measured. Fiber tracking demonstrated principle stromal fiber directions consistent with myocyte orientation seen on light microscopy of the same sample. Diffusion tensor microimaging may be valuable for investigation of variable results from attempts to measure diffusion anisotropy in the prostate in vivo. Magn Reson Med 000:000–000, 2012. © 2012 Wiley Periodicals, Inc.

Key words: diffusion; microimaging; prostate; anisotropy; stroma; tractography

An imaging method that generates contrast based on microscopic tissue structural properties would be expected to provide both sensitive and specific cancer detection if the image contrast can be made to reflect the structures that define cancer. Diffusion-weighted imaging (DWI) is an obvious candidate for this purpose because the free diffusion of water in tissue is known to be constrained by intra- and extracellular structures and cell walls. DWI can reveal both the scale and orientation of tissue structure because contrast depends on the net displacement of water over a specific time period in a specific direction. Two parameters are commonly used to describe the rate and relative directional freedom of water diffusion. These are the apparent self diffusion coefficient (ADC) or diffusivity, and the fractional anisotropy (FA) (1), respectively.

DWI studies of prostate tissue in vivo have demonstrated a decrease in the measured ADC in cancer tissue that correlates with Gleason grade (2,3). ADC has been posited to be consistent with both the loss of high ADC lumenal and ductal spaces and increased cell density characteristic of prostatic adenocarcinoma (4), however, recent evidence from diffusion microimaging studies of formalin fixed prostate tissue suggest that distinct diffusivity differences between the epithelial cells, the stromal matrix, and the acinar lumens are likely to contribute to changes in ADC measured at low spatial resolution in vivo (5–7).

Measurements of diffusion anisotropy in the prostate in vivo have produced equivocal results with widely differing FA values for similar tissue and no consistent correlation between pathology and FA (8–12). However, a study of formalin fixed radical prostatectomy specimens, performed at 4.7 T with spatial resolution 0.5 × 0.5 × 0.5 mm³, obtained diffusion anisotropy data consistent with gross tissue architecture (13). High FA was observed in regions of primarily fibromuscular stromal tissue with the primary diffusion axis parallel to the assumed main fiber axis.

The study reported here seeks to investigate the potential of diffusion microimaging of fixed tissue samples to clarify the biophysical basis of diverse findings from anisotropy measurements of the prostate.

METHODS

Tissue Collection

All tissue samples were collected from radical prostatectomy specimens with institutional ethics approval and written informed consent from tissue donors. Nine samples of normal tissue were collected from the left and/or right lateral peripheral zone of the prostate of six patients. Whole organs, immersed 72 h in 10% neutral buffered formalin post surgery, were sectioned for routine histopathology. Four-mm thick transverse slices were examined by a specialist urologic pathologist and full thickness tissue samples obtained with a 3 mm core punch (sample volume ~28 mm³). The selection of regions for sampling was based on visual assessment of the likely tissue type. In this preliminary study we focused on samples of normal glandular tissue. Cores were placed in vials of neutral buffered formalin and stored 1–2 weeks at room temperature before MR imaging.

MR Microimaging

Tissue cores were transferred from neutral buffered formalin to phosphate buffered saline containing 0.2% v/v gadolinium contrast agent (Magnevist, Schering AG, Germany). Dimeglumine gadopentetate 0.5 mg/mL) giving
Fractional Anisotropy of Tissue Compartments

<table>
<thead>
<tr>
<th>Sample</th>
<th>Patient</th>
<th>Epithelial FA ± SD (No. voxels)</th>
<th>Stromal</th>
<th>Ductal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.31 ± 0.14 (327)</td>
<td>0.47 ± 0.16 (283)</td>
<td>0.30 ± 0.11 (95)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.50 ± 0.19 (472)</td>
<td>0.58 ± 0.19 (1484)</td>
<td>0.45 ± 0.30 (132)</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.49 ± 0.20 (176)</td>
<td>0.53 ± 0.16 (500)</td>
<td>0.38 ± 0.15 (416)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.54 ± 0.19 (124)</td>
<td>0.60 ± 0.17 (804)</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>–</td>
<td>0.64 ± 0.23 (769)</td>
<td>0.38 ± 0.14 (154)</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>0.54 ± 0.22 (134)</td>
<td>0.57 ± 0.20 (1079)</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.45 ± 0.16 (481)</td>
<td>0.58 ± 0.18 (1753)</td>
<td>0.35 ± 0.14 (193)</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>–</td>
<td>0.63 ± 0.18 (538)</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>0.41 ± 0.23 (238)</td>
<td>0.66 ± 0.16 (255)</td>
<td>0.32 ± 0.13 (65)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>0.46 ± 0.08</td>
<td>0.58 ± 0.06</td>
<td>0.36 ± 0.05</td>
</tr>
</tbody>
</table>

aThe "epithelial" compartment is comprised of epithelium-containing voxels also containing unknown partial volumes of stroma and duct.

bBold values represents the epithelial FA significantly differing from stromal FA (P < 0.01, Student’s t-test).

cMean and SD of sample means.

ROI Selection

Voxel calculations were based on selection of imaging slices that most clearly permitted unequivocal selection of a large number of voxels composed primarily of a single compartment type (epithelium, stroma, or duct) (7). Adjacent slices were checked to minimize partial volume effects. A binary mask was made from the DWI slice and then used to select pixels from the FA data of the same slice. As the thickness of normal epithelium is variable (from a minimum of ~15 μm), and may include infolding, selected "epithelial voxels" may contain significant partial volumes of both ductal space and stromal tissue. In three of the normal tissue samples significant regions of ductal and/or epithelium-containing voxels could not be confidently selected.

Histopathology

The normal glandular status of samples was confirmed by histopathologic examination of the tissue immediately surrounding the sample core void. Following MR microimaging one of the tissue cores was embedded and sectioned for light microscopy and immunohistochemical staining.

RESULTS

Measurements of voxel FA based on ROI selection (7) are summarized in Table 1. The mean FA in stromal voxels was significantly higher than the mean FA of epithelium-containing voxels in the same sample in six of the seven samples in which both compartments could be confidently distinguished. As the ductal spaces were filled with phosphate buffered saline solution in which diffusion would be expected to be isotropic the "ductal" compartment FA measurements can be considered to represent background noise. The level of noise affecting estimates of epithelial and stromal FA would be lower than that seen in the ducts due to their lower diffusivity (Fig. 2a and Ref. 7) and consequently higher SNR in the b = 1000 s/mm² DW images.

Figure 1 shows light microscopy of sections of one of the samples of normal glandular tissue. The normal fibromuscular stromal tissue is characterized by a
distinct heterogeneity of myocyte cell orientations (Fig. 1a) with parallel orientation persisting only on a submillimeter scale. A similar heterogeneity of actin fiber orientation is evident in the smooth muscle actin stained adjacent section (Fig. 1b).

The diffusion-based properties of the same tissue core, imaged before light microscopy, are shown in Fig. 2. As reported previously (6,7) epithelium-containing voxels show low diffusivity relative to stromal tissue and ducts (Fig. 2a). This low diffusivity correlates with high cytokeratin density (Fig. 1b). Note that the correlation between light microscopy and MR images is imperfect as it is not possible to section the embedded tissue in planes exactly parallel to the MR imaging plane.
The FA image (Fig. 2b) suggests that the highest voxel anisotropies occur in stromal tissue and the principle eigenvector direction image (Fig. 2c) suggests that there are distinct local regions in which adjacent voxels have similar primary diffusion directions.

Fiber tractography (Fig. 2d) produces fibers (streamlines) with orientation consistent with the long axis of stromal cells seen in the H&E stained section (Fig. 1a) and actin filaments of the adjacent section (Fig. 1b).

DWI and fiber tracks in a second normal tissue sample are shown in Fig. 3. This sample is more densely glandular than the sample of Figs. 1 and 2. The generated fiber tracks are mainly confined to the regions of stromal tissue between glands and show minimal extension into the epithelium layer and ductal space.

Figure 4 illustrates the heterogeneous fiber track orientation found in three further cores of normal glandular tissue. In all samples the primary direction and spatial location of fiber tracks were robust to changes of propagation algorithm (FACT; 2nd order Runge-Kutta; interpolated streamline; tensorline), FA cutoff, and angle threshold. This heterogeneous microscopic anisotropy would most likely give rise to low ensemble anisotropy (13,15) when FA is measured in vivo at spatial resolutions where the voxel volume is typically similar to the tissue sample size used in this study (28 mm³). It is possible that microscopic heterogeneity of stromal fiber direction has contributed to diverse findings from attempts to measure FA in the prostate in vivo (8–12). Heterogeneity of microscopic anisotropy would also explain the generally lower FA reported at low spatial resolution in vivo compared with FA measured in the same organ at high spatial resolution ex vivo (13).

Noise due to the attenuation of the diffusion-weighted signal is a well-known problem in the estimation of diffusion anisotropy (16). We estimated the intrinsic SNR in our b = 0 images to be ~24 according to the method of Dietrich et al. (14). This exceeds the level of 20 suggested by Mukherjee et al. (16) to provide reliable DTI parameters. The good agreement between principle eigenvector direction, streamline (fiber track) direction, and stromal myocyte orientation seen on light microscopy of the same tissue section, together indicate that the SNR in stromal tissue was adequate for reliable diffusion anisotropy analysis.

Due to noise, the measured FA values are likely to be overestimates of the true tissue FA in both stromal and epithelial compartments (16). Despite much smaller voxel size (0.000064 mm³) compared with the fixed tissue study of Xu et al. (13) (0.125 mm³), we did not observe higher FA. The mean stromal FA of our samples (0.58 ± 0.06) was in the upper range of the values Xu et al. reported for “stromal BPH” (benign prostatic hyperplasia). From our fiber tracking images it is clear that, although fiber direction is heterogeneous, there are many large bundles of fibers such that a voxel of dimension 0.5 × 0.5 × 0.5 mm³ could be comprised of approximately parallel stromal fibers and would then be expected to have a measured FA similar to the stromal maximum we measured. Our maximum FA value of 0.66 is similar to that reported from a study of formalin-fixed

FIG. 3. Diffusion and fiber tracks in highly glandular tissue. a: Diffusion-weighted image illustrating multiple glands lined with low-diffusivity epithelium. b: Fiber tracks that traverse the same tissue slice as image (a). c: Overlay of fiber tracks onto diffusion-weighted image. Note that the generated tracks are primarily confined to stromal tissue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
rat heart (FA ~0.62) (17) and thus could be considered the maximum FA that can be expected in tissue comprised of densely packed muscle fibers. The maximum FA value (~0.72) that Xu et al. reported is possibly higher than this maximum muscle FA due to noise.

The FA measured in epithelium-containing voxels was generally lower than in stromal voxels but varied between samples. This variation is possibly due to variable partial volume effects as many voxels spanning the epithelial cell layer (minimum thickness 15 μm estimated on light microscopy) could be expected to contain as little as 40% “epithelial” cells (A distinction between epithelial and basal cells at this stage is not possible). Low FA in epithelium-containing voxels relative to stromal voxels may be the result of shorter average cell length or the absence of long actin filaments.

Although measurement of voxel mean diffusivity was not the primary focus of this report, the coincidence of high cytokeratin density seen on light microscopy and low diffusivity in the epithelial cell layer seen in DWI is noteworthy. The possibility that a dense intracellular cytokeratin matrix gives rise to hindered or restricted diffusion in epithelial cells warrants further investigation (18).

Limitations

The observations of this study are based on imaging of formalin fixed normal tissue. Water diffusivity in vivo is generally higher than in fixed tissue and it is possible that diffusion anisotropy may be altered or exaggerated by the fixation process. Although earlier low spatial resolution comparisons of fresh and fixed prostate tissue suggest that relative changes in anisotropy are minor (13), more extensive studies of both normal and cancer tissue will be required to unequivocally establish a relationship between diffusion anisotropy observed in fixed tissue and in vivo.

CONCLUSIONS

Diffusion tensor microimaging of a limited sample of normal glandular prostate tissue suggests that there are significant FA differences between stromal and epithelial tissue. Fiber tracks generated from diffusion tensor data from one sample were consistent with stromal myocyte and actin fiber orientation seen on subsequent light microscopy of the same sample. Diffusion tensor microimaging may be valuable for investigation of variable results from attempts to measure diffusion anisotropy in the prostate in vivo.

ACKNOWLEDGMENTS

This work was supported by an Australian National Imaging Facility access grant. The authors thank Ms Robyne Soper for preparation of the samples for light microscopy.

REFERENCES