Laser scanning confocal microscopy of cervical tissue before and after application of acetic acid

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OBJECTIVES: The use of high-resolution in vivo confocal imaging may offer a clinically useful adjunct to standard methods for the diagnosis and screening of epithelial precancers. This study assesses the feasibility of real-time confocal reflectance imaging of cervical tissue and the use of acetic acid as a contrast agent to increase visualization of cell nuclei.

STUDY DESIGN: A confocal microscope was used to image cervical cells and colposcopically normal and abnormal cervical biopsy specimens. Images were obtained before and after the application of 6% acetic acid.

RESULTS: The confocal imaging system resolved subcellular detail throughout the entire epithelial thickness. Normal and abnormal tissues were clearly able to be differentiated. Addition of acetic acid enhanced nuclear signal in all acquired images.

CONCLUSION: High-contrast reflected light images of cervical tissue are attainable in near real time. Acetic acid significantly increases light scattering from cell nuclei, which may partially explain why acetowhitening occurs. (Am J Obstet Gynecol 2000;182:1135-9.)

Key words: Acetic acid, cervix, confocal microscope

Optical technologies are being used increasingly to perform real-time assessment of tissue abnormalities. Many groups have demonstrated that quantitative optical spectroscopic approaches, including fluorescence spectroscopy[1-4] and reflectance spectroscopy,[5, 6] have the potential to deliver a highly sensitive, specific, and cost-effective diagnosis in real time and without the removal of tissue. These spectroscopic methods provide a means to extract diagnostically relevant structural and histochemical information on the basis of the interaction of light with chromophores within the tissue. Ramanujam et al[7-9] developed a diagnostic algorithm that could differentiate normal squamous tissue, normal columnar tissue, low-grade squamous intraepithelial lesions, and high-grade squamous intraepithelial lesions on the basis of laser-induced autofluorescence of cervical tissue, collected in vivo for 3 excitation wavelengths. The algorithm performed with a sensitivity of 82% and a specificity of 68% in a blinded 95-patient study conducted in a diagnostic setting.[9] Cantor et al[10] performed a decision analysis showing that a see-and-treat strategy combining fluorescence spectroscopy and colposcopy would be more effective and less expensive than the current standard of care of colposcopy. They[10] estimated that using fluorescence spectroscopy could save >$625 million annually in the United States.

Over the past several years, in an attempt to further improve sensitivity and specificity, new optical technologies have been introduced, which provide direct imaging of tissue structure on the basis of the backscattering characteristics of a tissue. One particularly promising new technology is confocal microscopy,[11] which samples small volumes of tissue, producing images with micron resolution at depths up to several hundred microns within the tissue. High-resolution confocal imaging can be used to obtain near real-time reflected light images of human epithelial tissue in vivo with micron resolution.[11] In vivo confocal imaging can provide information about subcellular morphologic and biochemical changes in epithelial cells, which may be useful in the recognition and monitoring of epithelial precancers in organ sites such as the uterine cervix. Much of the work demonstrating the potential of confocal microscopy to image cell morphologic features has been carried out in pigmented tissue in which melanin within cells provided the confocal signal and image contrast.[11] More recent work has demon-

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strated that confocal microscopy has the ability to visualize structure in amelanotic cells as well.[12] However, the level of native contrast between diagnostically important structures such as the nucleus and the remainder of the cell's contents can vary significantly among cell types as a result of differences in cell composition. To improve the ability to image the nucleus in epithelial tissue, it may be helpful to apply exogenous agents to enhance the intrinsic contrast.

Recently, we have shown that acetic acid increases contrast in reflected light confocal images of human breast epithelial cells.[12] On the basis of phase contrast microscopy experiments, we demonstrated that acetic acid induces spatial fluctuations in the nuclear index of refraction. We hypothesize that these fluctuations increase backscattering from the nuclear region. This results in clearer delineation of nuclear structure when cells are viewed with a reflected light confocal imaging system for which changes in refractive index are a primary source of image contrast. In this article we investigate whether cervical tissue contains sufficient intrinsic contrast to provide clinically informative confocal images. We then consider how image contrast may be improved through the use of acetic acid as a contrast agent. The results of this study are designed not only to demonstrate the diagnostic potential of confocal imaging but also to provide a plausible physical explanation to the clinically observed acetowhitening phenomenon.

Acetic acid is commonly used during colposcopic examination to identify atypical areas of the cervix that require biopsy. Addition of acetic acid in a concentration ranging from 3% to 6% causes acetowhitening of many cervical epithelial abnormalities, including cervical intraepithelial neoplasia, adenocarcinoma, and invasive squamous cell carcinoma.[13] Numerous studies have documented the use of acetic acid to improve detection of dysplastic regions of the cervix.[14-16] Although it has been shown that acetic acid can be used to augment detection of cervical dysplasia missed by Papanicolaou screening,[15] the mechanism through which acetic acid produces selective tissue whitening has never been fully elucidated. It has been suggested that acetic acid induces alterations in protein structure, preventing light from passing through the epithelium. Because light does not penetrate to the underlying vessels, the epithelium appears white rather than pink. The effect is more pronounced in dysplastic regions because these regions have a higher nuclear density and consequently a higher concentration of protein.[13] We hypothesize that some of the same mechanisms that cause the visual whitening effect of acetic acid may also help to improve contrast in confocal reflected light images. Thus we conducted a study to examine confocal images of live cervical cells and in vitro cervical biopsy specimens before and after addition of acetic acid.

Material and methods

Materials. Material for study was excised from patients undergoing colposcopic examination at M.D. Anderson Cancer Center in Houston, Texas. Informed consent was obtained from each patient, and the study was reviewed and approved by the Internal Review Board at M.D. Anderson Cancer Center. Additional cervical tissue specimens were obtained from the National Cancer Institute Cooperative Human Tissue Network. A protocol for the use of these specimens was reviewed and approved by the Internal Review Board at the University of Texas at Austin. Biopsy specimens from a total of 16 patients were imaged. The pathologist was blinded to the imaging data. The pathology results of the imaged specimens were as follows: normal cervix, 11; normal cervix with hyperkeratosis, 2; hyperkeratosis or mild dysplasia, 1; mild dysplasia, 1; and severe dysplasia, 1. Biopsy specimens measured 1 to 4 mm in diameter and ~1 mm in thickness. Two preparation methods were used. For the first preparation, biopsy specimens were snap-frozen and initially stored in liquid nitrogen and later at –70°C in an ultra-low-temperature freezer until the time of confocal imaging. After confocal imaging, transverse frozen sections were cut and stained with hematoxylin and eosin. Frozen sections were sent to an experienced pathologist for histologic examination. For the second preparation, biopsy specimens were immediately placed in chilled tissue culture media. Slices 200 μm thick were made with a Krumdieck tissue slicer, a microtome designed for rapid preparation of live tissue slices. With this device it is possible to produce tissue slices of a consistent thickness with minimal tissue trauma. Some slices were fixed for later histologic examination. Fixed slices were sectioned and stained with standard hematoxylin and eosin. Other slices were placed into chilled tissue culture media until the time of confocal imaging.

For the cell studies cervical cells from the SiHa cell line originally obtained from the American Type Cell Collection (Bethesda, Md) were grown in the laboratory of Professor Reuben Lotan at the University of Texas M.D. Anderson Cancer Center. Cells were harvested by trypsinization and washed 3 times in phosphate-buffered sodium chloride solution and resuspended in phosphate-buffered sodium chloride solution at concentrations of 1 to 6×10^6/mL. Images were recorded from 3 samples of SiHa cells.

Instrumentation. The confocal imaging system used to obtain the images presented in this article provides high-contrast reflected light images of unstained biologic samples at a near-video rate. The microscope consists of a laser, scanning system, intermediate optics, and a detector. Light from a titanium-sapphire laser (λ = 808 nm) was spatially filtered and recollimated to a diameter of 5 mm. A beam splitter transmitted 50% of the collimated light to a scanning system. A scan lens (f = 80 mm dou-

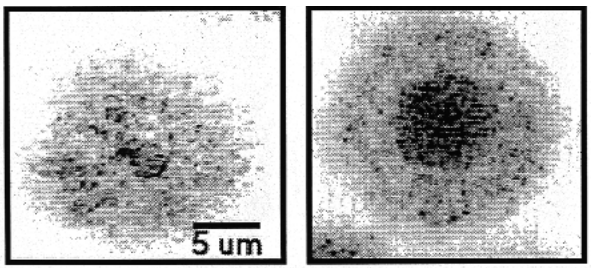

Fig 1. Confocal image (λ = 808 nm) of SiHa cervical cancer cell before *(left)* and after *(right)* application of 6% acetic acid.

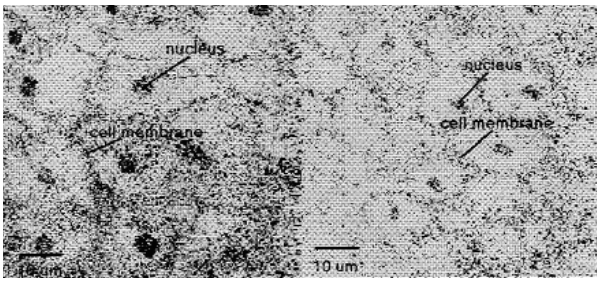

Fig 2. Analysis of acetic acid–treated biopsy specimen. Reflected light confocal images 50 μm below epithelial surface *(left)* and 200 μm below epithelial surface *(right).* Illumination wavelength was 808 nm.

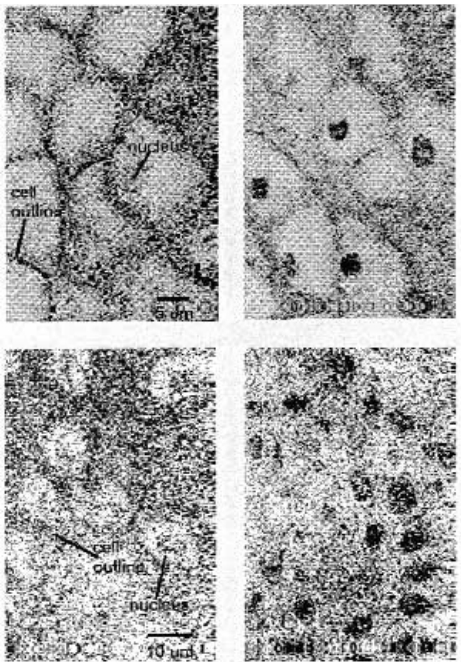

Fig 3. *Top row,* Images of normal cervical biopsy specimen before *(left)* and after *(right)* application of 6% acetic acid. *Bottom row,* Images of colposcopically abnormal cervical biopsy specimen before *(left)* and after *(right)* addition of 6% acetic acid.

blet) brought the light exiting the scanning system to a focus at the back focal plane of a ×25 water immersion objective. The light reflected backward from the focal plane of the objective, within the numeric aperture of the objective, returned through the scanning system. A beam splitter reflected half of the descanned light to a second lens, L2 (f = 50.4 mm). A pinhole 15 μm in diameter at the focus of L2, a conjugate image plane, rejected light backscattered from outside the focal volume illuminated. An avalanche photodiode (Hamamatsu C5460) converted the light passing through the pinhole to a voltage. A more detailed description of the instrumentation used has been previously presented.[12]

Imaging. Images were obtained by sampling avalanche photodiode voltage with a personal computer video card. Images of interest were saved to a 512 × 512 pixel file with 256 gray levels. Cervical cancer cells (SiHa) were imaged with the confocal microscope before addition of acetic acid. Before imaging, the cells were allowed to settle onto a layer of gelatin (n = 1.36) to stabilize the image and minimize background signal from the supporting surface. Then 10 μL of 6% acetic acid was added to 190 μL of a dilute suspension of cells (~10^6 cells/mL), and images of the cells were immediately recorded. Biopsy specimens and tissue slices were imaged before and several minutes after exposure to 6% acetic acid. To ensure reproducibility, multiple biopsy specimens and tissue slices were imaged on several different days.

Results

Confocal images of cervical cells before and after addition of acetic acid are shown in Fig 1. In the image of a native cell, the nucleus is difficult to resolve. The addition of acetic acid causes a dramatic increase in the signal from the nuclei, resulting in increased signal and image contrast. The whitening effect was present within seconds after the application of acetic acid. Images were acquired immediately after the effect became visible. In the cellular studies the effects of the acetic acid gradually diminished over a several-minute period.

Biopsy images from normal cervical tissue are shown in Fig 2. Fig 2 shows reflected light confocal images near the surface of the epithelium (50 μm deep) and near the basement membrane (200 μm deep). In both images the outlines of cells are clearly visible, as are the cell nuclei. The acetic acid penetrated the entire epithelial thickness. No significant size changes were noted in the biopsy specimens after the application of acetic acid. The most drastic increases in scattering occurred in the nucleus. In some biopsy specimens the cytoplasm appeared to be scattering slightly more after the application of acetic acid. Images obtained before the addition of acetic acid demonstrated similar features; however, signal strength and image contrast were reduced (data not shown).

Images of a colposcopically normal and abnormal biopsy specimen are shown in Fig 3. The top row shows images of the colposcopically normal biopsy specimen,

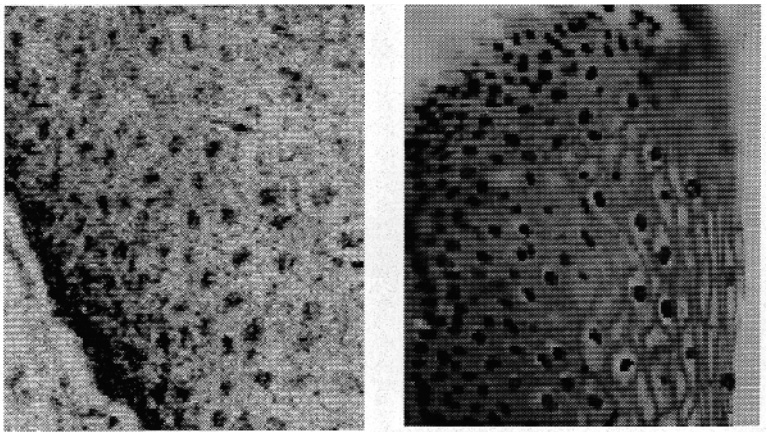

Fig 4. Confocal image of cervical tissue slice after addition of 6% acetic acid *(left)*. Corresponding hematoxylin and eosin–stained histologic section is shown *(right)*.

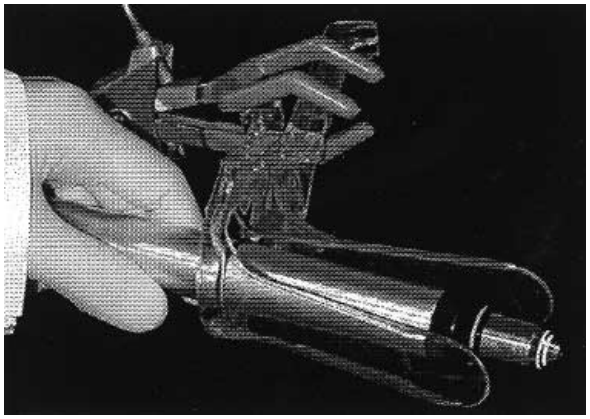

Fig 5. Fiber-optic confocal microscope shown inside standard speculum to demonstrate device dimensions. Widest portion of device is shown in image.

and the bottom row contains images of the colposcopically abnormal biopsy specimen, which contained a high-grade cervical intraepithelial neoplasia. In the left panel the pre–acetic acid images of the abnormal biopsy specimen show the cell outlines and an occasional nucleus. The pre–acetic acid images of the abnormal biopsy specimen show increased reflectivity of both the cell membranes and the nuclei. In addition, the cells are more crowded and irregularly spaced. Post–acetic acid images show increased signal from the nuclei in both the normal and abnormal biopsy specimens. Pathologic diagnoses confirmed clinical impressions at the time of colposcopy.

Images from normal tissue slices are shown in Fig 4. The image on the left is a confocal image of a tissue slice after addition of acetic acid. The image on the right is a corresponding image stained with hematoxylin and eosin. The tissue slice images presented here are perpendicular to the orientation in which tissue would be viewed during in vivo imaging applications. We show the images to demonstrate the optical sectioning principle of confocal microscopy, which permits viewing of thick sections without physical sectioning. In addition, the figure demonstrates the similarity between images obtained with confocal microscopy and those obtained from conventional histologic examination. Confocal images in the orientation presented here are attainable in in vivo situations by reconstructing 3-dimensional data sets consisting of slices imaged at multiple depths.

Comment

The results shown here provide the first evidence that confocal imaging of cervical tissue can yield images potentially useful for clinical diagnosis. The images illustrate that high-contrast reflected confocal images can be obtained in near real time, which clearly shows characteristic differences between normal and neoplastic tissue throughout the entire epithelial thickness. Without the application of acetic acid, it is difficult to distinguish the nucleus from the cytoplasm of a cell because of low contrast. This suggests that, to develop diagnostic applications of confocal microscopy, enhanced visualization of the nucleus may be required to facilitate the identification of morphologic changes indicative of dysplasia such as increased nuclear size and irregular shape.

Further work is needed to elucidate the underlying physical mechanisms responsible for the changes caused by acetic acid. Goniometric measurements have indicated increased scattering from cells after the addition of acetic acid.[18] Measurements of a scattering cross section of cells before and after exposure to acetic acid also offer further evidence of enhanced scattering.[19] In addition, simulations of light scattering from inhomogeneous cells using a finite difference time domain model indicate increased scattering when cells are modeled with alterations in nuclear refractive index structure, on the basis

of phase-contrast microscopic images of cells before and after the addition of acetic acid.[18] The combination of all of the available data suggests that increased scattering from cell nuclei is at least partially responsible for the visible whitening caused by acetic acid.

Because confocal images can be recorded in near real time without the need for tissue removal, high-resolution confocal imaging affords important advantages over traditional histopathologic detection techniques. Contrast agents such as acetic acid may offer a simple, inexpensive means to enhance the diagnostic utility of scattering-based imaging modalities such as confocal microscopy. Acetic acid may also be a valuable contrast agent for other optical imaging and spectroscopic techniques such as optical coherence tomography and reflectance spectroscopy.

Although the results presented in this study, particularly the tissue images after acetic acid application, are encouraging of future effort, a fiber optic version of the microscope will be required to make the confocal system suitable for in vivo imaging of the cervix. To this end a prototype fiber-optic confocal microscope is being constructed. A photograph of the fiber-optic microscope inserted through a speculum is shown in Fig 5. We believe such a tool will be highly useful for the recognition and monitoring of cervical precancers. Whereas colposcopy is very sensitive for identification of precancers, it is not very specific and many biopsies are necessary to confirm diagnosis. Confocal imaging guided by colposcopy could be used to image those areas that appear colposcopically abnormal. This would allow biopsy specimens to be obtained only from regions containing morphologic and architectural features consistent with premalignant changes, potentially reducing the number of unnecessary biopsies performed at colposcopy.

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