Erythrocytes, or red blood cells (RBCs), from individuals with sickle cell disease are less pliable and deformable than normal RBCs and, therefore, can be trapped within the lumen of blood vessels supplying various organs. Trapping or adhesion of sickle RBCs (SS-RBCs) has been suggested as the initiating event in the characteristic vaso-occlusions that occur in sickle cell retinopathy, but this hypothesis has never been directly demonstrated. In this study, fluorescent dye-labeled SS-RBCs were injected intravascularly into rats, and the retinas were analyzed by both traditional light microscopy and laser scanning confocal microscopy (LSCM) to determine vascular sites of adhesion and obstruction. The LSCM system optically sectioned the specimen through vertical focal planes, and the sections were overlaid to produce three-dimensional views not directly observable with a conventional microscope. Preliminary data suggest that the initial site for SS-RBC retention is within the precapillary arterioles of the peripheral retina.
The optical sectioning feature of the LSCM system allows three-dimensional (3D) volume visualizations to be constructed from serial two-dimensional (2D) slices of a specimen. As the specimen is translated vertically along the optical axis, the 2D images retain contrast and resolution wherever the specimen comes into focus. When serial 2D optical slices of the object are overlaid, a 3D volume representation with extended focus results. The confocal optical sectioning technique is free of the monumental problems of alignment and registration associated with classical sectioning methods. The conventional optical microscope is restricted in performing 3D sectioning by fundamental limitations from low signal-to-noise ratio; the light from a plane of focus deep in the sample is scattered and degraded as it passes through the sample matrix.

Images from the LSCM system contain digital information in the x, y, and z planes and intensity data for each volume pixel, known as a “voxel.” Volume renderings (made with Vital Images, Inc., Voxel View software) represent the 3D object as a collection of these cubelike voxels. A user-specified, measurable property from the original data set is used to reveal the regions of interest. Because the images are captured digitally, quantitative measurements can be obtained anywhere in the 3D data set, i.e., in any plane or direction or along some irregular path throughout the volume. This flexibility provides an extra dimension of data for (volume) analysis that is not possible with traditional 2D images. Thus, the researcher is free to browse through the volume from any orthogonal or oblique plane to obtain views not directly observable with the microscope alone. Using Vital Images Voxel Animator software, these views can be set into motion by creating animations to reveal otherwise obscure features.

STUDY OBJECTIVES

In this study we investigated how red blood cells (erythrocytes) from subjects with sickle cell disease interact with blood vessels in the sensory retina—the semitransparent vision tissue on which light is focused in the eye. Sickle cell disease is caused by single-point mutations in the hemoglobin gene. The presence of mutant hemoglobin in the red blood cell (RBC) causes hemoglobin tetramers to polymerize under hypoxic or acidic conditions, which gives the RBC the characteristic sickle shape (Fig. 3). Accelerated destruction of sickle RBCs (SS-RBCs), called hemolysis, results in the characteristic sickle cell anemia. The SS-RBCs can initiate blockage of blood vessels (vessel-occlusions) in most major organ systems of the body. Experimental models suggest that they either adhere to endothelial cells lining the blood vessels or become physically entrapped within the microvasculature, or both. In the retina, vessel-occlusions result in sickle cell retinopathy, which is characterized by the growth of new blood vessels—neovascularization—adjacent to areas of the retina made ischemic (blood deficient and therefore deprived of oxygen and nutrients) by vessel-occlusive processes. The site of the initial vessel-occlusions is thought to be at the level of the precapillary arterioles, arterial blood vessels immediately preceding the capillaries, but this theory has never been directly demonstrated. In this study we attempted to elucidate the sites of SS-RBC retention within the retinal vasculature.

EXPERIMENTAL METHODS

We injected either fluorescently labeled human sickle erythrocytes or normal human erythrocytes into anesthetized adult rats. The retinas were prepared by the adenosine diphosphatase (ADPase) histochemical technique for visualization of the retinal vasculature. The enzyme ADPase is almost exclusively localized to the vasculature in the retina and can be visualized by the lead reaction product that results from incubation for ADPase activity. Flat-mount preparations of ADPase-incubated retinas
were examined by both conventional microscopy and LSCM. Dark-field illumination was used to visualize the ADPase reaction product in blood vessels; fluorescence illumination was used to visualize labeled SS-RBCs.

Rat Model of Human Sickle Cell Adhesion within the Retinal Vasculature

Erythrocytes from human sickle cell patients (Fig. 3) were isolated and labeled with either fluorescein isothiocyanate or rhodamine isothiocyanate and then injected into the left ventricle of anesthetized rats. Cells were allowed to circulate for 5 min, the animals were sacrificed, and the retinas were prepared by the ADPase flat-mount technique. Normal human fluorescein isothiocyanate-labeled RBCs were injected into other rats as a control. The specimens were viewed under conventional dark-field and fluorescence microscopy using a Zeiss inverted microscope. Identical specimens were examined with a Zeiss LSCM.

Laser Scanning Confocal Microscopy

We collected LSCM optical sections through the inner (near the retinal surface) and outer retinal vascular networks using a Zeiss LSCM. A 63X/1.4NA oil-immersion Zeiss objective was used with a multiline argon laser illuminating source. Optical sections were taken with 0.45-μm separation through a depth range of 30.6 μm of retinal tissue. A dark-field image and fluorescence image were produced in one scanning series using independent detectors, and the two images were displayed simultaneously as overlays. Two-dimensional slices from the optical series were superimposed to produce a single, extended-focus image. Stored optical slices also were used to construct 3D volume renderings and stereo images, and the 3D representations were set into motion using a Silicon Graphics workstation and Vital Images animation software.

RESULTS

The entire retinal vasculature could be visualized by incubating the retinas for ADPase activity and then viewing the retina in flat mount under dark-field illumination. The lead ADPase reaction product was confined almost exclusively to the vasculature, as can be seen in the left panel of Fig. 4. Arteries and arterioles show the greatest ADPase activity and reaction product when compared with veins and venules; capillaries show the least activity. This differential staining and position in the vascular hierarchy permitted exact blood vessel type to be identified. Combining the ADPase method with injection of fluorescently labeled target cells allowed us to determine the location of the SS-RBCs in the vasculature with traditional fluorescent microscopy by simply changing the light source. Normal human RBCs injected into other rats were most often observed at precapillary arterioles, whereas the secondary vasculature near the surface of the retina has arteries, arterioles, veins, and venules, whereas the secondary vasculature in the inner nuclear layer contains almost all of the capillaries. This spatial segregation of microvasculature and large vessels necessitated a mechanical sampling of each plane in the retina by step-focusing each area to determine where in the vascular hierarchy the fluorescently labeled cells were retained.

We also combined fluorescent images with ADPase images to create a composite of SS-RBCs and the associated retinal vasculature by manually creating double exposures using photographic processes (Fig. 5). The SS-RBCs were most often observed at precapillary arteriolar bifurcations, suggesting that this site within the vascular hierarchy was the most vulnerable for retention of SS-RBCs. The normal human RBCs injected into control rats were not observed in the retinal vasculature (data not shown). Trapped SS-RBCs were also observed in clusters, apparently adhering to the walls of large veins in a few examples, and obviously not obstructing those vessels (Fig. 6).

The LSCM analysis of the retinal flat mounts enhanced our analytical capabilities primarily by improving the collection and visual presentation of the data. The spatial segregation of microvasculature and large vessels, which required a mechanical step-focus scan of each area with the traditional fluorescent microscope, was done automatically by the LSCM. All components of the bi-layered vasculature could be presented in one stereo image (Fig. 7). The LSCM system permitted the lead ADPase reaction product and the fluorescently labeled cells to be scanned simultaneously. The stacks of images from both scans could be easily aligned and presented...
Confocal Microscopic Imaging of Sickle Erythrocytes

Figure 4. (Left) Flat-mounted rat retina incubated for the histochemical demonstration of adenosine diphosphatase activity and viewed using dark-field illumination. (Right) The same area showing distribution of retained fluorescein-labeled sickle red blood cells in precapillary arterioles.

as a single data set using the Vital Images Voxel View software. A single LSCM 2D optical section through a precapillary arteriole of a test rat indicated less than total obstruction of blood flow (Fig. 8). When views were set in motion with animation, several perspectives demonstrated that the lumen was not totally obstructed (Figs. 9 and 10). The superior resolution of the LSCM system permitted the intimate relationship of SS-RBC and vascular endothelial cells to be observed. At the bifurcation shown in Fig. 10, the SS-RBCs appear to conform to the shape of the vessel wall, suggesting adhesion of the cells to the vascular endothelium.

DISCUSSION

Because the retinal vasculature is bilayered, the ADPase flat-mount retina method lends itself to the optical sectioning and 3D reconstruction permitted by LSCM. Combining fluorescent-dye labeling of SS-RBCs with the ADPase flat-mount technique permitted us to identify sites of adhesion and obstruction by SS-RBCs within the retinal vasculature. Using LSCM, we could collect the fluorescence and dark-field images in one scanning series and display them together as an overlay.

The LSCM optical series of 2D sections provides views not directly observable with the microscope alone. As a result, the extent of lumenal obstruction by SS-RBCs can be examined without the monumental problems of alignment and registration associated with conventional histological sectioning techniques, the only other method for observing the tissue in cross section.

Clinical fluorescein angiography of human subjects and ADPase flat-embedded analysis of retinas from young sickle cell subjects suggest that vaso-occlusions in sickle cell disease occur initially at the level of precapillary arterioles. Our animal model suggests that this echelon of retinal vascular hierarchy is particularly susceptible to the deposition of SS-RBCs. Therefore, our study confirms the precapillary arteriole as the initial site for SS-RBC retention and suggests that in some cases a single cell may not totally obstruct the blood vessel lumen. Kurantzin-Mills, Klug, and Lessin suggested that vortex
currents at the arteriolar bifurcation contribute to the deposition of SS-RBCs at this site. In nonocular tissue such as mesoappendix\textsuperscript{3} and mesocecum,\textsuperscript{4} SS-RBCs have been trapped at the level of postcapillary venules. Different sites of adhesion may occur in different tissues due to the surface molecules on the endothelial cells. Normal RBCs were not retained in the retinal vasculature, demonstrating the validity of our observations using SS-RBCs.

Subsequent work with this model will focus on demonstrating which class of SS-RBC initiates vaso-occlusions in the retinal vasculature. We also hope to show that the cells are truly adherent by flushing the vasculature before enucleation. This model provides a
system for determining the mechanism of adhesion and retention of SS-RBCs and for evaluating therapeutic modalities for preventing SS-RBC-initiated vaso-occlusions in the sickle cell patient.

REFERENCES


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