Permeability of Abnormal Blood-Retinal Barriers in Rat Phototoxic Retinopathy: A Clinicopathologic Correlation Study using Fluorescent Markers

Roy W. Bellhorn and Gary E. Korte

Phototoxic retinopathic rats were studied by vitreous fluorophotometry (VF) and histofluorescence microscopy using NaFl and FITC-Dextran molecules of selected molecular sizes as blood-retinal barrier permeability determinants. Retinopathic rats had significantly higher VF values than normal rats at a 35 mg/kg NaFl dosage; detectable levels were not obtained with 3.5 mg/kg NaFl nor with FITC-Dextran. However, as demonstrated by histofluorescence microscopy, NaFl and FITC-Dex 3 molecules readily passed the abnormal BRB tissues to enter neural retina and vitreous space whereas FITC-Dex 20, 40, 70 and 150 molecules did not, except under certain circumstances. These studies indicate a barrier defect of both the retinal vessels and RPE is responsible for the in vivo demonstrated permeability abnormality. Invest Ophthalmol Vis Sci 24:972-975, 1983

Late stage phototoxic rat retinopathy is characterized by complete loss of outer nuclear and photoreceptor layers and development of fenestrated retinal capillaries within the retinal pigment epithelial layer. Fundus fluorescein angiography demonstrated permeability abnormalities of the blood-retinal barrier (BRB) tissues to sodium fluorescein (NaFl) and to smaller sized fluorescein isothiocyanate labelled dextran molecules (FITC-Dex 3 and 20, but not FITC-Dex 40) in this experimental retinopathy. This present study concerns the usefulness of vitreous fluorophotometry (VF) in assessing abnormal BRB permeability in rat phototoxic retinopathy using NaFl and FITC-Dex molecules, and the use of histofluorescence microscopy to identify abnormally permeable BRB tissues.

Materials and Methods

Sixteen albino and eight pigmented rats, exposed to excessive light as previously described, were studied at times ranging from 44–46 weeks postexposure. Twelve albino and six pigmented age-matched control rats were studied similarly.

Vitreous fluorophotometry was performed on eight pigmented retinopathic and four pigmented control rats. Following anesthesia (sodium pentobarbital 35 mg/kg with ketamine HCL 15 mg/kg ip) and pupil dilation (1% atropine SO 4 with 10% phenylephrine HC1 drops), background midvitreous and plasma fluorescence readings were obtained prior to administering 5% NaFl (0.07 ml/100 g body weight = 35 mg/kg) via a femoral vein. For plasma fluorescein determinations, 50 μl of blood was added to 0.95 ml normal saline, centrifuged at 3,800 rpm for 5 min and the plasma “read” with the fluorophotometer at the center of the test tube. Thirty minutes later, midvitreous and plasma fluorescence levels were again ascertained and, after correcting for background fluorescence, vitreous/plasma ratios were calculated. In addition three retinopathic rats had VF performed using 0.5% NaFl (0.07 ml/100 g body weight = 3.5 mg/kg) and two retinopathic rats using FITC-Dex 3 (0.07 ml/100 g body weight of 33% solution) as fluorescent markers. The 0.5% NaFl solution was used since its fluorescence intensity is equivalent to that of the FITC-Dex solutions. The VF unit was a Haag-Streit slit lamp (model 900) fitted with a 450 μm diameter photometric probe (Gamma Scientific 700-10-14) and Spectrotech SE 40 excitation and SE 50 barrier filters. Slit-beam size was 0.5 × 3.0 mm and the incident light/ocular angle was 20°. Light signals, passed through a photomultiplier tube (Gamma Scientific D-46A), were converted by a digital radiometer.
Table 1. Histofluorescent abnormal permeability of retinopathic rat BRB

<table>
<thead>
<tr>
<th>Permeability marker</th>
<th>NaFl 0.5%</th>
<th>FITC DEX - 3</th>
<th>FITC DEX - 20</th>
<th>FITC DEX - 40</th>
<th>FITC DEX - 70</th>
<th>FITC DEX - 150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulation time (min)</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Number of animals</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Intra-RPE fluorescence</td>
<td>+</td>
<td>+</td>
<td>±*</td>
<td>±*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intra-retinal fluorescence</td>
<td>+</td>
<td>+</td>
<td>±*</td>
<td>±*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* 1 animal with a focal total retinal destruction had leakage. ESR = Einstein-Stokes Radius (theoretical molecular size).

Instrument calibration was accomplished prior to each VF session using freshly prepared serial dilutions ($10^{-4}$ to $10^{-9}$ g/ml) of NaFl.

For histofluorescence studies, all 24 retinopathic and 18 control rats were anesthetized and given 0.07 ml/100 g body weight of either 0.5% NaFl or a selected FITC-Dextran (Table 1), the eyes were enucleated and prepared for freeze-dry fluorescence microscopy as previously described. The posterior segments were divided into four quarters and sections from each quarter were examined. At least 7 days were allowed to pass before those rats having VF performed were prepared for histofluorescence microscopy.

Results

Fluorophotometry demonstrated significantly higher than control values in the vitreous of retinopathic rats receiving 35 mg/kg of NaFl (Table 2). Calculation of the V:P ratio also demonstrated significantly higher values for the retinopathic group. These calculations are necessary for ascertaining the effect of test-induced alterations of clearance of NaFl from the vascular system via the kidneys or into non-ocular tissues, or other factors affecting the level of intravascular fluorescein. Klein et al, for example, found a significantly lower level of plasma fluorescence in streptozotocin-induced diabetic rats, a factor that potentially could significantly alter interpretation of the vitreous fluorophotometric readings.

Histofluorescence microscopy of control rat tissues showed no evidence of any fluorescent marker passing the BRB into neural retina; FITC-Dex 70 and all smaller markers did pass from the choriocapillaris into the choroidal stroma (Fig. 1A) whereas FITC-Dex 150 did not.

The phototoxic retinopathic rat ocular tissues showed the characteristic loss of outer retinal layers and the presence of retinal vessel profiles within the proliferated RPE layer (Figs. 1B, C). Histofluorescence microscopy (Table 1) showed the presence of NaFl and FITC-Dex 3 in the neural retina adjacent to and within the vitreous, adjacent to some small retinal vessels in the inner plexiform and nuclear layers, and adjacent to the RPE (Fig. 1B). Intraretinal and vitreous fluorescence was focally present in two rats receiving FITC-Dex 20 or 40 (one rat each) where a marked focal damage to all retinal layers had occurred. In areas away from the focal lesion where the more characteristic outer retinal layer degeneration was present, those markers did not pass the BRB (Fig. 1C).

Except for the focal lesion present in one quadrant of the posterior segment of two eyes, the histofluorescent findings were otherwise similar in all quadrants of each retinopathic eye.

Discussion

A previous fluorescein angiographic study of this experimental retinopathy demonstrated BRB permeability abnormalities to NaFl and smaller sized FITC-Dex molecules, and several hypotheses were formed to interpret the angiographic findings. One was that dye from the choriocapillaris passed through a defective RPE barrier into the retina; another was that an increasing-in-size focal hyperfluorescence rep-

Table 2. Fluorophotometric values 30 min following IV injection of 5.0% NaFl (35 mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>Vitreous 10^{-7} g/ml</th>
<th>Plasma 10^{-7} g/ml</th>
<th>Vit/Pl Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control group</td>
<td>1.57 ± 0.34 (Mean ± SD)</td>
<td>250.75 ± 70.54</td>
<td>0.0066 ± 0.0021</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Retinopathic</td>
<td>12.56 ± 6.82</td>
<td>187.71 ± 36.54</td>
<td>0.0683 ± 0.0344</td>
</tr>
<tr>
<td>Significance A vs. B</td>
<td><em>P &lt; 0.001</em></td>
<td><em>P &gt; 0.1</em></td>
<td><em>P &lt; 0.001</em></td>
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<td>(Students t Test)</td>
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resented leakage from abnormal retinal vessels; a third was that both focal (nonincreasing in size) and diffuse fluorescence could represent choroidal fluorescence showing through hypopigmented RPE cells; and, fourth, abnormalities of retinal vessels within the RPE could give rise to either focal or diffuse intraretinal fluorescence. The present histofluorescent microscopic study has provided a basis for understanding the in vivo abnormalities.

Based upon the vitreous fluorophotometry and fluorescence microscopy data, the diffuse peripapillary fluorescence observed by fundus angiography was most likely intravitreal fluorescein. The fluorescence microscopic data indicates that the in vivo observed intraretinal fluorescence arises directly from abnormally permeable intraretinal vessels (Fig. 1B) and also from an apparent defect in the RPE barrier allowing dye to pass from the intra-RPE vessels and/or the choriocapillaris through the RPE into the adjacent neural retina. As in the previous in vivo study, the size of the FITC-Dextran molecule is instrumental in the presence or absence of dye leakage. The FITC-Dex 3 molecule was very similar to NaFl in its permeability characteristics (Table 1), but the larger FITC-Dextran molecules showed less evidence of abnormal BRB permeability as their size increased. What is interesting is that while molecules as large as FITC-Dex 70 freely passed from the fenestrated choriocapillaris into the choroidal stroma (Fig. 1A), neither FITC-Dex 70 nor FITC-Dex 40 (Fig. 1C) apparently penetrated the presumably present and previously described fenestrae of the intraRPE vessels. Freeze-dry preparation of tissues for fluorescence microscopy does not allow for demonstration of fenestrae; however, their widespread presence in other studies and the similarity of findings in all four ocular quadrants in this study indicates that fenestrae can be expected to be present in the intraepithelial neural retinal tissue. As in the previous in vivo study, the size of the FITC-Dextran molecule is instrumental in the presence or absence of dye leakage. The FITC-Dex 3 molecule was very similar to NaFl in its permeability characteristics (Table 1), but the larger FITC-Dextran molecules showed less evidence of abnormal BRB permeability as their size increased. What is interesting is that while molecules as large as FITC-Dex 70 freely passed from the fenestrated choriocapillaris into the choroidal stroma (Fig. 1A), neither FITC-Dex 70 nor FITC-Dex 40 (Fig. 1C) apparently penetrated the presumably present and previously described fenestrae of the intraRPE vessels. Freeze-dry preparation of tissues for fluorescence microscopy does not allow for demonstration of fenestrae; however, their widespread presence in other studies and the similarity of findings in all four ocular quadrants in this study indicates that fenestrae can be expected to be present in the intraepithelial

* Vessel walls and Bruch's membrane indicated by drawn lines since conversion of color transparencies to black/white prints causes loss of such details.
† Section thickness (10 microns) can give suggestion of extravascular fluorescence in photographs, but focusing up and down shows all fluorescence to be within vessel wall.
vessels. Since molecular size is the only dissimilar chemical characteristic, it can be said that the fenestrations of the intraRPE vessels may discriminate against dextran molecules of a moderate size whereas the choriocapillaris does not. It is also possible, however, that passage of these larger fluorescent tracers across the BRB into the perivascular space of the intraRPE vessels and thence into the retinal extracellular space is sufficiently slowed such that levels of the fluorescence suitable for detection by fluorescence microscopy are not attained. In the choroid, the greater volume of extracellular space would allow for enhanced opportunity to observe tracer by fluorescence microscopy.

It was disappointing that the preliminary VF study with FITC-Dex 3 was negative. Apparently the lower intensity of fluorescence (only about one fluorescein residue per 100–1,000 glucose residues) was beyond the minimal detectable limits of our VF instrumentation. Using NaFl serial dilutions, the minimal detectable level is in the $10^{-7}$ to $10^{-8}$ range, a range usually encountered using 5.0% NaFl for VF. The 10- to 100-fold decrease in fluorescence using 0.5% NaFl or FITC-Dextran for VF would, on the basis of this data, place the intensity level below the VF sensitivity levels. To overcome this drawback in the use of FITC-Dextran as VF clinicopathologic BRB permeability tracers would necessitate increasing the level of dextran molecule labeling with FITC and/or increasing the sensitivity of our VF instrumentation. The latter point was illustrated recently by Krupin et al in that they detected by VF levels of fluorescence at the $10^{-8}$ g/ml level in the vitreous of rats using FITC-Dex markers.

It is also possible that allowing the fluorescent markers to circulate for 60 min (as in the study by Krupin et al) rather than 30 min would have increased vitreous fluorescence to detectable levels since it has been demonstrated that vitreous fluorescence peaks at about 60 min. An important point of our study, however, is that the concomitant fluorescence microscopy showed that negative VF values were the result of technical factors, which emphasizes the importance of fluorescence microscopy to the interpretation of VF data.

In man, fenestrated vessels in diabetic vitreal proliferative retinopathy have been described as have both fenestrated and nonfenestrated vessels in sub-retinal neovascularization of choroidal origin in age-related macular degeneration. The reasons for the presence or absence of fenestrated vessels in these vasoproliferative retinopathies of man remains unclear. Therefore, continued studies of experimental retinopathies characterized by the development of fenestrated vessels are important for understanding more completely this phenomenon in man.

Key words: retinopathy, rat, blood-retinal barriers, retinal vessels, permeability, Dextran, fluorescent tracers

Acknowledgments

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References