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Effects of free and liposome-encapsulated hemoglobin on choroidal vascular plexus blood flow, using the rabbit eye as a model system

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ABSTRACT: Purpose. *This study investigated the vasoconstrictive effects of both stroma-free and liposome-encapsulated cross-linked hemoglobin (Hb) on vascular plexus hemodynamics, using the choroid of the rabbit eye as a model system.*
Methods. *Sequential subtraction of high-speed ICG fluorescence angiogram images facilitated visualization of the time-varying patterns of blood flow distribution in the choriocapillaris during the cardiac cycle. Differences between baseline and post-hemoglobin injection blood flow distributions were analyzed. Likewise, differences in the time-varying patterns of flow distribution between the particulate and liquid phases of blood during a cardiac cycle were investigated, since this may bear on differences in vasoactivity induced by circulating stroma-free vs. encapsulated Hb.*
Results. *Cross-linked Hb induced a transient, but marked, localized reduction in choriocapillaris blood flow. This effect was significantly attenuated when liposome encapsulated cross-linked hemoglobin was administered. Plexus blood flow distribution was different for particulate and liquid ICG.*
Conclusions. *Differences in particulate and liquid ICG flow patterns suggest that one contribution to the different plexus blood flow patterns observed in the encapsulated and free Hb experiments may be due to differences in liquid and particle-bound Hb distribution within the plexus. The observed choriocapillaris blood flow reductions may be attributable to an aggregate endothelial cell contractility induced by presence of extra-cellular Hb in the choriocapillaris plexus. (Eur J Ophthalmol 1999; 9: 103-14)*

KEY WORDS: Hemoglobin, Vascular plexus, Choroid, Hemodynamics, Blood

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INTRODUCTION

Considerable attention has been paid to the role of hemoglobin in regulating flow and oxygen delivery in the microcirculation (1-5). In recent years, the identification of nitric oxide as a key component of vascular tone regulation (originally identified as endothelium derived relaxation factor), and the well known strong interaction of nitric oxide with hemoglobin, have led investigators to examine the direct effect of hemoglobin on vascular tone in the microcirculation (6). To date, the majority of these studies have focused on the interaction of free hemoglobin in clinical indications such as subarachnoid hemorrhage and the application of acellular hemoglobin as an oxygen carrying fluid in indications in which red blood cells are currently used (blood substitutes) (7). In numerous animal and human studies, application of acellular hemoglobin has resulted in a substantial systemic pressor response (8-19). Yet, few studies have examined this pressor response and the resultant alteration of blood flow at the microcirculatory level.

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Investigations of hemoglobin action in the microcirculation to date have centered on end-arteriolar vascular systems. The most widely used model for investigating interactions of hemoglobin in the microcirculation has been the use of intravital microscopy in hamster skin fold studies. This model has been used to generate a "functional capillary density" which defines the number of capillaries observed to transit red blood cells. This model has been used to examine red blood cell velocity and oxygen delivery and to relate the capillary density to changes in blood flow induced by vasoconstrictor agents, including free and modified hemoglobins. The introduction of acellular hemoglobin has been shown to result in a marked reduction in functional capillary density, presumably due to the binding of nitric oxide and the subsequent inhibition of relaxation, although other factors such as increased arteriolar oxygen may also contribute to this effect (20).

The effects of encapsulation of hemoglobin on altering the well known vasoconstrictor properties of free hemoglobin recently have been examined . It was shown that encapsulation of hemoglobin attenuates (by 60-100 fold) vasoconstriction in both rabbit thoracic arterial segment and perfused ear artery preparations (21). The attenuation was hypothesized to be due to the reduced extravasation of the hemoglobin when encapsulated, with subsequent reduced interactions with nitric oxide. This hypothesis was supported by evidence which showed that the Hb-induced vasoconstrictor response was relieved following the infusion of a nitric oxide donor, s-nitrosylpenacillamine.

In the present study, we examined the effect of free and encapsulated hemoglobin introduction in the choroidal plexus of the rabbit eye. A unique morphological feature of the choroidal vasculature of the eye is that all of its capillaries lie in a single plane and form a vascular plexus. This vascular plexus, the choriocapillaris, lies immediately adjacent to the sensory retina, separated from it only by the single-cell thickness of the retinal pigment epithelium (22). The choroidal vasculature, which has one of the highest blood flows per gram of tissue anywhere in the body (23), consists of three layers: the outer-most (furthest from the retina) consists of large diameter arteries and veins which give rise to a middle layer of smaller diameter arteries and veins, and these in turn feed and drain the single-capillary thick choriocapillaris. The choriocapillaris is a true vascular plexus in that it is possible to move from any point within the choriocapillaris to any other point without traversing arteriolar or venular vessels. Viewed from its anterior side, the choriocapillaris is a fairly uniform tight meshwork composed of short segments of large diameter capillaries; the density of the meshwork decreases with radial distance from the posterior pole of the eye toward the periphery. Feeding arteriolar vessels from the underlying middle vascular layer enter the choriocapillaris approximately 90° to the capillary plane. Interspersed amongst the arterioles, draining venular vessels exit the choriocapillaris at oblique angles to the capillary plane as they enter the adjacent middle layer.

There are compelling morphological similarities between the vasculature of the choroid and that of the lung alveoli, similarities which serve as a catalyst to more intense investigation of the choroidal vascular plexus as a model system. The capillary blood vessels in the pulmonary alveoli particularly resemble those of the choriocapillaris in that they too are short, closely knit, and of such large diameter that they also can accommodate passage of several erythrocytes abreast (Fig. 1); in fact, it is clear by virtue of the close morphologic similarity of these two plexes that the concept of sheet flow (24), used to describe movement of blood through alveoli capillaries, applies equally well to the choriocapillaris. There is, however, a major dissimilarity between the capillary plexes of the cortex and alveoli and that of the choroid: whereas these two plexes are not readily accessible to non-invasive observation, the choroidal vasculature can be readily probed non-invasively by optical means.

In general, the complex angioarcitecture of plexes defies construction of simple models to describe blood flow through them, but since plexes are connected to multiple, interspersed feeding and draining vessels, an intuitive conclusion is that plexus blood flow must result from a complex network of local perfusion pressure gradients established amongst its feeding and draining vessels. Even though it may not be possible to define adequately all of the major parameters involved in regulating blood flow through a particular plexus, it is possible at least to characterize the overall distribution of blood flow through it, as well as time-varying changes in that distribution. Such a

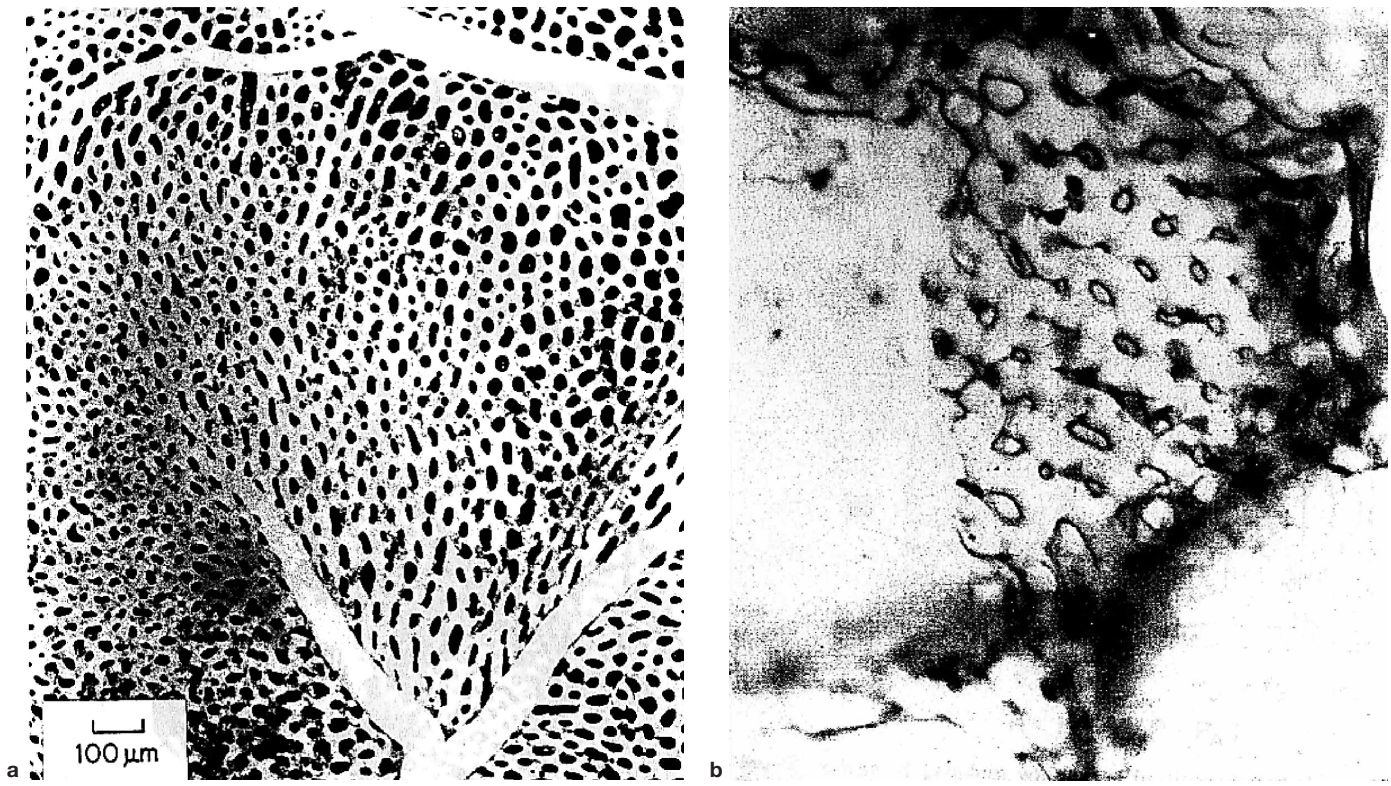

Fig. 1 - *Angioarchitecture of (***a***) the choriocapillaris (posterior view, showing draining veinoules) and (***b***) lung alveoli.*

characterization has been developed for the choroidal plexus of the eye.

MATERIALS AND METHODS

Visualization of choriocapillaris blood flow

The choroidal vasculature, which lies behind the sensory retina, is hidden from direct visualization by a monolayer of pigmented endothelial cells, sandwiched between the sensory retina and the choroid; moreover, interstitial tissue of the choroid itself is fairly densely pigmented also. High-speed indocyanine green (ICG) dye fluorescence angiography was developed by one of the present investigators to overcome the major problems encountered when attempting to visualize the rapid choroidal blood flow. ICG angiography utilizes near-infrared wavelengths, which penetrate the retinal pigment epithelium and choroidal pigment with relative ease. Since the majority of ICG molecules are bound to blood protein, ICG fluorescence arises from dye molecules in the blood volume moving through the choroidal vasculature (25). Although the technique of ICG dye fluorescence angiography makes it possible to routinely visualize choroidal blood flow, visualizing just choriocapillaris blood flow requires processing raw ICG angiogram images.

Circulating ICG dye first enters the layer of larger diameter vessels underlying the choriocapillaris layer, and, although the fluorescence from the two vessel layers is additive, the much greater fluorescence intensity of the underlying vessels obscures that of the choriocapillaris. However, a method whereby images of just the choriocapillaris circulation can be extracted from a sequence of raw ICG angiogram images has previously been developed and demonstrated (26-27). The technique derives from the facts that: (1) ICG fluorescence arising from the choriocapillaris is additive to that arising from the larger diameter, underlying vessels which feed and drain the choriocapillaris and to that arising from the vessels that permeate the overlying sensory retina, and (2) blood flow velocity through the choriocapillaris is pulsatile and significantly greater than the average velocities through ves-

sels in both the over- and under-lying layers. This means that, for any short time interval (e.g., 1/15 - 1/30 sec.), the percent change in fluorescence associated with the choriocapillaris is significantly greater than the percent change in fluorescence associated with the overlying or underlying vessel layers. Therefore, pixel-by-pixel subtraction of successive angiogram images produces a sequence of resultant angiogram images showing only the fluorescence arising from the choriocapillaris layer. However, by adjusting the span between subtracted images, the dye wave front moving through the arteriolar vessels feeding the choriocapillaris can be seen as well. That is , ordinarily image 1 is subtracted from image 2, 2 from 3, 3 from 4, etc., but instead image 1 can be subtracted from image 3, 2 from 4, 3 from 5, etc. This latter method was used throughout the images shown in this study so that arteriolar and capillary plexus blood flows could both be visualized.

To acquire the raw angiogram images, a small bolus (0.1 ml) of concentrated ICG dye (12.5 mg/ml) was injected intravenously and followed immediately by a larger rapidly injected bolus of isotonic saline; the saline pushes the dye bolus into larger vessels having more rapid flow. As the dye bolus travels to the eye, it becomes diluted to a point that its fluorescence is most efficient; ICG dye is subject to concentration fluorescence quenching, and its concentration in blood that produces the most efficient fluorescence (0.03 mg/ml) as well as the dilution an intravenously injected dye bolus undergoes in transit to the eye has been determined (28). Passage of dye-tagged blood through the choroid is recorded by a Zeiss fundus camera equipped with a pulsed (5 msec) laser diode light source to excite the dye to fluorescence; the laser is synchronized with a gated intensified CCD video camera (Xybion, Inc., San Diego, CA) that records the angiogram images at a rate of 30/sec. Typically, forty-eight to sixty-four 512 x 480 pixel images were digitized and stored in a PC computer during each dye transit period of approximately 2 seconds. Subsequently, the sequences of angiogram images were sequentially subtracted as described above.

Hb sample preparation

As much of the background data on the vasoconstrictor properties of Hb have indicated that vasoconstrictive effects are observed in normovolemic animals, we decided that administration of a top loading volume of either stroma-free cross-linked hemoglobin (XLHb) or encapsulated XLHb in the rabbit would be sufficient to generate data on Hb effects in the choroidal plexus. The characteristics of the Hb solutions that were used in our investigations are presented in Table I. The cross-linked hemoglobin used for these studies was prepared using bis (3,5-dibromosalicyl) fumarate (DBBF) as the cross-linking agents which cross-links the two alpha subunits of hemoglobin at the lysine 99 residues. The cross-linked hemoglobin was prepared under Good Laboratory Practices at the Armed Forces Blood Detachment facility (Gaithersburg, MD).

Rabbit preparation, Hb sample injection, and angiogram acquisition

For each test solution, 3 pigmented Dutch Belted rabbits were ketamine anesthetized (50 mg/kg) and had their eyes dilated with Tropicamide. Two 30 image/

TABLE I - PHYSICOCHEMICAL CHARACTERISTICS OF HUMAN HEMOGLOBIN SOLUTIONS

	Stroma-free XLHb	**Encapsulated XLHb**
Hemoglobin Conc. (g/dl)	3	3
metHemoglobin (%)	5	5-7
P_{50}	29	29
pH	7.28	7.28
Phospholipid (mg/ml)	4.5×10^{-4}	58
Free Iron (mole Fe/heme)	1.67×10^{-5}	1.67×10^{-5}
Endotoxin (Eu/ml)	0.25	<2.5
Diameter (microns)	—	0.18

sec. base-line angiograms were performed following injection of 0.10 ml of 1.2 mg/ml ICG dye and a 1 ml isotonic saline flush through an ear vein catheter. Each test solution was then administered through the ear vein at a volume equivalent to 10% of the rabbit's total blood volume (calculated on the basis of its weight) during a 3 to 4 minute period. Subsequently, ICG angiograms were acquired at approximately 15, 35, 65, and 85 minutes following infusion of the Hb solution as previously described. The angiogram sequences were then sequentially subtracted to produce images of dye transit through the isolated choriocapillaris plexus, and these resultant image sequences were compared.

The rates of dye-stained blood movement through individual vessels that feed the choriocapillaris plexus do not readily lend themselves to quantification, since the wave front of dye movement through those vessels is not perfectly delineated in the angiograms. Quantification of the plexus dye filling rate would be even more difficult, since plexus dye movement is multi-directional and not symmetrical. For these reasons, our data are presented in terms of differences in time-varying plexus dye filling patterns which readily can be seen in side-by-side comparisons of angiogram image sequences.

RESULTS

The effects of stroma-free XLHb on rabbit choroidal blood flow

Analysis of data consisted of side-by-side, image-by-image comparisons of angiograms. This revealed areas of markedly reduced choriocapillaris blood flow following XLHb infusion, the reduction in blood flow progressively decreasing throughout the periods of observation.

A summery of the typical blood flow changes that occurred during one of the periods of observation is presented in Figure 2. Each row consists of images from the same angiogram sequence: the first two rows are images from the two base-line angiograms acquired just prior to XLHb infusion, and the other rows are images from the angiograms acquired 15, 35, 65, and 85 minutes following infusion. All the rows of angiogram images are in phase with each other according to first appearance of dye in the same early-filling arteriole. Each column consists of images obtained at the same interval during dye transit through the choroidal vasculature. The first column, on the left, consists of images showing first appearance of dye in the same short arcuate vessel in the extreme upper left-hand corner of each image (because of density differences in the negative film strips used to make the figure, they are extremely faint in those first images of the last two rows). Then, moving left to right, the columns consist of images acquired 3/30 sec., 9/30 sec., and 15/30 sec. later. Using first appearance of dye in the same arteriolar vessel is a valid synchronization method because initiation of that event depends only upon arrival of a dye bolus wave front in the arterial vessels feeding the eye; it is a circulation event distal to the heart and is therefore independent of the phase relationship between venous dye injection and the cardiac cycle.

It should be noted that the overall time-varying dye-filling pattern changes recorded during the two baseline angiograms (the first two rows) are virtually identical; they were also consistent with other angiograms of the same eye made during earlier observations. Thus, the filling patterns become a microvasculature fingerprint of the animal from which to examine possible changes due to the infusion of the test materials. In the third row, acquired 15 minutes after XLHb infusion, a significant change in the dye filling pattern is evident in the second image (at 3/30 sec. later than the first image) and remains notable in the next two images. Specifically, a well demarcated, vertically-oriented band of reduced fluorescence, starts at the top and center of the field of view and extends down to the center; it is most pronounced in the third row, third column image; this is consistent with reduced regional choriocapillaris blood flow. (If during the time interval between which two sequential images were acquired, no change took place in the blood flow distribution within a particular area of a particular layer of choroidal vessels, then that area would appear dark in the resultant subtracted image. Thus, the vertically-oriented band is interpreted as such a lack of change in the choriocapillaris. But note that a large diameter underlying vessel in the center of the band is visible, indicating that during the same time interval a change in flow took place in it.) At the same time, filling of the two prominent, centrally-located arteriolar trees seen in the baseline an-

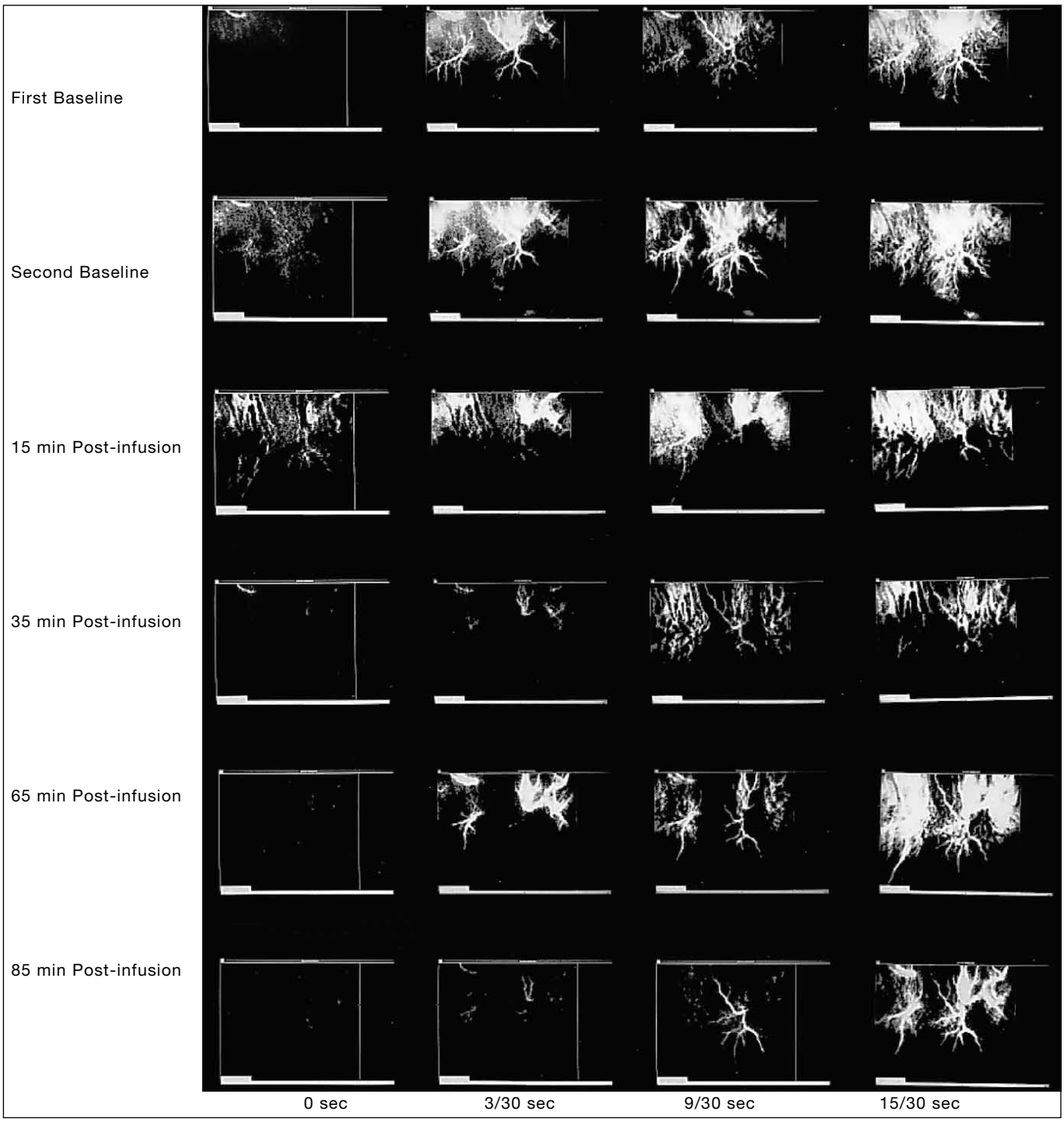

Fig. 2 - *ICG angiograms of the choriocapillaris following infusion of stroma-free XLHb. Each row consists of images from the same angiographic sequence: the first two rows of images from the two base-line angiograms acquired just prior to 10% XLHb top-load infusion, and the other rows are images from the angiograms acquired 15, 35, 65, and 85 minutes following infusion. All rows are in phase with each other according to the first appearance of dye in the same arcuate arteriole at the top left-hand edge of the 0 sec. images. Note that in the Second Baseline and 15 min. Post-infusion 0 sec. images that residual dye from the previous injections is visible in the venules that drain the plexus. Each column consists of images obtained at the same interval during dye transit through the choroidal vasculature. Each image shows a 10° fundus field of view, inferotemporal to the optic disc, just inferior to the margin of the medullary rays.*

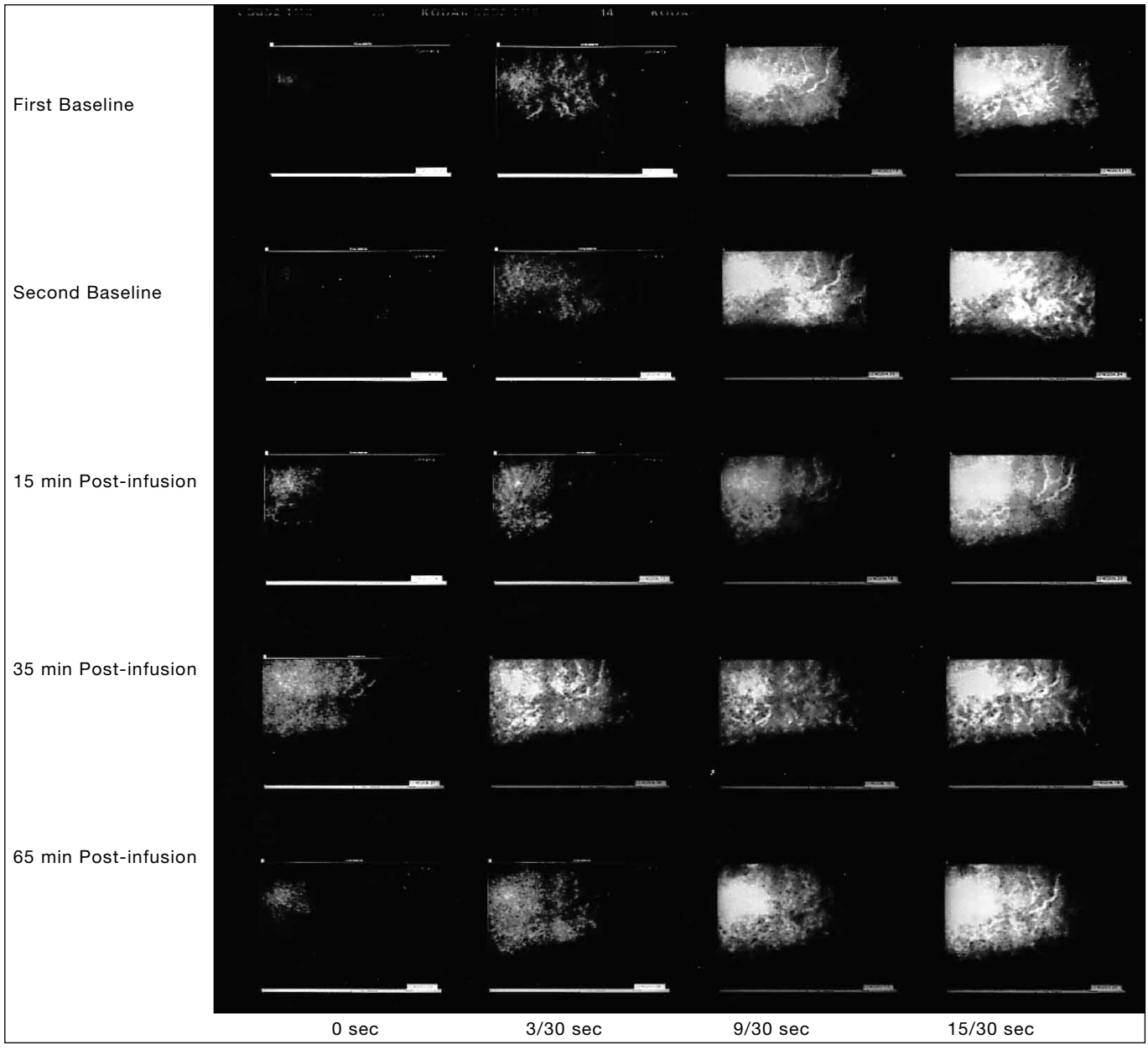

Fig. 3 - *ICG angiograms of the choroidal plexus following infusion of encapsulated XLHb. Each row consists of images from the same angiographic sequence: the first two rows of images from the two base-line angiograms acquired just prior to 10% encapsulated XLHb top-load infusion, and the other rows are images from the angiograms acquired 15, 35, and 65 minutes following infusion. All rows are in phase with each other according to the first appearance of dye in the same arteriole. Each column consists of images obtained at the same interval during dye transit through the choroidal vasculature. Each image shows a 10° fundus field of view, inferotemporal to the optic disc, just inferior to the margin of the medullary rays.*

giograms appeared to become retarded (compare the middle two images in rows 3 and 4 to those in the first two and last two rows). The inferior portion of each image in the figure remains dark, although the dark area diminishes moving left to right across each row. This is because during the approximately one-half second interval represented by the four images in each row, dye flow does not reach the most peripheral parts of the vasculature located at the bottom of the image field of view.

When this same top-loading experiment was repeated using an equivalent volume of isotonic saline instead

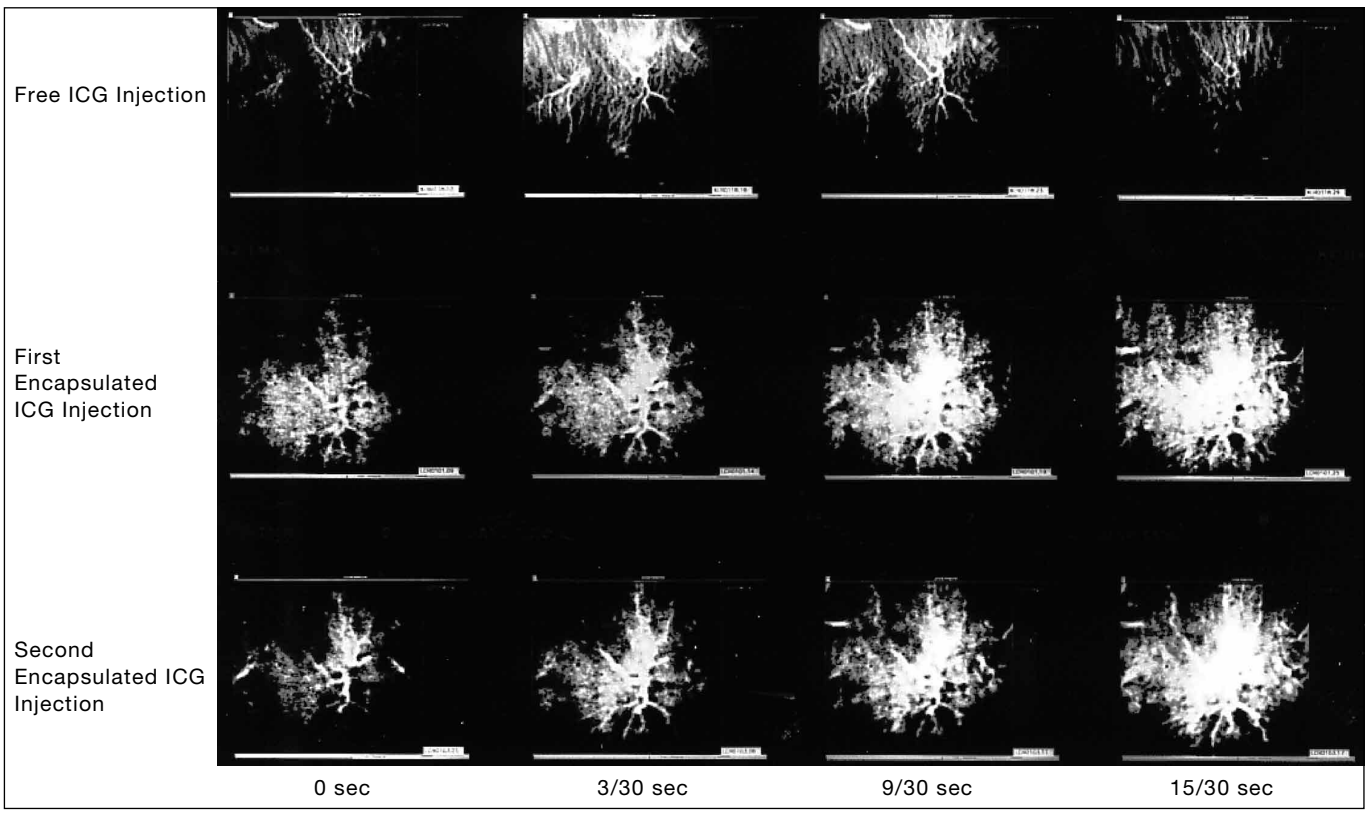

Fig. 4 - *Liquid vs. encapsulated ICG angiograms in the choroidal plexus. Liquid ICG (row 1) and liposome-encapsulated ICG (rows 2 and 3) angiograms. The columns of images are in phase, based on aligning images showing the same extent of filling of the predominant arterial vascular tree. Each image shows a 10° fundus field of view, inferotemporal to the optic disc, just inferior to the margin of the medullary rays.*

of XLHb, no change in filling patterns was detected in the angiograms made during the same time intervals following infusion. Thus, we are convinced by the preeminence and consistency of the dye-filling patterns we observed that infusion of stroma-free XLHb produces filling defects in choroidal plexus blood flow, relative to the normal baseline filling patterns. This finding is consistent with previous observations in an exchange transfusion study in the hamster skin fold preparation in which a transient but market reduction in functional capillary density was observed following the infusion of a similar hemoglobin preparation (6). This is the first demonstration of similar effects in the vasculature architecture of a plexus. The mechanism of this action is presently not defined but may be related to the potent interaction of nitric oxide and hemoglobin and the resultant inhibition of vasorelaxation. The spatial distribution of this effect within the choroid suggests that there are regions that are more susceptible to vasoconstrictor activity. This may be instructive in terms of spatially defining local regions which may be more involved with regulating global blood flow in this architecture.

Effects of liposome encapsulated XLHb on rabbit choroidal blood flow

Analysis of angiogram sequences following infusion of encapsulated XLHb revealed discrete areas of defective choriocapillaris filling that, as in the case of stroma-free XLHb infusion, appeared within 15 minutes following infusion, but the duration of the defects was relatively brief (less than 65), compared to that noted following infusion of stroma-free XLHb (more than 85 minutes). Interestingly, the location and shape of the defect was quite similar to that observed both times in the of stroma-free XLHb-infused rabbit. Figure 3 shows an array of ICG angiogram images, similar to that in Figure 2, from one of the animals. The rows are, from top to bottom, respectively: two base-

lines, 15, 35, and 65 minutes following infusion of liposome encapsulated XLHb; the columns are, from left to right, respectively: earliest filling of the small group of vessels in the upper left corner of the field of view (that small group, presumably consisting of end-on-viewed arterioles, filled concomitantly with a much larger patch of choriocapillaris in the 3rd and 4th rows), and then images acquired 3/30 sec., 9/30 sec., and 15/30 sec. later.

The two baseline angiograms are virtually identical except for the fact that the arterioles in the second frame of the first row appeared to fill a fraction of a second (less than 33 msec.) faster than in the second row, but the overall filling pattern is quite consistent. Beginning in the second frame of the third row (just 15 min. after blood substitute infusion, reduced filling of the right-hand side of the field of view is apparent, and in the 3rd and 4th frames, it is obvious that a defect similar to that pointed out in Figure 3 is present in these angiograms as well; note that as in the stroma-free infusion experiment, the vertically-oriented defect is immediately adjacent to an arteriolar tree feeding the choriocapillaris plexus. However, by 35 minutes following infusion, the dye filling-pattern begins to again resemble that of the baseline patterns, and by 65 minutes is virtually identical. Since this same time course of events occurred consistently, we concluded that although the same blood flow pattern change occurred as a result of infusing stroma-free and liposome-encapsulated XLHb, encapsulation attenuated the degree and duration of this effect, presumably due to the vasoconstrictor activity of Hb.

The encapsulation of hemoglobin in liposomes has been shown to attenuate the vasoconstrictor responses of hemoglobin in isolated *in vitro* aortic segments (21). This is the first report of attenuated vasoconstrictor activity in the microcirculation with encapsulated hemoglobin and supports the previous *in vitro* findings. The attenuated activity has been suggested to result from greater retention of hemoglobin in the vascular compartment, with reduced extravasation to sites of potential interaction with nitric oxide. This hypothesis is supported by stopped flow kinetic binding studies of NO-hemoglobin binding that have shown that similar binding constants for NO-hemoglobin in encapsulated and acellular forms. This also suggests that the lipid bilayer of the liposome does not retard diffusion of NO. The vasoconstrictor activity of acellular and encapsulated hemoglobin may thus be dependent on their spatial distribution in the vascular bed. This was the basis for our examination of the distribution of the particulate and solution phases within the choroidal plexus.

Visualization of choroidal plexus blood flow using particulate vs. liquid ICG

In order to investigate whether the differences in stroma-free and encapsulated XLHb vasoactivity are due to differences in the flow distribution of particulate vs. liquid phase in the plexus, we have investigated the differences in angiograms generated from free ICG and ICG encapsulated in liposomes (with no encapsulated hemoglobin). In these experiments, the same rabbits used in the XLHb experiments were subjects. To avoid any effects from the blood substitute experiments, more than two months elapsed before these experiments were performed. As before, ketamine anesthesia and Tropicamide dilation were employed. After baseline angiograms were acquired using the usual ear vein injection of 0.01 ml of liquid ICG dye, angiograms were obtained using ear vein-injected 0.01 ml boluses of 150 nm diameter liposomes encapsulating ICG at a concentration of 10^{-3} mg/ml of physiologic buffer.

Figure 4 compares angiograms made of the same rabbit eye, using liquid ICG (row 1), and liposome-encapsulated ICG (rows 2 and 3); again, the columns of images are all in phase, based on aligning images showing the same extent of filling of the predominant arteriolar tree. Note that the starting time shown is slightly later in the cardiac cycle than the one shown for the stroma-free infusion experiment; that is, the first two frames in row 1 of Figure 4 are equivalent to the last two frames shown in rows 1 and 2 of Figure 2. The columns again each contain images of identical intervals following those in the first column. Note also that the fields of view in the 2nd and 3rd rows of Figure 4 are slightly different from that in the 1st row; that is, in the ICG-liposome angiograms, the view is more centered on the predominant arteriolar tree. The most interesting difference in the ICG-liposome angiograms is that the choriocapillaris filling is more apparent than in the liquid ICG angiograms. We speculate that the phenomenon may be the result of differences in blood component (liquid vs. particulate)

flow distribution of particulate vs. free ICG. A simple explanation for the difference being that the dispersion of the ICG-containing liposomes throughout the plexus is much slower relative to dispersion of the free ICG molecules. The important point is that a significant difference in filling patterns is discernable.

DISCUSSION

We have investigated the effects of a vasoconstrictor agent, hemoglobin, on flow dynamics in the choroidal plexus in the eye. To our knowledge this is the first time such an investigation has been carried out in an *in vivo* vascular plexus model system, although Hahn, et al investigated the effects of stroma-free hemoglobin on arterial diameter and blood flow in rat tumors (29). Choroidal filling and emptying patterns were extremely reproducible, which provided a fingerprint for each animal from which to examine blood flow changes induced by the infusion of hemoglobin. Two hemoglobin solutions were intravenously injected into anesthetized rabbits, and their plexus blood flows were dynamically imaged over the course of 2 hours. Injection of acellular cross-linked hemoglobin resulted in significant defects in filling which were ameliorated over 90 minutes. Encapsulation of hemoglobin in liposomes attenuated this effect. As these effects may be due to partitioning of particulate and solute phases within the plexus, we used liposomes containing ICG to follow the particulate versus liquid phases of blood flow. Previous studies have shown that liposomes with hemoglobin behave as non-Newtonian fluids (30), like red blood cells, and therefore, we believe they demonstrate particulate behavior rather than fluid, or plasma flow behavior. These studies demonstrated that the plexus flow distribution was indeed different for particulate and liquid ICG, suggesting that one contribution to the different plexus blood flow patterns observed in the encapsulated and free hemoglobin experiments may be due to differences in liquid and particle-bound Hb distribution within the plexus. The retention of hemoglobin within the vascular compartment by encapsulating in liposomes may also contribute to this effect.

The vasoconstrictive effects of hemoglobin on choriocapillaris hemodynamics in our experiments were manifest as markedly altered blood flow patterns, but identifying the specific mechanism through which they were altered was beyond the scope of this study. There are, nevertheless, known aspects of the choroidal vascular plexus that help narrow the spectrum of possibilities. Foremost, it is important to recognize that blood flow patterns through the choriocapillaris plexus must be governed by whatever complex network of local perfusion pressure gradients is established amongst the interspersed feeding and draining vessels connected to its posterior side. These patterns have been shown to be unique to each eye because of the particular distribution of feeding and draining vessels in each eye, and those patterns have been shown to be very stable over long periods of time (26-27). Consequently, any perturbation within the perfusion pressure network of a given eye could change the plexus blood flow pattern, the most likely perturbing event being change in vascular resistance to flow. The most likely such change would be increased resistance due to vasoconstriction as opposed to decreased resistance due to vasodilatation, because given the density of the choriocapillaries, the small spaces between adjacent capillaries, and the relatively dense tissue milieu within which they lie, there is there is little room in which dilation could take place. Increased vascular blood flow resistance could be localized, as in vasoconstriction of feeding arteriolar vessels delivering blood to the plexus, or resistance increase could be diffusely distributed throughout a large area of the plexus, as produced collectively by very small changes in the capillary cell walls, or a combination of both of these could exist. The changes seen in the XLHb experiments, however, are consistent with the major component of increased vascular resistance being distributed throughout a segment of plexus area. For example, the decreased capillary plexus flow demonstrated in Figure 2 began in an isolated, vertically oriented segment (see row 3) before it became more generalized (as in row 4, 20 min. later). Trying to account for reduced flow to that segment on the basis of increased resistance localized to the underlying feeding arteriole is unconvincing, since if such an event were to have taken place, dye-stained blood from the adjacent plexus areas would have filled the deprived segment. Moreover, the images in both rows 3 and 4 indicate that dye-stained blood continued to be present in the underlying arteriolar vessels throughout.

Evidence for endothelial cell contractility appar-

ently started with Stricker's 1865 studies (31), and since then various other investigators have provided additional evidence (32-35). More recently, Aharinejad and co-workers elegantly demonstrated contraction of capillary endothelial cells in the mouse exocrine pancreas as a mechanism for flow regulation (36), and their work "suggests that capillaries may contribute to flow regulation when the whole microvascular bed of an organ is considered". In the context of the present study, it is conceivable that small contractions of the intra-cellular filaments within a majority of the choriocapillaris endothelial cells in any large segment of the choriocapillaris plexus would be manifest as a decrease in capillary wall flexibility and area, and hence, as an increased resistance to capillary blood flow through that segment. This concept certainly is consistent with the results of our XLHb experiments.

It seems reasonable also that the same changes in choriocapillaris plexus blood flow induced by the presence of extra-cellular hemoglobin can be induced in other plexes and by other vaso-active substances. Therefore, the choroidal vasculature of the eye should serve as a readily accessible capillary plexus model system for investigating the effects of a variety of vasoactive substances. And since the methodology for visualizing choriocapillaris blood flow is based on data acquired by an angiogram methodology already approved for and now widely used in human subjects, human vascular plexus hemodynamics can be routinely studied by our method.

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REFERENCES

1. Tsai A, Kerger H, Intaglietta M. Microvascular oxygen distribution: effects due to free hemoglobin in plasma. In: Winslow RM, Vandegriff KD, Intaglietta M, eds. Blood substitutes: New challenges. Boston: Birkhauser, 1996; 124-31.
2. Smiesko V, Lang DJ, Johnson PC. Dilator response of rat mesenteric arcading arterioles to increased blood flow. Am J Physiol 1989; H1958-65.
3. Jackson WF. Regional differences in mechanism of action of oxygen on hamster arterioles. Am J Physiol 1993; 265: H599-603.
4. Tsai AG, Friesenecker B, Intaglietta M. Capillary flow impairment and functional capillary density. Int J Microcirc Clin Exp 1995; 15: 248-3.
5. Winslow RM. Oxygen transport, oxygen carriers, and the transfusion trigger. In: Rudolph, Rabinovici, Feuerstein, eds. Red blood cell substitutes: basic principles and clinical applications. NY: Marcel Dekker, 1995; 37-69.
6. Tsai AG, Kerger H, Intaglietta M. Microcirculatory consequences of blood substitution with hemoglobin solutions. In: Rudolph, Rabinovici, Feuerstein, eds. Red blood cell substitutes: basic principles and clinical applications. NY: Marcel Dekker, 1998; 69-79.
7. Sanrdrzadeh SMH, Anderson DK, Panter SS. Hemoglobin potentiates central nervous system damage. J Clinc Invest 1989; 79: 662-4.
8. Schultz SC, Grady B, Cole F. A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med 1993; 122: 108-301.
9. Przbelshi RJ, Daily EK, Kisieki JC, Mattia-Goldberg C, Bounds MJ, Colburn WA. Phase I study of the safety and pharmacologie effects of diaspirin cross-linked hemoglobin solution. Crit Care Med 1996; 24: 1993-2000.
10. Winslow, RM. The toxicity of hemoglobin in hemoglobin based red cell substitutes. Baltimore: Johns Hopkins University Press, 1992; 136-62.
11. Savitsky JP, Doczi J, Black J, Arnold JD. A clinical safety trial of stroma-free hemoglobin. Clin Pharmacol 1978; 23: 73-80.
12. Zuck TF, Riess JG. Current status of injectable oxygen carriers. Crit Rev Clin Lab Sci 1994; 31: 295-324.
13. Przybelski J, Malcolm DS, Burris DG, Winslow RM. Cross-linked hemoglobin as a resuscitative fluid after hemorrhage in the rat. J Lab Clin Med 1991; 117: 143-51.
14. Hess JR, MacDonald VW, Brinkley WW. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. J Appl Physiol 1993; 74: 1769-78.
15. Thompson A, McGarry AE, Valeri R, and Lieberthal L. Stroma-free hemoglobin increases blood pressure and GFR in the hypotensive rat: role of nitric oxide. J Appl Physiol 1994; 77: 2348-54.
16. Murray JA, Ledlow A, Launspach J, Evans D, Loveday M, Conklind JL. The effects of recombinant human hemoglobin on esophogeal motor function in humans. Gas-

troenterology 1995; 109: 1241-8.
17. Collins P, Burman J, Chung HI and Fox K. Hemoglobin inhibits endothelium-dependent relaxation to acetylcholine in human coronary arteries *in vivo*. Circulation 1993; 87: 80-5.
18. Martin W, Villani GM, Designaro J, Furchgott F. Selective blockade of endothelium-dependent glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J Pharmacol Exp Ther 1985; 232: 708-16.
19. Furchgott RF, Zawadski JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-6.
20. Jackson WF, Duling BR. The oxygen sensitivity of hamster cheek pouch arterioles. *In vitro* and *in situ* studies. Circulation Res 1983; 53: 515-25.
21. Rudolph AS, Sulpizio A, Hieble P, MacDonald V, Chavez M, Feuerstein G. Liposome encapsulation attenuates hemoglobin induced vasoconstriction in rabbit arterial segments. J Appl Physiol 1997; 82: 1826-35.
22. Torczynski E, Tso MOM. The architecture of the choriocapillaris at the posterior pole. Am J Ophthalmol 1976; 81: 428-40.
23. Alm A, Bill A, Young FA. The effects of pilocarpine and neostigmine on the blood-flow through the anterior unea in monkeys. A study with radiactively labelled microspheres. Exp Eye Res 1978; 15: 31-6.
24. Fung YC, Sobin SS. Theory of sheet flow in lung alveoli. J Appl Physiol 1969; 26: 472-88.
25. Flower RW. Choroidal fluorescent dye filling patterns: a comparison of high speed indocyanine green and fluorescein angiograms. Int Ophthalmol 1980; 2: 143-9.
26. Flower RW. Extraction of choriocapillaris hemodynamic data from ICG fluorescence angiograms. Invest Ophthalmol Vis Sci 1993; 34: 2720-9.
27. Flower RW, Fryczkowski AW, McLeod DS. Variability in choriocapillaris blood flow distribution. Invest Ophthalmol Vis Sci 1995; 36: 1247-58.
28. Flower RW, Hochheimer BF. Quantification of the indicator dye concentration in ocular blood vessels. Exp Eye Res 1977; 25: 103-11.
29. Hahn JS, Braun RD, Dewhirst MW, et al. Stroma-free human hemoglobin A decreases R3230Ac rat mammary adenocarcinoma blood flow and oxygen partial pressure. Radiat Res 1997; 147: 185-94.
30. Jopski B, Pirkl V, Schmidt K H. Viscosity measurements of suspensions of hemoglobin containing liposomes of varying lipid composition. Biomaterials, Artificial Cells, & Artificial Organs 1990; 18: 345-58.
31. Stricker S. Studien uber den Bau und das Leben der kapillaren Blutgefasse. Sitzungsber. Wiener Akad Math Naturwiss 1865; 52: 379-93.
32. Zweifach BW. A micro-manipulative study of blood capillaries. Anat Rec 1934; 59: 83-108.
33. McCuskey RS and Chapman TM. Microscopy of the living pancreas *in situ*. Am J Anat 1969; 126: 395-408.
34. Weigelt H. Die spezialsierte Endothelzelle-erregbare Zelle und mechanischer Effektor der Mikrozirkulation. Funkt Biol Med 1982; 1: 53-60.
35. Dietrich HH and Weigelt H. Effect of adrenaline on capillary diameter in the frog mesentery. In: Lubbers DW, Acker H. Leninger-Follert E, Goldstick TK, eds. Oxygen transport to tissue. New York: Plenum Press 1984; 5: 747-53.
36. Aharinejad S, MacDonald IC, Schmidr EE, Bock P, Hagen D, Groom AC. Scanning and transmission electron microscopy and high resolution intravital video-microscopy of capillaries in the mouse exocrine pancreas, with special emphasis on endothelial cells. Anat Rec 1993; 237: 163-77.