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Advances in Brief

Effect of Red Blood Cell Rigidity on Tumor Blood Flow: Increase in Viscous Resistance during Hyperglycemia[1]

Eva Marie Sevick[2] and Rakesh K. Jain[3]

Tumor Microcirculation Laboratory, Department of Chemical Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213-3890

Abstract

Elevated glucose level and low pH have been shown to increase red blood cell (RBC) rigidity. This increased rigidity has been proposed as one factor which mediates the tumor blood flow (TBF) reduction during hyperglycemia by (*a*) causing RBC entrapment and hence increasing geometric resistance and (*b*) increasing viscous resistance to blood flow. However, due to the inability to measure these resistances *in vivo* in tumors directly, the relative contribution of RBC rigidity in TBF reduction has not been quantified. In the present study, blood flow resistance was measured in "tissue-isolated" mammary adenocarcinoma R3230AC perfused *ex vivo* with (*a*) normally deformable, (*b*) glutaraldehyde-hardened, and (*c*) glucose-incubated RBC suspensions. Flow resistance measured during tumor perfusion with Krebs-Henseleit buffer prior to and following perfusion with the glutaraldehyde-hardened RBC suspensions showed no significant change, suggesting constant geometric resistance and lack of RBC entrapment. Instead, our measurements indicated increased viscous resistance with loss of deformability due to glutaraldehyde and glucose incubation even though glucose incubation did not significantly alter the apparent blood viscosity measured *in vitro*. Thus, the TBF reduction during hyperglycemia may be due to subtle changes in RBC deformability. These results suggest the development of strategies to increase the delivery of drugs or oxygen must take into account any changes in intratumor viscous resistance. For example, the increase in the oxygen-carrying capacity of blood using RBC transfusion or fluorocarbon emulsions may be offset by the increase in viscous resistance and the corresponding reduction in TBF.

Introduction

Several investigators have shown that hyperglycemia causes a reduction in TBF[4] (for review see Ref. 1). While the pathophysiological mechanisms of this reduction in TBF are not completely understood, DiPette *et al.* (2) have shown that this reduction results from both systemic and local mechanisms. Systemic mechanisms include reduction and redistribution of the cardiac output (2, 3) and hemoconcentration in the case of i.p. injection (3-5). One local mechanism proposed to mediate TBF reduction under hyperglycemic conditions involves the loss of RBC rigidity (1, 6).

A number of investigators have shown that hyperglycemia causes a decrease in the extracellular pH of most tumors (1). This reduction in extracellular pH has been shown to decrease the vascular pH by approximately 0.2 pH unit (4). Crandall *et al.* (7) have shown that incubation of RBC for 30 min at low pH increases RBC rigidity, whereas Ward-Hartley and Jain (8) have shown that TBF reduction occurs immediately following glucose administration. Traykov and Jain (9) have shown that glucose itself can increase RBC rigidity almost immediately upon incubation. Hence, it is postulated that RBC rigidity increases primarily due to glucose and is further augmented by the acidic environment in the tumor microvasculature. The effect of increased RBC rigidity may be to reduce TBF by (*a*) facilitating RBC entrapment, thus increasing the geometric resistance to flow, and/or (*b*) increasing the viscous resistance to flow. To date, due to the inability to measure flow resistance in tumors directly, this hypothesis remains unproven. In this paper, we report changes in intratumor geometric and viscous resistances in response to the loss of RBC deformability caused by incubation in glutaraldehyde or glucose.

Materials and Methods

In this study, we have used a Poisueille-type relationship to directly monitor the intratumor flow resistance, F_R, from measurements of arteriovenous pressure drop, Δp, at varying perfusion rates, q, across the tumor microvasculature (10, 11). Previously, we have shown that the simple relationship

$$q = \frac{\Delta p}{F_R} \quad \text{(A)}$$

can be used to describe the F_R of the tumor at tumor arterial pressures above 30 mm Hg (10). For typical Poisueille flow, F_R can be further written as the product

$$F_R = \eta z_o \quad \text{(B)}$$

where z_o is the geometric resistance offered by the vasculature and η represents the viscous resistance (or commonly referred to as the apparent "*in vivo* blood viscosity") to blood flow. In order to monitor z_o and η, we have adopted the classic perfusion technique developed by Whittaker and Winton (12) to alternately perfuse isolated rat mammary adenocarcinoma R3230AC with a KH solution and RBC suspensions of varying hematocrit and deformability. In this technique, pressure-flow measurements are made (*a*) during perfusion with an acellular physiological solution of known, Newtonian viscosity and then (*b*) with suspensions of RBC. If it is assumed that the vascular structure, and hence z_o, remains constant with change of perfusate, then the relative intratumor viscosity, η, can be found from the ratio of F_R obtained from experimental measurements of q and Δp during perfusion with KH and during perfusion with RBC suspensions.

"Tissue-isolated" tumors consisted of rat R3230AC mammary adenocarcinoma (Biomeasure Laboratories, Hopkington, MA) grown as an "organ" with a single artery and a single vein (13). The site of tumor implant was the left ovarian fat pad which, upon ligating all contralateral vessels, is fed by the ovarian artery and vein. Upon exteriorizing from the peritoneal cavity, enveloping in a bag of Parafilm (American Can Co., Greenwich, CT), and closing within the s.c. space in the left lumbar region, the implanted tumor and its feeding vasculature re-

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[2] Recipient of a NIH predoctoral traineeship (1986-1988) and Individual NIH Postdoctoral Fellowship CA-08783 (1989-1991). Present address: Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6089.

[3] Recipient of the Humboldt Senior Scientist Award (1990-1991). To whom requests for reprints should be addressed.

[4] The abbreviations used are: TBF, tumor blood flow; HCT, hematocrit; KH, Krebs-Henseleit solution; z_o, geometric resistance; η, viscous resistance; RBC(s), red blood cell(s).

mained isolated for the duration of 7–10 days of growth. Following surgical extraction (10) the isolated tumor was placed within a moist, 37°C perfusion chamber and perfused via an arterial cannula with a low capacity peristaltic pump (Ismatec model 7618–30; Cole-Parmer, Chicago, IL) at calibrated flow rates between 1 and 60 ml/h and arterial pressures between 20 and 80 mm Hg (transducer model PG23XL; Spectramed, Inc., Oxnard, CA). Arterial pressures were maintained in this range since further reductions caused increased resistance to flow (10). The acellular and RBC perfusates used in this study were: (*a*) acellular KH solution with 5% bovine serum albumin, 1.5 mM papaverine (Sigma Chemical Co., St. Louis, MO), and 7 USP units/ml sodium heparin (LyphoMed, Inc., Rosemont, IL); (*b*) normal RBC suspended in the KH above; (*c*) glutaraldehyde-hardened RBC suspended in the KH above; and (*d*) glucose-incubated RBC suspended in hyperglycemic buffered media. The afferent perfusates were oxygenated by passing through Silastic tubing over which a 95/5% O_2/CO_2 mixture was passed. Drainage through the venous cannula occurred at atmospheric pressure allowing sample collection for viscosity (model LVTDCP; Brookfield Engineering Laboratories, Stoughton, NJ), hematocrit (microhematocrit capillary technique; Fisher Scientific, Pittsburgh, PA), and hemoglobin determinations (ABL3 blood gas analyzer; Radiometer, Copenhagen, Denmark).

RBC suspensions were prepared from fresh, heparinized blood obtained from healthy rat donors. The plasma and packed RBC were separated by centrifugation at 1500 × *g* for 30 min at room temperature. For glutaraldehyde incubation, 20 ml of packed RBC were added drop by drop into a 400-ml solution of 0.0125% glutaraldehyde in phosphate-buffered saline (pH 7.4; Sigma). This procedure for "minimal RBC hardening" with glutaraldehyde was adapted from the procedure of Simchon *et al.* (14). After 30 min of incubation at room temperature, the cells were washed 3 times with phosphate-buffered saline and resuspended in the KH medium to obtain the desired hematocrit. For normal and glucose-incubated RBC suspensions, the packed cells were washed 3 times with phosphate-buffered saline and resuspended in KH and in 50 mM glucose-KH media, respectively. The 50 mM glucose-KH medium were prepared by adding 5% bovine serum albumin, 1.5 mM papaverine, and appropriate portions of 308 mM glucose solution with 10 mM Tris (Sigma) for buffering, to the normoglycemic KH medium. The osmolarity and pH of KH and 50 mM glucose-KH were measured to be between 310 and 320 mosmol/kg (Osmomat 030; Gonotec, Berlin, Germany) and 7.3–7.4 pH units (ABL3 blood gas analyzer; Radiometer), respectively. Both media were filtered through 0.22-μm sterile filters (Corning, New York, NY) before cell resuspension. The cell suspensions were maintained at room temperature and used within 1 to 2.5 h of preparation. In this manner, the cells in the 50 mM glucose-KH medium were permitted enough incubation time for glucose-induced hardening (9). RBC suspensions were examined after each experiment at ×400 to confirm the absence of RBC aggregates and echinocytes.

Results and Discussion

Fig. 1*A* shows the typical pressure-flow behavior of a 2.6-g tumor alternately perfused with 13, 28, and 44% HCT glutaraldehyde-hardened RBC suspensions. The slopes of the linear portion of the acellular q-Δp data before and after alternate RBC perfusions were 0.65 ± 0.03 (SD) and 0.66 ± 0.03 mm Hg h/g/ml, respectively, indicating the lack of significant RBC entrapment within the tortuous yet dilated tumor microvasculature and hence unchanged z_o. We have obtained similar results with tumors perfused with suspensions of normally deformable RBC (11).

If it is assumed that the geometric resistance, z_o, is unchanged with altered perfusate, then the ratio of Δp-q slopes measured with KH and RBC perfusion represents the ratio of intratumor viscous resistance as calculated by Equations A and B (11). Table 1 lists the relative intratumor viscous resistance obtained from the perfusion results shown in Fig. 1*A* as well as the

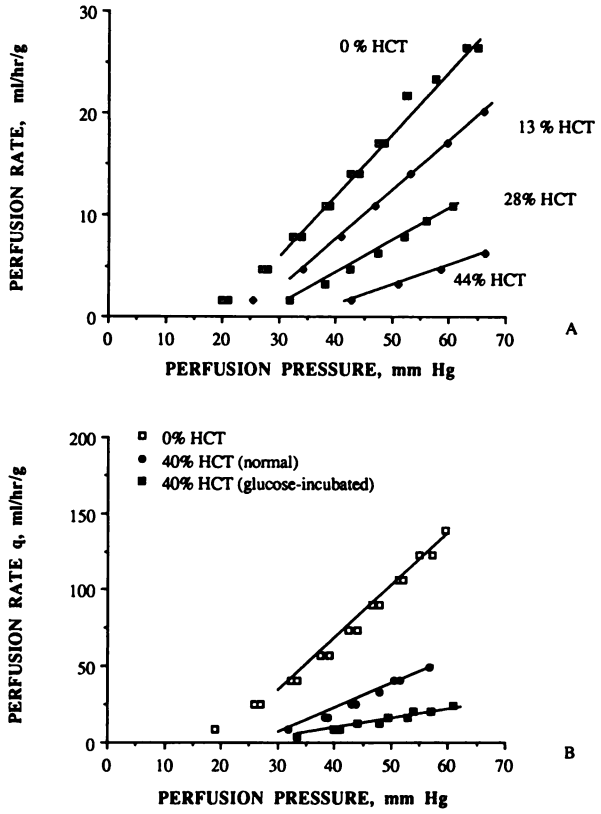

Fig. 1. Typical flow (ml/h/g) *versus* pressure (mm Hg) behavior for (*A*) a 2.6-g tumor alternately perfused with 0, 13, 28, and 44% hematocrit glutaraldehyde-hardened RBC suspensions; and (*B*) a 0.5-g tumor alternately perfused with normally deformable and glucose-incubated RBC suspensions at 40% hematocrit. ——, least squares fit of the data above 30 mm Hg.

relative viscosities of the venous fluid measured at 450 s^{-1} in a cone/plate viscometer. As shown in Table 1, the relative intratumor viscous resistance for glutaraldehyde-hardened RBC suspensions is less than the relative viscosity measured *in vitro*. Indeed, the relative intratumor viscous resistance monitored similarly in a total of 8 tumors (weight, 1.3 ± 0.6 g; range, 0.9–2.6) was found to be significantly less than the relative viscosity measured *in vitro* at 450 s^{-1} (Students' paired t test, $P = 0.0067$) as shown in Fig. 2. We interpret these results to be a consequence of the Fahraeus-Lindqvist phenomenon in which the viscous resistance to blood flow in capillary beds is reduced due to the hydrodynamic migration of RBC away from the vessel wall. We have previously demonstrated that the Fahraeus-Lindquist phenomenon may be responsible for the reduction in intratumor viscous resistance in the case of normally deformable cells (11). Here, we demonstrate that similar behavior occurs with minimally hardened RBC.

Fig. 2 also shows that when the RBC were mimimally hardened by dilute (0.0125%) glutaraldehyde incubation, η in eight solid tumors (weight, 1.3 ± 0.6 g; range, 0.9–2.6) was increased compared to those measurements obtained during perfusion of normal rat RBC suspensions. When alternately perfused with normal and glutaraldehyde-incubated RBC suspensions of 40% HCT, η increased from 2.4 ± 0.23 to 3.3 ±0.38 in a 0.2-g tumor and from 2.66 ± 0.35 to 3.43 ±0.49 in a 0.5-g tumor. These increases in tumor η are in agreement with the increased RBC suspension viscosity caused by glutaraldehyde incubation as measured *in vitro* (see Table 1). Yet does tumor η increase when more subtle changes in RBC deformability occur during hyperglycemia?

Table 1 *Ratios of intratumor flow resistance (or the relative viscous resistance) and relative blood viscosity for the glutaraldehyde-hardened, normally deformable, and glucose-incubated RBC suspensions in Figs. 1A, 1B, and 3.*

RBC suspension	HCT (%) (mean ± SD)[a]	Shear rate (s^{-1})	Relative blood viscosity[b] (mean ± SD)[a]	Viscous resistance[c] (mean ± SD)[d]
Glutaraldehyde-hardened	13.4 ± 0.2	450	1.6[e]	1.4 ± 0.1
	27.8 ± 0.5		2.9 ± 0.02	2.0 ± 0.1
	44.3 ± 0.9		6.3[e]	3.3 ± 0.2
Normally deformable	40.0	450	3.4 ± 0.01	
		225	3.9 ± 0.01	
		90	4.5 ± 0.01	
		45	5.2 ± 0.2	
Glucose-incubated	40.0	450	3.5 ± 0.1	
		225	4.0 ± 0.2	
		90	4.7 ± 0.2	
		45	5.3 ± 0.5	
Glutaraldehyde-fixed	40.0	450	5.8 ± 0.03	
		225	6.3 ± 0.05	
		90	7.0 ± 0.8	
		45	7.9 ± 0.2	

[a] N = 3–5 measurements.
[b] Measured *in vitro* using a cone/plate viscometer.
[c] Calculated from the ratio of flow resistances measured during KH and RBC tumor perfusion.
[d] Error derived from the propagation of SD arising from least squares regression of q-Δp curve.
[e] Efferent tumor sample size prevented more than one measurement.

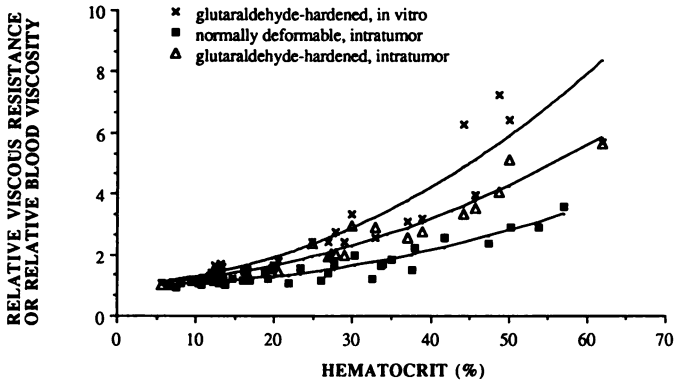

Fig. 2. Relative blood viscosity and viscous resistance *versus* hematocrit (%) for glutaraldehyde-hardened RBC suspensions measured *in vitro* using a cone/plate viscometer at 450 s^{-1} (×); glutaraldehyde-hardened RBC suspensions within the tumor preparation (△); and normally deformable RBC suspensions within the tumor preparation (■). ——, least squares fit to the equation

$$\eta = 1 + \alpha\phi + \beta\phi^2$$

For the *in vitro* measurement, $\alpha = 0.9 \pm 1.2$ and $\beta = 17.8 \pm 3.0$; for intratumor measurements $\alpha = 0.9 \pm 0.7$ and $\beta = 11.2 \pm 1.5$ where ϕ is the volume fraction of glutaraldehyde-hardened RBC suspensions; for the normally deformable suspensions, $\alpha = -0.1 \pm 0.8$ and $\beta = 7.4 \pm 1.9$.

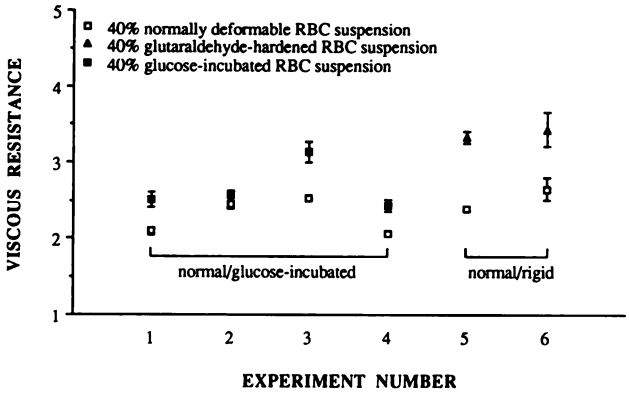

Fig. 3. Intratumor viscous resistance in 4 trials (experiments 1 through 4) of alternate perfusion with normally deformable (□) and glucose-incubated (■) RBC suspensions and in 2 trials (experiments 5 and 6) with normally deformable and glutaraldehyde-hardened (▲) RBC suspensions at 40% hematocrit. *Bars,* propagation of the standard deviation associated with the least squares fit of the linear pressure-flow curve.

When a 40% HCT RBC suspension was incubated in an isotonic, 50 mM glucose-KH medium buffered with 10 mM Tris (Sigma) (9), no significant change resulted in the relative blood viscosity as measured using a cone/plate viscometer (Table 1). Yet as shown in Fig. 1*B*, the pressure-flow relationship in a 0.5-g tumor was dramatically altered when the perfusate was changed from a normoglycemic to a 50 mM hyperglycemic RBC perfusate, each at 40% hematocrit. Reperfusion with KH shows identical pressure-flow behavior prior to and following RBC alternate perfusion. From the ratio of slopes obtained from KH and RBC perfusion, η for the normoglycemic RBC suspension is 2.1 ± 0.2 and 2.5 ± 0.3 for the hyperglycemic RBC suspension. Alternate perfusion in four tumors (0.8 ±0.2 g) shows trends of increased η (p = 0.03) obtained from the pressure-flow behavior monitored during perfusion with normal and glucose-incubated RBC suspensions at 40% hematocrit (Fig. 3). From measurements of hemoglobin concentration and hematocrit, we have shown that no gross changes in RBC volume occurred with glucose incubation (15). Yet when RBC incubated in the 50 mM glucose solution are resuspended in normoglycemic KH, no increase in tumor η occurs. This suggests that the reduced RBC deformability may result from the subtle and reversible swelling of RBC which may occur during hyperglycemia in addition to changes in the RBC membrane.

Implications. Our results suggest that η in the tumor microvasculature is susceptible to subtle changes in RBC deformability as may occur during hyperglycemia. It is unknown whether such moderate and physiological changes in RBC deformability can be an equally important rheological factor in the normal microvasculature. However, due to the metabolic environment and the unique vascular morphology of the tumor microvasculature (16, 17), one may expect loss of RBC deformability and increase in intratumor η to occur more easily and have a greater consequence in tumors than in some normal tissues. This observation should be taken into account when developing strategies to improve tumor oxygenation or blood flow for enhanced therapeutic benefit. For one example, the increase in intratumor η might offset the increased oxygen-carrying capacity with RBC transfusion or fluorocarbon emulsion administration.

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