Possible Role of Red Cell Deformability and Microvasculature in Microcirculation

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Abstract The roles of the deformability of red blood cells (RBC) and the microvasculature in the maintenance of blood flow were investigated in terms of the pressure (P)-flow rate ($\dot{Q}$) relationships in human RBC suspension perfusions of bullfrog hind limb. Although isotonicity for the bullfrog is ~ 215 mOsm/kgH₂O, perfusions in intact hind limbs showed no change in the P - $\dot{Q}$ relationship at test solution osmolalities ranging from ~ 150 to ~ 300 mOsm/kgH₂O. The deformability of RBC was examined in glutaraldehyde-fixed hind limbs. Perfusion of fixed limbs with RBC suspension revealed minimum resistance to flow at osmolalities of ~ 250 to ~ 420 mOsm/kgH₂O, whereas the same experiment in intact limbs revealed minimum flow resistance at osmolalities of ~ 200 to ~ 300 mOsm/kgH₂O. It was noteworthy that the reduction of RBC deformability was not observed in intact limbs at osmolalities of ~ 250 to ~ 200 mOsm/kgH₂O. Heinz body-forming RBC from a patient with unstable hemoglobin (Hb) disease (Hb Yokohama) exhibited a marked reduction in deformability as compared with normal RBC in fixed limbs, while there was no discernible difference between the two types of RBC in intact limbs, thereby suggesting that the microvascular bed can compensate, to an appreciable extent, for the impaired deformability of RBC, probably via its distensibility and/or a wall effect. The present study has considerable implications concerning the link between *in vitro* experiments and the *in vivo* situation, including the hemodynamic characteristics of RBC suspensions such as the effective viscosity.

Key words: red blood cells (RBC), deformability, Heinz body, microvasculature, osmolality.

The deformability of red blood cells (RBC) and the microvasculature are important factors in maintaining normal blood flow in the systemic circulation, in particular, in the microcirculation. Normal RBC have marked deformability; this allows their transit through the smallest vessels, which are narrower than the cell

diameter [1, 2]. The major determinants of RBC deformability are cell geometry, intracellular fluid viscosity, and the viscoelastic properties of the cell membrane [1–6]. Several useful methods for studying RBC deformability *in vitro* have been reported in the literature [1–5, 7], and, during the past few years, there have been considerable advances in our understanding of this phenomenon [1–13]. However, very little is known about any direct links between the roles of RBC deformability and the microvasculature in the maintenance of blood flow. In other words, there is growing need for understanding the link between *in vitro* experiments and the *in vivo* situation in connection with related fields of clinical medicine.

To elucidate these links, we used a system in which bullfrog hind limbs were perfused with human RBC suspension as a model for the microcirculation *in vivo*. The hemodynamic characteristics of RBC suspension transit through the microvasculature were examined in terms of the pressure (P)-flow rate ($\dot{Q}$) relationship, by determining the perturbing effects of osmolality on both RBC deformability and the vascular bed. We also investigated the possible role of the microvasculature in blood flow, using Heinz body-forming RBC from a patient with unstable hemoglobin (Hb) disease (Hb Yokohama) [14].

MATERIALS AND METHODS

Perfusion of isolated bullfrog hind limbs was performed by cannulating the abdominal aorta. The vertical tube method [15] with a minor modification [11] was used to construct the P - $\dot{Q}$ relationship. Bullfrogs (*Rana catesbeiana*) of both sexes, weighing 400–450 g, were used. The vascular bed of the bullfrog hind limbs was perfused with 215 mOsm/kgH₂O NaCl solution, the osmolality of which was isotonic for bullfrogs. There were variations in the rate of flow (flow rate) in different preparations when they were perfused with standard NaCl solution. The hind limbs that presented a consistent flow rate with NaCl solution were chosen for the experiments, and small variations of flow rate among preparations were normalized (standardized). Measurements made under one set of conditions were very reproducible, and, unlike mammalian models such as the isolated rabbit ear, the frog preparations were stable for at least 2 h. Both suspending medium and RBC suspension were perfused to distinguish the effects of the medium on the vascular bed from the effects on RBC deformability.

For the evaluation of RBC deformability and associated hemodynamic factors, the hind limbs were fixed by perfusion with glutaraldehyde (GA) solution containing 50 mM phosphate buffer (pH 7.4), at an osmolality adjusted to 215 mOsm/kgH₂O by adding NaCl. Perfusion with 100 ml of 0.4% GA solution was performed at a constant pressure of 35 cmH₂O for 15 min, followed by the perfusion with 1% GA solution at 35 cmH₂O for 15 min; the flow was then stopped for 30 min under these conditions. The GA solution was washed out by perfusing the preparation with 300 ml of 285 mOsm/kgH₂O NaCl solution at 40 cmH₂O. This washing with NaCl solution was sufficient to remove GA from the fixed hind limbs;

there were no residual effects of GA on fresh RBC. The same perfusion experiments were also carried out using a rigid glass tube (inner diameter: 650 μm ; length: 12.6 cm) in place of hind limbs.

Venous blood from the antecubital veins of healthy young male adults was collected into a disposable syringe with an 18-gauge needle, using 1/10 volume of 3.3% trisodium citrate as an anticoagulant. After centrifugation at $1,300 \times g$ for 10 min, the plasma and buffy coat were removed and replaced with 285 mOsm/kgH₂O NaCl solution. The RBC were washed three times by repeated resuspension in NaCl solution and centrifugation at 800, 600, and $500 \times g$, respectively.

RBC from a patient with unstable hemoglobin (Hb) disease were also investigated. The patient was a 43-year-old female affected by congenital hemolytic anemia. She had undergone splenectomy at the age of 21, but had no clinical improvement. Her blood sample was referred for specialized Hb studies, and the substitution of proline for leucine at position 31 of the β -chain was determined; the new unstable Hb was named Hb Yokohama after the city where it was discovered [14]. The patient exhibited a typical clinical course of unstable Hb disease with hemolytic anemia (Hb level: 8.3 g/dl; packed cell volume (Hct): 28%). Methylviolet staining showed that most of the RBC were Heinz body-positive.

An aliquot of the RBC suspension was fixed with 1% GA solution containing 50 mM of phosphate buffer (pH 7.4); the osmolality was adjusted to that of each test sample by adding adequate amounts of NaCl. The shape of the RBC was examined with a differential interference contrast microscope (Nikon, TMD, Tokyo, Japan) at a $400\times$ magnification. The osmolality of the suspending medium was measured with a freezing point depression type osmometer (Osmette A, precision System Inc. Natick, MA). The viscosity of dextran (M.W. 50,000–70,000) solution was measured with a cone-plate type E viscometer (Tokyo Keiki Co. Ltd., Tokyo, Japan). All solutions, except for the GA solution, were adjusted to pH 7.4 with 10 mM HEPES-Na buffer. The reagents used were of analytical grade. Experiments were carried out at room temperature ($22 \pm 1.5^\circ\text{C}$).

RESULTS

Perfusion of fixed hind limbs

Figure 1A shows the P - $\dot{Q}$ relationship of test solutions at several viscosities in the perfusion of fixed hind limbs. The flow rate decreased with rises in viscosity. All P - $\dot{Q}$ relationships were linear passing through the origin, which is characteristic of the P - $\dot{Q}$ relationship of a Newtonian fluid in the perfusion of a rigid tube [16]. Thus, in spite of a complicated microvascular network, these fixed hind limbs can be regarded as a rigid tube, as far as perfusion with a Newtonian fluid is concerned.

Figure 1B shows the P - $\dot{Q}$ relationship of NaCl solution and RBC suspension at several osmolalities in the perfusion of fixed limbs. No osmolality-related change in the P - $\dot{Q}$ relationship was shown when the fixed limbs were perfused with NaCl solution at different osmolalities. Both hypertonically and hypotonically altered

Fig. 1. Effects of viscosity and osmolality on the P - $\dot{Q}$ relationship in the perfusion of fixed hind limbs. A: Perfusion with NaCl and dextran solutions at several viscosities. The viscosity of the NaCl solution is 1.0 ($\circ$) centipoise (cP) and those of the dextran solutions are 1.5 ($\bullet$), 1.8 ($\blacktriangle$), and 2.5 ($\blacksquare$) cP, respectively. B: Perfusion with RBC suspensions at several osmolalities. Osmolalities of the RBC suspensions are 300 ($\bullet$), 218 ($\blacktriangle$), and 731 ($\blacksquare$) mOsm/kgH₂O. Experiments were performed at constant cell numbers; the Hct value of the suspension was 10% at 285 mOsm/kgH₂O. Perfusion with NaCl solutions is also shown; osmolalities are 300 ($\circ$), 218 ($\triangle$), and 731 ($\square$) mOsm/kgH₂O.

RBC showed a marked decrease in the flow rate. Thus, it was shown that changes in the deformability of RBC can be detected in the perfusion of fixed limbs, since the osmotic stress impairs RBC deformability [7, 9, 10]. All P - $\dot{Q}$ relationships of the RBC suspension were in the form of a smooth curve convex to the pressure axis (P -axis) at low P ($< \sim 12$ cmH₂O), tending to a straight line at high P ($> \sim 15$ cmH₂O). This indicates that the RBC suspension behaves like a Newtonian fluid at high P , and that the non-linear part of the P - $\dot{Q}$ relationship at low P arises from the non-Newtonian properties of the RBC suspension [16]. The asymptotic lines (extrapolated linear segments) of the P - $\dot{Q}$ relationship had the same intercept on the

Fig. 2. Effects of osmolality on the P - $\dot{Q}$ relationship in the perfusion of intact hind limbs. A: Perfusion with NaCl solutions at several osmolalities. Osmolalities of the test solutions are 180 (●), 215 (▲), 300 (☆), 394 (△), and 749 (■) mOsm/kgH₂O. B: Perfusion with RBC suspensions at several osmolalities. Osmolalities of the RBC suspensions are 300 (☆), 390 (△), and 731 (■) mOsm/kgH₂O. Experiments were performed at constant cell numbers; the Hct value of the suspension was 10% at 285 mOsm/kgH₂O. Perfusion with NaCl solution (300 mOsm/kgH₂O) is also shown (○).

negative flow rate axis ($\dot{Q}$ -axis). In this experiment and all other perfusion experiments, RBC exhibited no discernible morphological changes or hemolysis after perfusion.

The osmotically altered RBC did not show any change in the P - $\dot{Q}$ relationship when the perfusion experiments were performed with the rigid glass tube (inner diameter: 650 μ m; length: 12.6 cm); these experiments produced results consistent with those for the fixed limbs used in this study with regard to the P - $\dot{Q}$ relationship of standard NaCl solution.

Perfusion of intact hind limbs

Figure 2A shows the P - $\dot{Q}$ relationship of NaCl solution at several osmolalities

in the perfusion of intact limbs. The flow rate decreased with a rise of osmolality in the hypertonic region. However, hypotonic stress (180, 215 mOsm/kgH₂O) did not lead to any change in the P - $\dot{Q}$ relationship. Unlike the perfusion of fixed limbs (Fig. 1A), the perfusion of intact limbs with NaCl solution (Newtonian fluid) showed a non-linear P - $\dot{Q}$ relationship. This finding indicates the intrinsic compliance (distensibility) of the intact vasculature; the non-linear part of the P - $\dot{Q}$ relationship arises from the distension of the vascular tree, which may be determined by a steady state condition operating between applied pressure and the resultant change in vascular tone [17]. All asymptotic lines had the same intercept on the negative $\dot{Q}$ -axis.

Figure 2B shows the P - $\dot{Q}$ relationship of the RBC suspensions at several osmolalities in the perfusion of intact limbs. The flow rate decreased, due to the osmotic stress on both the RBC and the vascular bed. The P - $\dot{Q}$ relationships were non-linear, and the asymptotic lines met at the same point on the negative $\dot{Q}$ -axis.

Effects of osmolality on RBC deformability and vascular bed

Figure 3 shows the asymptotic resistance to flow plotted against the osmolalities of the RBC suspension. The following equation can be written for the asymptotic P - $\dot{Q}$ lines, such as those shown in Figs. 1 and 2, replacing the vasculature with a single rheologically equivalent vessel [17]:

$$\dot{Q} = (\pi^4/8\eta l)P - I, \quad (1)$$

where η is the asymptotic viscosity, r and l are the effective radius and length, respectively, of such a "single equivalent vessel" for the vascular tree of the limbs, and I is the intercept on the negative $\dot{Q}$ -axis. Therefore, the reciprocal of the slope exhibits asymptotic resistance to flow (flow resistance). Also, Eq. (1) gives the asymptotic relative viscosity when changes in r and l can be ignored. When the asymptotic lines have the same intercept on the $\dot{Q}$ -axis, the decrease in the flow rate at high P quantitatively corresponds to the increase in the flow resistance and the relative viscosity.

No osmolality-related change in the flow resistance was observed in the perfusion of fixed hind limbs. The perfusion of intact hind limbs with NaCl solution did not show any change in the flow resistance at osmolalities ranging from ~ 150 to ~ 300 mOsm/kgH₂O; the flow resistance increased as the osmolality increased to above ~ 300 mOsm/kgH₂O. Consistently, the hind limb preparations did not show any discernible weight changes after perfusion at the osmolalities of ~ 150 to ~ 300 mOsm/kgH₂O. Thus, intact bullfrog hind limbs can be perfused at an osmolality that is isotonic for human RBC (~ 285 mOsm/kgH₂O), even though isotonicity for the bullfrog is lower (~ 215 mOsm/kgH₂O). The perfusion of fixed limbs with RBC suspension revealed minimum flow resistance at osmolalities ranging from ~ 250 to ~ 420 mOsm/kgH₂O, whereas, in intact limbs, RBC encountered minimum flow resistance at ~ 200 to ~ 300 mOsm/kgH₂O.

Fig. 3. Effects of osmolality on asymptotic flow resistance. The flow resistance was obtained from the $P-\dot{Q}$ relationship, as shown in Figs. 1 and 2. Perfusion with NaCl solution performed in fixed ($\blacktriangle$) and intact ($\bullet$) hind limbs and perfusion with the RBC suspension in fixed ($\triangle$) and intact ($\circ$) limbs are shown. Experiments were performed at constant cell numbers; the Hct value of the suspension was 10% at 285 mOsm/kgH₂O.

Effect of Hct value on flow behavior of RBC suspensions

The effect of Hct value on the $P-\dot{Q}$ relationship of the RBC suspension was examined in glass tube, intact and fixed limb perfusions. All asymptotic $P-\dot{Q}$ lines met at the same point on the negative $\dot{Q}$ -axis in the perfusions of glass tube, intact limbs, and fixed limbs, respectively; the intercepts were independent of Hct value. Figure 4 shows the effect of Hct value on the asymptotic flow resistance obtained from the $P-\dot{Q}$ relationships. The flow resistance increased as Hct value increased and the flow resistance in the perfusion of glass tube and fixed limbs were greater than that in intact limbs. However, the flow resistance in glass tube was not so greater than that in fixed limbs. Hypertonicity altered RBC exhibited a marked increase in the flow resistance along with a rise of Hct value; the reduction of RBC deformability was enhanced with a rise in Hct value.

Perfusion with Heinz body-forming RBC

Figure 5B illustrates RBC from the patient with unstable Hb disease (Hb

Fig. 4. Effects of Hct value on asymptotic flow resistance. The flow resistance was obtained from the P - $\dot{Q}$ relationship of the RBC suspension. Perfusion of intact limbs (●) and of a glass tube (□) was performed at 300 mOsm/kgH₂O. Perfusion of fixed limbs was performed at 300 mOsm/kgH₂O (○) and 500 mOsm/kgH₂O (△). The inner diameter of the glass tube was 650 μ m.

Yokohama). Larger, often solitary formation of Heinz bodies was observed in these cells. Figure 6A shows the P - $\dot{Q}$ relationship of the Heinz body-forming RBC suspension in the perfusion of fixed hind limbs. The Heinz body-forming RBC revealed a marked flow rate reduction at a Hct value of 10% as compared with normal RBC; Heinz body formation impaired the deformability of RBC. However, there was no discernible difference between the two types of RBC, even at a Hct value of 28%, when the perfusion experiments were performed in intact limbs, as shown in Fig. 6B.

DISCUSSION

To elucidate the possible roles of RBC deformability and the microvasculature in the maintenance of normal blood flow, we investigated the hemodynamic characteristics, in terms of P - $\dot{Q}$ relationships of human RBC suspensions perfused through the vascular network of bullfrog hind limbs. The deformability of RBC

Fig. 5. RBC shape. A: Normal discocytes (young male adult). B: Heinz body-forming RBC (Hb Yokohama patient).

Fig. 6. P - $\dot{Q}$ relationship of RBC suspension from patient with Hb Yokohama. A: Perfusion of fixed hind limbs. The Hct value of the suspension was 10%. $\circ$, NaCl solution; $\bullet$, normal RBC; $\triangle$, RBC from Hb Yokohama patient. B: Perfusion of intact hind limbs. The Hct value of the suspension was 28%. Symbols are the same as those in Fig. 6A.

was examined in perfusions of fixed limbs, which diminished compliance and acted like a rigid tube with regard to perfusion with a Newtonian fluid (Fig. 1A). The reduced flow rate was detected in the perfusion of fixed limbs with osmotically altered RBC (Fig. 1B), but not with perfusion of the glass tube, which produced a flow rate consistent with that for fixed limbs. Thus, the reduced flow rate may be related to the impaired deformability of RBC and to the geometrical (mechanical) hindrance of the vascular tree. In other words, the mechanical hindrance may enlarge on the impaired deformability. Since fixed limbs have a reasonable similarity to the anatomy of the microvasculature *in vivo*, except for diminished distensibility, it would seem that this perfusion system is useful for investigating RBC deformability in terms of relating deformability *in vitro* to deformability *in vivo*, as discussed below.

The osmotic stress of the suspending medium influences RBC deformability in all major determinants such as cell geometry and intracellular viscosity [7, 9, 10]. In the perfusion of fixed limbs, a marked decrease was demonstrated in the deformability of osmotically stressed RBC; the flow resistance increased at osmolalities above ~ 420 mOsm/kgH₂O or below ~ 250 mOsm/kgH₂O (Fig. 3). This result essentially agreed with the results of experiments employing filtration with microsieves, although there were some differences [7, 9, 10]. For example, filtration with a nickel mesh filter (5- μ m pores) showed reduced deformability at osmolalities above ~ 320 mOsm/kgH₂O or below ~ 260 mOsm/kgH₂O [7], thereby indicating that the nickel mesh filtration system is more sensitive than the fixed limb system for the detection of reduced deformability due to increased intracellular viscosity at osmolalities of ~ 320 to ~ 420 mOsm/kgH₂O. In general, capillary diameter in the frog is greater than that in humans; in the case of *Rana pipiens*, the range of diameter in the capillaries from 21 frogs was 6 to 31 μ m, the mean value ($\pm$ SD) being 16.7 ± 4.4 μ m [18]. It is therefore likely that the difference between the two systems (nickel mesh vs fixed limb) may be due to differences in their diameters.

When RBC are placed in a hypertonic solution, they lose water, thereby leading to a reduction in cell volume and an increase in intracellular viscosity; this is accompanied by changes in shape, in which the RBC become crenated and shrunken, at above ~ 420 mOsm/kgH₂O, i.e., they maintain their normal disc shape up to a level of ~ 420 mOsm/kgH₂O [7]. Thus, the perfusion of fixed limbs emphasized that additional shape changes in response to increased intracellular viscosity facilitated reduction in the deformability of RBC. Indeed, nickel mesh filtration also showed that the degree of the reduction in deformability increased more at above ~ 420 mOsm/kgH₂O [7].

When RBC are exposed to a hypotonic solution, water diffuses into them, thus increasing cell volume and reducing intracellular viscosity, while the surface area remains constant; this is accompanied by dome-shaped and spherical shape changes [7]. Filtration with microsieves, especially those with small (~ 3 μ m) pores [9, 10], has demonstrated that hypotonically reduced deformability was mainly due to a

decrease in the ratio of surface area to volume; smaller pores were more sensitive in detecting this reduced deformability. It is of note that the decreased deformability was detected at ~ 250 mOsm/kgH₂O in the perfusion of fixed limbs (Fig. 3), while it was detected at ~ 260 mOsm/kgH₂O with 5- μ m-pore nickel mesh filtration [7]. This therefore suggests that the RBC perfused through the smallest capillaries of the frog, the diameters of which may be near 6 μ m.

The osmotic stress of the suspending medium also plays a crucial role in clinical medicine, particularly, in radiologic examinations. Water-soluble radiologic contrast media (CM) are very hypertonic; the osmolality of a conventional CM, iohalamate, being $\sim 1,500$ mOsm/kgH₂O. Even the recently developed so-called low osmolality CM, iohexol, is hypertonic (~ 600 mOsm/kgH₂O). Thus, the osmotic effects of CM on RBC deformability and vasculature are greater than the other effects of CM, except for their chemotoxicity, which is closely related to the occurrence of side effects such as pain and hemodynamic changes [13, 19].

The formation of Heinz bodies, i.e., the presence of precipitated, denatured Hb within RBC, is the fundamental event leading to hemolysis in unstable Hb disease [20]. RBC must be highly deformable to pass through the narrow splenic sinusoid, and processes that reduce deformability can lead to accelerated destruction [21]. Thus, it has been suggested that the presence of Heinz bodies brings about a decrease in the deformability of RBC and impedes their passage. Indeed, Lubin and Desforges [22] have reported the reduced filterability of acetylphenylhydrazine-treated (i.e., Heinz body-forming) RBC. Flynn *et al.* [23], using a micropipette technique, demonstrated reduced membrane deformability in a patient with Hb Köln disease (unstable Hb disease). In contrast, another study with phenylhydrazine-induced Heinz bodies has shown that RBC filterability was not appreciably altered until the Heinz body material covered almost the entire cell endosurface [24]. The present study supports the former findings, in that the perfusion of fixed limbs showed a marked reduction in the deformability of RBC from the patient with Hb Yokohama (Fig. 6A), although the perfusion of fixed limbs is less sensitive than nickel mesh filtration in detecting intracellular viscosity. Considering that hemolytic anemia had not improved after splenectomy in this patient [14], it is possible that the impaired deformability arising from Heinz body formation may be related to intravascular destruction, i.e., to hemolysis *in vivo*, which does not always require splenic sequestration.

The filtration of RBC suspensions through microsieves has been effective when used for investigating RBC deformability, both experimentally [2, 6, 7, 9–11, 13] and theoretically [8, 12]. It is, however, to be noted that when the filtration method is used, the link between the roles of RBC deformability and the microvasculature in blood flow cannot be examined. In our study, the perfusion of intact limbs with RBC suspensions showed minimum flow resistance at osmolalities ranging from ~ 200 to ~ 300 mOsm/kgH₂O (Fig. 3), while the same experiment in fixed limbs showed the minimum at osmolalities of ~ 250 to ~ 420 mOsm/kgH₂O (Fig. 3). Since the intact limbs were not impaired at test solution osmolalities of ~ 150 to

~ 300 mOsm/kgH₂O (Fig. 3), these results suggest that an intact vascular bed can compensate for the impaired deformability of RBC at osmolalities of ~ 200 to ~ 250 mOsm/kgH₂O. Further, perfusion with Heinz body-forming RBC exemplified compensation for the impaired deformability by intact limbs, as shown in Fig. 6. Since the perfusion of fixed limbs showed substantially the same results as filtration with the nickel mesh [7], it can be concluded that this compensatory effect of the intact vasculature on RBC deformability is probably due to its distensibility and/or to a wall effect. However, since capillary diameter in humans is less than that in frogs [18], these results cannot be directly extrapolated to the pathogenesis of circulatory abnormalities in human patients. Nevertheless, the results of the present study have considerable implications regarding the roles of changes in the vascular bed and in the deformation of RBC in the maintenance of the microcirculation; these findings should contribute to investigations of the link between *in vitro* experiments and the *in vivo* situation.

Although many features of RBC suspension flow behavior have been described in the literature, several questions remain to be resolved [25]; typical of these are the effects of inertial pressure losses on P - $\dot{Q}$ relationships in isolated dog hind limbs [26]. Benis *et al.* [26] have maintained that Whittaker-Winton's finding [27] that the effective viscosity of RBC suspensions was about half that measured for these suspensions in a large bore capillary viscometer, was due to incorrectly ignoring the inertial effect contribution to the total pressure loss through the isolated preparation. Whittaker and Winton [27] attributed this difference to the Fåhræus-Lindqvist effect [28]. Similar disagreements have been noted in experiments with cat calf muscle [29], isolated rabbit ear [30, 31], and bullfrog hind limbs [17]. Our treatment (Eq. (1)) [17] is based on the empirical treatment described by Haynes and Burton [16] and the marginal zone theory of Gordon [32]; this treatment implicitly ignores the inertial effect. Contrary to Benis' argument, Eliassen *et al.* [33] demonstrated that what could be interpreted as an effect of inertial forces was, at least in their preparation of the cat calf muscle, actually due to a change in vascular geometry (distensibility), which occurred naturally when the perfusion fluid was changed from a poor oxygen-carrying medium, such as saline, to a good one, such as the RBC suspension. Our results also showed an importance of the distensibility of the vascular bed; unlike intact limbs [17], the flow resistance (accordingly, the relative viscosity) in fixed limbs was not so smaller than that in glass tube (Fig. 4). Thus, it can be seen that the detailed analysis of the P - $\dot{Q}$ relationships is very complicated. Furthermore elaborate experimental and theoretical treatments will be required to elucidate these relationships.

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