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The endothelial glycocalyx affords compatibility of Starling's principle and high cardiac interstitial albumin levels

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Abstract

Objective: To test the role of an oncotic pressure gradient across the endothelial glycocalyx with respect to extravasation of fluid and colloids and development of tissue edema in a whole organ setting.
Methods: We measured filtration in the intact coronary system of isolated guinea pig hearts, comparing colloid-free perfusion and perfusion with 1.67% albumin or 2% hydroxyethylstarch (oncotic pressures 5.30 vs. 11.10 mm Hg, respectively). Heparinase was used to alter the endothelial glycocalyx.
Results: Extremely high net organ hydraulic conductivity was obtained with colloid-free perfusion (9.14 μl/min/g tissue). Supplementing perfusate with albumin caused a significant decrease, also vs. hydroxyethylstarch (1.04 vs. 2.67 μl/min/g, $p<0.05$). Albumin also lowered edema formation vs. the other perfusion modes ($p<0.05$). Stripping the glycocalyx of heparan sulfate reduced the effect of colloids, especially that of albumin. The steady-state concentrations of hydroxyethylstarch and albumin in the mixed interstitial fluid leaving the intact coronary bed averaged about 95% of the intravascular level. Electron and light microscopy indicated that colloid extravasated mainly in the venular sections.
Conclusion: We propose a low-filtration model for the coronary system with different barrier properties in arteriolar/capillary and venular sections. Arteriolar/capillary: very little fluid and colloid extravasation due to the endothelial surface layer formed by the glycocalyx and albumin plus the endothelial strand barrier; venular: little net extravsation of fluid and colloids despite large pores, because of low hydrostatic and oncotic pressure differences between intra- and extravascular spaces. The latter sites provide physiological access of large solutes (colloids) to the tissue.

Keywords: Capillaries; Capillary permeability; Coronary circulation; Microcirculation; Endothelial function

1. Introduction

According to the classic Starling view, the main determinants of the filtration rate per unit area across the capillary wall are the hydrostatic (HP) and colloid osmotic pressures (COP) in the capillary lumen and in tissue, respectively. Recent evidence indicates that tissue COP have less influence on fluid balance than the other three parameters. Indeed, most tissues would not be in fluid balance if Starling's equation in its classic form was correct; especially the measured lymph flow seems far too low [1]. In a rat mesenteric microvessel model, the effective COP difference opposing filtration was near 70% of luminal COP though the colloid tissue concentration equalled that in the lumen of the microvessel [2]. It was proposed that the endothelial glycocalyx (EG) acts as a primary molecular filter and generates the effective oncotic gradient within a very small space [3,4].

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Hu and Weinbaum developed a detailed model of these concentration gradients across the EG [5], using size and frequency of junction strand breaks in frog mesenteric vessels measured by Adamson and Michel [6]. Pertinently, in various capillary preparations, the tissue oncotic force had no influence on the COP exerted by the colloid-containing perfusate [2,7]. The upshot of these observations is that albumin is filtered at the EG and the ultrafiltrate, with its low colloid osmotic force, is funelled into small, infrequent breaks in the junction strand. Consequently, the velocity within these breaks is relatively high, even if the filtration rates are low, and there is practically no flux of albumin from the tissue back toward the EG.

Rehm and coworkers were able to show in an isolated heart preparation that the endothelial cells (EC) and the EG act as two competent components of the vascular barrier. However, despite low rates of net fluid filtration, the concentrations of colloids in the extravasated fluid approched the levels within the coronary vessels [8,9]. This raises the question, where in the vascular bed do the colloids gain access to the interstitial space?

Most of the relevant previous studies dealing with the new filtration concept represent investigations of single vessels. Apparently, one has to distinguish between capillaries with continuous endothelium [2], in which the interstitial COP seems to have little influence on the colloid osmotic difference, and fenestrated capillaries with a much less enclosed exit from the EG "ultrafilter" [1]. In an isolated microvessel, when both the perfusate and the interstitial space are protein free, the relationship between the filtration rate per unit area and the hydrostatic capillary pressure is linear and extrapolates through the origin. This condition was taken to represent a negligible hydrostatic tissue pressure, an artificial situation probably different to that in vivo.

We aimed to test the new concept involving the EG in a whole organ setting, namely the isolated perfused heart (guinea pig). This preparation gives direct and easy access to the net fluid filtration in an intact vascular bed: sampling of the "transudate" appearing on the epicardial surface provides a quantitative measure of the net amount of fluid loading the lymphatic system and the pericardial space. Analysis of the transudate yields the composition of the mixed extravasated fluid, averaged regionally and over 200–240 cardiac cycles [9]. For a better approach to the conditions in vivo we compared the filtration behaviour of the coronary bed under varying perfusion pressures at different COPs. Additionally, we investigated the competence of a glycocalyx-denuded barrier by applying the enzyme heparinase to remove heparan sulfate groups [8,10]. This approach has been described previously also for isolated coronary arterioles and venules [11].

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the official ethics committee (Government of Upper Bavaria, #209.1/211-2531.3-3/99).

2.1. Heart preparation

Guinea pig hearts were isolated and perfused in a modified Langendorff mode. The animals (male; weight 200–250 g) were stunned by neck dislocation with a specially designed instrument. Immediately after opening of the thorax, the hearts were arrested with ice-cold isotonic saline. Within 30–60 s the coronaries were perfused in situ at a constant flow rate of 6 ml/min. After removal from the thorax all veins entering the right and left atria were ligated and a cannula inserted into the pulmonary artery [8,9]. The perfusion pressure was recorded in the aortic feed line.

Interstitial and lymphatic fluid formed by net fluid filtration of the coronary system appeared at the epicardial surface. This "transudate" was collected from the apex of the heart in timed aliquots [12], coronary effluent was collected from the pulmonary artery.

A temperature of 37±0.5 °C, controlled on the epicardial surface and in the coronary effluent, was maintained by heating the perfusate and a warming chamber surrounding the preparation.

2.2. Experimental protocols

2.2.1. Protocol 1: the intact vessel/tissue barrier (Fig. 1A)

Hearts were perfused with Krebs–Hensleit buffer (KHB: 116 mM NaCl, 23 mM $NaHCO_3$, 3.6 mM KCl, 1.16 mM KH_2PO_4, 1.25 mM $CaCl_2$, 0.58 mM $MgSO_4$, 5.4 mM glucose, 0.3 mM pyruvate, and 2.8 U/l insulin, gassed with 94.5%O_2 and 5.5% CO_2) from the onset of preparation. After an equilibration interval of 20 min we continued perfusing KHB in one group or altered the composition of the perfusate by replacing 1/3 volume of KHB by either 0.9% NaCl, 5% human albumin, or 6% hydroxyethylstarch (HES) 130/0.4. We measured aortic pressure (cmH_2O) and transudate formation (ml/min/g dry weight) at four different flow rates (3, 4, 6 and 8 ml/min; f. 1A: "Perfusion Phase"), each rate maintained for 5 min to establish steady-state conditions of perfusion pressure. Pressure measurements and timed collection of transudate and effluent were conducted in the fifth minute, i.e., at protocol times 25, 30, 35 and 40 min (Fig. 1A). Samples of every perfusate were taken to exclude relevant differences in resulting PO_2, PCO_2, concentration of glucose and chief electrolytes (Na^+, K^+, Ca^{2+}). Colloid concentrations in transudate and effluent were determined at every measuring point, tissue edema at the end of the protocol (see below).

The groups are:

"*KHB*"($n=17$): perfused throughout with normal KHB.
"*NaCl*"($n=5$): 1/3 of the KHB replaced by 0.9% saline ("Isotone Kochsalzlösung™ 0.9%"; Braun Melsungen AG, Melsungen, Germany).

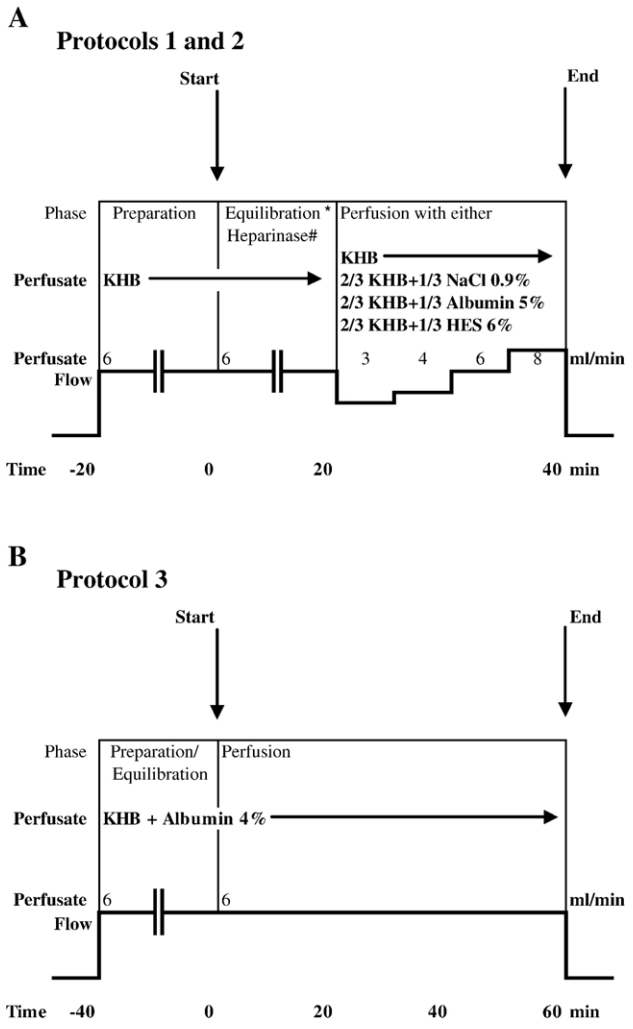

Fig. 1. Perfusion protocols. A: Protocols 1(*) and 2(#). B: Protocol 3.

"*1.67% Albumin*" ($n=5$): KHB containing human albumin at a subphysiological concentration of 16.67 g/l, 1/3 of the KHB replaced by 5% albumin ("Humanalbin™", ZLB Behring, Marburg, Germany).
"*2% HES*" ($n=5$): KHB containing 20 g/dl HES, 1/3 of the KHB replaced by 6% HES130/0.4 ("Voluven™", Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany).

Two additional hearts in every group were prepared for electron microscopy at the end of the protocol (see below).

2.2.2. Protocol 2: the glycocalyx-denuded vessel/tissue barrier (Fig. 1A)

A total of 10 IU of heparinase ("Heparinase I", Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were applied throughout the first 15 min of the equilibration phase (perfusion with KHB, constant perfusate flow 6 ml/min). This has been shown to strip the negatively charged heparan sulfates from the glycocalyx [8,10]. To exclude nonspecific enzyme activity, e.g., against the basement membrane, enzymograms were run, testing the heparinase for collagenase activity. None was found. Additionally, we have recently measured the simultaneous release of syndecan-1 and heparan sulfate in the coronary effluent of hearts treated with heparinase under the same conditions as described here. Only heparan sulfate moieties were detected, not the core proteins [13]. These results speak against any substantial non-specific protease activity of the applied enzyme. After heparinase, KHB perfusion was maintained for another 5 min (washout), before continuing as in Protocol 1.

The groups are: "KHB+H", "NaCl+H", "1.67% Albumin+H", and "2% HES+H" ($n=5$ each).

Replacing 1/3 volume of KHB by 0.9% NaCl in groups "NaCl" (Protocol 1) and "NaCl+H" (Protocol 2) served as vehicle controls. As the concentration of extracellular Ca^{2+} has a relevant impact on vascular permeability, it is important to generate a "Ca^{2+}-diluted" control group for comparison with the situation when 1/3 volume of colloid is added to KHB. This indeed resulted in similar free Ca^{2+}-levels in all groups with diluted KHB (Table 1), and in an exclusion of Ca^{2+}-effects on intergroup differences.

2.2.3. Protocol 3: application of KHB containing albumin at physiological concentration

To prevent damage to the EG possibly incurred during colloid free perfusion and tissue edema formation during ex vivo perfusion in Protocol 1, we developed a KHB-based perfusate containing physiological concentrations of albumin (40 g/l) and electrolytes. Albumin concentrate (20% stock solution, "Humanalbin™", ZLB Behring, Marburg, Germany) was admixed via peristaltic pump to a modified KHB solution in the aortic feed line. The volume of albumin stock added comprised 1/5th of the total flow. To compensate for dilution of electrolytes, the KHB, in contrast to Protocols 1 and 2, was modified by raising concentration levels to 120% of normal. Because albumin binds Ca^{2+} and acts as a charge dependent buffering system, the albumin stock solution was pre-titrated with $CaCl_2$-concentrate (8.08% solution) and 5N NaOH until a physiological free Ca^{2+} level and pH 7.4 were attained ("Rapidlab™ 348", Bayer AG,

Table 1
Characteristics of the applied perfusates

Perfusate	Measured colloid concentration (g/l)	Colloid osmotic pressure (mm Hg)	Free calcium (mmol/l)
KHB	~	~	1.31
2/3 KHB+1/3 NaCl 0.9%	~	~	0.83
2/3 KHB+1/3 Albumin 5%	16.67	5.30	0.78
2/3 KHB+1/3 HES 6%	20.32	11.10	0.81
KHB−Albumin 4%	39.88	15.50	1.30

Results are averages of triplicate determinations each; for abbreviations see materials and methods; ~ = below detection.
Colloid osmotic pressure determined with Oncometer BMT 923 (BMT Messtechnik GmbH, Berlin, Germany) at 37 °C.

Leverkusen, Germany). Stability of the pH over 90 min needed to be established before the titration was deemed adequate. The resultant values were: Na^+ 138±2 mmol/l; K^+ 4.6±0.3 mmol/l; free Ca^{2+} 1.31±0.05 mmol/l; pH 7.37±0.02; PO_2 389±21 mm Hg; PCO_2 41±3 mm Hg; glucose 99±11 g/dl.

Perfusion of the hearts with this perfusate (group: "4% Albumin"; perfusate: "KHB+Albumin 4%") commenced from the first moment of preparation at a constant flow rate of 6 ml/min, maintained throughout equilibration and another 60 min (Fig. 1B). After that we determined tissue edema (n=4, see below) or fixed hearts for electron microscopy (EM; n=2). Alternatively, hearts were perfused with colloid-free KHB for 80 min before we switched to albumin at physiological concentration. We determined tissue edema 30 min later (n=4, group: "4% Albumin after KHB").

2.2.4. Protocol 4: time dependence of albumin washout from the EG

We perfused the coronary system with KHB+Albumin 4% during an equilibration interval of 15 min to replace guinea pig albumin of the ESL by human albumin. We then switched to colloid-free KHB and, after 1, 5, 10 or 20 min washout, fixed the hearts for light microscopy (LM; see below).

2.3. Measurements of tissue edema

At the end of each protocol, tissue edema was assessed in some hearts by measuring ventricular wet weight (at once) and dry weight (after 24 h drying at 60 °C). The quotient "wet weight (WW)×dry weight $(DW)^{-1}$" was then calculated. This numeric parameter rises with myocardial edema formation from a mean value of 4.76 in vivo [14].

2.4. Electron microscopy

EM (Phillips CM, Aachen, Germany) was performed as published [8]. According to Vogel and coworkers [15], binding of La^{3+} to the negative charges of the glycocalyx (heparan and chondroitin sulfates) stabilizes the structure during the fixation. The coronary vessels were perfused with fixation solution (2% glutaraldehyde, 2% sucrose, 0.1 M sodium cacodylate phosphate, 2% $La(NO_3)_3$, 4 min). Diced pieces of the left ventricular wall were immersed at 20 °C in the fixation solution for 2 h, then for 12 h in 2% H_2O_2, 2% sucrose, 0.1 M sodium cacodylate phosphate, 2% $La(NO_3)_3$,

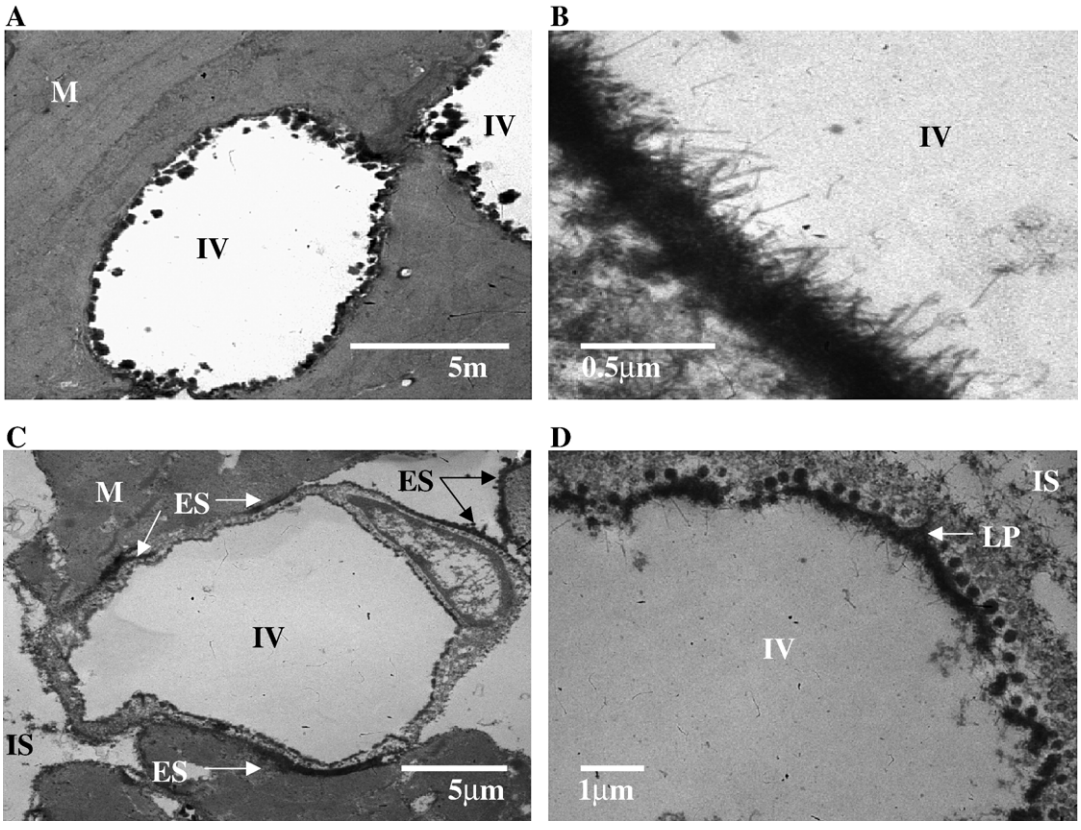

Fig. 2. Electron microscopic view of the endothelial glycocalyx. A: Examplary picture taken from a heart perfused for 20 min with 2/3KHB+1/3HES (group "2% HES"). B: Endothelial glycocalyx at high magnification. C: Extravascular staining of cell glycocalyx in the vicinity of a venule. D: Close-up of a venular capillary depicting glycocalyx-positive cavaeolae and a "large pore". IV = intravascular space; IS = interstitial space; M = myocardium; ES = extravascular staining; LP = stain extravasation at a possible large pore.

and washed in 0.03 N NaOH+2% sucrose. Contrast was enhanced with a solution containing 2% osmium tetroxide and 2% $La(NO_3)_3$. 50 microvessels of two hearts, presented in 3 slices of myocardial tissue each, were screened for every group. Only sections showing perfusion fixation (non-ruffled vessel lumina) were considered.

2.5. Light microscopy and immunohistochemistry

Hearts were perfusion-fixed by adding formaldehyde to the flowing KHB to a resulting concentration of 1%. After 4 min hearts were stored in 4% formaldehyde solution for 24 h. Paraffin-embedded tissue slices were stained using a monoclonal mouse antibody against human albumin (Clone HSA-11, Sigma, Missouri, USA) and a secondary antibody (Anti-mouse IgG, goat, biotinylated, Vektor, California, USA), together with a reaction kit (Vectastain, Vektor California, USA).

2.6. Statistical analysis

The measured data are presented as mean±SD, with n indicating the number of experiments. Comparisons were made using the Student-*t*-test or, for multiple comparisons, analysis of variance with the Bonferroni correction. *Post hoc* testing was performed using Student–Newman–Keuls' method for multiple comparisons. $p<0.05$ was considered to be significant.

3. Results

3.1. Physico-chemical properties of the perfusates

Table 1 lists colloid concentration, COP and free Ca^{2+} level of different perfusates. HES exerted a 70% higher COP in relation to albumin when both were at a concentration of approximately 2 g%. This is surprising, because the average molecular weights are 66 kDa for albumin vs. 130 kDa for HES. Presumably, HES has considerable molecular dispersion. The lower Ca^{2+} levels in the groups "NaCl", "1.67% Albumin" and "2% HES" vs. KHB largely reflect the dilution of KHB caused by adding 1/3volume of the three, relatively Ca^{2+}-free solutions to generate the final perfusates. However, as intended, the diminished Ca^{2+} levels of the colloid groups were comparable to that of the vehicle control ("NaCl").

3.2. Electron microscopy

EM conducted at the end of the protocol revealed an EG with a width of 0.2–0.3 μm in hearts of all groups without heparinase-pretreatment (see examplary Fig. 2A+B). Intracoronary application of 10 IU heparinase/15 min led to reliable destruction of the EG. The extent of tissue edema formation differed, with tissue of hearts perfused with 2/3KHB+1/3Albumin 5% appearing more dense than that of preparations perfused with 2/3KHB+1/3NaCl 0.9%. Perfusion with 4% albumin resulted in a heart tissue presenting packed muscle cells without any edema and a highly preserved glycocalyx. 2/3KHB+1/3HES 6% led to an intermediate appearance (results not shown). Interestingly, the more edemateous hearts allowed us to detect sites of extravascular staining of cell gylcocalyx following brief perfusion with the fixative. Extravasation of La^{3+} only occurred in the proximity of venular segments of the coronary bed, never in capillary or arteriolar sections (Fig. 2C). Insight into the "birth" of the glycocalyx may have been found. Fig. 2D gives an example of an endothelial staining observed repeatedly: cavaeolae tightly packed with glycocalyx, perhaps on its way to the cell surface. Evidence for the glycocalyx filling caveolae has been given before [16,17].

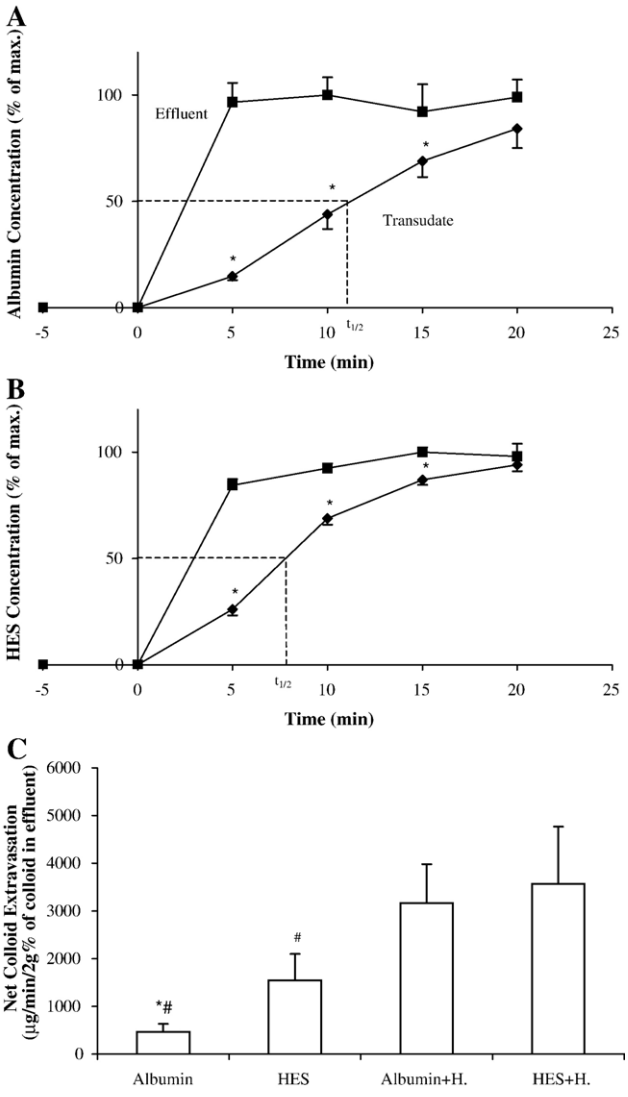

Fig. 3. A: Albumin concentration in effluent and transudate (mean±SD); *$p<0.05$ vs. effluent. B: Hydroxyethylstarch concentration in effluent and transudate (mean±SD); *$p<0.05$ vs. effluent. C: Net colloid extravasation per unit of time, normalized to a perfusate content of 2 g% of colloid (coronary flow 6 ml/min, steady state; mean±SD, $n=5$); *$p<0.05$ vs. HES; #$p<0.05$ vs. Albumin+H and HES+H.

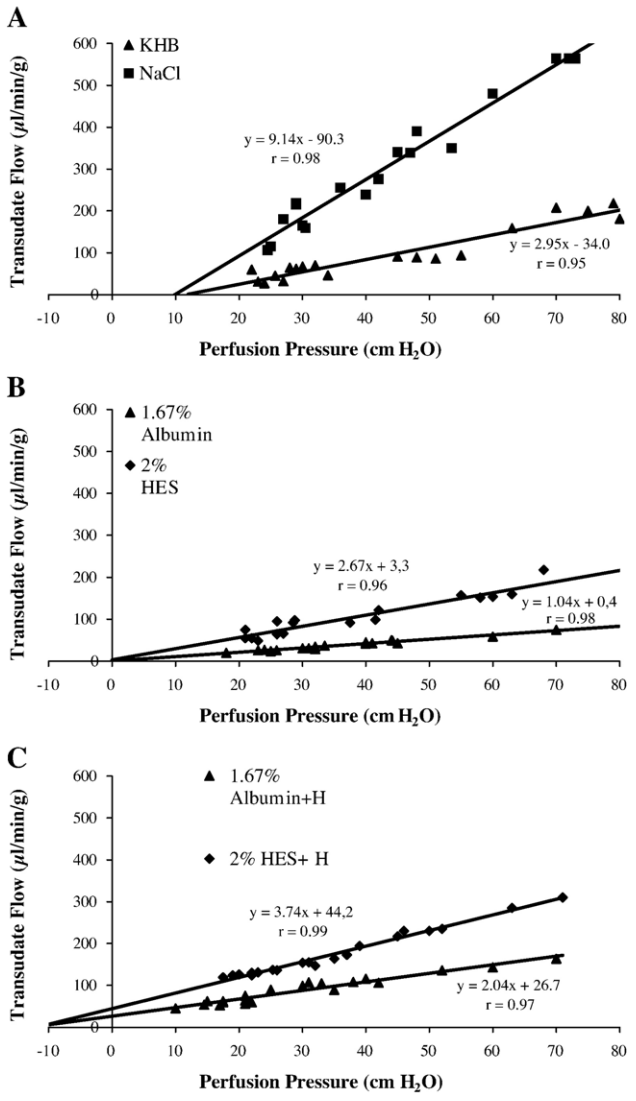

Fig. 4. Dependence of transudate flow on coronary perfusion pressure. A: Colloid-free perfusion (Groups "KHB" and "NaCl"). B: Colloidal perfusion (Groups "1.67% Albumin" and "2% HES"). C: Colloidal perfusion after degrading the endothelial glycocalyx (Groups "1.67% Albumin+H" and "2% HES+H"). r = correlation coefficient, 5 hearts/group, 3–4 distinct pressures/heart.

This figure also illustrates what may be a very "large pore" [18] or interendothelial gap. We encountered such structures several times in venular segments of the hearts investigated here.

3.3. Extravasation of colloids

The rate of appearance and concentration of albumin and HES in coronary effluent and transudate of the hearts are compared in Fig. 3A+B. In both instances, transudate levels of colloid had approached those in the effluent after about 20 min continuous infusion. Thus, little effective reflection of colloid seems to exist at the vessel wall in the isolated perfused heart when the coronary bed is taken as a whole. Fig. 3A+B include an estimate of the half-time ($t½$) required for the colloid concentration in transudate to rise to the equilibrium level. Unexpectedly, $t½$ was shorter for HES than for albumin (7.83±0.16 vs. 11.09±1.33 min, respectively, $n=5$ each, $p<0.01$ by t-test), despite albumin's smaller molecular weight. Taking the amount of transudate formation per unit of time into consideration, we observed a significantly higher outflow of HES than of albumin. The distinction between the two colloids was lost after denudation of the EG with heparinase. Fig. 3C shows this for the coronary flow of 6 ml/min.

Heparan sulfate levels in the coronary effluent rose immediately upon application of heparinase from about 0.18 µg/ml (basal) to approximately 1.2±0.3 µg/ml and remained at this level throughout application (ELISA: Seikagaku Corp., Tokyo; results not shown).

3.4. The pressure dependence of transudate flow

Fig. 4A illustrates the pressure dependence of transudate flow in hearts perfused with "KHB" and with "2/3KHB+1/3NaCl 0.9%". A highly correlated linear relationship existed in both cases within the pressure range examined here. There was an average pressure dependent increase in transudate formation ($\Delta TF/\Delta P$) of 2.95 µl min^{-1} g^{-1} (wet weight) per cm H_2O in group "KHB". Extrapolation of the line back to zero, i.e., to a fictitious state of stopped transudate formation (stopping pressure = SP), yielded a value of +12 cm H_2O. Diluting KHB by 1/3 with isotonic saline (group "NaCl") markedly heightened leak, as evidenced by a $\Delta TF/\Delta P$ value of 9.14 µl min^{-1} g^{-1} per cm H_2O, whereas the value for SP (+10 cm H_2O) was not altered. In groups "KHB+H" and "NaCl+H", pretreated with heparinase, we found unchanged $\Delta TF/\Delta P$ and SP values with respect to groups "KHB" and "NaCl", respectively (not shown).

Dilution of KHB by 1/3 with 5% albumin solution (group "1.67% Albumin") reduced leak by almost one order of magnitude vs. NaCl vehicle (Fig. 4B). Transudate formation theoretically stopped at a mean perfusion pressure of 0 cmH_2O. KHB diluted by 1/3 with the artificial colloidal solution 6% HES130/0.4 (group "2% HES") led to a $\Delta TF/$

Table 2
Characteristics of transudate formation related to perfusion pressure

Group	$\Delta TF/\Delta P$ (µl×min^{-1}×g^{-1}×cm H_2O^{-1})	SP (cm H_2O)
KHB	2.95	+12
KHB+H	2.95	+12
NaCl	9.14	+10
NaCl+H	9.41	+11
1.67% Albumin	1.04	0
1.67% Albumin+H	2.04	−13
2% HES	2.67	−1
2% HES+H	3.74	−12

Values are derived from the mean linear regression line of transudate flow vs. perfusion pressure in the respective group. $\Delta TF/\Delta P$=average pressure dependent increase in transudate formation; SP = "stopping pressure" (extrapolated mean perfusion pressure at which there is a stop of transudate formation).

Table 3
Tissue edema resulting from the respective perfusion mode

Group	Wet weight Dry weight
KHB	8.33±0.25
NaCl	8.25±0.23
1.67% Albumin	7.13*±0.41
1.67% Albumin+H	8.31±.0.28
4% Albumin	5.41‡±0.36
4% Albumin after KHB	5.92‡±0.09
2% HES	7.70±0.38
In vivo (14)	4.76±0.24

Values are mean±SD, $n=5$ each; * $p<0.05$ with respect to "NaCl", "KHB", "1.67% Albumin+H"; ‡ $p<0.05$ with respect to "NaCl", "KHB", "1.67% Albumin", "2% HES".

ΔP of 2.67 μl min^{-1} g^{-1} per cm H_2O (Fig. 4B). The theoretical perfusion pressure below which transudate formation stopped was −1 cm H_2O.

Application of colloidal solution after altering the EG (groups "1.67% Albumin+H" and "2% HES+H") revealed an increased ΔTF/ΔP in both cases. The respective extrapolated SP values were −13 and −12 cm H_2O (Fig. 4C).

The values of ΔTF/ΔP and SP are compiled for all groups in Table 2. The extremely low resistance of the endothelial barrier against intravascular pressure in the case of the "NaCl" groups presumably originates from the low extracellular Ca^{2+} level (Table 1). The change in slope seen upon application of colloids suggests a change in hydraulic conductivity of the vascular barrier, with albumin having a better "sealing" capability than HES.

3.5. Tissue edema

In vivo, dry weight amounts to approximately 21% of total heart weight in the guinea pig (wet-to-dry-weight ratio 4.75). The ratio for hearts perfused with KHB according to Protocol 1 amounted to 8.33±0.25 (Table 3). The edema resulting with 2/3KHB+1/3NaCl 0.9% was not significantly different. KHB with 1.67 g% albumin significantly reduced edema formation, an effect completely lost following pretreatment with heparinase. Supplementing perfusate with albumin further to the physiological level of 4 g% and additionally maintaining electrolyte levels, mainly Ca^{2+} (group "4% Albumin"), lowered edema even more. This supplementation also restored tissue integrity after 1 h of perfusing with colloid-free buffer (Table 3). Augmenting KHB with the artificial colloid HES at an effective concentration of 2 g%, resulting in a COP of 11.10 mm Hg, left hearts with higher edema than 1.67% albumin at COP of 5.30 mm Hg.

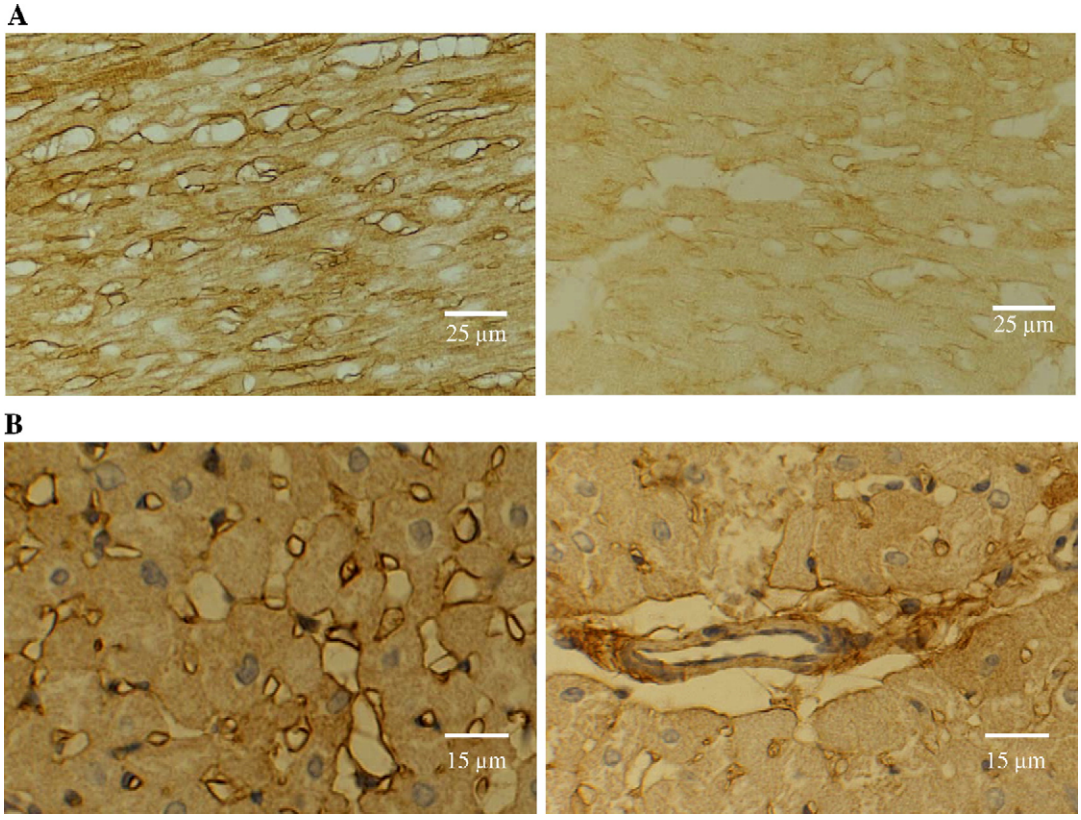

Fig. 5. A: Light microscopic distribution of albumin after 15 min of perfusion with human albumin (4 g%) and washout with KHB for 1 min (left hand panel) or 20 min (right hand panel). B: Comparison of albumin extravasation from capillary (left hand panel) and large venular (right hand panel) microvessels (3 min of perfusion with human albumin and washout for 1 min; tissue counterstained with hematoxylin-eosin).

3.6. Light microscopy

Changing perfusion mode to albumin-free KHB caused effluent levels of albumin to fall below the level of chemical detection within 4 min, suggesting almost complete washout from the coronary vessels. Transudate formation increased significantly during colloid-free perfusion with respect to the situation before (not shown).

As shown by antibody reaction, a relevant amount of albumin was attached to the endothelial surface, even 20 min after onset of washout (Fig. 5A). The antibody against human albumin did not cross-react with guinea pig albumin (not shown). Human albumin was also observed within the interstitial space after 15 min of perfusion and some of this resisted washout, at least during our time of observation (Fig. 5A). Interstitial detection agrees with the appearance of albumin in the coronary transudate. When hearts were perfused only briefly with albumin (3 min), the albumin distribution within the interstitial space shown in Fig. 5B indicates that albumin extravasation occurs mainly from the larger venular microvessels.

4. Discussion

In this and previous studies on the heart we have made the controversial observation that, under steady-state conditions, the concentration of colloid in the bulk extravasated fluid approaches that of the coronary perfusate [8,9]. Several authors [3,5,19] have presented evidence that the colloid concentration of bulk interstitial and lymphatic fluid does not have the strong impact on the generation of lymphatic fluid that Starling's law of microvascular filtration presumes. Studies on isolated microvessels demonstrated that selective retention of colloids in the EG, in combination with the structural features of the interendothelial clefts, generates a steep COP gradient localized to the endothelial surface across the glycocalyx [2]. We substantiated the role of this structure as a second, competent barrier against extravasation of fluids and colloids in the coronary vascular bed, additional to the ECs [8].

The spontaneously beating heart preparation facilitates investigation of key aspects of fluid handling in an intact organ. First, a complete vascular network with arteries, capillaries, venules and veins can be subjected to varying, predetermined pressure and flow. Second, net fluid extravasation can be directly quantified by collecting the transudate, dripping from the epicardial surface. Transudate, a mixture of interstitial and lymphatic fluid, represents that part of fluid filtered from the intact coronary system which is not stored interstitially. Third, the extent of tissue edema can be determined by comparing wet and dry weight of the myocardium. Fourth, by varying perfusate composition, it is possible to assess the impact of physiological and synthetic colloids in conjunction with the EG on properties of the endothelial surface layer (ESL). Furthermore, degradation of the glycocalyx can be implemented by applying enzymes such as heparinase [8–11]. From previous work we know that general tissue morphology appears unaltered after application of heparinase as described here, while the ultrastructural appearance of the glycocalyx changes dramatically [9,10]. Washout of heparan sulfate from the coronary system occurs during application of heparinase (results not shown). Application of atrial natriuretic peptide to hearts initiated shedding of the syndecan scaffold of the glycocalyx, accompanied by changes in colloid passage similar to those seen after heparinase treatment [9]. Again, there appeared to be no principle effects on morphology.

4.1. Synopsis and interpretation of the new findings.

While Adamson et al. assumed a thickness of the EG of about 150 nm [2], our EM findings suggest an extension of 200–300 nm in the coronary system. Pries and coworkers even found a physical thickness of this layer of about 0.8 μm, as visualized by intravital microscopy in rat mesentery microvessels [20].

Transudate flow proved to be linearly dependent on coronary perfusion pressure in our model, just as described previously for the isolated microvessel [21], but it did not inevitably extrapolate through the origin, even in a protein-free perfusion mode (Fig. 4). This may well be due to the fact that we are unable to assess the perfusion pressure pertaining at the actual sites of filtration, presumably located chiefly in the microvascular segments of the coronary bed. However, with falling perfusion pressure coronary flow also falls, so that the difference between aortic and microvascular pressure will become increasingly negligible, giving some meaning to the extrapolation.

The characteristics of transudate flow at varying coronary perfusion pressures observed here fall into three classes:

1.) Colloid-free buffer yielded the highest rates of whole organ hydraulic conductivity ($\Delta TF/\Delta P$ = pressure dependent increase in transudate formation) and a positive *x*-axis intercept at a hydrostatic pressure of about +11 cm H_2O. It is tempting to speculate that this intercept reflects build-up of pressure opposing filtration, i.e., it indicates accumulation of interstitial fluid. Appropriately, a high level of tissue edema was observed in these hearts. Stripping the glycocalyx did not further aggravate the situation in the colloid-free perfusion mode. The importance of extracellular Ca^{2+} for determining hydraulic conductivity is readily apparent from comparison of KHB with KHB+NaCl (Fig. 2A).
2.) Applying colloids significantly lowered hydraulic conductivity vs. saline vehicle, i.e., at identical Ca^{2+} level. Interestingly, no pressure opposing filtration seemed to be generated in the tissue under this condition (x-axis intercept at about 0 cm H_2O) and edema was low.
3.) After degrading the glycocalyx, net organ hydraulic conductivity in the presence of colloids was at an

intermediate level. However, the x-axis intercept became negative. Assuming again that the extrapolation in some way reflects the hydrostatic conditions pertaining at zero net fluid flux, a kind of "suction" seems to have been generated in the tissue of the isolated organ, drawing fluid from the intravascular space. Tissue edema was comparable to that developing in the colloid-free perfusion mode, suggesting a limitation to the extent of expansion of the interstitial space in the beating heart.

The net amount of fluid and colloid leaving the intravascular space per unit of time was approximately 0.1–1.0% of the coronary load of the isolated heart preparations, in good agreement with literature [12,22]. This is presumably larger than cardiac lymphatic flow in vivo, although the latter has never been totally quantified, least of all for small mammals, where the lymph flow per gram of myocardial tissue may well be high, paralleling the higher rate of myocardial perfusion in these species. Furthermore, in the isolated heart preparation, pericardial run-off is absolutely unrestricted, in contrast to the situation in vivo, where pericardial fluid needs to drain via mediastinal vessels. Additionally, lymphatic vessels normally leading interstitial fluid away along the aorta were unavoidably ligated. The major difference, however, is presumably the composition of the perfusate as opposed to plasma.

Adding colloids to the perfusate led to an impressive decrease in transudate formation. At first sight, this appears to confirm the classical Starling hypothesis, but the effect of the colloid containing perfusates did not correlate with the colloidal osmotic pressures: Albumin prevented extravasation much more successfully than HES, despite a much lower intravascular COP at comparable concentrations. A discrepancy between sealing-behaviour of individual colloidal substances and their molecular weight has been reported in numerous older studies (see, e.g., literature cited in Ref. [23]). This "COP paradox" is of practical importance, due to a very frequent but indiscriminant use of different colloids in clinical settings, all being assumed to maintain plasma volume simply on the basis of their COP [24].

There was no measurable difference in colloid concentration between the intravascular and transudate fluid 20 min after beginning of perfusion and irrespective of the applied colloid. As reported previously, this was no "artifact" due to macro leakage [8,9]. Immunohistochemical staining now revealed that the albumin molecules entered the interstitial space on the way to the epicardial surface within a very short period of time. Pertinently, extravasation of albumin did not seem to occur in capillaries (Fig. 5B). Rather, the stain spread into the myocardium from around larger venular and venous vessels.

In a totally colloid-free perfusion mode, maintained for 40 min, stripping the glycocalyx away had no measurable impact on pressure dependent transudate flow (Table 2). Thus, the EG alone does not seem to have much competence as a barrier against fluid extravasation. Colloid-free perfusion with intact glycocalyx or perfusing colloids after removal of the EG caused impressive myocardial edema. On the other hand, perfusing albumin at physiological concentration prevented tissue edema. Additionally, the natural colloid had the power to restore interstitial integrity after colloid-free perfusion. The only prerequisite seemed to be an intact glycocalyx. This can develop into a competent ESL in the presence of a suitable colloid at sufficient concentration. According to LM, the albumin molecule seems to be strongly bound to the endothelial surface, as well as to structures within the interstitial space, resisting colloid-free washout for a relevant period of time (Fig. 5A).

The superiority of the natural colloid albumin to HES in limiting edema and net organ hydraulic conductivity is readily explained by the respective binding properties. The charges exposed by the molecules forming the EG are mainly negative (heparan, dermatan and chondroitin sulfates, etc.). In contrast to HES, the molecules of which are uniformly negative, albumin is amphiphilic, carrying some positive charges (arginine, lysines), besides negative ones, at physiological pH. In fact, previous work suggested these arginine groups to relevantly influence the effects of albumin on vascular permeability [21]. This natural colloid should, thus, be superior to HES in restoring a complete ESL. Albumin should also be retained more completely by the glycocalyx, leading to a lower colloid loss per unit of time in the steady state. Indeed, this was demonstrated in the present work, as was the increased passage after degradation of the EG (Fig. 3C). Consequently, the difference in COP across the glycocalyx-presumably responsible for attenuating fluid extravasation in vivo should be greater with albumin than with HES. As shown repeatedly by others, it may well be that constituents of plasma other than albumin further augment the glycocalyx barrier ([11,23] and literature therein).

This new information concerning the distribution of colloid between the intravascular, interstitial and epicardial compartments serves to explain the three different situations distinguished by the *x*-axis intercept of the relation between transudate flow and perfusion pressure.

1. *Physiological conditions:* When perfusate contains colloid, fluid flux outwards is low, attenuated by a COP gradient across the glycocalyx. This small fluid flux is accompanied by a colloid flux which enables the fluid to enter, but also to leave the colloid-saturated interstitial space at equal COP. In the heart, this COP does not seem to differ significantly from that of the intravascular space, possibly it is slightly less. Low filtration rate, combined with lack of any relevant COP difference between interstitial and epicardial compartments mitigates development of any relevant HP in tissue. In this balanced state, no fluid is stored interstitially above the level of interstitial saturation (see x-axis intercept in Fig. 4B). In vivo, the lymphatic system should be able to adequately

remove the small amount of fluid entering and no edema should result.

2. *Hypoalbuminemia*: Perfusion in a colloid-free mode overloads the interstitial space with a large amount of fluid due to an incompetent ESL, as evidenced by impressive edema (see Table 3) [14]. Presumably, the interstitial pressure rises and, in the steady state, counteracts filtration from the vasculature. In our preparations, a pressure of 12 cm H_2O seemed to represent the limit of interstitial capacity (Fig. 4A). Intravascular HP above this forced fluid outwards, the excess colloid-free fluid appearing at the epicardial surface. In this situation it is irrelevant for fluid filtration whether the glycocalyx is intact or not and a high hydraulic conductivity of the coronary vascular bed is revealed.
3. *Altered glycocalyx*: Applying heparinase leads to a glycocalyx scaffold having far inferior colloid-binding properties. Though albumin seems to be somewhat more able to interact with this than HES, the result is an interstitial space overwhelmed with fluid containing colloid, extravasation enforced by HP. It is questionable if this effect is as impressive in vivo as our experiments on the isolated heart suggest (Fig. 4C), where excess extravasated fluid appearing at the epicardial surface is removed with great efficiency. However, in vivo the outflow of colloid should still lead to more fluid storage and increased interstitial HP. Due to the interstitially stored albumin molecules, any acute decrease in plasma colloid concentration should lead to transient increases of tissue edema (oncotic "suction").

The classic Starling equation takes COPs and HPs of the intravascular and the interstitial space into consideration. This equation, however, is unable to explain the "low lymph flow paradox" [25] and it is also unable to explain the "COP paradox" seen here for transudate flow and edema formation when comparing albumin and HES. As proposed by Adamson et al. [2] the term "COP in tissue" needs to be replaced by "COP beyond the EG". The "modern" Starling equation for microvascular segments reads:

$$J_v/A = L_p((P_c - P_t) - (\pi_e - \pi_g)),$$

where J_v/A = filtration rate per unit area; L_p = hydraulic conductivity of the vessel wall; $P_c - P_t$ = difference of HPs between the capillary lumen [c] and tissue [t]; π_e = COP in the ESL and π_g = COP directly below the ESL.

Though the concept of Starling that HPs and COPs determine fluid balance in the vascular system is still principally valid, evidence for a variability of L_p confounds the issue. Hydraulic conductivity seems to depend on the nature of the applied colloid, with albumin better able to seal the barrier than HES. Special effects of plasma constituents beyond their pure oncotic potential have been described repeatedly ([3,23] and literature therein).

A question remaining to be discussed concerns the phenomenon of near identical colloid concentrations in the intracoronary fluid and transudate observed here in the steady-state. It is also important to reconcile colloidal reflection coefficients of <0.1, found here in the isolated organ, of approximately 0.2–0.3 for cardiac lymph in vivo

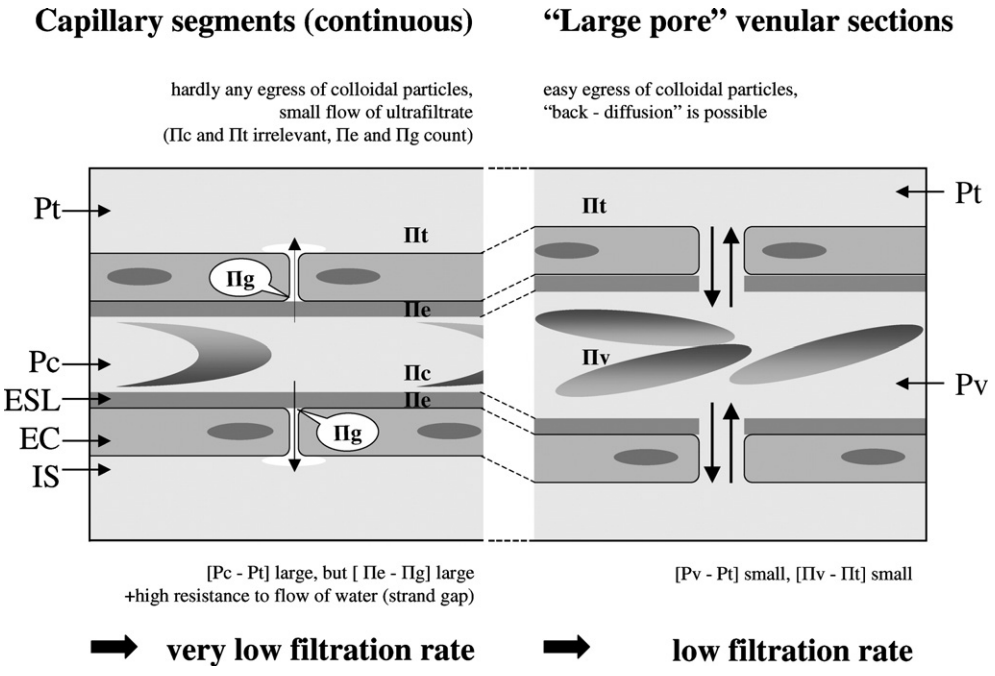

Fig. 6. Low-filtration concept of lymph production. Pt, Pc and Pv = hydrostatic pressure in tissue, capillary and venule, resp.; Πt, Πc, Πv, Πe and Πg = colloid osmotic pressure in tissue, capillary, venule, endothelial surface layer and beyond the endothelial glycocalyx, respectively; ESL = endothelial surface layer (glycocalyx + bound colloid), EC = endothelial cell, IS = interstitial space.

[26,27], and of "zero" for perivascular fluid [28], with the high reflection coefficients, e.g. of albumin, repeatedly determined in measurements on single microvessels [23] and some whole organs [29]. The EM visualisations of the glycocalyx throw some light on this issue. The fixation solution applied into the coronaries for 4 min contains the polyvalent cation La^{3+}, able to bind not only to negatively charged groups of the EG but also to those on the surface of parenchymal cells. However, because of sequestration by the luminal glycocalyx, rapid escape of La^{3+}into the interstitial space should only be possible at sites at which also large colloidal molecules readily extravasate. Such sites have previously been described in venular segments of the vasculature and termed "large pores" [18]. In hearts with intact EG, La^{3+}-staining of tissue did not occur outside of arterioles and capillaries. However, in the vicinity of larger "venular" capillaries electron-dense covering of the adjacent cardiomyocytes was sometimes seen (Fig. 2C). Localisation of albumin in the interstitial space gave a similar picture (Fig. 5B). In studies comparing isolated coronary vessels, venules proved to be considerably more permeable to albumin than arterioles [30]. Accordingly, the coronary vascular bed does not seem to exhibit a uniform permeability: arteriolar and capillary domains are relatively impermeable, while venular pores or gaps allow facile extravasation. The latter property is in accordance with the newly realised fact that, in contrast to Starling's concept, there is no net reabsorption of fluid in the venular segments of the microcirculation [1,3].

Since the concentration of colloid in transudate approximates 90–95% of that in coronary effluent, the relative proportion of fluid filtration in the capillary as opposed to the venular sections must be dominated by the latter. Moreover, allowing colloid to extravasate in the venular segments serves to mitigate net fluid movement in this section, because both HP and COP differences will be very small. All in all, a situation of "low net filtration" will prevail, affording low rates of formation of lymphatic fluid despite near equilibration of intravascular and bulk interstitial colloid concentration. The scenario is schematically illustrated in Fig. 6. For such venular beds, the classic Starling equation is principally valid. However, there is no real filtration barrier. To appreciate the value of such a regulation in a physiological context, it should be pointed out that extravasation of hormones, vitamins, lipoproteins, etc. to supply the parenchymal cells is no problem at all, and back diffusion of substances from the tissue into the vasculature is possible. It also complies with the clinically established fact that retroperfusion of the heart via the coronary sinus affords excellent fluid and solute transport into the myocardium [31]. Still, the glycocalyx seems to be of importance throughout, since clustering of anionic cell-surface molecules at fenestrations and gaps was reported early by Simionescu et al. [32].

Perfusion with artificial colloids or without any colloid, as well as perfusing a damaged glycocalyx in the presence of colloids are unphysiological conditions. Tissue edema ensues due to an overwhelmed lymphatic drainage system. The possibility of dynamic regulation of the regionally heterogeneous double barrier of ESL and ECs needs further exploration.

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