Age-related changes in rat dermal extracellular matrix composition affect the distribution of plasma proteins as a function of size and charge

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Age-related changes in rat dermal extracellular matrix composition affect the distribution of plasma proteins as a function of size and charge. Am J Physiol Heart Circ Physiol 308: H29–H38, 2015. First published October 31, 2014; doi:10.1152/ajpheart.00545.2014.—Collagen and glycosaminoglycans (GAGs) constituting the ECM may limit the space available and thus exclude macromolecules from a fraction of the interstitial fluid (IF) phase. This exclusion phenomenon is of importance for transcapillary fluid and solute exchange. The purpose of the study was to examine the range of interstitial exclusion in rat skin by using probes within a span of molecular weights and electrical charge and also to test if a change in interstitial composition, occurring as a consequence of aging, affected exclusion. To this end, we used a novel approach, involving the exact determination of albumin concentration and mass in IF and tissue eluate by HPLC and thereafter, expressing the corresponding numbers relative to albumin for a set of probe proteins assessed by quantitative proteomics. Albumin was excluded from 55 ± 4% (n = 8) of the extracellular fluid phase. There was a highly significant, positive correlation between probe Stokes-Einstein (SE) radius and fractional excluded volume (VEF), described by VEF = 0.078·SE radius + 0.269 (P < 0.001), and oppositely, a negative correlation between probe isoelectric point (pI) and exclusion for proteins with comparable size, VEF = −0.036·pI + 0.719 (P = 0.04). Aging resulted in a significant reduction in skin hydration and sulfated GAGs, a moderate increase in 0.036·pI

interstitial fluid; extracellular matrix; plasma protein exclusion

THE INTERSTITIUM OR INTERSTITIAL space is the physical and biochemical environment of the cells in the body, consisting of connective and supporting tissues located outside of the blood and lymphatic vessels and parenchymal cells. In essence, the interstitium can be divided into two phases: the interstitial fluid (IF) and the structural molecules of the interstitial or the ECM, and can be thought of as a three-dimensional “meshwork” composed of a complex aggregation of protein fibers, glycogen, and carbohydrate polymers. The interstitial macromolecules constituting the ECM, particularly collagen and glycosaminoglycans (GAGs; i.e., hyaluronan and proteoglycans), may sterically limit the space available and thus exclude macromolecules from a fraction of the IF phase. This is because two interstitial macromolecules cannot occupy the same space simultaneously, and their centers cannot come closer than the sum of their radii (3, 7, 12). Additional to steric effects, electrostatic factors might be involved in selectively excluding other negatively charged macromolecules residing in or being transported through the interstitium, since GAGs are negatively charged at physiological pH values (31). The magnitude of the excluded volume (VEF) has important consequences in the dynamics of transcapillary exchange of fluid and solutes and will, e.g., influence equilibration rate and mass transfer of macromolecules in the interstitium and thus plasma volume regulation (3, 31). Furthermore, studies of exclusion phenomena may give information on the functional consequences of the anatomical organization of the structural elements of the ECM.

As reviewed recently (31), many previous studies have focused on steric exclusion and showed that albumin, an important determinant of plasma and interstitial colloid osmotic pressures, is excluded from a large fraction of most interstitia (4, 5, 24). Interestingly, the more positive IgG, having a molecular weight approximately twice that of albumin, was found to be excluded from a similar fraction of the skin interstitium (27), suggesting that the charge is an important determinant of macromolecular distribution volume, in agreement with data from the lung (20). Such assumptions have been supported by studies in vitro (28) and in vivo (9), thus indicating that GAGs have a significant effect as electrostatic-excluding agents.

Because of the presumed importance of interstitial exclusion for transcapillary fluid exchange and that previous studies have mainly focused on albumin, we wanted to examine the range of interstitial exclusion by using probes within a span of molecular weights and electrical charges. Due to a lack of data for skin, an important reservoir for IF (3), we also wanted to test whether and if so, how much a change in interstitial composition, occurring as a consequence of aging, affected exclusion. To this end, we used a novel approach that allows for testing of exclusion properties of multiple protein probes simultaneously. We found a highly significant, positive correlation between probe Stokes-Einstein (SE) radius and VEF and oppositely, a negative correlation between probe isoelectric point (pI) and exclusion. Moreover, we found that structural changes associated with aging influenced the distribution volume of macromolecules in skin, suggesting that our findings have significant implications for fluid homeostasis and the ability for salt storage.

METHODS

Experimental Animals and Housing

The experiments were performed in female Sprague-Dawley rats, either 8–12 wk, here defined as young (total n = 29), or 12–13 mo (total n = 14), here defined as aged, obtained from Taconic M&B (Lille Skensved, Denmark), which were fed a standard laboratory diet.
Hypnorm (fentanyl/fluanisone)/Dormicum (midazolam), 2.5 ml kg⁻¹ as described in detail previously (24), except using a 1:1 mixture of 4°C and dialyzed for at least 24 h before use. containing 0.02% azide. The stock solution was stored in the dark at 5–10% of the total radioactivity, as estimated by TCA precipitation, was removed by dialyzing the tracer against 1 liter of 0.9% saline solution. 0.1 ml of this solution was dispersed in a 1.8-ml Nunc vial (Nunc-Elutability of Plasma Proteins from Skin
To determine whether plasma proteins were elutable from excised skin, we measured recovery of labeled proteins that had been brought to a steady-state concentration in the extracellular compartment. Initially, we intended to measure VE for several abundant plasma proteins after equilibration of radioactively labeled plasma for 192 h (8 days) using a modified version of a continuous infusion method (23). We would then measure their concentration and mass in skin IF and tissue by counting their respective fractions after HPLC fractionation (see below). Whereas this was possible for albumin, we were not able to identify other proteins uniquely in tissue eluate and therefore, had to use an alternative approach. Thus these chronic infusion experiments served as documentation of elutability of plasma proteins in general.

Isolated rat plasma was labeled with 125I by Iodo-Gen. Briefly, 5 mg 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Product #T0656; Sigma-Aldrich, St. Louis, MO) was dissolved in 5 ml chloroform, and 0.1 ml of this solution was dispersed in a 1.8-ml Nunc vial (Nunc-Kamstrup, Roskilde, Denmark). A film of the virtually water-insoluble Iodo-Gen was formed in the Nunc vial by allowing the chloroform to evaporate to dryness under nitrogen. Then, 1.5 mg dried rat plasma, dissolved in 1 ml 0.05 M PBS solution, pH 7.5, containing 10 MBq 125I (Institute for Energy Technology, Kjeller, Norway) and 15 µl 0.01 M NaI, was added, and the iodinating tube gently agitated for 10 min before the reaction was terminated by removing the albumin solution. Unincorporated iodine-isotope, accounting for 5–10% of the total radioactivity, as estimated by TCA precipitation, was removed by dialyzing the tracer against 1 liter of 0.9% saline containing 0.02% azide. The stock solution was stored in the dark at 4°C and dialyzed for at least 24 h before use.

The labeled plasma was infused for 8 days at a constant flow rate of 1 µl h⁻¹ with an implantable Alzet osmotic pump (Model 2001), as described in detail previously (24), except using a 1:1 mixture of Hypnorm (fentanyl/fluanisone)/Dormicum (midazolam), 2.5 ml kg⁻¹ ip as an anesthetic agent.

Measurement of Extracellular Fluid Volume
In pentobarbital-anesthetized rats, both kidney pedicles were ligated via flank incisions. Through a PE-50 catheter, inserted into the right jugular vein, a bolus of 51Cr-EDTA (0.5 MBq in a volume of ~0.15 ml) was injected intravenously for measurements of extracellular volume (Vex). The tracer was allowed to equilibrate for 2 h. A final blood volume of ~1 ml was collected by cardiac puncture, and the rat was killed with saturated KCl. Tissue samples were taken for determination of radioactivity, as described above using window settings for 51Cr of 530–690 keV. Vex was found as plasma equivalent space, as described in previous publications [e.g., Wiig et al. (24)].

Isolation of IF from Skin by Tissue Centrifugation
Immediately after euthanasia, the rats were transferred to an incubator with 100% relative humidity to prevent evaporation during tissue handling. Excised skin samples were removed carefully and folded with their visceral side facing a nylon mesh basket (pore size ~15 × 20 µm) and put into a 1.5-ml microcentrifuge tube, as described by Wiig et al. (23). The preweighed tube with the nylon basket containing the sample was immediately capped, reweighed, and centrifuged at 424 g (2,000 rpm) for 10 min at 4°C. A final blood volume of ~1 ml was collected by cardiac puncture, and the rat was then euthanized with saturated KCl. Skin was harvested, as described above, and 125I radioactivity in plasma and tissue samples was determined in a gamma counter (Model 1282; Compugamma; LKB Instruments, Mount Waverley, Melbourne, Victoria, Australia) using window settings of 120–320 keV. Standards were counted in every experiment, and spillover, as well as background and decay during the period of measurement, was automatically corrected.

Following counting, tissue samples were subjected to elution with a mixture containing 0.02% sodium azide in 0.9% saline solution, as described in detail in a previous publication (32). Tissues were minced, an aliquot of 10 ml eluent was then added to each sample, and the mixture was left in an agitator at room temperature for 24 h. After centrifugation, the supernatant was removed, a new aliquot of 10 ml saline-azide solution was supplemented, and the agitation procedure was repeated. After another 24 h of elution, the individual supernatant-free tissues were counted again and corrected for isotope decay, allowing for estimation of elutability of the tracer. It turned out that proteins eluted from skin could not be uniquely identified by HPLC, and therefore, we turned to a mass spectrometry (MS) approach as described below.

Extraction of Tissue Protein by Elution
Proteins processed for later MS analysis were extracted from skin by elution using a somewhat different procedure than for the isotope experiments described above and optimized for MS analysis. Carefully minced skin samples were transferred to 15 ml centrifuge tubes containing 5 ml, 1 M sodium-phosphate buffer with 0.5% azide (25% Na3HPO4, 25% NaH2PO4, 50% Na2SO4, pH 6.7). The tubes were gently rotated for 48 h at 4°C to extract tissue proteins (28). After

Anesthesia and Surgery
Anesthesia was induced with pentobarbital sodium, 50 mg/kg body wt, given intraperitoneally unless otherwise specified. While anesthetized, the body temperature was maintained at 37 ± 1°C using a heating pad and lamp. If needed for the particular experiment, polyethylene (PE-50) catheters were placed in one femoral vein for injection of tracers and substances (see below) and in one femoral artery for blood sampling and monitoring of blood pressure. Upon termination of the experiment, the rat was killed by cardiac arrest, induced under anesthesia with an intravenous/intracardiac injection of saturated KCl.

After euthanizing the rat, the skin from the medial and lateral part of both thighs, while avoiding the ankle, was closely clipped, and skin (0.12–0.22 g) was harvested inside of a 100% humidity chamber at room temperature. Thigh was chosen above back skin because of a lower content of subcutaneous fat that makes thigh samples more homogenous (28). Uniform, paired samples from each thigh were transferred into preweighed tubes, such that each tube contained tissue from both legs.

Measurement of Extracellular Fluid Volume
In pentobarbital-anesthetized rats, both kidney pedicles were ligated via flank incisions. Through a PE-50 catheter, inserted into the right jugular vein, a bolus of 51Cr-EDTA (0.5 MBq in a volume of ~0.15 ml) was injected intravenously for measurements of extracellular volume (Vex). The tracer was allowed to equilibrate for 2 h. A final blood volume of ~1 ml was collected by cardiac puncture, and the rat was killed with saturated KCl. Tissue samples were taken for determination of radioactivity, as described above using window settings for 51Cr of 530–690 keV. Vex was found as plasma equivalent space, as described in previous publications [e.g., Wiig et al. (24)].
elution, the samples were centrifuged in an Eppendorf 5810R centrifuge at 3,220 \( g \) at 4°C. The supernatant was transferred to a new tube, and the centrifugation procedure was repeated. The supernatant was finally filtered through a filter with a cutoff of 22 \( \mu \)m, and 4 \( \mu \)l filtrate was processed, as described, for the IF sample above.

**Measurement of Albumin in Eluate and IF**

Albumin in eluate and IF was determined by size-exclusion chromatography (SEC) (19). Filtered eluate (50 \( \mu \)l) was diluted 1:2 in phosphate buffer (0.1 M \( \text{Na}_2\text{SO}_4 \)), 0.05 M \( \text{Na}_2\text{PO}_4 \), 0.05 M \( \text{H}_2\text{NaPO}_4 \), pH 6.8), whereas IF was diluted 1:336 in phosphate buffer. All samples were analyzed by HPLC using a size-exclusion column (Super SW2000, 4.6 \( \times \) 300 mm, 18674; Tosoh Bioscience, Tokyo, Japan), and a reversed-phase column (ProSwift RP-4H, 1 \( \times \) 50 mm; Dionex, Sunnyvale, CA) was coupled in the series. Albumin was separated from IgG and other larger plasma proteins in the first dimension. Subsequently, the sample that eluted in the retention time of albumin was loaded onto the second column by an inline switch at a flow rate of 0.35 ml/min and with an 8-min acetonitrile gradient (5–60%) for separation from proteins with similar molecular weight and quantification of the albumin peak. The albumin concentration was determined based on the area under the curve for standards and samples (19).

**Size and Charge Distribution of Proteins in Eluate and IF**

To characterize the probes in eluate and IF, the size and charge of selected proteins of interest (see below) were determined by SEC and gel fractionation. Samples (\( n = 6 \)) were fractionated by size into 16 fractions by SEC, as described above. All fractions were washed with 3 ml distilled water using Amicon Ultra-4 Centrifugal Filter Units (cut-off 3 kDa; Millipore, Milford, MA). The retained fluid was buffer exchanged further to 100 mM ammonium bicarbonate in the same Amicon filters to a total volume of \( \sim 40 \mu l \) for LC-MS/MS analysis.

Gel fractionation was used to separate the pooled (\( n = 6 \)) samples into 24 fractions based on pI using an Offgel fractionator (Model 3100; Agilent Technologies, Santa Clara, CA), in accordance with the protocol provided by the manufacturer. Because of low-protein content, fractions 1–5 and 20–24 were pooled, resulting in 20 samples that were washed with ammonium bicarbonate, as described for SEC samples.

Fractions isolated based on size and charge separations were analyzed by MS. The molecular weight and pI of the selected molecular probes were found by gel filtration compared with relevant standards, and the SE radius was calculated as described previously (1).

**Protein Identification and Quantification by LC-MS/MS**

The protein concentrations in samples from IF, Amicon-filtered eluate, SEC, and Offgel fractions were determined by the Qubit Quant-IT Protein Assay Kit (Invitrogen, Carlsbad, CA), and 30 \( \mu \)g protein from plasma, IF, and eluate samples was diluted to 30 \( \mu \)l with 100 mM ammonium bicarbonate, digested by trypsin, and desalted, as described previously (10, 14). Desalted and purified peptides were dissolved in 0.1% formic acid and analyzed further using LC-MS/MS.

Proteins were identified and quantified using LC-MS/MS, as described in detail in previous publications (10, 14). IF and eluate peptide samples (1 \( \mu \)g) were analyzed in two separate rounds by LC-MS/MS, each time with three technical replicates. An Agilent Technologies 1100 LC/MSD Trap XCT Plus system with a HPLC-Chip contained a 0.075\( \times \)1 \( \mu \)m column and an integrated 9-mm, 160-nl enrichment column packed with the same material (Part #G4240-63001 SPQ110; Agilent Technologies).

To validate the relative protein quantitation using an independent tool, the proteins of interest in IF and eluate were also quantified by selected reaction monitoring (SRM), analyzing the peptide samples (0.3 \( \mu \)g) on a 6410 Triple Quadrupole LC/MS, coupled to a 1,200 Cap/Nano system via the HPLC-Chip cube electrospray ionization interface (Agilent Technologies), as described previously (8).

**Composition of Skin ECM**

Since collagens and GAGs are major ECM elements determining exclusion, these substances were quantitated. Skin used for analysis was freeze dried and incubated in digestion buffer for 18 h at 65°C. The digestion buffer consisted of 0.150 ml papain (Sigma-Aldrich) in 0.2 M phosphate buffer (0.1 M \( \text{Na}_2\text{PO}_4 \), 0.1 M \( \text{H}_2\text{NaPO}_4 \), pH 6.4), with 0.1 M sodium acetate, 0.01 M NaEDTA, and 0.005 M cysteine HCl. The samples were centrifuged at 20,000 \( g \) for 20 min, and the supernatant was pipetted off and stored at \(-20^\circ\text{C} \) for further analysis.

The concentration of hyaluronic acid (HA) in skin samples was analyzed using a sandwich protein-binding assay (HA Test Kit 029-001; Corgenix, Broomfield, CO), according to the procedure described by the manufacturer. The optical density was read spectrophotometrically at 450 nm, and HA concentrations were calculated from the linear regression curves of the HA standards.

Sulfated GAG (sGAG) concentration was measured using the Blyscan sGAG assay (BC-B1000; Biocolor, Carrickfergus, UK), according to the procedure described by the manufacturer. Absorbance was measured spectrophotometrically at 656 nm.

The collagen content was determined, as described originally by Woessner (34) and as used by us in previous publications [e.g., Wig et al. (33)]. This method is based on the determination of hydroxyproline content, assuming the conversion factor of 6.94 \( \mu \)g hydroxyproline/mg collagen (11).

**Data Analysis and Calculations**

**Analysis of MS data.** Eluate (E) and IF from aged (A; \( n = 6 \)) and young (Y; \( n = 6 \)) rats were analyzed with LC-MS/MS in triplicate in two rounds, meaning six replicates for each rat, resulting in 36 LC-MS/MS runs for each of the conditions AIF, AE, YIF, and YE for each series. Hence, the four conditions resulted in 144 LC-MS/MS runs in total. That generated 144 Agilent raw files that were extracted, searched, and auto validated using Spectrum Mill (Rev A.03.02.060; Agilent Technologies), as described previously (14). Proteins were identified in Spectrum Mill using a "protein-protein comparison column," such that, for the identified proteins, the number of spectra from each run was obtained. The spectral count data from all replicates were exported to Microsoft Excel, where quantitative values were calculated [Log2 (average AIF/average YIF) – median] and [Log2 (average AE/average YE) – median]. Furthermore, the number of spectra for all of the replicates from each condition was imported into GraphPad Prism 6 (GraphPad Prism Software, La Jolla, CA) to evaluate proteins differently expressed in the aged vs. young rats for IF and eluate (AIF/YIF and AE/YE, respectively). Multiple\( -\text{tests, followed by the Holm-Sidak method (} \alpha = 0.001\), were used to calculate statistical significance without assuming constant SD (14).

**Analysis of SRM MS data.** Two to three unique peptides were used for quantification of each protein, as described previously (8). Heavy isotope-labeled peptides, corresponding to unique sequences in rat albumin (LVQETDFAK, K + 8 Da) and rat α1-antitrypsin (TLSS-LGITR, R + 10 Da), were provided by Thermo Fischer Scientific (Waltham, MA). These heavy peptides were spiked into eluate and IF at \( \sim 50\) fmol/\( \mu l \) to control for any asymmetrical matrix effects. The heavy peptides were analyzed by LC-MS/MS, coupled to a 1,200 Cap/Nano system via the HPLC-Chip cube electrospray ionization interface (Agilent Technologies), as described previously (8).
E/IF ratios of the peak area of all of the respective qualifications were <10% among the samples. The peak areas for the quantifiers of the respective protein in IF and eluate were then used to calculate available volume (VA) in skin (see below, Calculations, Eqs. 3–6).

Calculations. The VX in skin was found as the plasma equivalent space of $^{51}$Cr-EDTA, i.e., a virtual extracellular space having the same concentration of the tracer as plasma, as:

$$V_X = \frac{(^{51}\text{Cr-EDTA cpm/g tissue})}{(^{51}\text{Cr-EDTA cpm/ml plasma})} \times \text{(ml plasma)} \times \text{(g tissue)}$$

To calculate protein VA, tissue protein mass (m) and IF protein concentration ($C_i$) are needed. We quantified albumin by HPLC in IF and eluate. By assuming equilibrium between eluent fluid and tissue, we could calculate albumin mass. The absolute VA for albumin, as

$$V_{A} = m_{(alb)}(C_i)$$

and the absolute VE for albumin as

$$V_{E} = V_{A} - V_{X}$$

The relative or fractional VA for the protein of interest, here named P, was then found as

$$V_{A (P)} = m_{(P)}(C_i)$$

and the fractional VE for the protein P calculated from Eqs. 4 and 5, respectively.

The present calculations do not take into account the intravascular plasma proteins, including hemoglobin, that appeared in the MS assay. We have shown previously that the intravascular volume in skin is low [−0.5% of wet weight (wwt)] (30). Intravascular proteins will thus not affect the calculated protein-distribution volumes and can be neglected.

Statistics

Data and statistical analysis was performed using GraphPad PRISM, Version 6.0. Results were compared using two-tailed t-tests unless specified otherwise. Values are given as mean ± standard error, and $P < 0.05$% was considered statistically significant.

RESULTS

Elutability of Plasma Proteins from Skin

The present method is based on the assumption that soluble proteins can be extracted almost completely and proportionally to albumin from the extracellular fluid phase to the surrounding buffer by elution. To examine protein extraction, we labeled rat plasma with $^{125}$I and infused labeled plasma intravenously for 8 days in $n = 5$ rats to achieve steady-state tissue concentration using an osmotic pump, as described previously for albumin (24). The plasma level of the tracer during infusion was checked every other day from day 3 and was not different from the final concentration measured when ending the experiment.

To investigate whether the tracer was degraded during the infusion period, we assessed the amount of free $^{125}$I by TCA precipitation of the tracer remaining in the pump after terminating the experiment. We found that this fraction was <0.5% in all experiments and therefore, negligible. We also examined whether there was alteration in the size distribution of proteins during the infusion period by subjecting harvested tracer solution to SEC. As evident from the representative example from one experiment in Fig. 1, the elution curves were similar before and after the infusion period, suggesting that the tracer was not degraded in the experimental period. Moreover, by simultaneous gamma detection, we found that the gamma intensity reflected exactly the corresponding optical density, showing that the radioactively labeled proteins reflected plasma (Fig. 1).

The recovery of equilibrated tracer ranged from 94% to 98% ($n = 5$) when estimated by elution of skin samples. As for plasma, the gamma intensity in the eluate reflected exactly the corresponding optical density, showing that the radioactively labeled proteins were representative for eluate proteins and were extracted proportionally to albumin found to be 94% eluted in hind-limb skin (9). Collectively, these radioactive tracer experiments demonstrated that extracellular plasma proteins could be eluted quantitatively and proportionally from the skin interstitium.

Validation and Characteristics of Plasma Proteins Used As $V_E$ Probes

IF and eluate from skin were processed for separation by size using SEC, followed by gel fractionation. Thereafter, candidate proteins were identified with MS and proteomics. We were searching for proteins with a span in size and charge to be used as probes for estimation of their VA and VE in the interstitium. Candidate proteins should meet the following criteria: 1) be present in all IF and eluate samples with a minimum of three spectra/sample; 2) have a similar charge (pI) and SE radius in IF and eluate; and 3) be extracellular (i.e., elutable) without local production and binding and thus an abundant plasma protein.

Whether the candidate protein was elutable was tested in the radioactive plasma-infusion experiments described above, and a lower or similar concentration in IF and plasma, either determined by HPLC or by relating spectra, verified that there

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Fig. 1. HPLC of plasma after continuous infusion. Radioactivity in successive collections of eluate from a size-exclusion gel-chromatography (SEC) column after application of plasma stock solution (red line) and plasma from 1 rat sampled after in vivo continuous infusion for 192 h of $^{125}$I-labeled plasma (dashed line). Also shown is the UV signal for plasma stock solution (blue line). Overlapping curves suggest that the tracer was not degraded during the experiments and that the labeled proteins reflected plasma. Ve, elution volume.
was no local production. We found seven proteins listed in Table 1 that met all criteria, also listing estimated values for their size (molecular weight and SE radius) and charge. As evident from Table 1, most of the plasma proteins were negatively charged with a pI value less than seven, ranging from 3.9 for α1-antitrypsin and murinoglobulin-1 to 5.0 for albumin. Only serotransferrin was close to its neutral state, with a pI of 6.8. These seven proteins had similar elution patterns in IF and eluate, as demonstrated in Fig. 2A, showing an example of SEC of the two fluids and their corresponding elution volumes. The molecular weight and SE radius of these proteins were estimated from their elution volume by comparing with standards displayed in Fig. 2, B and C, respectively, and as exemplified for hemopexin in Fig. 2D. Again, with the use of hemopexin as an example, Fig. 2E shows determination of pI by combining gel electrophoresis and MS.

### Table 1. Characteristics of abundant plasma proteins

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Accession Number</th>
<th>MW, kD</th>
<th>SE, nm</th>
<th>pI</th>
</tr>
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<tbody>
<tr>
<td>Serum albumin</td>
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<td>3.6</td>
<td>5.0</td>
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<tr>
<td>Serotransferrin</td>
<td>P12346</td>
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<td>3.4</td>
<td>6.8</td>
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<td>3.4</td>
<td>4.4</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>Q63041</td>
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<td>3.1</td>
<td>3.9</td>
</tr>
<tr>
<td>Hemopexin</td>
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<td>4.6</td>
</tr>
<tr>
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<td>4.9</td>
<td>3.9</td>
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<tr>
<td>Murinoglobulin-1</td>
<td>Q03626</td>
<td>295</td>
<td>6.1</td>
<td>3.9</td>
</tr>
</tbody>
</table>

MW, molecular weight; SE, Stokes-Einstein; pI, isoelectric point.

### Interstitial VE of Plasma Proteins

Interstitial VE was estimated as described in METHODS. Central elements in this calculation are the total VX in skin, as determined by 51Cr-EDTA, which averaged 0.49 ± 0.02 ml/g wwt (n = 8), and albumin, having an IF concentration of 25.5 ± 2.6 mg/ml (n = 12) and a content of albumin of 5.5 ± 0.7 mg/g wwt (n = 12). These data allowed for the calculation of an absolute VA (alb), averaging 0.22 ± 0.02 ml/g wwt (n = 12), and thus a VE, averaging 0.27 ± 0.02 ml/g wwt (n = 8), corresponding to a VEF (alb) of 0.55 ± 0.04 (n = 8).

The albumin data enabled us to calculate VA and VE in the same rats (n = 6) for the other plasma proteins listed in Table 1, and VEF as a function of SE radius is shown in Fig. 3. Compared with albumin (molecular weight 67 kD and SE radius of 3.6 nm in our experiments), there was a modest increase in VEF with increasing molecular size, averaging 0.63 for ceruloplasmin and 0.79 for murinoglobulin, with respective SE radiuses of 4.9 and 6.1 nm (Fig. 3 and Table 1). The relationship between VEF and the protein’s SE radius in the range between 3.1 and 6.1 nm can be described by the equation, \( VEF = 0.078 \cdot SE \text{ radius}^{0.269} \).
Since quantification of the actual proteins, except albumin, was based on spectral counts and thus stochastic correlation only, we used an independent peptide quantification method in an attempt to substantiate the data. As shown in Fig. 3, the SRM method gave practically identical results as the label-free LC-MS/MS method, suggesting that proteins can be reliably quantified based on spectral counts assessed by MS.

Proteins with SE radius similar to albumin (Table 1) had a variable VEF that may be related to variation in pl. Therefore, we plotted VEF as function of pl. As shown in Fig. 4, there was a weak, but statistically significant, negative correlation between pl and VEF that can be described by the equation, 

\[ \text{VEF} = -0.036 \cdot \text{pl} + 0.719 \quad (P = 0.04) \]

**Composition of Skin ECM**

VA and VEF are dependent on the major components of the ECM. Whereas our previous exclusion studies have mostly been performed on back skin (28, 33), here, we chose hind-limb skin that appears more homogenous and has less fat than back skin. To assess excluding structures in skin from this region, we measured collagen, hyaluronan, and sGAGs in thigh skin (n = 6), averaging 174 ± 16, 1.1 ± 0.1, and 3.6 ± 0.3 mg/g dry weight (dwt), respectively.

We were searching for a physiological model of altered skin structure to study whether such change would influence distribution volume. Since age influences skin structure, we assessed the major excluding elements of thigh skin in aged animals (12–13 mo). Interestingly, whereas collagen was not significantly different in young and aged rat hind-limb skin, hyaluronan was significantly higher, averaging 1.45 mg/g dwt (n = 6; P = 0.04, Mann-Whitney U-test), and sGAGs significantly lower, averaging 2.3 mg/g dwt (n = 8; P = 0.001), than in young rats (Fig. 5).

**Interstitial VEF of Plasma Proteins in Skin with Altered ECM**

Since there was a change in skin structure involving sGAGs and hyaluronan, both representing major excluding elements, we decided to quantify a potential distribution volume effect by measuring VEF in aged rats (n = 6). Aged rats had reduced total tissue-water content when compared with young rats, averaging 1.07 ± 0.05 and 1.29 ± 0.07 ml/g dwt, respectively (P = 0.038). Their VX was 0.42 ± 0.03 ml/g wwt.

With the application of the same approach and the same probes as described for young rats, we measured VEF for aged rats, and the results for the same seven proteins are displayed in Fig. 6A. In general, the VA for all probes were higher and accordingly, the VEF lower in aged rats, e.g., averaging 0.29 for albumin, 0.49 for ceruloplasmin, and 0.52 murinoglobulin. As for young rats, there was a linear relationship between VEF and SE radius in the range between 3.1 and 6.1 nm, as described by the equation, 

\[ \text{VEF} = 0.059 \cdot \text{SE radius} + 0.163 \quad (P = 0.0016) \]

A comparison of all of the seven proteins included with the respective proteins in young animals showed that VEF was lower in aged animals (P < 0.01, Wilcoxon matched-pairs signed-rank test). A comparison of the slopes for the respective curves, correlating VEF values and molecular weight (35), showed that slopes were not significantly different. When comparing the elevations (vertical position on the graph) or intercepts (35), however, these were found to be highly, significantly different (P = 0.0001; Fig. 6B), supporting the analysis above that VEF was lower in aged animals. Collectively, these data show that the loss of excluding elements occurring with age results in a decrease in volume exclusion and thus an increase in VA for macromolecules in skin.

**DISCUSSION**

Here, we have presented a novel approach to estimate VA and VEF of a range of differently sized and charged plasma proteins in the interstitial space of skin in vivo. We exploited the fact that albumin can be assessed by high accuracy in IF and tissue by HPLC and could express the mass and concentration of other proteins relative to albumin in the same compartments by MS. We could show that there is a positive correlation between VEF and macromolecular size and oppositely, a negative correlation between pl and SE radius. Interestingly, aging led to a reduction in sGAGs and an increase in HA with a concomitant reduction in VEF, allowing for a quan-
titiation of functional effects of the structural elements in the ECM on VE in relation to fluid homeostasis. Similar structural changes have been observed in humans (22), suggesting that our observations can be translated to the clinic.

Evaluation of the Method—Assumptions and Limitations

Here, we used a novel and improved approach, enabling the assessment of VE for several macromolecular probes in the same sample. A major aim of the present study was to determine the effect of size and charge on VE using as probes several plasma proteins with a span in size and charge. We assumed that this could be achieved by continuous infusion of radioactive-labeled probes (27, 30) using the entire plasma as substrate and later separating out the probe proteins by gel filtration. Whereas this worked for IF, it was not possible to identify proteins uniquely in tissue eluate, and therefore, we turned to a method using native plasma proteins that could be identified by MS.

Central elements in the calculation of VE are that we can reliably estimate the probe concentration in IF and its mass in the corresponding tissue. Implicit in this assumption is that we isolate representative IF. We sampled IF by centrifugation, a method that we have validated extensively in this context. Notably and of particular relevance here, the method will not result in any sieving of macromolecules (23). The avoidance of radioactive tracers by using native plasma proteins already in equilibrium in the tissue is an obvious advantage of the present method. This will, however, require that the proteins are elutable quantitatively and to the same extent as albumin, shown to occur at ≥94% in all samples based on recovery of radiolabeled plasma. Our previous finding of 94% recovery of albumin in hind-limb skin (9) supports that this assumption is valid. We are well aware that the quantification of proteins based on spectral counts will be relative only, but the quantification was made absolute by relating the spectral counts of the respective proteins to those of albumin in the same sample, again quantified with two-dimensional SEC with high accuracy and precision. Moreover, that our approach was validated was substantiated by an independent MS method giving practically identical results. The present method can be used with any protein that can be identified by the sufficient number of spectra, although dependent on the capacity of the mass spec-

Fig. 5. Composition of the ECM in young and aged rats. The content of collagen (A), hyaluronan (B), and sulfated glycosaminoglycans (sGAGs; C) in young and aged rats, showing a marked reduction in sGAGs in aged rats. Values are mean ± 1 SE. *P = 0.04; ***P = 0.001. dwt, dry weight.

Fig. 6. Relationship between VEF and probe size in aged rats. A: relationship between VEF and size, expressed as SE radius of the probes in aged (12–13 mo) animals. Albumin was quantified by SEC and all other probes expressed relative to albumin based on label-free MS. Also shown is the regression line: VEF = −0.059·SE radius + 0.163 (confidence interval for slope 0.024–0.095). The slope was significantly different from 0 (P = 0.002). B: relationship between VEF in aged (data transferred from A) and young rats (data transferred from Fig. 3) and SE radius. ***P < 0.0001 when comparing the elevations of the curves (35), showing lower VEF for the selected probe proteins in aged rats. Values (except for albumin) are mean ± 1 standard error.
trometer, and in any species where IF concentration and tissue mass can be determined.

No proteins with SE radius <3.1 nm (molecular weight < 46 kD) were used. This is due to our strict criteria and inherent limitations in the MS analysis used. Smaller probes (proteins and peptides) will be found in lower concentrations than plasma proteins, will have fewer peptides that can be used for identification, and will be masked by the abundant proteins. Specific proteins can, however, be identified by SRM and enter an analysis of V_E. A low number of spectra were the reason for excluding IgG that we have used as a probe in several previous studies (26, 27). This fact notwithstanding, when potential candidate proteins are established and relatively quantified, potential errors involved in tissue extraction, chemical or immunoassays, evaporative losses, and measurement of very small weights or volumes are minimized.

Comparison with Previous Studies

Since albumin served as a reference in our new approach, a comparison with other studies having assessed V_E for albumin with alternative methods is relevant. Our observed V_E for skin of 0.55 is within the, although in the upper, range of previous V_E values, ranging from 0.40 to 0.58 in entire skin or dermis (9, 17, 24, 26, 28). Although this rather large range in V_E might seem problematic, we should remember that the V_E may depend strongly on hydration (31), as well as composition of the tissue, as also exemplified here in the experiments in aged rats discussed below. Moreover, here, we chose skin from the thigh instead of the back because of a lower content of fat. Such tissue-specific factors likely explain the somewhat high value. The above data suggest that the new method reflects V_E reliably for albumin and accordingly, for the other probes that rely on albumin for their V_E calculation.

Previously, we have compared V_E of albumin with IgG and found them not to be different [e.g., Wiig et al. (26)]. This occurred in spite of the fact that IgG is twice the size of albumin, which should result in an increased exclusion; however, this was interpreted to result from a more positive charge compensating for the size effect (9). Unfortunately, IgG had to be excluded from the calculation because it did not meet the strict criteria set for unique detection. With knowledge of the SE radius of 5.3 nm (1) and the pI of 7.6 (26), we may estimate whether this is the case using the equations derived above for size and charge effects on V_E. Doing so, we arrive at a potential V_E for IgG, due to size only of 0.68, clearly exceeding V_E of albumin. If we assume that albumin, upon cationizing, would follow a path depicted in Fig. 4, then bringing the pI of albumin to 7.6 (that of IgG) would reduce the V_E for albumin by 0.1 only. Apparently, from such extrapolations, it appears that charge variation is not sufficient to result in an equal V_E for these two proteins and thus that the influence on V_E of charge is lower than might be anticipated from our previous data. Accordingly, additional factors must be involved to explain the observed, similar V_E of albumin and IgG in previous studies. Variable interaction effects with the ECM, depending on size and charge distribution of the probe, as suggested by recent mathematical modeling (13), are likely candidates.

Interestingly, we found an increased macromolecular distribution volume and thereby, a reduced V_E upon aging. Addi-

Physiological Implications

The present finding of a reduced V_E in aged rats has consequences for our understanding of fluid distribution and transcapillary fluid exchange in the interstitium with implications for plasma volume and blood-pressure regulation. As discussed previously (3, 33), the extent of exclusion has little effect on the steady-state IF protein concentration and thereby, colloid osmotic pressure. A reduced V_E will, however, influence plasma volume regulation by prolonging the tissue re-
response time to perturbations in IF volume and increase the transfer of interstitial protein to plasma for a given capillary hyperfiltration. Because the skin contains 30–40% and thus a substantial part of the total body IF volume (2), our data, showing reduced exclusion with age, indicate that the response time will increase and that adjustments to perturbations in fluid filtration will be slower in the aged animal.

Such adjustment delay may actually be estimated using previous modeling data. In a model simulation of microvascular exchange in the rat, Reed et al. (16) studied the effect of altering \( V_E \). In virtual fluid perturbation studies, starting out from the normal situation, they found that ~20 h were needed to re-establish a new steady state, agreeing well with data from Bert and Pinder (6) in comparable experiments. Increasing exclusion resulted in a proportional reduction in the time needed to reach a new steady state after a step perturbation. If we use our albumin-exclusion data as an example, then \( V_E \) in aged (0.29) was ~50% of that in young animals (0.55). If we assume that the opposite applies when reducing exclusion, then these models, together with our albumin data, suggest that the time needed to reach a new steady state is doubled, i.e., 40 h, in the aged animals (Fig. 7). Another implication of the modeling data is that larger fluid shifts are occurring during this process, making aged animals more prone to fluid accumulation. The quantitative influence of volume-exclusion phenomena on fluid homeostasis may therefore be substantial.

Interestingly, the change in composition and organization of the ECM that affected \( V_E \) may moreover have consequences for blood-pressure regulation by influencing the ability of the skin to accumulate Na\(^+\). In a series of papers that was summarized recently (21), Titze and collaborators suggested a new and more active role of skin GAGs in total-body Na\(^+\) and fluid-volume regulation. This study found that large amounts of Na\(^+\) are stored in the skin interstitium without commensurate water retention and suggested that GAGs and particularly sGAGs may provide an actively regulated cation-exchange mechanism, regulating fluid volume and blood pressure. Our finding of a moderately increased HA and markedly reduced sGAGs in aged rats may thus indicate that there is a decreased charge density of GAGs (18) with a concomitant, reduced tissue osmolality (29). An implication of our data would therefore be that the skin has a reduced capacity for salt storage, recorded as reduced tissue osmolality, and therefore, an increased propensity for salt-sensitive hypertension in aged animals. Whether this is actually occurring will have to be addressed in future experiments. The amount of skin GAGs may accordingly be a target for therapeutic interventions to preserve the ability to counteract fluid-volume changes and the development of salt-sensitive hypertension.

To summarize, we have presented a new method to measure interstitial exclusion of macromolecules in rat skin based on relating concentrations and masses of probes determined by a proteomic approach to that of albumin assessed exactly by HPLC. This approach can be used in any species where IF and tissue proteins can be assessed. By comparing young and aged animals, we found that the aged animals had a lower \( V_E \) that may be ascribed to a lower amount of sGAGs and a different organization of ECM. Implications of our findings are that these changes in aged skin may strongly affect fluid homeostasis, indicated by a doubling of the time needed to reach a new steady state after fluid perturbations and increased propensity to salt-sensitive hypertension. We suggest that the amount of sGAGs may be a target for therapeutic interventions to preserve the ability to counteract fluid-volume changes and salt-sensitive hypertension. Similar structural changes, occurring with aging in humans, indicate that our findings can be translated to the clinic.

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DISCLOSURES

The authors declare that there is no competing interest.

AUTHOR CONTRIBUTIONS


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