Specific adsorption of some complement activation proteins to polysulfone dialysis membranes during hemodialysis

Jan Mares[1], Visith Thongboonkerd[2], Zdenek Tuma[3], Jiri Moravec[3] and Martin Matejovic[1]

[1]Department of Internal Medicine I, Charles University Medical School and Teaching Hospital, Plzen, Czech Republic; [2]Medical Proteomics Unit, Office for Research, and Development Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand and [3]Proteomic Laboratory, Charles University Medical School, Plzen, Czech Republic

Dialyser bioincompatibility is an important factor contributing to complications of hemodialysis with well known systemic consequences. Here we studied the local processes that occur on dialysis membranes by eluting proteins adsorbed to the polysulfone dialyser membranes of 5 patients after 3 consecutive routine maintenance hemodialysis sessions. At the end of each procedure, a plasma sample was also collected. These eluates and their accompanying plasma samples were separated by 2-dimensional gel electrophoresis; all proteins that were present in all patients were analyzed by tandem mass spectrometry; and a ratio of the relative spot intensity of the eluate to plasma was calculated. Of 153 proteins detected, 84 were found in all patients, 57 of which were successfully identified by mass spectrometry as 38 components of 23 unique proteins. In 10 spots the relative eluate intensity differed significantly from that in the plasma, implying preferential adsorption. These proteins included ficolin-2, clusterin, complement C3c fragment, and apolipoprotein A1. Our finding of a selective binding of ficolin-2 to polysulfone membranes suggests a possible role of the lectin complement pathway in blood-dialyser interactions.

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KEYWORDS: biocompatibility; complement; dialyser elution; ficolin; lectin pathway; proteome

Correspondence: *Jan Mares, Department of Internal Medicine I, Charles University Teaching Hospital, Alej Svobody 80, 30460 Plzen, Czech Republic. E-mail: mares@fnplzen.cz*

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Hemodialyser bioincompatibility has long been established as an important factor in various complications accompanying both chronic and acute hemodialysis (HD). Several aspects of biological response triggered by blood exposure to a dialyser, namely coagulation, complement level, and leukocyte activation, were recognized shortly after the introduction of the artificial dialysis membrane into clinical practice.[1,2] In the past decades, we gathered comprehensive information covering metabolic consequences of these processes; that is, chronic micro-inflammation, enhanced oxidative stress, and pro-coagulant condition.[3–6] Although difficult to link directly with increased mortality, bioincompatibility is held (at least partially) responsible for accelerated atherosclerosis, malnutrition, and thrombotic diathesis found in HD patients.[7–9]

Although we have ample data regarding systemic effects of the interaction between artificial material surface and blood, its molecular substrates remain obscure. Yet, understanding the mechanisms responsible for foreign pattern recognition and launching a subsequent reaction cascade could help us to develop better-tolerated materials, and to prevent the adverse sequelae. Most studies addressing this issue were accomplished under simplified laboratory conditions (by means of phantoms and pre-fractionated plasma),[10–14] or targeted onto a predefined set of molecules.[15–22] However, to investigate the subject in its full complexity and in an unbiased manner, the elution of dialysers used in regular HD and in the proteomic approach is crucial.

Therefore, the aim of our study was to verify the elution algorithm eligible for proteomic analysis, to highlight the eluate proteome, and to qualify the potential molecules involved in the blood–dialyser interaction. To the best of our knowledge, this is the first trial studying with proteomic method the full spectrum of proteins adsorbed by a hemodialyser in a clinical setting.

RESULTS

The protein concentrations of both eluate (acetic acid) (449 (98.9, 2487.3) mg/l) and washout (ethylenediaminetetraacetic acid in phosphate buffered saline, PBS/EDTA) (260.3 (156, 779.4) mg/l) were significantly higher ($P<0.001$) than

in the final 10-ml flush (Plasmalyte) (24.4 (19.2, 27.6) mg/l). The difference in protein concentrations between eluate and washout was not statistically significant ($P = 0.17$). Although the variation in protein content was relatively low in the flush (coefficient of variation, CV = 22%), it was considerably higher in the washout (CV = 152%) and in the eluate (CV = 154%). Nevertheless, protein concentrations were closely correlated in the latter two samples ($r = 0.82$, $P < 0.001$).

In total, 45 gels derived from 15 dialyser eluates and the corresponding plasma samples were analyzed. A representative two-dimensional (2D) gel-scanned image is shown in Figure 1 to demonstrate the typical eluate proteome map. There were 153 spots detected in at least one eluate sample, 84 of them were found in all 5 patients, and 44 were present in all procedures. Protein spots were distributed along the molecular weight (MW) range of 15–175 kDa and pI range of 3.4–8.9. However, 95% of the overall protein spot intensity was localized inside the interval of MW of 18–85 kDa and pI of 3.4–7.7. Besides that, samples of the PBS/EDTA washout from three dialysers were used to prepare another nine gels and compared with corresponding plasma and eluate spot patterns. As all the spots detected in the washout were present in the acetic eluate as well, and the quantitative analysis of relative spot intensities did not show any significant differences between the washout and plasma, only acetic eluates were submitted for further analysis.

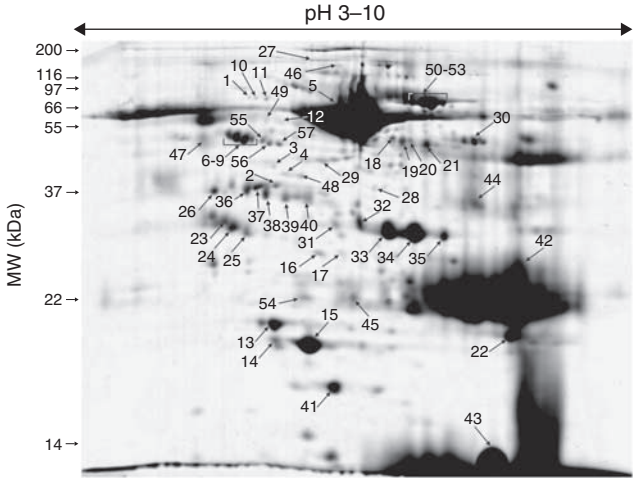

Figure 1 | Representative two-dimensional gel image of dialyser eluate. Spots labeled with numbers were successfully identified by tandem mass spectrometry analyses (see Table 1). MW, molecular weight.

Spot distribution is shown in Figure 2. Spots detected in all patients ($n = 84$) were excised from gels; 57 spots (those labeled with numbers in Figure 1) were successfully identified by mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analyses as 38 components of 23 unique proteins (Table 1). Some of the identified spots contained multiple isoforms of one protein (most likely because of post-translational modifications), for example, ficolin-2 (spot nos. 31–35) or clusterin (spot nos. 23–25). The protein identifications were further verified by the comparison of measured pI and MW (estimated by the coordinates of the spots in gel) with their theoretical values (as predicted by the protein database). Confirmed identifications are marked with an asterisk in Table 1. Discrepant MWs were common particularly in albumin (spot nos. 28, 29, 38–40) and in hemoglobin (spot nos. 30, 42, 44) co-migrated with another protein.

In several protein spots (that is, complement C3, mannose-binding lectin-associated serine proteases (MASP) 1 and 2), the measured MWs were lower than the theoretical ones. This difference could be explained by the enzymatic cleavage of their inactive forms. Figure 3 illustrates peptide fragments and amino acid sequences of these proteins established with MS and MS/MS analyses, and indicates the presence of proteolytic cleavage products rather than full-length or native forms. Moreover, MW and pI measured in 2D gels corresponded better with the theoretical values of their respective fragments: C3 complement (spot no. 26; measured MW: 37 kDa, pI: 4.8) vs C3c α-chain fragment 2 (theoretical MW: 39.5 kDa, pI: 4.8); MASP-1 (spot no. 47; measured MW: 44 kDa, pI: 4.0) vs MASP-1 heavy chain (theoretical MW: 49 kDa, pI: 4.9); and MASP-2 (spot no. 48; measured MW: 39 kDa, pI: 5.5) vs MASP-2A (theoretical MW: 47.7 kDa, pI: 5.4).

The variability of eluate intensities of the corresponding spots among patients (CV inter-individual) ranged from 6 to

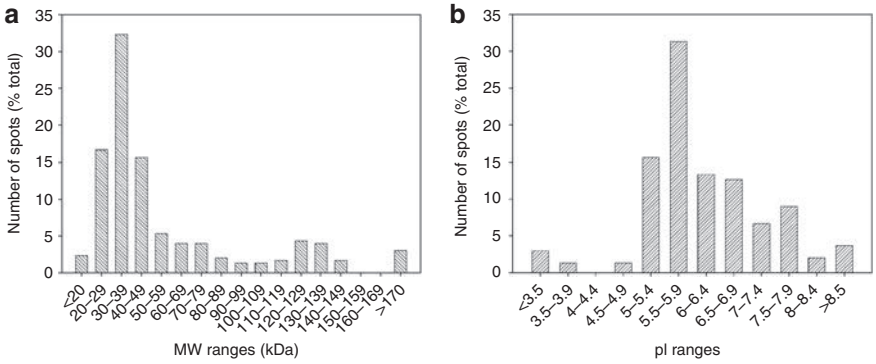

Figure 2 | Basic characteristics of proteins detected in dialyser eluate. (a) Molecular weight (MW) and **(b)** pI distributions of proteins in dialyser eluate. Percentage per category of total spot number are given.

Table 1 | Proteins identified in eluates from dialysers used in clinical hemodialysis

Spot no.	Protein name	Swissprot entry	PMF score/sequence coverage	MSMS peptide no./total ion score	CV inter-individual (%)	E/P ratio
1	78 kDa glucose-regulated protein[a]	P11021	107/29%	4/110	36	0.65
2	Actin, α-cardiac muscle 1[a]	P68032	60/25%	NA	81	1.74
	Actin, cytoplasmic 1[a]	P60709	174/51%	9/723		
3	Actin, cytoplasmic 1[a]	P60709	64/45%	4/203	53	NM
4	Actin, cytoplasmic 1[a]	P60709	NA	1/56	26	1.19
5	Albumin[a]	P02768	260/69%	10/833	6	0.83
6	α-1-Antitrypsin[a]	P01009	162/52%	4/413	13	0.93
7	α-1-Antitrypsin[a]	P01009	194/54%	6/542		
8	α-1-Antitrypsin[a]	P01009	226/52%	6/425		
9	α-1-Antitrypsin[a]	P01009	140/43%	4/238		
10	α-1B-glycoprotein	P04217	113/33%	5/273	25	0.82
11	α-1B-glycoprotein	P04217	180/41%	8/261	17	0.92
12	Antithrombin-III	P01008	NA	1/52	25	NM
13	Apolipoprotein A-I[a]	P02647	77/22%	6/234	45	0.73
14	Apolipoprotein A-I[a]	P02647	121/50%	5/327	18	2.06[#]
15	Apolipoprotein A-I[a]	P02647	NA	1/37	38	29.89[#]
	Peroxiredoxin-2[a]	P32119	132/41%	7/426		
16	Apolipoprotein E[a]	P02649	240/71%	9/962	176	11.44
17	Apolipoprotein E[a]	P02649	113/41%	5/133		
18	β-2-Glycoprotein 1	P02749	95/48%	5/292	66	0.70
	Ig α-1 chain C	P01876	NA	4/72		
19	β-2-Glycoprotein 1	P02749	83/47%	6/401	52	0.27[#]
	Ig α-2 chain C	P01877	NA	4/221		
20	β-2-Glycoprotein 1	P02749	59/20%	3/132	22	0.24[#]
	Ig α-2 chain C	P01877	NA	1/31		
21	β-2-Glycoprotein 1	P02749	NA	1/44	49	0.52
22	Carbonic anhydrase 1[a]	P00915	NA	4/279	69	NM
	Flavin reductase	P30043	NA	1/48		
23	Clusterin[a]	P10909	71/34%	2/112	90	9.08[#]
24	Clusterin[a]	P10909	NA	3/61		
25	Clusterin[a]	P10909	NA	1/76		
	Apolipoprotein E[a]	P02649	132/29%	6/135		
26	Complement C3	P01024	66/13%	8/452	55	3.20[#]
27	Complement factor H[a]	P08603	103/18%	6/394	79	NM
28	Complement factor H-related protein 1	Q03591	NA	2/109	152	NM
	Albumin	P02768	95/25%	5/186		
29	Fibrinogen γ-chain[a]	P02679	94/47%	3/37	88	0.52
	Albumin	P02768	118/22	2/84		
30	Fibrinogen β-chain[a]	P02675	NA	3/81	31	0.275
	Hemoglobin subunit-α	P69905	NA	2/152		
	Hemoglobin subunit-β	P06727	NA	4/181		
31	Ficolin-2[a]	Q15485	NA	2/49	60	26.12[##]
32	Ficolin-2[a]	Q15485	132/52%	6/554		
33	Ficolin-2[a]	Q15485	93/44%	7/470		
34	Ficolin-2[a]	Q15485	119/44%	NA		
35	Ficolin-2[a]	Q15485	85/28%	5/419		
36	Haptoglobin	P00738	91/28	6/353	29	0.88
	Apolipoprotein A-IV[a]	P06727	45/17%	NA		
37	Haptoglobin[a]	P00738	NA	1/31	14	0.94
	Apolipoprotein A-IV[a]	P06727	206/44%	8/441		
38	Haptoglobin[a]	P00738	88/17%	3/112	41	1.41
	Albumin	P02768	159/34%	6/336		
39	Haptoglobin[a]	P00738	NA	4/217	41	1.37
	Albumin	P02768	177/41%	7/451		
40	Haptoglobin[a]	P00738	NA	5/60	46	0.91
	Albumin	P02768	122/37%	5/261		
41	Haptoglobin	P00738	NA	4/224	57	1.82
42	Hemoglobin subunit-α	P69905	59/58%	5/382	32	4.82[#]
	Hemoglobin subunit-β	P06727	101/78%	2/82		
	Ig κ-chain C	P01834	NA	4/407		
43	Hemoglobin subunit-α[a]	P69905	NA	2/152	39	39.22[#]
	Hemoglobin subunit-β[a]	P06727	NA	4/181		
44	Ig γ-1-chain C	P01857	74/41%	2/86	72	6.04
	Ig γ-2-chain C	P01859	89/34%	4/130		

Table 1 continues on following page

Table 1 | Continued

Spot no.	Protein name	Swissprot entry	PMF score/sequence coverage	MSMS peptide no./total ion score	CV inter-individual (%)	E/P ratio
	Hemoglobin subunit-α	P69905	NA	2/152		
	Hemoglobin subunit-β	P06727	NA	4/181		
	Complement factor H-related protein 1[a]	Q03591	91/31%	5/300		
45	Ig κ-chain C region	P01834	56/75%	3/269	21	0.44[#]
	Ig λ-chain C regions	P01842	NA	1/100		
46	Lysozyme C	P61626	NA	1/47	116	NM
	Proline-rich protein 4	Q16378	NA	1/30		
47	Mannan-binding lectin serine protease 1	P48740	81/21%	6/374	77	NM
48	Mannan-binding lectin serine protease 2	O00187	NA	2/70	17	NM
49	Plastin-2[a]	P13796	70/27%	3/245	29	0.75
50	Serotransferrin[a]	P02787	NA	2/62	19	1.04
51	Serotransferrin[a]	P02787	65/13%	2/92		
52	Serotransferrin[a]	P02787	82/20%	NA		
53	Serotransferrin[a]	P02787	170/27%	8/561		
54	Serum amyloid P[a]	P02743	60/24%	4/134	19	1.10
55	Vimentin[a]	P08670	289/65%	10/726	16	1.04
56	Vitamin D-binding protein[a]	P02774	NA	4/339	28	1.13
	Vimentin[a]	P08670	193/46%	6/271		
57	Vitamin D-binding protein[a]	P02774	105/31%	8/536	91	1.46

CV, coefficient of variance; E/P, eluate-to-plasma ratio of relative spot intensities; MSMS, tandem mass spectrometry; MW, molecular weight; NA, not applicable (identification by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) was not done or was unsuccessful); NM, not matched (eluate spot could not be matched with plasma unambiguously); PMF, peptide mass fingerprinting.
Mean E/P ratios (from five patients, three hemodialysis procedures each) are given for all proteins matched to plasma. Relative protein spot intensities were compared in eluates and plasma ($^{\#}P<0.01$, $^{\#\#}P<0.001$).
For proteins represented by multiple spots, cumulative intensities of all spots were used to calculate CV and E/P. Therefore, only one value is given for each variable covering all spots containing a specific protein.
[a]Spots whose measured MW and pI were in agreement with their theoretical values.

176% (Table 1), whereas the method variability (mean CV inter-gel) reached 27%. A comparative analysis of the eluate proteome to matched plasma profile of the respective patients revealed significant differences (with >two-fold changes in the eluate-to-plasma (E/P) ratio, $P<0.01$) in levels of 10 proteins (Table 1). Relative intensities of high-abundant plasma proteins (albumin, transferrin, haptoglobin, α-1-antitrypsin) in eluates were similar to plasma (E/P = 0.5–2) (Table 1). Only fibrinogen β- and γ-chains were depleted in eluates (spot no. 30, E/P = 0.28 and spot no. 29, E/P = 0.52, respectively), whereas immunoglobulin light chains seemed enriched (spot no. 42, E/P = 4.8) (Table 1). Nevertheless, the latter spot was co-migrated with hemoglobin α- and β-chains, and the spot intensity varied profoundly among samples. In several low-abundant plasma proteins, pronounced differences in eluate and plasma intensities were detected ranging from E/P = 3.2 ($P=0.007$) for complement C3 (spot no. 26; Figure 4) to E/P = 26.1 ($P<0.001$) in the case of ficolin-2 (spot nos. 31–35 cumulative intensity; Figure 4). Figure 5 shows quantitative data of plasma proteins, the levels of which were significantly higher in eluates than those in the plasma. Naturally, several intracellular proteins, for example, hemoglobin (spot no. 43) or peroxiredoxin (spot no. 15), reached an even higher E/P ratio (39 and 29, respectively), or were not matched to plasma at all (spot no. 22; carbonic anhydrase, flavin reductase). Therefore, hemoglobin was considered the major contaminant released from erythrocytes entrapped within the dialysers. To avoid an error in the assessment of eluate protein quantities (and consequently E/P ratios), spots containing hemoglobin were excluded from the integrated spot density while calculating relative intensities.

DISCUSSION

Proteins adsorbed to the polysulfone dialysis membrane during clinical HD were investigated using proteomic tools in five patients treated with chronic HD. A stepwise elution of the dialysers was performed following routine procedures, thrice in each patient. Proteins thus obtained were separated by electrophoresis on two-dimensional polyacrylamide gels (2-DE), and their relative intensities were measured. The results were then compared with the corresponding plasma protein patterns. Prominent spots were excised and identified by MS.

To minimize the contamination of desorbed proteins, we adopted a sequential elution algorithm. As soon as the patient was disconnected, the dialyser was flushed with saline to remove the residual plasma and cells. Protein concentration in the final 10 ml of this flush was negligible (compared with plasma) and similar in all patients, suggesting simple kinetics of plasma washout. In the next step, we applied EDTA solution, which had been shown earlier to detach adhering leukocytes more effectively than PBS alone.[23] Finally, elution with acetic acid was performed, because it had been used successfully to solubilize adsorbed proteins while preserving them for proteomic analysis.[24] Protein denaturation and solubilization, necessary for 2D electrophoresis, can

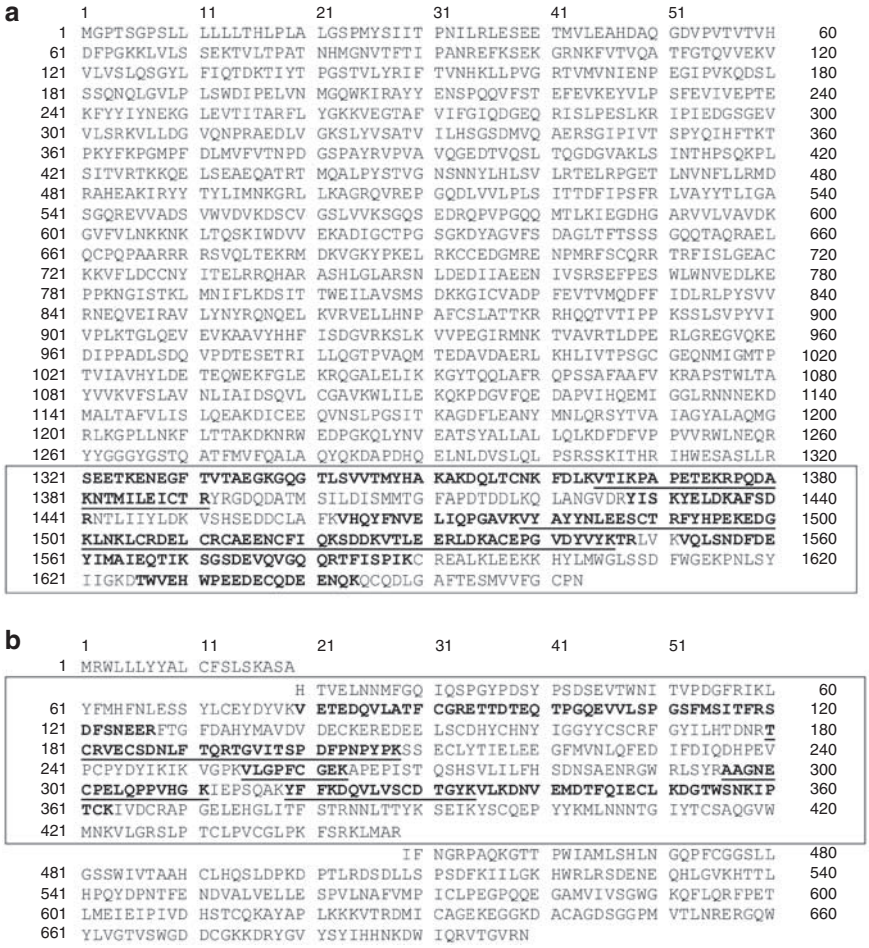

Figure 3 | Peptide fragments and sequence coverage of complement C3 and mannose-binding protein-associated serine protease-1 (MASP-1) identified in dialyser eluate. Complement C3 (**a**) and MASP-1 (**b**) amino acid sequences (in one-letter code). C3c α-chain fragment 2 (1321–1663) and MASP-1 heavy chain (20–448) are framed within a box. Peptides covered by peptide mass fingerprinting are highlighted in bold; sequences confirmed by tandem mass spectrometry are underscored.

be achieved by means of acidic, alkaline, chaotropic, or detergent agents. Ishikawa *et al.*[24] report, having tested acetic acid, bicarbonate, urea, and sodium dodecylsulfate (SDS) to find, acetic elution to be the most efficient. This observation has been confirmed by us; acetic acid has been shown to be superior to SDS in a preliminary sequential elution study (see Supplementary Table 1).

In our experiment, recirculation with PBS/EDTA yielded a protein at a concentration an order of magnitude higher than the final portion of PBS flush. This protein gain can be explained by the release of proteins attached to the dialyser, considering the inert nature of PBS, probably because of convective forces and equilibrium shift between adsorption and desorption occurring in a protein-free medium. Moreover, the protein profile of the PBS/EDTA washout resembled that of acetic eluate (see Supplementary Figure 1). The following elution with acetic acid induced an additional discharge of more firmly bound proteins, which even slightly exceeded the first one. Taking into account the high variation of the protein content, the adsorption seems to be patient- and procedure-dependent. The described advantage of acidic eluent over other solvents can be theoretically attributed to the surface negativity of polysulfone membrane (ζ-potential −5 mV), which would be suppressed by strong anions thus dissociating electrostatic bindings.

In eluates, the complete spectrum of MW and pI fractions detectable with the 2-DE technique was discovered, thus substantiating the use of proteomic rather than the targeted approach. Of the 153 protein spots, 84 were present in all patients constituting the typical proteome adsorbed to a hemodialyser during clinical HD. Among them, 23 unique proteins were identified with MS. Most of them were plasma proteins, both high (serum albumin, transferrin, α-1-antitrypsin, haptoglobin, immunoglobulin chains) and low abundant (MASP-1 and MASP-2, ficolin-2, clusterin, etc.). However, several proteins arose from the blood cells, mainly erythrocytes (hemoglobin α- and β-subunits, peroxiredoxin, carbonic anhydrase).

The only available trial addressing a similar issue with proteomic technology was performed *ex vivo*, using minidialysers

composed of cellulose diacetate or ethylenevinyl alcohol, and blood treated with EDTA.[13] Nine proteins, mostly high abundant, were identified in this model, all of which were also detected in our setting, except aspartyl-tRNA synthetase.

Yet, none of these proteins were clearly related to complement, coagulation, or leukocyte activation.

Mulzer and Brash [20] selected 16 plasma proteins, mainly those engaged in blood clotting and complement activation, and tested their presence in various dialyser eluates using specific immunoassays. All high-abundance proteins reported in this experiment were also identified by us, namely albumin, transferrin, immunoglobulin, fibrinogen, α-1-antitrypsin, apolipoproteins, and haptoglobin. Mulzer and Brash[20] found the adsorption of low-abundance proteins to be largely dependent on the dialyser type. Cuprophane, which is considered standard in terms of bioincompatibility, bound substantially various cleavage products of HMWK (high MW kininogen), antithrombin III, C3 (mainly C3c and C3d), fibronectin, and α2-macroglobulin. Of these, the C3c fragment and antithrombin III stained as strong bands during immunoblot and were also confirmed in our study. The absence of HMWK, fibronectin, and α2-macroglobulin fragments in our experiment may reflect different properties (biocompatibility) of polysulfone, as well as the lower sensitivity of MS detection (respective bands were assessed as moderate or weak by Mulzer and Brash [20]).

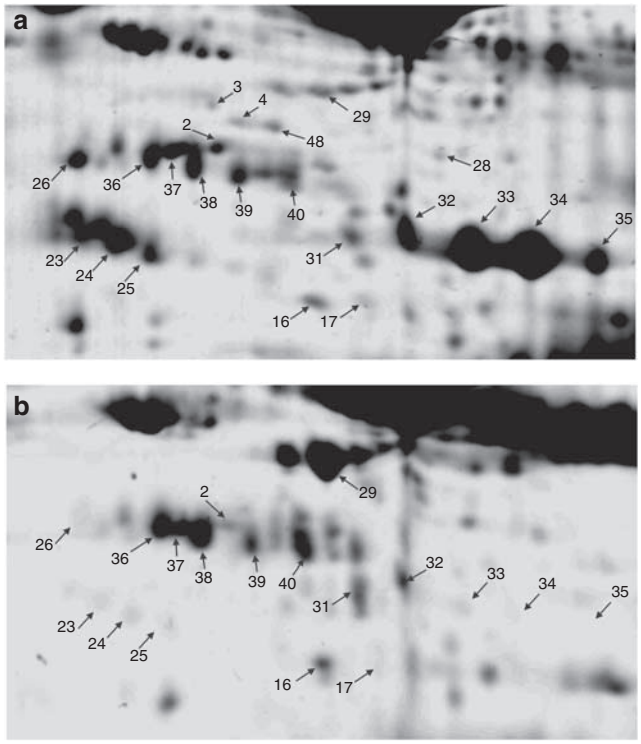

Figure 4 | Enlarged images of corresponding sections in gels derived from dialyser eluate and plasma. Significant differences in relative intensities of plasma proteins identified in **(a)** dialyser eluate compared with **(b)** plasma. The highest eluate-to-plasma ratio (E/P) of spot intensity was detected in ficolin-2 (spot nos. 31–35), clusterin (spot nos. 23–25), and complement C3c fragment (spot no. 26).

Several lines of references indicate that many of the adsorbed proteins identified in this study are involved in blood–dialyser interaction. These included complement C3, ficolin-2, clusterin, MASP-1, MASP-2, complement factor H, and complement factor H-related protein 1, which participate in complement activation, as well as[25–30] fibrinogen, antithrombin, and β-2-glycoprotein-1, which control blood coagulation,[31,32] and serum amyloid P, which mediates leukocyte adhesion and activation.[33] To ascertain proteins undergoing selective adsorption, and hence possibly a specific interaction with the dialyser membrane, we calculated E/P ratios. Although 2-DE allows only estimation of protein quantities, and low E/P may be caused by imperfect elution, as well as by lack of affinity to the dialyser; it is reasonable to consider high E/P as evidence of preferential binding. In this manner, the relevance of ficolin-2 and clusterin has been emphasized.

Ficolin-2 (MW = 34 kDa, its synonyms include L-ficolin and hucolin) is a lectin family member secreted in the plasma, and complexed with serine proteases, particularly MASP-1 and MASP-2. It acts as a soluble pattern recognition

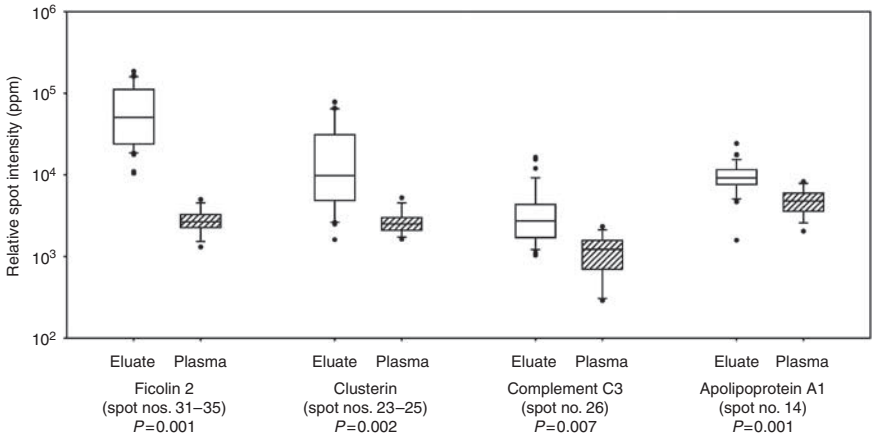

Figure 5 | Plasma proteins identified in dialyser eluate and that reached significantly different relative spot intensities in eluate vs plasma. Statistical significance was tested using Mann–Whitney rank sum test.

protein, which upon binding to polysaccharide bacterial wall promotes auto-cleavage of MASPs, and thus initiates the lectin complement pathway.[34] In addition, it has an opsonic activity,[35,36] and both MASP proteases cleave prothrombin and trigger blood clotting.[37,38] The role of the lectin pathway in biomaterial-induced inflammation has not been evaluated so far, and both alternative and classical pathways have been attributed major significance. According to the accepted models, the classical pathway accounts for the initiation of the process, whereas the alternative pathway (demonstrated by complement factor B conversion) is responsible for its amplification.[39] Nevertheless, it may be difficult to discern the classical from the lectin pathway, as most studies dealing with this issue determine complement activation according to C5a or C4a levels, and this part of the cascade is shared by both pathways. Namely, the activated MASP cleaves C4, which in turn activates C2 thus, giving rise to C3 convertase (C4b2a); C3b is central to all the three pathways. Substrate specificity of the ficolins has been addressed before, and includes various kinds of acetylated oligosaccharides;[40,41] however, a possible capacity of polysulfone membrane to activate ficolin-2 and associated MASP should be confirmed directly.

Our data show excessive binding of ficolin-2 (spot nos. 31–35; E/P = 26.1) in a clinical setting together with C3c fragment (spot no. 26; E/P = 3.2). C3c is a degradation product of activated C3b providing evidence of both C3 activation and subsequent C3b inactivation. Relative abundance of C3c in eluates from cuprophane dialysers was reported in early studies, whereas only minimal amounts of C3d were obtained.[20,42] This is easily comprehensible as the latter fragment contains the thioester site covalently binding to the surface,[43] and its release is not probable under the described elution conditions. Moreover, we documented the concurrent presence of the activated forms of MASP-1 (spot no. 47) and MASP-2 (spot no. 48)[44,] which infer a possible connection between ficolin adsorption and C3 activation. Therefore, it is conceivable that ficolin-2 adsorption and lectin pathway contribute to complement activation, as well as leukocyte adhesion and perhaps even blood coagulation.

Factor H adsorption to polyanionic surfaces recognized as 'self' and the subsequently enhanced degradation of C3b have been shown to play a role in tissue protection from improper complement-dependent lysis.[29,30,45] Yet, complement factor H and its related protein-1 (key regulatory molecules of the alternative pathway) detected in our experiment showed low relative quantities, and were hardly matched with the plasma (complement factor H has MW >130 kDa, and was located in a low-resolution area on 13% gels). Quite speculatively, adsorption of factor H and its related protein 1, evidenced in our study, could be, in part, responsible for the accelerated turnover of C3b (we showed the adsorption of its cleavage fragment C3c only; no intact C3b was observed).

Another candidate biocompatibility factor derived from our results is clusterin (complement cytolysis inhibitor, spot nos. 23–25; E/P = 9.1), which is a plasma protein consisting of two non-identical subunits (each ~35 kDa). Clusterin has been shown to interfere with the soluble membrane attack complex (C5b-9) formation, and to prevent (or limit) cytolysis in this manner.[46] Biological consequences of its adsorption are difficult to predict; we may hypothesize that enhanced clearance of clusterin by binding to the dialyser membrane could increase the burden brought about by complement activation due to lack of circulating clusterin (as observed in experiment).[47]

In several other proteins, their role in the blood–dialyser interaction (while plausible) is difficult to define. The β-2-glycoprotein-1 is an important inhibitor of the contact phase system neutralizing negatively charged surfaces and suppressing the intrinsic blood coagulation pathway. Binding of the β-2-glycoprotein-1 to a dialyser might have a favorable effect on thrombogenicity, yet its eluate quantity was significantly decreased (E/P below 0.5). Nevertheless, its spots on the 2D gels overlapped with the immunoglobulin heavy chains fraction, which might have substantially corrupted the quantification. It has been proposed that actin and vimentin co-assemble under the guidance of fimbrin (L-plastin) at the cell adhesion sites of macrophages.[48] All these proteins were identified in the dialyser eluate; however, it is questionable whether their presence in the eluate predicates more than leukocyte lysis.

In summary, we report an elution technique allowing the preparation of proteins adsorbed to a dialyser during clinical HD. The proteins obtained were subjected to gel electrophoresis and subsequently to MS to show their applicability for proteomic profiling. To screen out proteins undergoing preferential adsorption, and hence presumably a specific interaction with the dialyser, we calculated the E/P ratios of relative spot intensities. The proteins concentrated significantly in the eluate were considered to be potentially engaged in the blood–dialyser interaction. These criteria were met, especially in ficolin-2 and clusterin, both of which play an important role in complement activation (ficolin-2 is also a potent opsonic agent). With respect to our data, we hypothesize that the lectin pathway could contribute to complement activation occurring on contact of human blood with the polysulfone hemodialyser.

MATERIALS AND METHODS

Patients and treatment

Patients were recruited at the Hemodialysis Center of the Charles University Teaching Hospital in Plzen, and the study protocol was approved by the Institutional Ethics Committee. Inclusion criteria were as follows: regular HD for more than 3 months, established 4-h dialysis thrice a week with F6 dialyser (polysulfone, 1.3 m^2, Fresenius Medical Care, Bad Homburg, Germany), native arteriovenous shunt allowing blood flow for 300 ml/min or more, and heparin anticoagulation with a total dose (bolus + continuous) 4000–6000 i.u. as per procedure. Patients meeting the following criteria were excluded from the study: current or past anticoagulation treatment (oral or parenteral) other than during the dialysis session, history of thrombotic complication or known coagulation disorder, diabetes mellitus, active inflammation, malignancy, or liver disease. Within a 3-week screening period,

the heparin dose was maintained, and the proportion of the dialyser filled with coagulum was assessed visually at the end of every procedure. A semi-quantitative scale commonly used in the center was applied (1 = no visible clot; 2 = up to 50% capillaries in the dialyser filled with clot; 3 = more than 50% capillaries filled with clot; 4 = macroscopic coagulum in the lines, outside the dialyser; 5 = complete obstruction of the extracorporeal circuit), and only patients with a score of 1 or 2 were considered eligible.

Finally, five patients aged 58–82 years, treated with HD for 5–42 months were included in the study. In this group, hemodialysers were eluted on three consecutive procedures, following the protocol described below. All HD sessions were performed routinely; that is, blood flow (300 ml/min), dialysate flow (500 ml/min), and ultrafiltration rate (250–500 ml/h). At the end of the HD session, 4 ml of plasma was collected, and blood residuum left in the lines was retrieved using 250-ml rinse (Plasmalyte, Baxter, Deerfield, IL, USA). Thereafter, the patient was disconnected from the system and the dialyser was flushed immediately with another 1000 ml Plasmalyte solution. The final 10 ml of this flush was gathered from the dialyser outlet and stored at −80°C.

Elution protocol and sample preparation

The F6 dialyser has a declared priming volume of 78 ml. Initially, it was emptied and directly filled with 80 ml of 3 mM EDTA (EDTA/PBS, pH = 7.4), thereafter it was re-circulated with the peristaltic pump (flow rate: 80 ml/min) at 24°C for 30 min to detach and wash out adhering leukocytes. The dialyser was drained thereafter; the obtained fluid was centrifuged at 1200 *g* at 4°C for 10 min. The supernatant, denoted as washout, was separated from the sediment containing cells and stored at −80°C. The dialyser was immediately loaded with 80 ml of 40% acetic acid, re-circulated again with a flow rate of 80 ml/min at 24°C for 30 min, emptied, and the resulting eluate was centrifuged at 4000 *g* at 4°C for 10 min to remove cellular detritus, and stored at −80°C.

To minimize salt contamination, eluates were dialyzed against ultra-high quality water at 4°C (10 exchanges, 1.5 l each) using a semi-permeable membrane with a molecular mass cutoff at 7 kDa (Serva-Electrophoresis, Heidelburg, Germany) for 5 days. Dialyzed samples were concentrated by vacuum evaporation (SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA), and the proteins were dissolved in a lysis buffer (7 M urea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 40 mM Tris base, 2 M Thiourea, 2% immobilized pH gradient, IPG, buffer pH 3–10, 120 mM dithiotreitol, DTT). Blood samples (4 ml) were processed by centrifugation only (at 4000 *g* and 4°C for 10 min) to separate plasma (supernatant). Protein concentrations in all samples were assessed using Bradford's dye-binding assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

To assess the effectivity of the elution process, we performed additional studies evaluating a sequential elution with an SDS detergent. Two dialysers were eluted consecutively either with 40% acetic acid followed by 10% SDS or vice versa. The protein quantities obtained were 9.33 and 0.014 mg using the eluent sequence acetic acid–SDS, whereas 6.99 and 2.85 mg were eluted when the inverse order was used. However, in case of SDS subsequent to acetic elution, the protein concentration (0.21 μg/ml) was at the detection limit of the method.

Two-dimensional electrophoresis

Urea, CHAPS, Tris base, thiourea, SDS, DTT, IAA (iodacetamide), and bromphenol blue used during the preparation were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany); immobilized IPG buffer (ZOOM carrier Ampholytes 3–10) was purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). Equally, 200-μg proteins in both the eluate and plasma samples were mixed with rehydration buffer (7 M urea, 4% CHAPS, 40 mM Tris base, 2 M Thiourea, 2% IPG buffer pH 3–10, 120 mM DTT, and a trace of bromophenol blue) to obtain the final volume of 140 μl. Samples were then rehydrated in IPG strips (7.7-cm length, pH 3–10 nonlinear; Invitrogen), and focused in a MiniProtean cell (Bio-Rad). IPG strips were rehydrated passively for 1 h and actively for 10 h at 30 V, followed by a stepwise isoelectric focusing (IEF) as follows: 200 V until 400 Vh were reached, 450 V for another 500 Vh, 750 V for another 900 Vh, and finally 2000 V for the other 10,000 Vh. After isoelectric focusing, the IPG strips were equilibrated in the equilibration buffer 1 (112 mM Tris-base, 6 M urea, 30% v/v glycerol, 4% w/v SDS, 130 mM DTT, and a trace of bromophenol blue) for 30 min, and subsequently alkylated in buffer 2 (112 mM Tris-base, 6 M urea, 30% v/v glycerol, 4% w/v SDS, 135 mM IAA, and a trace of bromphenol blue) for 30 min. Each equilibrated IPG strip was placed on the top of a 13% polyacrylamide gel (9 × 7 cm) and covered with 0.5% agarose. Second-dimension separation was performed with 65 mA per gel at 20°C until the bromophenol blue dye front reached the bottom of the gel. At the end of each run, the 2D gels were stained with Simply Blue (Invitrogen), and scanned with an Epson Perfection 4990 Photo scanner (Epson, Long Beach, CA, USA).

2-DE pattern analysis and statistics

Computer-aided analysis of 2-DE gel images was carried out using PDQuest 2D software version 8.1 (Bio-Rad). A synthetic image was constructed out of the triplicated gels processed from each sample, using only the spots constantly present in at least two gels. The protein quantity was determined relative to the integrated spot density. The intensity levels of the protein spots in the eluate samples were compared with those of the corresponding or matched spots of the plasma samples collected from the same patients, and expressed as E/P ratios. Quantitative differences were considered significant when showing at least a two-fold intensity variation. The statistical analysis was performed using the SigmaPlot/SigmaStat data analysis software (version 12.0, SPSS Inc., Chicago, IL, USA). The inter-gel variability was determined as the CV of relative spot intensities across triplicates. Moreover, the inter-individual variability (CV among patients) was assessed in identified spots. Mann–Whitney rank sum test was used to compare the continuous variables. Data are given as medians (ranges), differences were considered statistically significant if the *P*-values were below 0.01.

In-gel tryptic digestion

Acetonitrile (ACN), ammonium bicarbonate, DTT, IAA, TFA (trifluoroacetic acid), formic acid, and CHCA (α-cyano-4-hydroxy-cinnamic acid) were purchased from Sigma. Spots detected in all patients were excised manually. The SimplyBlue stain (Invitrogen Corporation, Carlsbad, CA, USA) was removed by washing with 50 mM ammonium bicarbonate and ACN. Proteins in the gel were reduced with 10 mM DTT/50 mM ammonium bicarbonate at 56°C for 45 min and alkylated with 55 mM IAA/50 mM ammonium bicarbonate (for 30 min, in the dark at room temperature). Gel plugs were washed with 50 mM ammonium bicarbonate and ACN and dried by SpeedVac. Dried gel particles were rehydrated with a digestion buffer containing 12.5 ng/μl sequencing grade trypsin (Roche, Basel, Switzerland) in 50 mM ammonium bicarbonate at 4°C. After 45 min, the remaining solution was removed and replaced by 0.1 M ammonium bicarbonate.

Tryptic digestion was performed overnight (37°C). After digestion, proteolytic peptides were subsequently extracted with 25 mM ammonium bicarbonate, ACN, and 5% formic acid. The three extracts were pooled and 10 mM DTT solution in 50 mM ammonium bicarbonate was added. The mixture was then dried using SpeedVac and the resulting tryptic peptides were dissolved in 5% formic acid solution and desalted using ZipTip μC18 (Millipore, Bedford, MA, USA).

MALDI (matrix-assisted laser desorption/ionization) TOF (time-of-flight) tandem MS and protein identification

Proteolytic peptides were mixed with CHCA matrix solution (5 mg/ml CHCA in 0.1% TFA/50% ACN 1:1, v/v) in a 1:1 ratio, and 0.8 μl of this mixture was spotted onto the MALDI target. All mass spectra were acquired at a reflectron mode using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Framingham, MA, USA). A total of 2000 and 3000 laser shots were acquired and averaged to MS and MS/MS spectra, respectively. The MS/MS analyses were performed using collision energy of 1 kV and collision gas pressure of 1.3×10^{-6} Torr. MS peaks with a signal-to-noise ratio above 15 were listed, and the 15 strongest precursors with a signal-to-noise ratio above 50 among the MS peaks were automatically selected for MS/MS acquisition (see Supplementary Data 1–3). A mass filter was used to exclude autolytic peptides of trypsin.

The resulting data were analyzed using the GPS Explorer 3.6 (Applied Biosystems) software. Proteins were identified by searching against the human subset of the Swissprot protein database (Swiss Institute of Bioinformatics, Basel, Switzerland) (release 54.6; 4 December 2007) using the MASCOT 2.1.0 search algorithm (Matrix Science, London, UK). The general parameters for the peptide mass fingerprinting search were considered to allow maximum one missed cleavage, ±50 p.p.m. of peptide mass tolerance, variable methionine oxidation, and fixed cysteine carbamidomethylation. Probability-based molecular weight search scores were estimated by comparison of the search results against the estimated random match population, and were reported as $-10\log_{10}(p)$ where p is the absolute probability. MOWSE scores >55 were considered significant ($P<0.05$) for peptide mass fingerprinting. A peptide charge state of $+1$ and fragment mass tolerance of ±0.25 Da were used for the MS/MS ion search. Individual MS/MS ions scores >28 indicated identity or extensive homology ($P<0.05$) for the MS/MS ion search.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Comparison of sequential elution protocols.
Supplementary Figure 1. Spot patterns in plasma, EDTA washout and acetic eluate.
Supplementary Data 1. Ficolin-2
Supplementary Data 2. clusterin.
Supplementary Data 3. C3 complement.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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