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Compstatin, a peptide inhibitor of C3, prolongs survival of ex vivo perfused pig xenografts

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Compstatin, a newly described C3-binding peptide, inhibits complement activation by blocking C3 convertase-mediated cleavage of C3. As the complement activation is an essential part of the rejection reaction, we evaluated the ability of Compstatin to delay or prevent hyperacute rejection in an ex vivo xenograft model. Pig kidneys were perfused with fresh human blood containing either Compstatin (n=6) or a control agent (n=6). Graft survival and activation of complement, leukocytes and platelets both in the fluid-phase and in the tissue were examined. The survival of the Compstatin-perfused kidneys (median, 380 min) was significantly (P=0.0036) longer than that of the controls (median, 90 min). The classical complement pathway (C1rs-C1inhibitor and C4bc) was significantly and equally activated in both groups during the first 60 min. C3 activation products increased fivefold and terminal complement complex eightfold in the control group, but no increase occurred in the Compstatin group during this period. Immunohistochemistry showed less C3 and fibrin deposition and immune electron microscopy showed less terminal SC5b-9 complement complex deposition in the Compstatin group. A significant change in total white cells, neutrophils, myeloperoxidase, and expression of the surface activation markers CD11b (CR3) and CD35 (CR1) and CD62L (L-selectin) was observed in both groups. Leukocyte activation was lower in the Compstatin group but the difference was not statistically significant. There were no differences in platelet counts, thrombospondin, soluble P-selectin or β-thromboglobulin between the groups. We conclude that Compstatin prolongs graft survival and suggest that it may be a useful agent for attenuating hyperacute rejection by inhibiting C3 and thus terminal complement pathway activation.

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Introduction

The potential for clinical application of xenotransplantation has generated increasing interest in recent years [1]; hyperacute rejection (HAR), however, remains a major obstacle to successful clinical use of this approach. The complement system has been shown to play a central pathophysiological role in HAR [2] and to contribute to the inflammation and organ injury associated with transplantation [3].

Extended survival of grafts in primates has been achieved with pig organs transgenic for human regulators of complement activation such as human decay accelerator factor (DAF) which inhibits complement activation on cell surfaces [4]. However, it is also of value to inhibit the fluid-phase complement activation. Therefore, fluid-phase complement inhibitors, including soluble complement receptor type 1 (sCR1) [5], a monoclonal anti-C5 antibody [6], and a peptide-blocking C1q that binds to xenoantibodies [7], have been used to

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attenuate HAR-associated complement-dependent damage.

Recently a novel phage-displayed C3-binding peptide (Compstatin) has been identified that suppresses complement activation and, therefore, may be of therapeutic value in clinical situations such as xenotransplantation, which involve complement-mediated tissue damage. This peptide binds reversibly to the C3c portion of native C3 and inhibits both the classical and alternative pathways of complement activation [8]. Reduction and alkylation of this synthetic peptide destroys its inhibitory activity. Analysis of the mechanism of inhibition has revealed that the peptide binds to C3 to prevent its activation. This inhibition was not the result of sterically hindered access to the C3a/C3b cleavage site [8].

The aim of the present study was to evaluate the effect of Compstatin on discordant xenograft survival and to assess the ability of the peptide to modulate the activation of complement, leukocytes and platelets and, furthermore, to locate C3 and terminal SC5b-9 complement complex (TCC) in the kidneys. Our results suggest that inhibition of complement can be achieved with Compstatin and that this inhibition significantly can prolong xenograft survival.

Materials and methods

Animals

Six Norwegian landrace pigs of either sex, with a median body weight of 20 kg (range 18–23 kg), were used for these experiments. The outbred animals, obtained from a local farmer, were supplied with food and water ad libitum. This study was carried out at a Laboratory Animals Unit approved by the National Animal Research Authority for such work on pigs. The protocol was approved by the Unit's Competent Person, according to the regulations (1996) established by the Norwegian Animal Welfare Act of 1974.

Anesthesia and surgical preparation

Kidneys were removed using sterile procedures as previously described [9]. Immediately after isolation, the organs were perfused with precooled Ringer's acetate and stored on crushed ice. Prior to ex vivo perfusion, cannulas were inserted into the renal artery and the renal vein.

Reagents

N-(9-fluorenyl)methyloxycarbonyl (Fmoc) amino acids and 1-hydroxybenzotriazole were purchased from Novabiochem (San Diego, CA, USA); Rink amide resin, trifluoroacetic acid (TFA), 2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethyluroniumhexafluorophosphate and piperidine were purchased from Perkin Elmer/Applied Biosystems (Foster City, CA, USA). Ether, thallium(III) trifluorophosphate, thallium(III) trifluoroacetate and p-hydroxymercuribenzoic acid were purchased from Aldrich (St Louis, MO, USA). Dimethylformamide, N-methylpyrrolidone and dichloromethane were purchased from VWR (Rochester, NY, USA). Phenol and acetonitrile were purchased from Fisher Scientific (Philadelphia, PA, USA).

Synthesis and extraction of C3-binding peptide (Compstatin)

Compstatin (ICVVQDWGHHRCT-NH_2), a cyclic peptide with a molecular weight of 1551 Da and an in vitro half-life in human blood of about 2 hr, was initially identified using a phage-displayed random peptide library. Extensive ex vivo analysis of Compstatin required the synthesis of several grams of the peptide. The actual assembly and cleavage of the linear form (free thiol) of the molecule using Fmoc-based strategies in automated solid-phase peptide synthesis methods were straightforward and non-problematic. The rate-limiting step was the cyclization of the free thiol material through the oxidation of the two internal thiols provided by the cysteine residues in the polypeptide chain. Oxidations performed at dilute concentration in ammonium bicarbonate buffer led to problematic side reactions, resulting in moderate amounts of multimeric material such as dimers, trimers, tetramers, and pentamers. Time consuming lyophilization and purification steps were also required for preparation of material. The following method of preparation overcame these obstacles. Compstatin was synthesized using a peptide synthesizer (Perkin Elmer Applied Biosystems 433 A, Foster City, CA, USA) with Fmoc chemistry programmed by the manufacturer (Applied Biosystems) who provided the FastMoc Chemistry software (Applied Biosystems User Bulletin no. 32, FastMoc Chemistry: HBTU Activation in Peptide Synthesis. November 1990 pp 1–40). Rink amide resin (4-(2′, 4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin) was used to provide a carboxamido C-terminus. Unwanted aspartamide formation during Fmoc deprotection was prevented by adding 0.1 M 1-hydroxybenzotriazole to piperidine [10]. The side-chain protecting groups for the Fmoc amino acids were Cys(Acm), Gln(Trt), Asp(OtBu), Trp(Boc), His(Trt), Arg(Pmc) and Thr(tBu). The peptide was cylized by an on-resin method involving treatment of the peptide-resin with 1.5 Eq of thallium(III) trifluoroacetate in dimethylformamide for 3 h at room temperature; this approach effects a selective

deprotection of the two Cys residues and subsequent oxidation of the Cys-thiols to a disulfide [11]. The peptide-resin was then successively washed with dimethylformamide, and methanol, methanol:dichloromethane (60 : 40) and dried under vacuum. The peptide was cleaved from the peptide-resin by treatment with 87.5% TFA with 5% phenol, 5% water, and 2.5% triisopropylsilane for 3 h at room temperature [12]. The peptide was harvested from the reaction mixture by filtration and precipitated with cold ether then extracted three times with cold ether. The final peptide pellet was dissolved in 50% acetonitrile containing 0.1% TFA and lyophilized.

Peptide purification and characterization

The lyophilized crude peptide was dissolved in 10% acetonitrile containing 0.1% TFA and purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The preparative RP-HPLC was performed on an automated system (Waters Prep-LC 4000, Milford, MA, USA) equipped with a C18 column (Vydac, 300 Å pore size, 15 μm particle size, 22 × 250 mm, Heperia, CA, USA). In a typical preparative RP-HPLC run 70 mg of the peptide were loaded onto the column. The column was initially developed with buffer A (0.1% TFA in water) for 10 min, and peptide fractions were eluted with a linear gradient of 500 ml of 5–90% buffer B (0.1% TFA in acetonitrile) at a flow rate of 20 ml/min. The elution profile of the peptide fractions was monitored by ultra violet (UV) detection at 230 nm and the major peak containing the desired peptide was collected and lyophilized. The purity of the final product was assessed critically by a combination of analytical RP-HPLC and by matrix-assisted laser desorption mass spectrometry (MALDI-MS) using a time-of-flight mass spectrometer (MicroMass TofSpec, [formerly Fisons Instruments], Beverly, MA, USA). Cyclization of the peptide was verified with a MALDI-MS assay using p-hydroxymercuribenzoic acid and by monitoring the product by MALDI-MS [13].

Control peptide

C3-binding peptide 1–13,2/12-Ala (amino acid substitutions at position 2 and 12) with a molecular weight of 1486 Da was constructed as a linear control peptide (I<u>A</u>VVQDWGHHR<u>A</u>T-NH_2) and used in same concentration as Compstatin in the perfusion set-up.

Blood preparation

Pigs type as either A or 0 with human reagents. To eliminate possible interference from anti-A and anti-B, AB blood was used. Fresh whole blood (500–ml samples) drawn from informed, healthy human volunteers (Red Cross and National Hospital Blood Bank, Oslo, Norway), was heparinized (5 IE/ml) and used within 3 hr after collection. To ensure that we had a sufficient quantity of blood to fill both the Compstatin and the control perfusion circuits, and to compensate for the blood loss resulting from sampling during the perfusion, we used pooled, cross-matched blood from two donors in each set-up.

Perfusion device and procedure

The ex vivo perfusion system with physiological conditions of the pig kidneys and the endothelium, as well as the biocompatibility of the system used in this study have previously been described in detail [9]. Conditions of oxygen, carbon dioxide, electrolyte content, pH, and temperature were allowed to stabilize before the perfusion was begun, thus imitating a clinical situation. Compstatin and control agent were diluted in Ringer's acetate and added to a final concentration of 0.088 mg/ml, a concentration that was found to effectively inhibit C3 and terminal C5-C9 pathway activation in preliminary experiments. The flow rate at the start of the perfusion was kept at 50–100 ml/min, and the mean arterial pressure (MAP) was 80–120 mmHg in all experiments.

Graft monitoring

Renal vascular resistance was calculated as MAP/flow. Rejection was defined as a 100% increase in the vascular resistance and was characterized by marked macroscopic pathologic changes, with patchy hemorrhage and swelling of the perfused kidneys. Graft survival time was defined as the time elapsed from start of perfusion until rejection.

Human blood studies

Blood samples were collected from the perfusion circuits before the addition of Compstatin or control agent, after the addition of these agents, at 5, 15, 60 and 180 min after the start of perfusion, and at the time of rejection.

Urine samples

Urine samples were collected at 5, 15, 60 and 180 min after the start of perfusion, and at the time of rejection.

Complement activation products

Specific complement activation products were quantitated by use of enzyme immunoassays (EIA) based on neoepitope-specific monoclonal antibodies that

recognize particular activation products [14]. The general principles of these assays and guidelines for the collection, preparation and storage of samples have been described previously [15]. Blood samples were drawn into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). The samples were cooled on ice, and plasma was prepared immediately and stored at – 70°C until analyzed in one batch. Activation of the classical complement pathway was measured by assays detecting complexes between C1r, rs and C1-inhibitor (C1rs-C1inh) and C4 activation products, whereas activation of the final common pathway was measured using assays for C3 activation products and the TCC.

C1rs-C1inh complexes

C1rs-C1inh complexes were measured using a monoclonal antibody (KOK-12) specific for a neoepitope that is generated in the C1-inhibitor when it is complexed with the proteases [16]. Microtiter plates were coated with the antibody, reacted with plasma or control samples, and the complex was detected using a mixture of anti-C1r and anti-C1 s antibodies. Human serum activated with heat-aggregated IgG was used as a standard and was defined as containing 1000 arbitrary units (AU)/ml.

C4bc

C4bc (i.e. the sum of C4b, iC4b and C4c) was measured essentially as previously described [17]. The same standard was used as for the C1rs-C1inh assay.

C3bc

C3bc (i.e. the sum of C3b, iC3b and C3c) was measured as described previously [18]. The standard was normal human serum activated with zymosan and was defined as containing 1000 AU/ml.

Tcc

Terminal SC5b-9 complement complex (TCC) was measured as described [19] and later modified [20]. The same standard was used as for the C3bc assay.

Myeloperoxidase

Samples were drawn in tubes containing EDTA and were immediately cooled on ice, then centrifuged shortly thereafter. The plasma was stored at – 70°C until assayed in one batch. Myeloperoxidase, released by activation of leukocytes, was assayed by EIA as described [21].

β-thromboglobulin (BTG)

Blood was collected into tubes containing platelet inhibitors (Diatube H, Diagnostica Stago, Asnier-sûr-Seine, France) and kept on ice before centrifugation at 10 000 × g at 4°C to produce a platelet-poor-plasma. The mid-portion of the plasma was removed and stored at – 70°C. BTG, released by activation of platelets, was determined in a competitive EIA: The plates were coated with partially purified BTG from activated human platelets. Samples and biotinylated (biotinylation kit, Sigma, St. Louis, MO, USA) anti-human BTG antibody (Biogenesis, Poole, Dorset, UK) were added, and peroxidase-conjugated avidin (Zymed Laboratories, San Fransisco, CA, USA) was added in the final step. The substrate was o-phenylenediamine dihydrochloride (Sigma), and purified BTG from human platelets (Celsus Laboratories, Cincinnati, OH, USA) was used as a standard. The absorbency was read at 490 nm in a microtiter plate reader (ELX800, Bio-Tec Instruments, Winooski, VT, USA).

Thrombospondin

Thrombospondin released by activated platelets and endothelial cells, was quantitated using a double-antibody EIA. Microtiter plates were coated overnight with a monoclonal anti-thrombospondin antibody (clone P12, Immunotech S.A., Marseilles, France) at 0.4 μg/ml. The standard for this assay was a pool of serum from 20 healthy blood donors that had been calibrated. Plasma samples were obtained as described for BTG and used at a 1 : 2 dilution. A biotinylated monoclonal antithrombospondin antibody (clone P10, Immunotech) was used for detection, and final development was performed using streptavidin-conjugated horseradish peroxidase (Amersham, Buckinghamshire, UK) and 2,2′ -azino-di-(3-ethyl)-benzthiazoline sulfonic acid (ABTS) (Boehringer Mannheim, Mannheim, Germany) as substrate.

sCD62P (soluble P-selectin)

Samples were obtained as described for BTG and assayed with an sP-selectin EIA kit (Bender MedSystems, Vienna, Austria).

Flow cytometry

Samples for flow cytometry were drawn from the arterial line and ejected into sodium-heparin tubes in the absence of vacuum. The heparinized blood (0.1 ml) was mixed with 1 ml of 0.4% formaldehyde in phosphate-buffered saline (PBS) for fixation at room temperature. Fixed, whole blood specimens were used to avoid in vitro upregulation of the

markers during sample preparation. After fixation, lysis of the red blood cells was accomplished by adding 2 ml of 156 mmol/l NH_4Cl containing 10 mmol/l $NaHCO_3$ and 0.12 mmol/L EDTA. After centrifugation for 5 min at 1200 × g, the pellets were resuspended in 1 ml of PBS containing 2% bovine serum albumin and appropriate number of cells were stained with fluorochrome labeled antibodies. Staining was performed for 20 min at room temperature. The specimens were transferred to flow tubes and fixed in 1% paraformaldehyde in PBS solution. The cells were analyzed by a FACScan (model 440) flow cytometer with PCLYSIS software (Becton-Dickinson, San Jose, CA, USA). Neutrophils and platelets were identified by their distinct patterns on forward scatter and 90-degree sidescatter and by appropriate surface markers. Neutrophils were identified with a monoclonal PE-conjugated anti-CD 15 antibody (Sigma Chemical Company, St. Louis, MO, USA) and platelets with a monoclonal PE-conjugated anti-CD41 (DAKO A/S, Glostrup, Denmark). Fluorescein isothiocyanate (FITC)-conjugated antibodies were used to quantitate the molecule of interest. We used FITC-conjugated monoclonal antibodies against CR1 (CD35), CD11b and P-selectin (CD62P) (Central Lab Netherlands Red Cross, Amsterdam, the Netherlands) and against L-selectin (CD62L) (Pharmingen, San Diego, CA, USA). IgG1-PE Control (Becton-Dickinson) and IgG1-FITC (DAKO) monoclonal antibodies were used for negative controls.

Hematology and blood gases

Hemoglobin, hematocrit, platelet and leukocyte counts were determined in an automated counter (H-I Technicon, Miles, Tarrytown, NY, USA). Blood gases were analyzed in a blood gas analyzer (Radiometer ABL4, Copenhagen, Denmark).

Correction for hemodilution

The concentrations of the complement activation products, myeloperoxidase, BTG, soluble CD62P and thrombospondin and the blood cell counts were corrected for hemodilution by using hematocrit values according to the method described by van Beaumont [22].

Immunohistochemical studies

Pig kidney biopsies, obtained ex vivo by excision at rejection, were immediately immersed in isotonic saline and kept at 4°C until frozen for storage. The biopsies were frozen in Tissue-Tek OCT compound (Lab-Tek Products Division, Miles Laboratories, Naperville, IL, USA), using dry ice and 2-methylbutane, and stored at – 70°C. Sections (5 μm) were cut using a cryostat model 1720 (Leitz, Wetzlar, Germany), fixed for 10 min in acetone and washed twice in PBS. The sections were examined directly with the following FITC-labelled polyclonal rabbit antisera: anti-human IgG, anti-human IgM, anti-human C3c, anti-human C4c, anti-human fibrinogen (DAKO); or indirectly with unlabelled mouse monoclonal antibody against TCC (clone aE11, produced in our own laboratory) and FITC-labelled goat antimouse Ig (Sigma). For direct staining, sections were incubated with FITC-labelled antibody for 30 min, washed twice in PBS, and then mounted in polyvinyl alcohol/PBS. For indirect staining, the sections were first incubated with the unlabelled antibody, then washed and stained with the secondary antibody as described above. Sections were examined under a microscope equipped for white light and incident light immunofluorescence microscopy (Model DMRB, Leitz). For most of the experiments, the FITC-labelled antisera were used in dilutions that reacted negatively or gave a very weak granular fluorescence in the glomeruli of unperfused pig kidney and gave negative results with various types of normal human tissue. The results were recorded separately for larger vessels; glomeruli (at least 10 glomeruli were examined); interstitial tissue mainly consisting of capillaries between tubuli; and tubules. The pattern and intensity of the immunofluorescence were recorded, with the intensity arbitrarily graded from 0 to 3+.

Immune electron microscopy

The kidney biopsies were obtained in the same way as described for the immunohistochemical studies. About 0.5 mm^3 cubes of cortical tissue were fixed by immersion in 2% paraformaldehyde and 0.05% glutaraldehyde for 4 h. Dehydration was performed in graded ethanols with progressive lowering of the temperature [23]; 30 min in 30% ethanol at 0°C, 60% ethanol at – 15°C, and 70% ethanol at – 20°C, 60 min in 90% ethanol at – 20°C, and 120 min in absolute ethanol at – 20°C. Immersion in the resin (Unicryl, British Biocell, Cardiff, UK) at – 20°C was performed over night and polymerization of the blocks was accomplished by UV illumination for 1 day at + 4°C. Semithin sections were stained with toluidin blue and examined by light microscopy for selection of glomeruli. Immunolabelling was carried out according to the method described by Robertson et al. [24]. The primary antibody was polyclonal antibody to human TCC, rabbit antiserum produced in our laboratory. The primary antibody was detected by anti-rabbit IgG conjugated to 5 nm gold particles (Amersham, Buckingshare, UK) diluted 1 : 50. Silver enhancement was obtained

using a silver enhancing kit (Amersham) for 6 min. After washing, the sections were contrasted by standard procedures with uranyl acetate and lead citrate. Control studies were carried out omitting the primary antibody.

Experimental groups

Compstatin-perfused circuit (n=6): pig xenografts were perfused with human blood containing Compstatin (final conc. 0.088 mg/ml). Control group (n=6): pig kidneys perfused with human blood and control agent. The total fluid volume, protein concentration and amount of blood were equal in both the Compstatin and control perfusion circuits, and the perfusion was performed in both circuits simultaneously with pooled blood from the same two donors.

Statistics

Results are given as medians and 95% confidence intervals. Data were first analyzed using two-way repeated measures analysis of variance (ANOVA) using the SPSS-PC program package. As a result of differences in the duration of individual experiments and the occurence of non-normal variables and unequal variances, the conditions for anova were only partly met. Therefore, the Friedman test was used for further comparisons of parameter changes over time within each group, and the Mann–Whitney U-test was employed for further comparisons between groups. In order to achieve an overall P-value for significance below 0.05 and correct for multiple comparisons, any P-values from the Friedman or Mann–Whitney U-tests below the corresponding P-values from the repeated measures two-way analysis of variance were regarded invalid. In the control group, complete analysis of complement activation products in urine was not possible because of missing data. Instead, the concentration of complement products in urine at 5 min was compared to that at 60 min by the Wilcoxon Signed Rank Test, and the two groups were compared only at 60 min by the Mann–Whitney U-test.

Results

Graft survival and gross morphology

In all six experiments graft survival was significantly longer (P=0.0036) in the Compstatin perfused group [380 (297–441) min] than in the control group [90 (58–120) min]. The pig kidneys were normal in color at the start of the experiments, then gradually became mottled in appearance; in all cases, the color shift occured earlier in the control than in the Compstatin perfused kidney. Contractions of the ureters were observed in all experiments during the perfusion and continued until rejection occurred.

Complement activation products (Table 1).

Classical pathway activation

The concentrations of C1rs-C1inh complexes and of C4bc increased significantly ($P < 0.001$) in both groups from the beginning of perfusion to rejection, with no significant differences in concentration between the groups.

C3bc

There was no consistent change in the concentration of C3bc within the Compstatin group during the first 60 min. In contrast, in the control group a twofold increase occured at 15 min and a fivefold increase ($P < 0.001$) at 60 min. The concentration of C3bc was significantly higher (P=0.03) in the control group than in the Compstatin group at 60 min.

TCC

There was no consistent change in the concentration of TCC within the Compstatin group during the first 60 min. In the control group a gradual increase occured, obtaining an eightfold increase ($P < 0.01$) at 60 min. The concentration of TCC was significantly higher (P=0.006) in the control group than in the Compstatin group at 60 min.

Urinary C3bc and TCC

The concentrations of urinary C3bc and TCC increased significantly within the control group (P=0.02) and in the Compstatin group ($P < 0.05$) from the start of urinary production to 60 min. The concentrations of C3bc and TCC were markedly higher ($P < 0.02$) in the control group than in the Compstatin group at 60 min.

Leukocytes (Table 2)

In both groups the leukocyte and neutrophil counts decreased significantly ($P < 0.001$) from the start of perfusion to rejection, with no significant intergroup differences. Myeloperoxidase and leukocytes increased significantly ($P < 0.001$) with time in both groups. The increase was less pronounced in the Compstatin group than in the control group, but this difference was not statistically significant (P=0.08). In both the Compstatin and the control group flow cytometry analysis of neutrophils revealed a significant increase in the neutrophil surface activation markers CD11b (CR3) ($P < 0.01$) and CD35 (CR1) ($P < 0.01$), and significant downregulation of

CD62 L (L-selectin) ($P < 0.05$), without significant intergroup differences.

Platelets (Table 3)

The platelet counts decreased significantly ($P < 0.001$) and thrombospondin increased significantly ($P < 0.001$) in both the Compstatin and control groups. Soluble CD62P (sP-selectin) increased significantly ($P < 0.01$), although numerically little, in the control group but not in the Compstatin group, whereas BTG did not change significantly in either group. There were no significant differences between the two groups with respect to platelet activation.

Immunohistochemistry

At rejection the biopsy samples obtained from kidneys perfused with Compstatin showed less deposition of C3 and fibrin than did the control kidneys (Fig. 1). The extent of C1q, TCC, IgG and IgM deposition did not differ between the two groups at rejection. The TCC

Table 1. Complement variables before, at the beginning (T0) and after 15 and 60 min of perfusion and at rejection in kidneys perfused with Compstatin or control agent

	Compstatin group (n=6)		Control group (n=6)		Compstatin vs. control group	
	Median (95%CI)	P-value[a] vs. before	Median (95%CI)	P-value[a] vs. before	P-value[b]	P-value[c]
C1rs/C1inh (AU/ml)						0.87
Before	24 (19–30)	–	19 (13–27)	–		
T0	26 (19–35)	NS	27 (19–33)	<0.01		
T15	23 (17–54)	NS	29 (14–44)	<0.05		
T60	40 (27–62)	<0.001	46 (30–62)	<0.001		
Rejection			46 (23–67)	<0.001		
Rejection	52 (28–76)	<0.001				
C4bc (AU/ml)						0.63
Before	11 (6–15)	–	8 (6–11)	–		
T0	11 (9–13)	NS	12 (10–16)	<0.01		
T15	11 (7–13)	NS	15 (6–19)	<0.05		
T60	20 (13–26)	<0.001	23 (15–29)	<0.001		
Rejection			29 (17–42)	<0.001		
Rejection	39 (23–61)	<0.001				
C3bc (AU/ml)						0.03
Before	20 (12–30)	–	16 (7–29)	–		
T0	12 (7–20)	<0.001	24 (15–37)	<0.05	0.03	
T15	17 (11–22)	NS	34 (22–44)	<0.01		
T60	25 (12–66)	NS	82 (33–133)	<0.001	0.03	
Rejection		–	110 (46–217)	<0.001		
Rejection	50 (19–156)	<0.001			0.09	
TCC (AU/ml)						0.005
Before	0.6 (0.4–0.7)	–	0.5 (0.3–0.6)	–		
T0	0.7 (0.4–1.2)	NS	1.3 (0.9–4.6)	<0.01	0.03	
T15	0.8 (0.4–1.1)	<0.05	1.2 (0.8–1.6)	<0.01		
T60	0.7 (0.5–1.1)	NS	4.5 (2.4–7.3)	<0.01	0.006	
Rejection			4.2 (2.1–7.2)	<0.01		
Rejection	1.5 (0.8–2.2)	<0.01			0.01	
Urinary C3bc (AU/ml)						
T5	1 (1–1)	–	3 (1–8.9)			
T15	1 (1–2.4)	NS	12.5 (3.9–50.8)			
T60	7 (1–14.4)	<0.05	28 (7–111)	0.02[d]	0.02	
Rejection			17 and 25			
Rejection	5 (3.3–40.8)	<0.05				
Urinary TCC (AU/ml)						
T5	0.06 (0.04–0.08)	–	0.16 (0.05–0.45)			
T15	0.08 (0.04–0.32)	NS	1.98 (0.29–6.72)			
T60	1.54 (0.12–3.07)	<0.001	10.12 (0.20–13.6)	0.003[d]	0.02	
Rejection			5.2 and 8.6			
Rejection	0.84 (0.24–4.48)	<0.001				

[a]Friedman test. [b]Mann-Whitney U-test. [c]ANOVA. [d]Wilcoxon Signed Rank Test. In the control group, complete analysis of complement activation products was not possible because of missing data. Instead the concentration of complement products in urine at 5 min was compared to that of 60 min by the Wilcoxon Signed Rank Test, and the two groups were compared only at 60 min by the Mann-Whitney *U*-test, TCC AU, arbitrary unit; terminal SC5b-9 complement complex.

staining was almost negative in both groups. When present the fluorescence was seen in the arteries as granular deposits in the vessel walls and in the kidney tubules as a granular pattern, mainly near the basal membrane. In the glomeruli, the immunofluorescence appeared as a granular pattern along the basal membrane; in the capillary loops it assumed a more diffuse pattern in the cytoplasm of the endothelial cells.

Table 2. White cell variables before, at the beginning (T0) and after 15 and 60 min of perfusion and at rejection in kidneys perfused with Compstatin or control agent

	Compstatin group (n=6)		Control group (n=6)		
	Median (95%CI)	P-value[a] vs. before	Median (95%CI)	P-value[a] vs. before	Compstatin vs. control group P-value[c]
Leukocytes ($\times 10^9$/l)					0.51
Before	5.7 (4.1–7.5)	–	5.5 (4.1–7.4)	–	
T0	5.9 (3.4–8.3)	NS	5.7 (3.7–8.3)	NS	
T15	4.9 (2.6–7.9)	NS	3.1 (1.6–5.7)	<0.001	
T60	3.2 (1.9–6.6)	<0.001	2.3 (1.2–4.4)	<0.001	
Rejection			1.9 (0.6–3.0)	<0.001	
Rejection	2.8 (1.3–3.5)<0.001				
Neutrophils ($\times 10^9$/l)					0.53
Before	3.1 (2.2–4.9)	–	3.1 (2.2–4.8)	–	
T0	3.2 (1.8–5.5)	NS	3.2 (1.9–5.4)	NS	
T15	2.8 (1.3–5.0)	NS	1.2 (0.4–3.4)	<0.001	
T60	1.5 (0.9–4.3)	<0.001	0.7 (0.2–2.6)	<0.001	
Rejection			0.5 (0.2–1.6)	<0.001	
Rejection	0.8 (0.4–2.0)	<0.001			
Monocytes ($\times 10^9$/l)					0.21
Before	0.4 (0.3–0.6)	–	0.4 (0.3–0.6)	–	
T0	0.5 (0.3–0.7)	<0.01	0.4 (0.3–0.7)	NS	
T15	0.4 (0.2–0.7)	NS	0.1 (0.01–0.3)	<0.001	
T60	0.1 (0.1–0.4)	<0.001	0.03 (0.01–0.3)	<0.001	
Rejection			0.3 (0.01–0.3)	<0.001	
Rejection	0.02 (0.02–0.03)	<0.001			
Myeloperoxidase (μg/l)					0.08
Before	367 (282–446)	–	373 (231–444)	–	
T0	537 (235–1219)	NS	899 (520–1393)	<0.001	
T15	883 (318–1568)	<0.01	1349 (645–2067)	<0.001	
T60	1174 (830–1666)	<0.001	1702 (1023–2350)	<0.001	
Rejection			2236 (1558–3135)	<0.001	
Rejection	1617 (1054–2839)	<0.001			
Neutrophil CD11b MFC					0.74
Before	37 (26–131)	–	40 (28–156)	–	
T0	66 (46–197)	<0.01	86 (41–355)	<0.05	
T15	72 (45–257)	<0.01	154 (67–490)	<0.01	
T60	102 (46–306)	<0.01	126 (79–493)	<0.01	
Rejection			156 (94–477)	<0.01	
Rejection	238 (111–549)	<0.01			
Neutrophil CD35 MFC					0.52
Before	117 (51–299)	–	122 (59–336)	–	
T0	158 (66–339)	<0.01	169 (86–421)	<0.01	
T15	140 (63–327)	<0.05	194 (101–375)	<0.01	
T60	146 (80–341)	<0.01	206 (63–417)	<0.01	
Rejection			257 (73–443)	<0.01	
Rejection	256 (125–388)	<0.01			
Neutrophil CD62L MFC					0.91
Before	21.5 (15.9–58.1)	–	21.3 (17.1–42.8)	–	
T0	21.2 (15.9–41.3)	NS	21.8 (17–45.4)	NS	
T15	20.5 (15.9–36.6)	<0.05	18.1 (14.0–31.5)	<0.05	
T60	18.8 (13.8–33.0)	<0.05	18.4 (13.9–31.8)	<0.05	
Rejection			18.4 (14.4–29.4)	<0.05	
Rejection	14.3 (12.1–31.6)	<0.05			

[a]Friedman test. [c]ANOVA. MFC, mean fluorescence channel.

In the interstitial tissue, the fluorescence seemed mostly confined to the intertubular capillaries.

Immune electron microscopy

Ultrastructurally TCC was demonstrated in sections from the control kidneys, and labelling was observed both in the glomeruli and tubuli. In the glomeruli labelling was found in the capillary basement membrane as well as in the lumina of many capillaries and also in the mesangial regions (Fig. 2A,B). Furthermore, labelling was seen in the urinary space in varying amounts from case to case. In the tubular cells labelling was found in phagolysosomes in the same way as demonstrated in Fig. 4 in all kidneys.

In the Compstatin perfused kidneys the glomerular labelling varied from case to case. In two kidneys there was almost no labelling of the glomeruli and the difference when compared with the control kidneys, was evident (Fig. 3A,B). The labelling of phagolysosomes in tubular cells was present in all the Compstatin infused kidneys as demonstrated in Fig. 4.

Discussion

The present study indicates that the use of Compstatin to inhibit complement activation may significantly prolong graft survival and reduce HAR of pig kidneys perfused with human blood. As expected, classical pathway complement activation, indicated by the levels of C1 and C4 activation products was similar in the Compstatin-and control agent perfused groups, since Compstatin inhibits at a later step in the cascade. The significantly greater increase in the concentrations of C3bc and TCC in the control group than in the Compstatin group from the start of perfusion till rejection indicates that Compstatin did indeed act at the level of C3 in this xenograft model. The difference in complement activation between the two groups from start to rejection was more pronounced for TCC, which previously has been shown to be more sensitive than C3bc in its differential response to various complement-activating extracorporeal devices under various conditions [25].

The differences between the two groups in the fluid-phase with respect to C3bc and TCC were

Table 3. Platelet variables before, at the beginning (T0) and after 15 and 60 min of perfusion and at rejection in kidneys perfused with Compstatin or control agent

	Compstatin group (n=6)		Control group (n=6)		
	Median (95%CI)	P-value[a] vs. before	Median (95%CI)	P-value[a] vs. before	Compstatin vs. control group P-value[c]
Platelets ($\times 10^9$/l)					0.82
Before	237 (201–278)	–	239 (207–273)	–	
T0	203 (162–298)	NS	215 (145–283)	NS	
T15	140 (62–252)	<0.001	143 (80–200)	<0.01	
T60	126 (61–180)	<0.001	154 (76–191)	<0.001	
Rejection			125 (67–153)	<0.001	
Rejection	115 (50–164)	<0.001			
S-CD62P (ng/ml)					0.67
Before	70 (51–92)	–	68 (58–79)	–	
T0	60 (48–73)	NS	58 (45–69)	<0.05	
T15	61 (49–73)	NS	56 (51–73)	NS	
T60	75 (60–90)	NS	78 (61–92)	<0.05	
Rejection			98 (78–143)	<0.01	
Rejection	103 (64–150)	NS			
Thrombospondin (ng/ml)					0.28
Before	91 (74–114)	–	57 (30–85)	–	
T0	154 (122–200)	<0.01	171 (109–187)	<0.001	
T15	68 (59–152)	NS	56 (41–99)	NS	
T60	129 (91–148)	NS	175 (119–205)	<0.001	
Rejection			222 (164–281)	<0.001	
Rejection	292 (206–359)	<0.001			
BTG (ng/ml)					0.99
Before	249 (54–1025)	–	258 (48–1017)	–	
T0	681 (44–1924)	NS	259 (49–1309)	NS	
T15	582 (35–1715)	NS	201 (49–1533)	NS	
T60	361 (38–1183)	NS	381 (52–1447)	NS	
Rejection			416 (52–1448)	NS	
Rejection	714 (34–1527)	NS			

[a]Friedman test. [c]ANOVA.

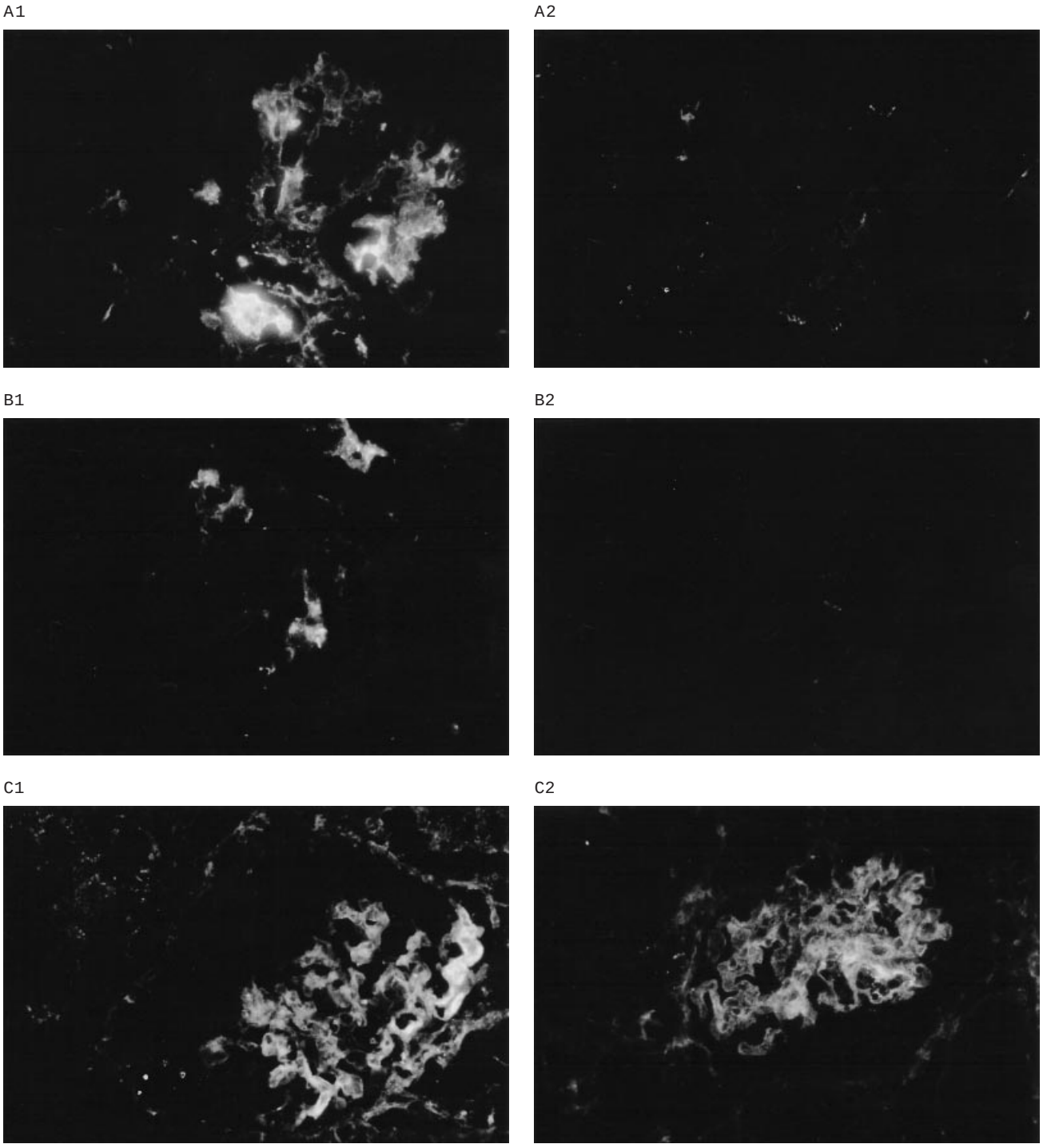

Fig. 1. Immunohistochemical analysis of pig kidneys perfused with human AB blood. Left panel: specimen from control experiment, showing deposition of fibrin (A1), C3 (B1) and IgG (C1), mainly in the glomeruli. Right panel: corresponding specimen (A2-C2) from the Compstatin experiment showing less deposition of fibrin and C3, but similar amounts of IgG.

supported by the immunohistochemistry studies relating to C3. Likewise, there was a difference in intensity of TCC labelling showed by the electron microscopy studies, especially in two kidneys, where TCC was almost only located in the tubular cells. These findings support the fluid phase that Compstatin inhibits the formation of TCC. Furthermore, the findings clearly demonstrate that TCC is taken up into phagolysosomes in the tubular cells. This probably represents either a transport-or degradation mechanism. It is important to note, however, that both the immunohistochemistry and the electron microscopy studies were made on biopsies at rejection, which occured at markedly

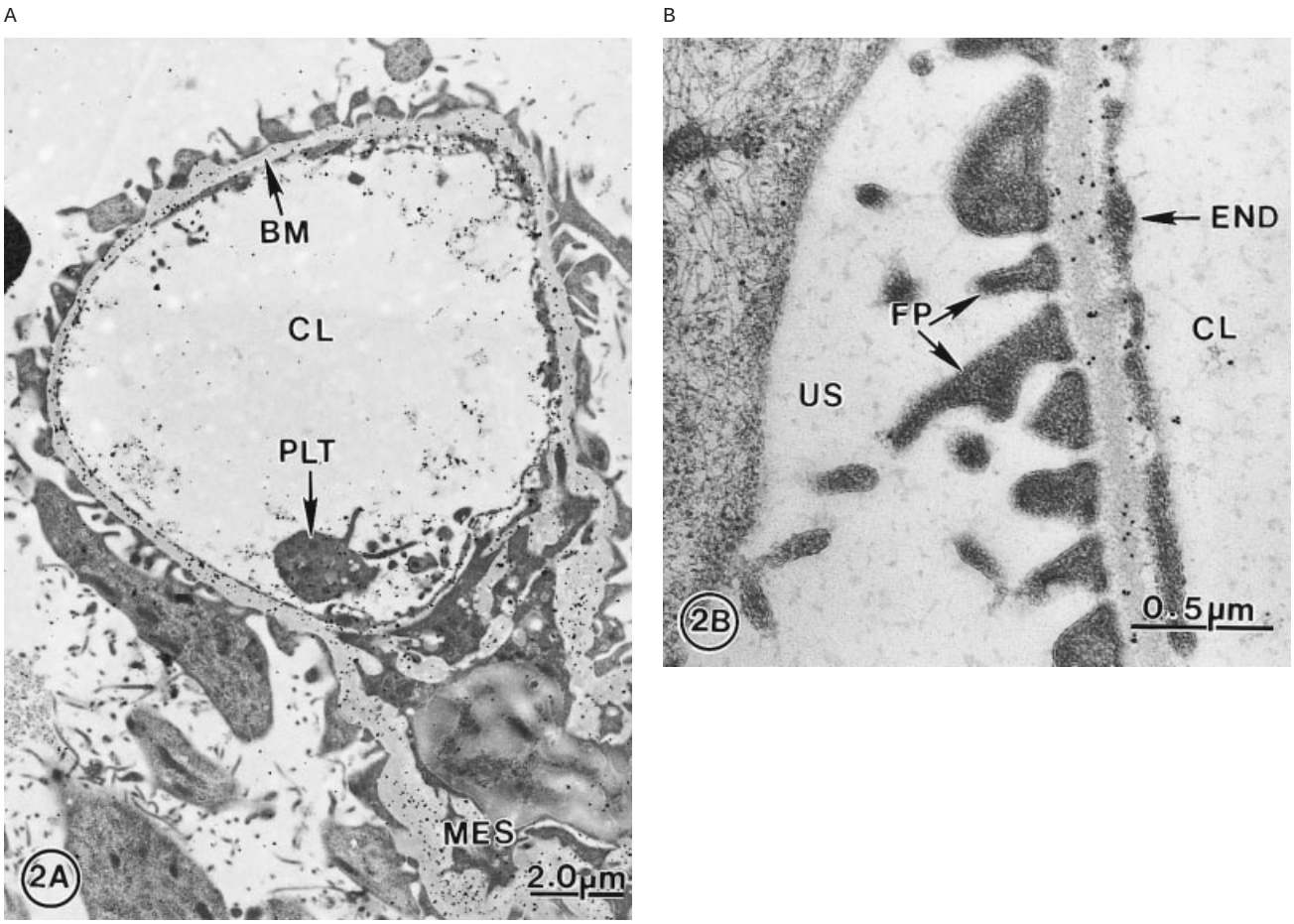

Fig. 2. (A) Electron micrograph of a mesangial region (MES) and a glomerular capillary from a control kidney. Note the labelling demonstrating TCC present in the mesangial matrix as well as in the basement membrane (BM) of the capillary and within the capillary lumen (CL). Blood platelet (PLT). Perfusion-time 60 min. (B). Detail from the glomerular capillary wall demonstrating TCC labelling of the basement membrane. Endothelium (END), foot processes (FP), urinary space (US).

different times in the two groups. We avoided taking biopsies during the perfusion period, since preliminary experiments had shown technical problems with bleeding because of biopsying. These problems would have influenced the survival and fluid-phase data, which were the main data to be collected in the present investigation. We hypothesize that if biopsies had been obtained during the first 60 min, there would have been a more pronounced difference between the two groups in favor of less complement deposition in the Compstatin group.

There was a substantial increase in urine of C3bc and TCC in the control group, significantly more than in the Compstatin group. It is unclear whether these complement activation products accumulated in the urine as a consequence of increased leakage of preformed products, or were activated in the tubular system. The urinary data may suggest that Compstatin efficiently protect the kidney against complement-mediated damage during the perfusion period.

The number of leukocytes, neutrophils and monocytes declined significantly with time from start to rejection in both groups, indicating increased cell adhesion, extravasation and/or destruction during perfusion. Flow cytometry analysis revealed inflammatory response-related changes in a number of surface adhesion molecules on neutrophils in both Compstatin and control set-ups: CD11b (CR3), the receptor for iC3b, was markedly upregulated; CD35 (CR1), a receptor for C3b that also acts as a cofactor for factor I-mediated C3b cleavage, was moderately upregulated; and CD62L (L-selectin), an important adhesion molecule, was downregulated. Studies in humans have indicated that upregulation of CD11b and downregulation of CD62L (L-selectin) on neutrophils is related to endothelial changes leading to capillary leakage [26].

Although it was not statistically significant ($P=0.08$), we observed a trend toward a less myeloperoxidase release in the Compstatin group than in the control group up to 60 min. However, the

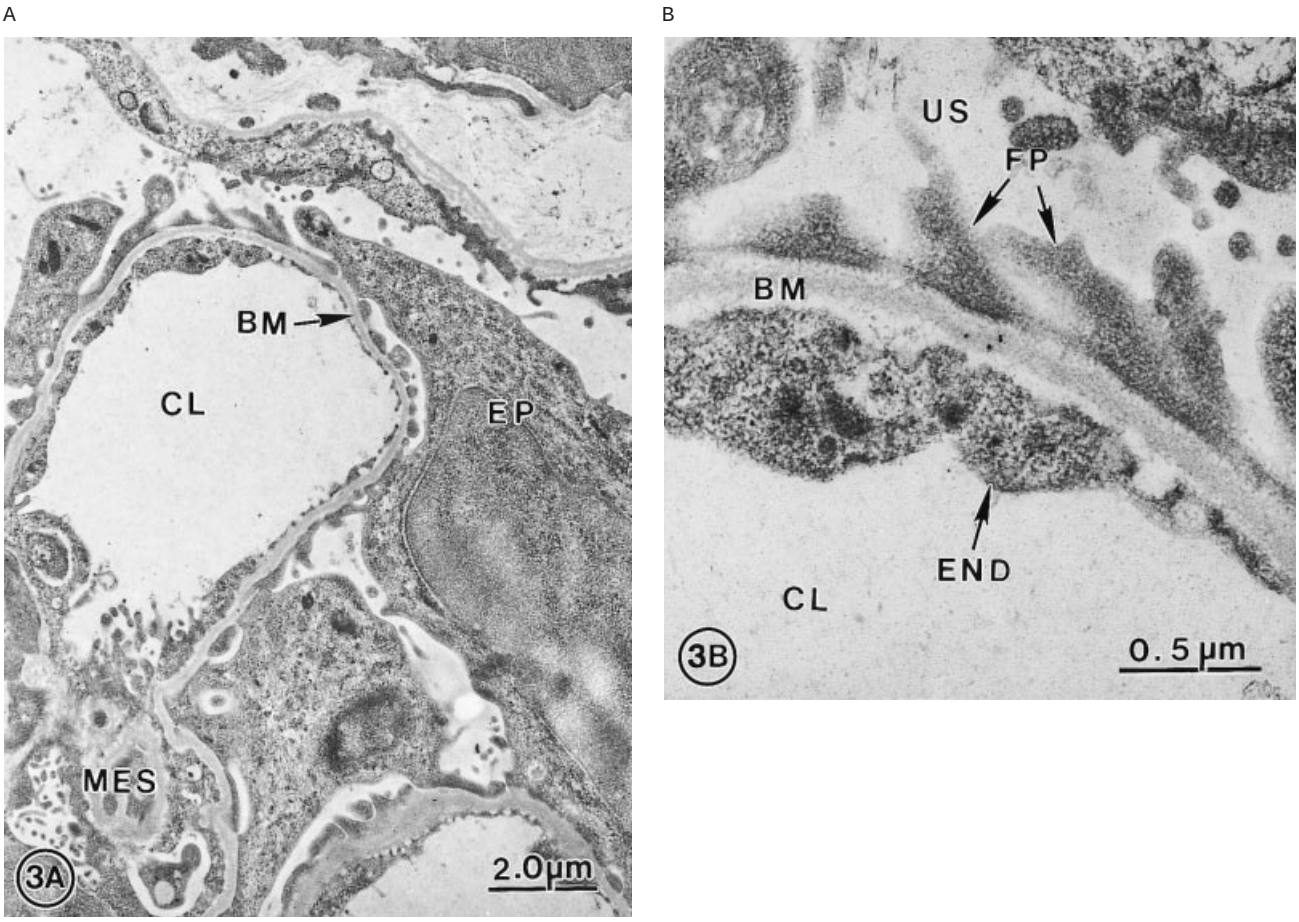

Fig. 3. (A) Mesangium (MES) and glomerular capillaries from the Compstatin perfused kidney corresponding to the control kidney in Figure 2 and immunolabelled in parallel with the control kidney specimen. Almost no TCC labelling present. Basement membrane (BM), capillary lumen (CL), epithelial cell (EP). Perfusion-time 410 min. (B) Detail from the capillary wall.

other leukocyte activation markers studied (number of leukocytes, CD11b, CD35, CD62L (L-selectin) were not statistically different between the two groups. Whether Compstatin indirectly by attenuating the complement activation had a modulatory effect on these inflammatory cells or not is, therefore, unclear.

The number of platelets declined significantly with time from start to rejection in both groups, and microthrombi were observed in small vessels in both groups at rejection by electron microscopy, consistent with the important role of thrombosis as an important process in HAR. However, no difference in number of platelets or in the fluid-phase activation markers soluble CD62P (sP-selectin), BTG, or thrombospondin was found between the groups. Thus, from the data presented here it seems that Compstatin in our model efficiently inhibits complement activation, and may also thereby attenuate leukocyte activation, but it probably does not influence platelet activation [27].

All ex vivo perfusion systems have limitations for physiological reasons [9]. We used Ringer's acetate, a mild vasodilator, and heparin in our set-up to prevent thrombosis. These two agents may have contributed to the prolonged perfusion before rejection in our control group. Heparin enhances the inactivation of C3b [28] and inhibits the formation of C3 amplification convertase [29], and its use may therefore have contributed to the previously reported failure of C3 activation products to correlate with levels of TCC during perfusion [30]. Furthermore, heparin can exert a direct effect on neutrophils [21,30], and it can also activate them either directly or indirectly through platelet release [31]. Thus, one might speculate that the neutrophil activation we observed during ex vivo perfusion was also related to the systemic use of heparin in our set-up. With regards to platelet function, the effect of heparin on platelet function during ex vivo perfusion is not yet clear [32]. In any case, the possible effects of heparin that we have described should be the same for each of our experimental groups and thus should not be responsible for the differences we observed between the two groups. The low number of observations increased the probability of statistical

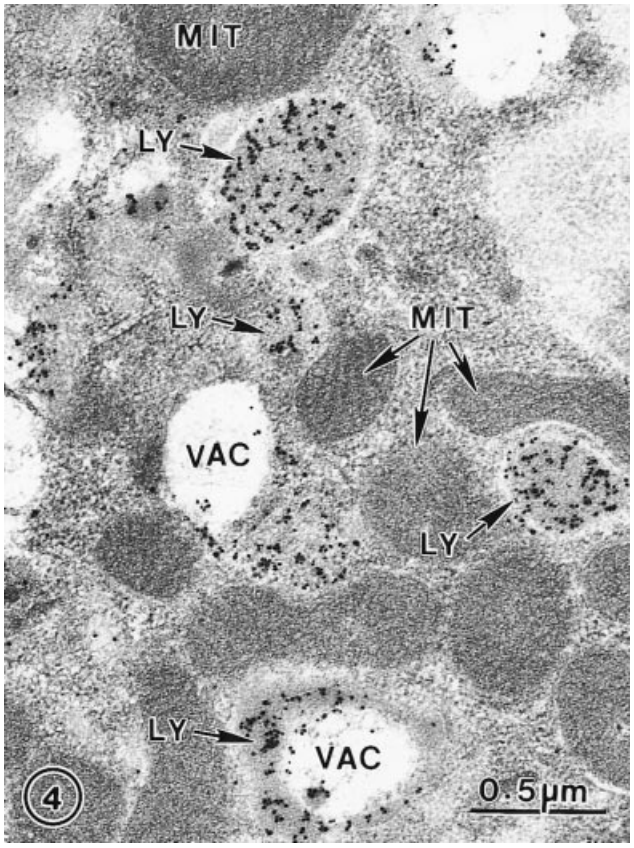

Fig. 4. Electron microscopic detail from a proximal tubular cell from the same thin section as shown in Figure 3. Note the pronounced TCC labelling of the phagolysosomes (LY) and vacuoles (VAC), but no labelling of the mitochondria (MIT).

type II errors (failure to detect a real significance). The amount of peptide and the total resources required indeed limited the number of experiments that could be performed. There are currently no published data to indicate whether sustained systemic complement inhibition with Compstatin will increase the susceptibility of a recipient to infection, which otherwise presumably would limit the use of this peptide for any period of time in humans.

In summary, the present study has demonstrated that addition of a new complement inhibitor, Compstatin, to a human blood perfusate, can produce a substantial increase in the survival time of the pig kidneys in a pig-to-human ex vivo perfusion model of xenotransplantation. The effect is apparently mediated by inhibition of C3 and the terminal C5-C9 pathway.

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References

1. Platt JL. The immunological barriers to xenotransplantation. Crit Rev Immunol 1996; 16: 331.
2. Dalmasso AP. The complement system in xenotransplantation. Immunopharmacology 1992; 24: 149.
3. Baldwin WM III, Pruitt SK, Brauer RB, Daha MR, Sanfilippo F. Complement in organ transplantation. Contributions to inflammation, injury, and rejection. Transplantation 1995; 59: 797.
4. Cozzi E, White DJ. The generation of transgenic pigs as potential organ donors for humans. Nat Med 1995; 1: 964.
5. Pruitt SK, Kirk AD, Bollinger RR, Marsh HC Jr, Collins BH, Levin JL, Mault JR, Heinle JS, Ibrahim S, Rudolph AR, Baldwin WM III, Sanfilippo F. The effect of soluble complement receptor type 1 on hyperacute rejection of porcine xenografts. Transplantation 1994; 57: 363.
6. Kroshus TJ, Rollins SA, Dalmasso AP, Elliot EA, Matis LA, Squinto SP, Bolman RM III. Complement inhibition with an anti-C5 monoclonal antibody prevents acute cardiac tissue injury in an ex vivo model of pig-to-human xenotransplantation. Transplantation 1995; 60: 1194.
7. Fryer JP, Blondin B, Stadler C, Ivancic D, Rattner U, Kaplan B, Kaufman D, Abecassis M, Stuart F, Anderson B. Inhibition of human serum mediated lysis of porcine endothelial cells using a novel peptide which blocks C1q binding to xenoantibody. Transplant Proc 1997; 29: 883.
8. Sahu A, Kay BK, Lambris JD. Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. J Immunol 1996; 157: 884.
9. Fiane AE, Videm V, Foerster A, Scholz T, Pedersen TH, Karlsen H, Svennevig JL, Geiran OR, Aasen AO, Mollnes TE. An ex vivo model to evaluate hyperacute rejection in a discordant pig-to-human combination. Eur Surg Res 1998; 30: 341.
10. Lauer LL, Fields CG, Fields GB. Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. Lett Peptide Sci 1994; 1: 197.
11. Edward WB, Fields CG, Anderson CJ, Pajeau TS, Welch MJ, Fields GB. Generally applicable, convenient solid-phase synthesis and receptor affinities of octreotide analogs. J Med Chem 1994; 37: 3749.
12. Solé NA, Barany G Optimization of solid-phase synthesis of [Ala8]-Dynorphin Al-8. J Org Chem 1992; 57: 5399.
13. Moore WT. Laser desorption mass spectrometry. Methods of Enzymology 1997; 289: 520.
14. Mollnes TE, Harboe M. Neoepitope expression during

complement activation – a model for detecting antigenic changes in proteins and activation of cascades. Immunologist 1993; 1: 43.
15. Mollnes TE. Analysis of in vivo complement activation. In: Herzenberg LA, Weir DM, Herzenberg LA, eds. Weir's Handbook of Experimental Immunology. Boston: Blackwell Science Inc., 1997.
16. Fure H, Nielsen EW, Hack CE, Mollnes TE. A neoepitope-based enzyme immunoassay for quantification of C1-inhibitor in complex with C1r and C1s. Scand J Immunol 1997; 46: 553.
17. Wolbink GJ, Bollen J, Baars JW, ten Berge RJ, Swaak AJ, Paardekooper J, Hack CE. Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA for the quantification of complement activation via the classical pathway. J Immunol Methods 1993; 163: 67.
18. Garred P, Mollnes TE, Lea T Quantification in enzyme-linked immunosorbent assay of a C3 neoepitope expressed on activated human complement factor C3. Scand J Immunol 1988; 27: 329.
19. Mollnes TE, Lea T, Frøland SS, Harboe M. Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex. Scand J Immunol 1985; 22: 197.
20. Mollnes TE, Redl H, Høgåsen K, Bengtsson A, Garred P, Speilberg L, Lea T, Oppermann M, Götze O, Schlag G. Complement activation in septic baboons detected by neoepitope-specific assays for C3b/iC3b/C3c, C5a and the terminal C5b-9 complement complex (TCC). Clin Exp Immunol 1993; 91: 295.
21. Videm V. Heparin in clinical doses 'primes' granulocytes to subsequent activation as measured by myeloperoxidase release. Scand J Immunol 1996; 43: 385.
22. Van Beaumont W. Evaluation of hemoconcentration from hematocrit measurements. J Appl Physiol 1972; 32: 712.
23. Armbruster BL, Carlemalm E, Chiovetti R, Garavito RM, Hobot JA, Kellenberg E, Villiger W. Specimen preparation for electron microscopy using low temperature embedding resins. J Microsc 1982; 126: 77.
24. Robertson D, Monaghan P, Clarke C, Atherton AJ. An appraisal of low-temperature embedding by progressive lowering of temperature into Lowicryl HM20 for immunocytochemical studies. J Microsc 1992; 168: 85.
25. Videm V, Fosse E, Mollnes TE, Garred P, Svennevig JL. Time for new concepts about measurement of complement activation by cardiopulmonary bypass? Ann Thorac Surg 1992; 54: 725.
26. Elliott MJ, Finn AH. Interaction between neutrophils and endothelium. Ann Thorac Surg 1993; 56: 1503.
27. Mammen EF, Koets MH, Washington BC, Wolk LW, Brown JM, Burdick M, Selik NR, Wilson RF. Hemostasis changes during cardiopulmonary bypass surgery. Semin Thromb Hemost 1985; 11: 281.
28. Sahu A, Pangburn MK. Identification of multiple sites of interaction between heparin and the complement system. Mol Immunol 1993; 30: 679.
29. Maillet F, Petitou M, Choay J, Kazatchkine MD. Structure-function relationships in the Inhibitory effect of heparin on the complement activation: independency of the anti-coagulant and anti-complementary sites on the heparin molecule. Mol Immunol 1988; 25: 917.
30. Faymonville ME, Pincemail J, Duchateau J, Paulus JM, Adam A, Larbuisson R, Limet R, Lamy M. Myeloperoxidase and elastase as markers of leukocyte activation during cardiopulmonary bypass in humans. J Thorac Cardiovasc Surg 1991; 102: 309.
31. Cairo MS, Allen J, Higgins C, Baehner RL, Boxer LA. Synergistic effect of heparin and chemotactic factor on polymorphonuclear leukocyte aggregation and degranulation. Am J Pathol 1983; 113: 67.
32. Kestin AS, Valeri CR, Khuri SF, Loscalzo J, Ellis PA, MacGregor H, Birjiniuk V, Ouimet H, Pasche B, Nelson MJ, Benoit SE, Rodino LJ, Barnard MR, Michelson AD. The platelet function defect of cardiopulmonary bypass. Blood 1993; 82: 107.