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ORIGINAL ARTICLE

Influence of hypersulfated and low molecular weight heparins on ischemia/reperfusion: injury and allograft rejection in rat kidneys

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Summary

The aim of the study was to evaluate the effect of the hypersulfated nonanticoagulant heparin derivative LU 51198 (LU) and of the low molecular weight heparin reviparin (REVI) on ischemia/reperfusion (I/R) injury, acute rejection (AR) and chronic allograft nephropathy (CAN) in rats. Organs were harvested 5 days after 60 min of renal I/R injury. For investigation of AR and CAN we used the allogeneic Fisher–Lewis model. Kidneys were harvested at one respectively 32 weeks after transplantation. Rats were treated with either vehicle, LU or REVI. After I/R injury, treatment with REVI or LU reduced infiltration with MHC II and R73-positive cells, whereas only REVI reduced ED1-positive cells and expression of monocyte chemoattractant protein-1. There was no effect of REVI and LU on acute allograft rejection. Treatment with LU or REVI reduced glomerular infiltration with ED1 and MHCII-positive cells and renal expression of transforming growth factor-beta 32 weeks after transplantation. Only REVI treatment reduced albuminuria, interstitial infiltration and histological signs of CAN. LU, and in a more potent manner REVI, reduce signs of CAN and renal inflammation after I/R injury. Chemically modified heparins without anticoagulatory effects may offer a new treatment option in preventing I/R injury and CAN in human kidney transplantation.

Introduction

Chronic allograft nephropathy (CAN) is the most common cause for late graft loss besides death with functioning graft. The clinical course is characterized by gradual deterioration of graft function as manifested by slowly rising plasma creatinine, increasing proteinuria and worsening hypertension [1]. Both alloantigen-dependent and -independent mechanisms such as brain death, cold preservation, or ischemia/reperfusion (I/R) injury [2,3], play a role in the development of CAN [4]. Delayed graft function due to ischemic renal damage has been associated with late renal allograft deterioration and failure [5,6]. Although the precise mechanisms of I/R injury have not been clarified, a large number of cytokines and mediators have been suggested to play a pathogenetic role.

Besides the clinical use for prevention and treatment of thromboembolic diseases, heparins possess anti-inflammatory, antiproliferative, and antioxidative properties [7–11]. Heparins inhibit the interferon-gamma induced upregulation of MHC II on tubular and endothelial cells [8] and reduce the expression of transforming growth factor-beta (TGF-beta) in kidney tissue of diabetic rats [12]. Antiproliferative effects of heparins

have been shown in experimental models of mesangial cell proliferation and mesangioproliferative glomerulonephritis [7,13]. In addition heparins can reduce tissue damage caused by free oxygen species [14,15], which has been demonstrated in an experimental model of heart I/R injury [16,17].

Several studies have shown that treatment with low molecular weight heparins (LMWH) or pentosan polysulfate reduces allograft rejection in heart transplantation in rats [18–20]. In humans it has been shown that heparin administration improves biopsy proven chronic rejection [21] and may reduce severe proteinuria after transplantation [22]. We have recently shown that treatment with the LMWH reviparin reduces signs of CAN in an allogeneic rat model [23]. We hypothesized that the protective effects of reviparin on CAN might be attributable to a reduction of either I/R injury, acute allograft rejection, or both. As the grade of sulfatation plays a major role in the anti-inflammatory effects of heparins we investigated the hypersulfated heparin derivative LU 51198 devoid of any anticoagulatory effects compared with the LMWH reviparin in rat models of I/R injury, acute rejection (AR) and CAN.

Methods

Animals

Inbred male Fisher (F344, $RT1^{1vr}$) and Lewis (LEW, $RT1^{1}$) rats, all weighing between 180 and 200 g, were used as graft donors (Fisher for allografts; Lewis for isografts) or recipients (Lewis), respectively. Animals were kept under standard conditions and fed standard rodent chow and water *ad libitum*. Animals were purchased from Harlan Winkelmann GmbH, Borchen, Germany. All procedures were performed according to the 'Guide for the Care and Use of Laboratory Animals' published by the National Academy of Sciences and have been approved by the local animal care committee (Regierungspräsidium Karlsruhe; AZ 37–9185.81/145/97).

Surgical procedures

All surgical procedures were performed under isoflurane anesthesia. For induction of I/R injury a right nephrectomy was performed in Fisher rats, and the vessels of the remaining left kidney were clamped for 60 min. After an ischemic period of 60 min the clamps were removed and reperfusion was started. Serum creatinine was measured daily after I/R injury and organs were harvested on day 5. In the allogeneic renal transplantation model, Fisher rats served as donors and Lewis rats as recipients, Lewis–Lewis served as isogeneic controls. Operation procedures were performed as previously described [24]. In the model of AR kidneys were harvested after 7 days and no immunosuppression was given to the recipients in the treatment groups. In the model of CAN kidneys were transplanted into unilaterally nephrectomized recipients, the second native kidney was removed after 10 days and animals were subsequently observed for 32 weeks. All recipients received immunosuppression with cyclosporin A 5 mg/kg (Sandimmun®, Novartis, Nürnberg, Germany) for 10 days.

Experimental protocols

Ischemia/reperfusion injury

Rats received either isotonic saline (IR-VEH), reviparin 2 mg/kg (IR-REVI) or LU 51198 2 mg/kg (IR-LU) by daily subcutaneous injection (s.c.) starting 1 h before induction of I/R injury until organs were harvested after 5 days ($n = 6$ each group).

Acute allograft rejection

Four groups of allogeneic transplanted animals were studied ($n = 4$ each group). In group 1 (AR-VEH) recipients received vehicle s.c., no immunosuppression was given. In group 2 (AR-CsA) animals received vehicle s.c. and cyclosporin A 5 mg/kg/day intramuscularly. Animals of group 3 (AR-REVI) received reviparin 2 mg/kg/day s.c. without immunosuppression. In group 4 (AR-LU) animals received LU 51198 2 mg/kg/day s.c. without immunosuppression. In group 1, 3 and 4 no immunosuppression was given to allow the development of AR in a minor MHC-mismatch model (Fisher to Lewis).Treatment was started 1 h before transplantation and continued until harvesting of organs on day 7 after transplantation in all groups.

Chronic allograft nephropathy

Six groups of animals were studied ($n = 12$ each group). Group 1 (ISO-VEH) served as isogeneic control (Lewis to Lewis), animals were treated with vehicle daily s.c. for 32 weeks. Group 2 was transplanted allogeneically (Fisher to Lewis) and recipients received vehicle (ALLO-VEH). Animals of group 3 (UNI-VEH) were not transplanted, a right nephrectomy was performed on day 10, the left kidney was mobilized but not subjected to ischemia and recipients received vehicle. Animals of group 4 and 5 were allogeneically transplanted and recipients were treated with reviparin 2 mg/kg/day (ALLO-REVI) or LU 51198 2 mg/kg/day (ALLO-LU-L) s.c. for 32 weeks. In group 6 animals received a short-time treatment with LU 51198. Recipients were treated with LU 51198 2 mg/kg s.c. starting 3 days before surgery, and treatment was continued until day 5 after transplantation (ALLO-LU-K). In all groups animals received immunosuppression with cyclosporin A

5 mg/kg/day for 10 days after surgery to prevent AR episodes. All organs were harvested after 32 weeks.

Functional studies

Blood samples for determination of serum creatinine (Dimension®; Dade-Behring, Frankfurt, Germany) were taken daily after induction of I/R injury and organs were harvested on day 5 for further investigation. In the model of CAN rats were housed for 24 h in individual metabolic cages on weeks 8, 20, 28 and 32 after transplantation. Urine volume was determined gravimetrically, protein concentration in 24 h urine samples was determined using the method of Coomassie, and blood samples for measurement of serum creatinine (Dimension®; Dade-Behring) were taken at the end of each 24 h period.

Histology

Kidneys were removed at the end of the experiment after retrograde *in situ* perfusion at systemic pressure with 2% buffered formalin for 3 min. Before the start of the perfusion, the upper poles of the renal graft were dissected and snap-frozen in liquid nitrogen for the preparation of frozen sections to perform immunohistochemistry and PCR analysis. The remainder of the graft was immersion-fixed in 5% neutrally buffered formalin. Paraffin embedding was performed using routine procedures. Paraffin sections were stained with hematoxylin/eosin, periodic acid-Schiff, and trichrom. A Zeiss Axioscope Light microscope (Carl Zeiss, Zena, Germany) was used for microscopic evaluation. A minimum of 20 microscopic fields per each graft was assessed at ×100–×400 magnification. Several histomorphological variables derived from the Banff system that reported on renal transplant biopsies [25] were evaluated and graded by a pathologist (V.N.) masked for the different treatment groups. Histologic evaluation and grading included transplant glomerulopathy, tubulo-interstitial fibrosis, and tubular atrophy. The histologic grading scale was from 0 to 3 (0 = not present, 1 = mild alteration, 2 = moderate alteration, and 3 = severe alteration).

Immunohistology

At the end of the observation period upper poles of kidneys were snap-frozen in liquid nitrogen. Sections of 6 μm were fixed in ethanol and incubated with mouse anti-rat antibodies. Sections were then stained with biotinylated horse anti-mouse antibody with the avidin-biotinylated-peroxidase immunolabeling method (Vector Laboratories Inc., Burlingame, CA, USA) and counterstained with hematoxylin. Cell populations and cell surface markers were assessed with monoclonal antibodies to macrophages and monocytes (ED1), T cells (R73), and MHC II (F17–23–2). Negative controls included omission of the first antibody or murine control immunoglobulin (all antibodies were purchased from Serotec Ltd, Kidlington, England). Positive cells were expressed as mean ± SEM of cells per field of view. More than 20 fields of view per section were evaluated at ×400 magnification.

TGF-beta and MCP-1 PCR

For molecular studies, upper poles of the kidneys were immediately frozen in liquid nitrogen and stored at −80 °C. Frozen tissue was homogenized (IKA Labortechnik, Fischer Scientific, Schwerte, Germany), RNA-isolation and c-DNA-synthesis was performed by a protocol of GIBCO BRL/Life Technologies (Gaithersburg, MD, USA). For TGF-beta the following primer sequences were used: forward 5′-CTAGTCTCTGTCATACT-3′, reverse 5′-CTGCGAGCTAGTGA-3′. For monocyte chemoattractant protein-1 (MCP-1) the following primer sequences were used: Forward 5′-ATGCAGTCTCTGTCACG-3′, reverse 5′ CTAGTTCTCTGTCATACT-3′. For the house keeping gene porphobilinogen-deaminase (PBGD) the following primer sequences were used: forward 5′CAG-TCAGCATCGCTACA-3′, reverse 5′ATGTCGTACGCGC-3′. PCR products (10 μl) were analyzed by gel electrophoresis using a 4% agarose gel (Sigma, Munich, Germany). Electrophoresis was performed with a constant voltage of 80 V for 90 min in Tris-borate-EDTA (TBE)-buffer (PAN Biotech, Aidenbach, Germany). DNA was visualized on an UV-table and digitalized using specific software (Bio Doc I/NT, Biometra, Hannover, Germany). The density of the band was analyzed with the same system.

Data calculation and statistical evaluation

Data are shown as mean ± SEM. Kruskal–Wallis test and Mann–Whitney *U*-test were applied for comparison of means between different groups (StatsDirect version 2.2.2, StatsDirect Ltd., Altrincham, UK). Comparison of the histological data was performed by Fisher's-exact test. Statistical significance was defined as a P-value <0.05.

Results

Ischemia/reperfusion injury

In all groups I/R injury induced a significant impairment of renal function as measured by an increase in serum creatinine. Treatment with reviparin (I/R-REVI) or LU 51198 (I/R-LU) significantly prolonged increase of serum creatinine after I/R injury (Fig. 1a). Five days after I/R injury renal function was not different between the

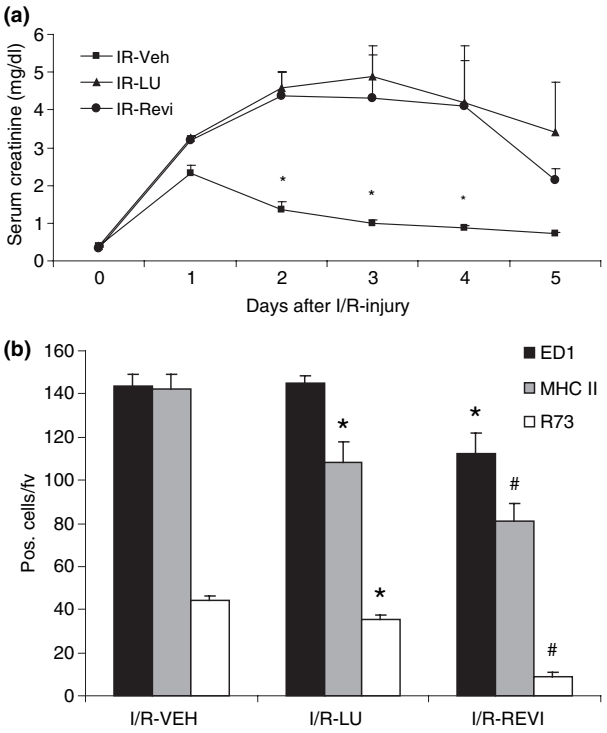

Figure 1 (a) Serum creatinine (mg/dl) on days 0–5 after 60 min of I/R injury. Animals treated with isotonic saline (I/R-VEH), reviparin (I/R-REVI) or LU 51198 (I/R-LU) for 5 days after I/R injury. * = $P < 0.05$ I/R-VEH vs. I/R-REVI and IR/LU; ANOVA. (b) ED1-, MHC II-, and R73-positive cells 5 days after I/R injury. Animals treated with isotonic saline (I/R-VEH), reviparin (I/R-REVI) or LU 51198 (I/R-LU) for 5 days after I/R-injury. * = $P < 0.05$ I/R-LU vs. I/R-VEH and I/R-REVI (ED1) vs. IR-VEH, # = $P < 0.001$ I/R-REVI vs. IR-VEH.

groups. Reviparin significantly reduced infiltration with ED1-, MHC II-, and R73-positive cells, whereas LU 51198 only reduced, to a smaller extend, MHC II- and R73-positive cells (Fig. 1b). Five days after I/R injury treatment with reviparin significantly decreased expression of MCP-1 in renal tissue (Table 1), whereas LU 51198 had no influence on MCP-1 expression.

Acute allograft rejection

Five days after allogeneic transplantation treatment with LU 51198 (AR-LU) or reviparin (AR-REVI) did not influence infiltration with MHC II-, ED1-, or R73-positive cells in renal tissue compared with vehicle (AR-VEH) (Fig. 2). Treatment with cyclosporin A (AR-CsA) prevented renal inflammation as measured by a marked decrease in MHC II-, ED1-, or R73-positive cells in renal tissue. Light microscopically reviparin and LU 51198 did not significantly reduce signs of AR, whereas treatment with cyclosporin A prevented the appearance of signs of AR after 5 days. Expression of MCP-1 was reduced after treatment with both reviparin and cyclosporin A. LU 51198 did not reduce MCP-1 expression compared with vehicle (Table 1).

Chronic allograft nephropathy

Treatment with reviparin significantly reduced albuminuria 28 and 32 weeks after transplantation (Fig. 3). LU 51198 treatment for 32 weeks or for 1 week after transplantation had no influence on albuminuria. Isogeneic transplanted animals (ISO-VEH) showed a slight but insignificant rise in albuminuria after 32 weeks compared with uninephrectomized rats (UNI-VEH). Albuminuria was significant lower in ISO-VEH and UNI-VEH groups compared with all other groups at all time points. Both LU 51198 or reviparin treatment over 32 weeks reduced glomerular infiltration with ED1- and MHCII-positive cells down to the level of isogeneic controls (Fig. 4a). Only reviparin treatment significantly reduced interstitial infiltration with ED1-and MHCII-positive cells down to the level of isogeneic controls (Fig. 4b). In contrast to vehicle or LU 51198, reviparin treatment significantly reduced both glomerulopathy and tubular atrophy after 32 weeks (Fig. 4c–h). Renal expression of TGF-beta after 32 weeks was significantly reduced after treatment with both LU 51198 (0.85 ± 0.27; relation TGF-beta/PBGD) and reviparin (0.67 ± 0.056) compared with vehicle (1.85 ± 0.24) (Table 2). Expression of MCP-1 was not influenced by reviparin or LU 51198 treatment (Table 1).

Discussion

This study shows that both treatment with the LMWH reviparin as well as with the hypersulfated LMWH derivative LU 51198 reduce signs of CAN and renal inflammation after I/R injury, without any influence on acute allograft rejection in rats. These results confirm and

Table 1. MCP-1 in renal tissue. Relation MCP-1/PBGD 5 days after I/R injury (I/R), 5 days (AR) and 32 weeks (CR) after allogeneic transplantation in renal tissue of animals treated with isotonic saline (VEH), reviparin (REVI) or LU 51198 (LU).

MCP-1/PBGD	VEH	REVI	LU	$P < 0.05$
I/R	3.16 ± 0.34	1.67 ± 0.06	2.45 ± 0.21	IR-REVI vs. IR-VEH
AR	3.67 ± 0.54	2.01 ± 0.25	2.96 ± 0.67	AR-REVI vs. AR-VEH
CR	0.81 ± 0.33	0.93 ± 0.16	0.85 ± 0.27	Not significant

MCP, monocyte chemoattractant protein-1; PBGD, porphobilinogen-deaminase; I/R injury, ischemia/reperfusion injury; AR, acute rejection.

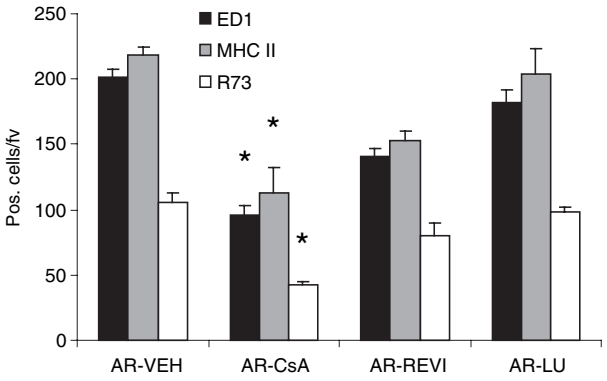

Figure 2 ED1-, MHC II-, and R73-positive cells 5 days after allogeneic transplantation. Animals treated with isotonic saline (AR-VEH), cyclosporine A (AR-CsA), reviparin (AR-REVI), LU 51198 (AR-LU) for 5 days after transplantation. Animals treated with cyclosporin A showed a significant reduction of infiltration with ED1-, MHC II-, and R73-positive cells compared with all other groups. * = $P < 0.05$ AR-CsA vs. all other groups.

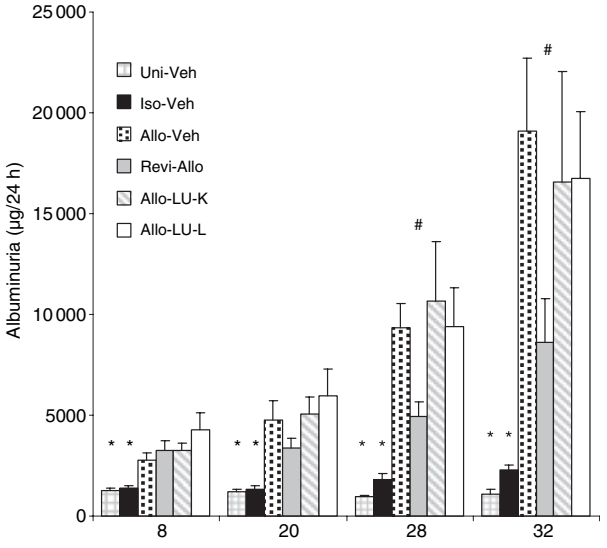

Figure 3 Albuminuria (µg/24 h) at 8, 20, 28 and 32 weeks after transplantation. Allografts treated with reviparin (ALLO-REVI), LU 51198 (ALLO-LU-L), or isotonic saline (ALLO-VEH) for 32 weeks or 7 days (ALLO-LU-K), and isografts treated with isotonic saline (ISO-VEH) or uninephrectomized animals treated with vehicle (UNI-VEH) for 32 weeks. # = $P < 0.05$ ALLO-REVI vs. ALLO-VEH, * = $P < 0.01$ ISO-VEH and UNI-VEH vs. all other groups.

expand findings of a prior study showing that treatment with reviparin over a period of 12 or 32 weeks reduces CAN in rats [23].

The protection provided by either reviparin or LU 51198 treatment seems not to be mediated by an influence on acute allograft rejection, as no relevant effects with respect to the presence of inflammatory cells in grafts were observed. Therefore, our data indicate that the protective effect of reviparin and LU 51198 treatment on CAN is more likely to be mediated by a reduction of renal inflammation after initial I/R injury, reduction of TGF-beta expression, and long-term reduction of interstitial infiltration with ED1- and MHC II-positive cells. Compared with LU 51198, reviparin more effectively prevented renal inflammation after I/R injury as well as MCP-1 expression and monocyte infiltration after 32 weeks, lending further support for an important role of early intervention for preventing long-term graft damage. Despite an initial increase in serum creatinine after I/R injury in LU and REVI treated rats, both treatment groups showed a significant reduction in renal inflammation as shown by a reduction of renal infiltration with ED1, MHC II and R73 positive cells. The effect of reduced nephron mass due to ischemia induced tubular necrosis leading to a decline in renal function, has been emphasized in both animal experiments [26] and observations in transplanted patients [27], but we and others favor a role for monocytes/macrophages in the chronic response to acute I/R injury [28]. Nevertheless, the reason for the increase in serum creatinine in both treatment groups remains to be obscure and has to be investigated in future studies. MCP-1 is a potent chemoattractant protein for monocyte recruitment. Treatment with reviparin reduced expression of MCP-1 in renal tissue 5 days after both I/R injury and allogeneic transplantation. This was paralleled by a reduced infiltration of ED-1 positive cells in renal tissue. The inhibition of macrophage activation has been shown to decrease functional and histological signs of chronic rejection in a rat model of renal transplantation confirming the importance of macrophages and macrophage-derived factors in chronic rejection [29].

In our CAN model in both treatment groups the main reduction of infiltrating cells was observed in the glomerula. This phenomenon has also been observed in prior studies, where the protective effects of heparins were investigated in experimental models of proliferative glomerulonephritis and glomerulosclerosis. Thus, heparin treatment reduced glomerular injury in rats with

Table 2. TGF-beta in renal tissue. Relation TGF-beta/PBGD in renal tissue 32 weeks after allogeneic transplantation and treatment with isotonic saline (ALLO-VEH), reviparin (ALLO-REVI) or LU 51198 (ALLO-LU-L) and isogeneic transplantation and treatment with vehicle (ISO-VEH).

	ISO-VEH	ALLO-VEH	ALLO-LU-L	ALLO-REVI
TGF-beta /PBGD	0.23 ± 0.043*	1.85 ± 0.24	0.85 ± 0.27**	0.67 ± 0.056**

*$P < 0.05$ vs. all other groups (anova), **$P < 0.05$ ALLO-REVI or ALLO-LU-L vs. ALLO-VEH.

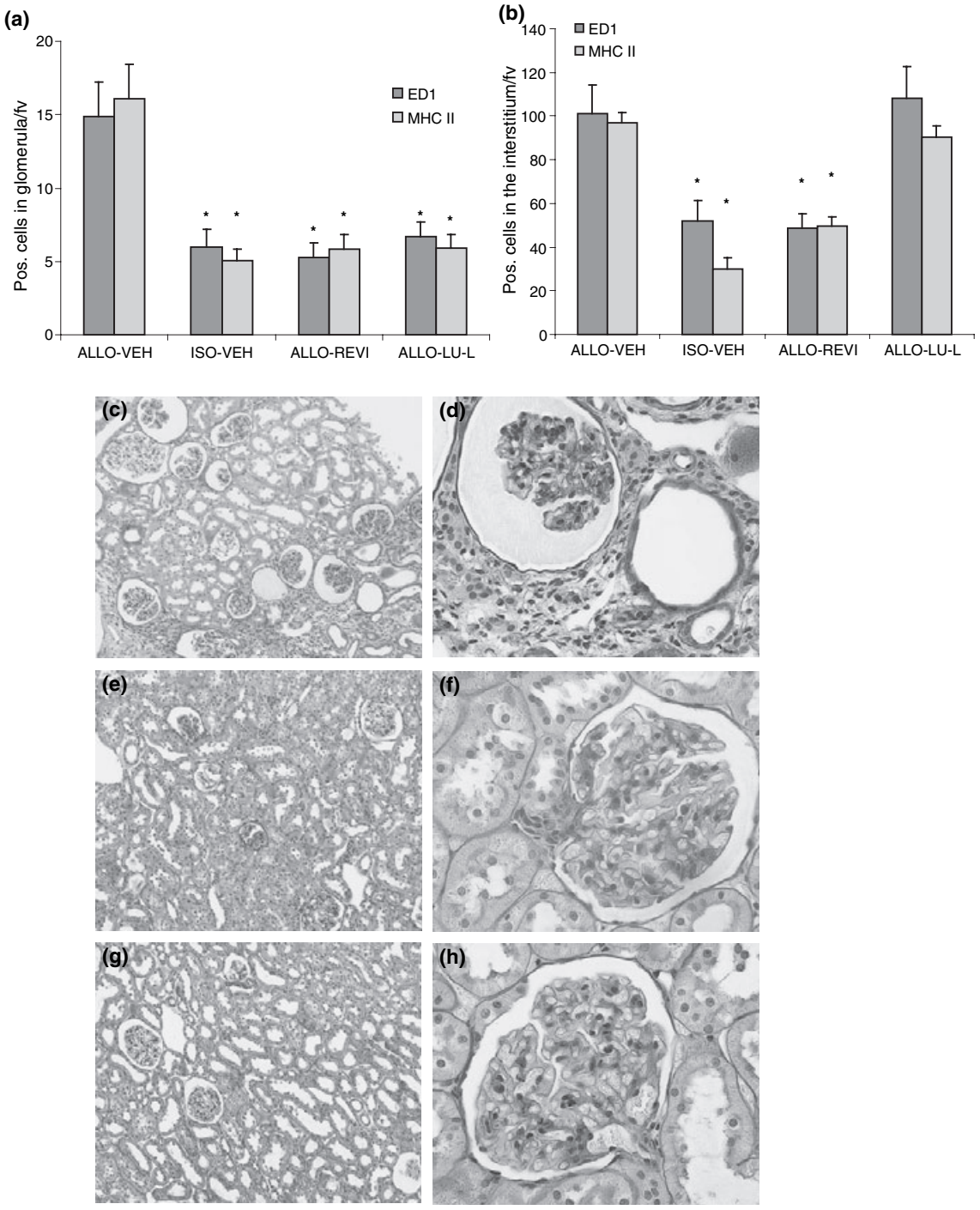

Figure 4 ED1-, MHC II-, and R73-positive cells 32 weeks after transplantation in the glomerula (a) or in the interstitium (b). Allografts treated with isotonic saline (ALLO-VEH), reviparin (ALLO-REVI) or LU 51198 (ALLO-LU-L), or isografts treated with isotonic saline (ISO-VEH) for 32 weeks after transplantation. * = $P < 0.001$ vs. ALLO-VEH; ANOVA. (c–h) Light microscopy (hematoxilin/eosin staining) of interstitial area (c, e, g) and glomerula (d, f, h) 32 weeks after transplantation. Animals treated with isotonic saline (c and d), LU 51198 (e and f) or reviparin (g and h). Significant signs of chronic allograft nephropathy as interstitial fibrosis, tubular atrophy and glomerulosclerosis in vehicle treated rats (c and d), compared with animals treated with reviparin (g and h) with only marginal signs of tubular atrophy and glomerulosclerosis, $P < 0.05$, Fisher's exact test; magnification ×100 (c, e, g) and ×400 (d, f, h).

adriamycin-induced nephropathy, but did not modify tubulointerstitial damage [30]. Furthermore, treatment with modified heparins prevented puromycin- and diabetes-induced glomerulosclerosis in rats by reducing the glomerular infiltration with inflammatory cells as well as by reducing TGF-beta expression in renal tissue [12,25].

Transforming growth factor-beta is a potent growth factor capable to mediate many of the events that are involved in later stages of renal repair [31]. It has been shown previously that TGF-beta inhibits proliferation of renal proximal tubule cells *in vitro* and stimulates extracellular matrix synthesis, tubulogenesis, cell clustering, and apoptosis [24,31,32]. Both TGF-beta mRNA and protein expression is rapidly enhanced in damaged and regenerating proximal tubules and remains elevated for up to 14 days after renal ischemia [33]. Moreover, recent studies have shown a decrease of TGF-beta expression after heparin treatment in mesangial cells in high glucose conditions [34], in proximal tubular epithelial cells cultured in human serum albumin under basal conditions [35], and in an experimental model of puromycin induced glomerulosclerosis [25]. An elevation of renal TGF-beta expression has been demonstrated in rats as well as in human grafts biopsies with chronic rejection, suggesting that TGF-beta may play a role in the pathogenesis of fibrosis in CAN [36,37]. We have shown a significant decrease in renal TGF-beta expression 32 weeks after allogeneic transplantation and treatment with the LMWH reviparin or its derivative LU 51198. Therefore, down regulation of renal TGF-beta expression might be responsible for both the reduced glomerular infiltration with inflammatory cells as well as the decrease of glomerulosclerosis observed in our treatment groups.

Treatment with reviparin resulted in a significant reduction of albuminuria 32 weeks after allogeneic transplantation. We reported previously on two patients with marked proteinuria after renal transplantation, in whom treatment with a LMWH led to a significant and long-lasting reduction of proteinuria, indicating that LMWH may possess renoprotective properties, thus confirming previous data from experimental nephropathies [22].

The degree of sulfation of heparins plays an important role in the modulation of inflammatory cascades [8]. However, the hypersulfated heparin derivative LU 51198 was less effective than the LMWH reviparin in reducing renal inflammation after I/R injury and CAN in our model. This suggests that the degree of sulfation does not correlate with the protective properties of heparins in this model. In conclusion we have shown that treatment with both the hypersulfated LMWH derivative LU 51198 and in a more potent manner the LMWH reviparin reduces CAN and renal inflammation after I/R injury without an influence on acute allograft rejection in rats. Protection may be mediated by reducing MCP-1 and TGF-beta expression in the kidney. LMWH with or without anticoagulatory effects might be a treatment option in preventing I/R injury and CAN in human kidney transplantation.

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