Dimethylarginine metabolism during acute and chronic rejection of rat renal allografts

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Abstract

**Background.** Dimethylarginines are inhibitors of NO synthesis and are involved in the pathogenesis of vascular diseases. In this study, we ask the question if asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels change during fatal and reversible acute rejection, and contribute to the pathogenesis of chronic vasculopathy.

**Methods.** The Dark Agouti to Lewis rat strain combination was used to investigate fatal acute rejection. Fischer 344 kidneys were transplanted to Lewis rats to study reversible acute rejection episode and the process of chronic rejection. Isograft recipients and untreated Lewis rats were used as controls. L-arginine derivatives were determined by HPLC, and ADMA-metabolizing enzymes were studied by quantitative RT–PCR and western blotting.

**Results.** Renal transplantation transiently increased dimethylarginine levels independent of acute rejection. ADMA plasma levels did not importantly differ between recipients undergoing fatal or reversible acute rejection, whereas SDMA was even lower in recipients of Fisher 344 grafts. In comparison to isograft recipients, ADMA and SDMA levels were slightly elevated during reversible, but not during the process of chronic rejection. Increased dimethylarginine levels, however, did not block NO synthesis. Interestingly, protein methylation, but not ADMA degradation, was increased in allografts.

**Conclusions.** Our data do not support the concept that renal allografts are protected from fatal rejection by dimethylarginines. Dimethylarginines may play a role in triggering chronic rejection, but a contribution to vascular remodelling itself is improbable. In contrast, differential arginine methylation of yet unknown proteins by PRMT1 may be involved in the pathogenesis of acute and chronic rejection.

Keywords: ADMA; kidney transplantation; L-arginine; rat; SDMA

Introduction

Dimethylarginines have moved into the spotlight of scientific interest as endogenous inhibitors of nitric oxide synthesis. They are potential mediators of endothelial dysfunction, hypertension and vascular remodelling, and seem to be involved in chronic kidney diseases [1–7]. NO synthesis involves cellular uptake of L-arginine (L-arg) by y+ transporters, which is inhibited by asymmetric dimethylarginine (ADMA) and by symmetric dimethylarginine (SDMA) [1,6]. These transporters are also needed for renal L-arg absorption and contribute to the maintenance of systemic L-arg levels. L-arg can be converted to NO and citrulline by NOS [2].

Dimethylarginine metabolism is also of outstanding interest in the context of transplantation, predominantly because the NOS isoforms, endothelial NOS (eNOS) and...
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inducible NOS (iNOS), play protective and deleterious roles in acute and chronic allograft rejection [8–14]. Acute and chronic allograft rejection are linked to each other, as acute rejection is an important clinical risk factor for the development of chronic rejection [15] and experimental evidence suggests that chronic rejection is irreversibly triggered during acute rejection episodes [16]. An important hallmark of chronic rejection of kidneys is the development of allograft vasculopathy, a severe intimal hyperplasia of renal arteries. The idea suggests itself that increased levels of dimethylarginines may play a role in the pathogenesis of these lesions. Indeed, clinical and experimental data evidenced that dimethylarginines contribute to cardiac allograft vasculopathy [17,18], which resembles vasculopathy of renal allografts [19].

ADMA and SDMA are elevated in patients suffering from renal disease [3,20–24]. Moreover, ADMA levels correlate negatively with renal function, and positively with mortality and cardiovascular complications [25]. In patients undergoing dialysis, ADMA and SDMA are also increased [23]. Transplantation reduces SDMA [26], whereas its effect on ADMA is disputed [23,24,26,27].

ADMA synthesis starts with the methylation of protein arginine residues by protein arginine methyltransferases (PRMTs) [28], which catalyse the formation of mono- methylarginine (MMA) from L-arg [29]. These enzymes are classified into type I (PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8) and type II (PRMT5, PRMT7 and FBXO10). Type I PRMTs produce ADMA, while type II PRMTs produce SDMA [28]. After proteolytic degradation of methylated proteins, MMA, SDMA or ADMA are released and in part cleared by renal excretion [30]. In addition, ADMA, but not SDMA, is degraded in the liver, kidney, and other organs to citrulline and dimethylamines by dimethylarginine dimethylaminohydrolases (DDAH), DDAH1, and DDAH2 [31,32].

The purpose of this study is to answer two questions: (i) do dimethylarginine levels change during fatal and reversible acute allograft rejection, and (ii) do dimethylarginines and their metabolism contribute to the pathogenesis of chronic vasculopathy? Therefore, we investigate dimethylarginine metabolism during renal allograft rejection as well as NOS expression and activity. An experimental model of fatal acute rejection of rat renal allografts is compared with a model for chronic allograft rejection, which involves a reversible rejection episode: (i) kidney transplantation in the fully allogenic, Dark Agouti (DA) to Lewis (LEW) rat strain combination leads to acute graft destruction 4–5 days post-transplantation. Graft recipients are investigated on Day 4. (ii) Kidneys transplanted in the Fischer 344 (F344) to LEW combination remain functional for at least 6 months but undergo reversible acute rejection around Day 9 [33], and develop allograft vasculopathy in the long run [34]. Those recipients are investigated on Day 9 and during vascular remodelling on Day 42.

Materials and methods

Details of materials and methods used in these studies are given in the online supplementary data.
ADMA (A), SDMA (B) and L-arg (C) were quantified by HPLC in the blood plasma from healthy control rats (C, white), isograft recipients (iso, dotted), and allograft recipients of DA and F344 donors (allo, grey). Graft recipients were investigated on Day (d) 4, 9 and 42 post-transplantation. Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers); circles indicate data beyond 3 × standard deviation; P-values ≤0.05 and number of animals (n) used in the study are indicated.
ADMA in renal allograft rejection

Table 1. L-arg/ADMA, L-arg/SDMA, and ADMA/SDMA ratios in the plasma of control rats and renal graft recipients

<table>
<thead>
<tr>
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<th>L-arg/ADMA</th>
<th>L-arg/SDMA</th>
<th>ADMA/SDMA</th>
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<tbody>
<tr>
<td>Control</td>
<td>201 (137–229)</td>
<td>548 (360–602)</td>
<td>2.6 (2.6–2.9)</td>
</tr>
<tr>
<td>Iso, Day 4</td>
<td>164 (110–178)</td>
<td>265 (139–345)</td>
<td>1.5 (1.1–2.0)</td>
</tr>
<tr>
<td>Allo DA, Day 4</td>
<td>97 (93–113)</td>
<td>104 (66–175)</td>
<td>1.1 (0.6–1.5)</td>
</tr>
<tr>
<td>Iso, Day 9</td>
<td>156 (144–177)</td>
<td>592 (485–768)</td>
<td>3.7 (3.1–4.6)</td>
</tr>
<tr>
<td>Allo F344, Day 9</td>
<td>138 (123–149)</td>
<td>354 (267–389)</td>
<td>2.5 (2.1–2.6)</td>
</tr>
<tr>
<td>Iso, Day 42</td>
<td>174 (161–263)</td>
<td>432 (385–653)</td>
<td>2.4 (2.3–2.6)</td>
</tr>
<tr>
<td>Allo F344, Day 42</td>
<td>138 (121–159)</td>
<td>322 (123–351)</td>
<td>2.1 (1.0–2.5)</td>
</tr>
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</table>

The number of animals (n) used in the study is indicated.

\(^a\)Isograft recipient versus control.

\(^b\)Allograft versus isograft.

\(^c\)Allograft Day 4 versus allograft Day 9.

\(^d\)Allograft Day 9 versus allograft Day 42.

<table>
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<tr>
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<th>Median (min–max) (P-value)</th>
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<tr>
<td></td>
<td>Urea</td>
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<tr>
<td>Control</td>
<td>36 (30–41) (n = 5)</td>
</tr>
<tr>
<td>Iso, Day 4</td>
<td>49 (42–101) (0.008) (n = 5)</td>
</tr>
<tr>
<td>Allo DA, Day 4</td>
<td>58 (53–565) (0.310) (n = 5)</td>
</tr>
<tr>
<td>Iso, Day 9</td>
<td>57 (48–664) (0.008) (n = 5)</td>
</tr>
<tr>
<td>Allo F344, Day 9</td>
<td>98 (77–108) (0.016) (0.286) (n = 4)</td>
</tr>
<tr>
<td>Iso, Day 42</td>
<td>39 (35–49) (0.151) (n = 5)</td>
</tr>
<tr>
<td>Allo F344, Day 42</td>
<td>66 (59–178) (0.008) (0.286) (n = 5)</td>
</tr>
</tbody>
</table>

The number of animals (n) used in the study is indicated.

\(^a\)Isograft recipient versus control.

\(^b\)Allograft versus isograft.

\(^c\)Allograft Day 4 versus allograft Day 9.

\(^d\)Allograft Day 9 versus allograft Day 42.

parametric exact Mann–Whitney U-test (SPSS software version 15). Data were analysed in exploratory manner.

Results

Histopathology

Day 4 renal allografts (DA to LEW) were characterized by severe perivascular and interstitial mononuclear infiltrates, and isografts were almost unimpaired [36]. After transplantation in the F344-to-LEW rat strain combination, which results in chronic rejection in the long run, severe but reversible acute rejection developed peaking around Day 9 after surgery [33]. Day 9 histopathology resembled Day 4 DA-to-LEW kidneys, and the infiltrate was predominantly composed of macrophages and T cells (Supplementary Figures 1–3). On Day 42 (F344 to LEW), the infiltrate was reduced, and macrophages and T cells still surrounded blood vessels and exhibited a patchy distribution in the renal interstitium (Supplementary Figures 1–3). Day 9 and Day 42 isografts were almost normal (Supplementary Figures 1–3; see online supplementary material for a colour version of these figures).

ADMA, SDMA and L-arg plasma levels

In healthy control rats, the ADMA concentration was 2.10 (1.78–2.21)μmol/L, similar to previously published observations [40,41]. In comparison to controls, ADMA levels were increased in isograft recipients on Day 4 and 9 after transplantation, but returned to control levels on Day 42 (Figure 1A). Plasma ADMA levels of Day 9 allograft (F344) recipients were higher compared with Day 9 isograft and Day 42 allograft recipients (Figure 1A).

On Day 4, plasma SDMA levels were increased in isograft recipients compared with controls (Figure 1B). However, our data did not suggest that Day 4 allograft recipients differ from isograft recipients. SDMA levels attained control levels in Day 9 and 42 isograft recipients, but were elevated in Day 9 F344 allograft recipients compared with isograft recipients.

L-arg plasma levels tended to be lower 4 days after allogenic transplantation compared with isograft recipients (P = 0.095) and with F344 allograft recipients on Day 9 (Figure 1C). L-arg/ADMA and the L-arg/SDMA ratios are indicated in Table 1. L-arg/ADMA ratios as well as L-arg/SDMA ratios were decreased in plasma samples from allograft recipients during fatal acute rejection compared...
with the respective isograft recipients and with recipients of F344 allografts on Day 9 (Table 1).

Renal function

Plasma creatinine and urea concentrations were increased in all isograft recipients, except in Day 42 allografts, compared with untreated controls (Table 2). In comparison to isograft recipients, both urea and creatinine levels were increased in recipients of F344 kidneys on Day 9 and 42 (Table 2).

DDAH mRNA expression and activity

Quantitative RT–PCR did not confirm the assumption that DDAH1 mRNA expression in renal tissue differs between experimental groups (Figure 2A). In contrast, DDAH2 mRNA levels were lower in Day 4 allografts compared with isografts (Figure 2B). Renal DDAH function was analysed in vitro (Figure 2C and D), and equally high rates of ADMA degradation were observed in all experimental groups (Figure 2C and D). As expected, SDMA was not degraded (Figure 2C, lower panel).

PRMT1 protein expression

PRMT1 protein levels were examined by western blotting (Figure 3A, C and E). In both models, kidney allografts were characterized by an increase in PRMT1 expression, compared with appropriate isografts (Figure 3B, D and F). Of note, also Day 42 F344 allografts expressed higher PRMT1 levels (Figure 3F).

Immunohistochemistry using the same antibodies to PRMT1 was performed on renal isografts and allografts (Figure 4, Supplementary Figures 2 and 3). PRMT1 immunoreactivity was ubiquitously seen in all renal grafts. Besides a moderate cytoplasmic staining, a more conspicuous nuclear signal was present in numerous but not all cells. Structures morphologically compatible with distal tubules and collecting ducts as well as single glomerular cells were strongly immunopositive. Additionally, intensely stained cells were detected in perivascular regions and in the renal interstitium of all allografts. Numerous macrophages and T lymphocytes were detected in the same regions, suggesting that these strongly PRMT1-positive cells belong to the infiltrate (Figure 4, Supplementary Figures 2 and 3). PRMT1-positive infiltrates were less abundant in Day 42 F344 allografts (Supplementary Figures 2 and 3).

Protein methylation in renal tissue

To estimate renal PRMT function, methylated proteins were analysed by western blotting using antibodies to dimethylarginine–glycine repeats [42] (Supplementary Figure 4). Protein methylation increased during acute rejection. Most strikingly, in Day 42 allografts undergoing chronic rejection, several proteins exhibited impaired methylation. In addition, differences in the methylation of individual proteins were observed between control kidneys, isografts and allografts (details are given in Supplementary Tables 1–3).

Expression of iNOS and eNOS

Protein levels of iNOS and eNOS were examined by western blotting. As expected, iNOS was induced in DA allografts during fatal acute rejection (Figure 5A). No iNOS was detected in Day 9 and 42 F344 grafts (Figure 5B and C). In contrast, eNOS was equally expressed by Day 4 isografts and allografts (Figure 5D and E). A slight increase in eNOS expression was detected in Day 9 isografts, which in turn did not differ from Day 42 isografts (Figure 5F–I).

Tissue and plasma NOx

Our experiments did not detect differences in NOx plasma concentrations between controls and all isograft recipients (Figure 6A). During acute rejection, however, NOx levels markedly increased in allograft recipients of both models compared with isograft recipients (Figure 6A). As expected, NOx levels were lower in recipients of F344 grafts compared with recipients of DA grafts. Our data suggest that NOx returns to isograft recipient levels in F344 allograft recipients on Day 42.

Also, NOx isograft tissue levels did not differ from controls (Figure 6B). NOx levels increased in allografts undergoing fatal acute rejection (Figure 6B), but in Day 9 and 42 F344 kidneys, NOx levels were not elevated compared with isografts (Figure 6B).

Discussion

The most important findings of this study are (i) shortly after renal transplantation and recipient nephrectomy, ADMA and SDMA are increased, even in the absence of acute rejection. (ii) ADMA levels did not importantly differ between recipients undergoing fatal and reversible acute rejection, whereas SDMA concentrations are lower during reversible rejection. (iii) During reversible acute rejection preceding chronic allograft rejection, ADMA and SDMA plasma levels are slightly elevated in comparison with isograft recipient plasma. (iv) PRMT1 protein expression and function are increased in DA and in F344 allografts compared with isografts, probably due to graft-infiltrating leucocytes. (v) In spite of elevated systemic dimethylarginine concentrations, NOS is at least partially functional.
Concerning the question whether changes in dimethylarginine levels and their metabolism are involved in the pathogenesis of acute or chronic kidney rejection, only differences between isograft and allograft recipients are relevant (Supplementary Figure 5). The most striking differences are seen for PRMT1 expression and protein arginine methylation, which strongly increase in allograft recipients during fatal and reversible acute rejection and slightly increase during the process of vascular remodelling on Day 42. The resulting increased protein methylation, however, does not consistently lead to elevated dimethylarginine levels, which are only seen during reversible acute rejection. Accordingly, increased dimethylarginine levels may be involved both in reverting acute rejection and in triggering chronic rejection in F344 to LEW allografts at Day 9.

Fig. 3. PRMT1 expression in renal tissue. Homogenates of healthy control kidneys (C, white), isografts (iso, dotted), and allografts of DA and F344 donors (allo, grey) were separated by SDS–polyacrylamide (12%) gel electrophoresis. PRMT1 expression was (A, C, E) analysed by western blotting on Day (d) 4, 9 and 42 post-transplantation. The intensity of the resulting bands (B, D, F) was quantified by densitometry and divided by the values obtained for the house-keeping protein GAPDH. The mean of the PRMT1/GAPDH ratio of the controls was set to 1, and each individual value including the control values was calculated accordingly. Data are displayed as median (bar), 25–75 percentiles (box), and the highest and lowest data point (whiskers); P-values ≤0.05 and number of animals (n) used in the study are indicated.
However, be argued that they are not of functional relevance. Further interventional studies would answer these questions. Certainly, more studies are needed to understand the relevance of the vigorous changes in PRMT1 expression and protein methylation during organ rejection.

Increased ADMA and SDMA levels like those seen early after transplantation may interfere with the cellular uptake of L-arg by impairing NOS activity and renal re-uptake of L-arg [6]. During fatal but not during reversible acute rejection, systemic L-arg levels are reduced. As iNOS protein is strongly expressed concomitantly, probably more L-arg is consumed. Increased circulating ADMA inhibits NOS activity directly [22]. However, this does not seem to happen in our experimental setting: although dimethylarginine levels are increased in Day 4 allograft recipients, NOx levels are elevated in plasma and in graft tissue, suggesting that iNOS is at least partially functional. Furthermore, on Day 9 after allogenic transplantation, when ADMA levels are increased in comparison to Day 42, plasma and tissue levels of NOx are in the same range.

Possibly, high L-arg levels enable NOS function in spite of increased ADMA. It is indeed a matter of debate whether modest changes in dimethylarginine levels modulate NO production [43,44]. Previously, Cardonuel et al. [43] demonstrated in vitro that the L-arg/dimethylarginine ratio must at least decrease to 10 to elicit a physiological effect, and experiments in vivo demonstrated that elevated ADMA levels do not necessarily result in impaired NO production [45,46]. We observe L-arg/ADMA and L-arg/SDMA ratios above 100, suggesting that NOS is unimpaired. However, ADMA and SDMA may contribute to acute renal allograft rejection by NOS-independent mechanisms such as stimulation of monocyte adhesion [5,47] and production of reactive oxygen species [5,48].

The modulation of systemic dimethylarginine levels during acute rejection is a complex process (Supplementary Figure 5). Systemic dimethylarginine concentrations are regulated by at least four variables: (i) renal function [30], (ii) ADMA degradation by DDAHs [4], (iii) protein methylation by PRMTs [29] and (iv) degradation of methylated proteins [49].

(i) Renal dysfunction may contribute to increased dimethylarginine levels in graft recipients, which are typically nephrectomized resulting at least in a 50% reduction of the functional renal mass. Graft function is indeed impaired in all recipients, but as expected, isograft function on Day 9 and 42 is superior to allograft function. Clinical studies demonstrated that ADMA and SDMA levels are elevated in patients with renal disorders [23,24,50], and dimethylarginine levels are known markers of renal function [1,51,52].
(ii) ADMA, but not SDMA, is degraded by DDAHs [32,53]. As ADMA is enzymatically degraded in the kidney [54], we analysed DDAH expression and function in graft tissue. DDAH2 mRNA slightly decreased during fatal acute rejection, which was, however, not reflected in DDAH function. In contrast, early after ischaemia/reperfusion injury of rat kidneys, total DDAH function is impaired [46]. Our data suggest that DDAH function is restored within 4 days. We conclude that changes in ADMA levels are not due to impaired renal degradation. DDAH activity, however, may change in other organs such as the liver. (iii) PRMT1, the dominating enzyme resulting in asymmetric protein methylation [55], is increased in all allografts. Immunohistochemistry suggests that this is due to infiltrating leucocytes expressing high PRMT1 levels. This notion is also supported by western blot experiments revealing a much stronger expression of PRMT1 in normal rat spleens compared with kidneys (data not shown). Hence, increased PRMT1 expres-
sion in allografts may contribute to increased systemic ADMA levels. Changes in renal PRMT1 expression, however, cannot be responsible for increased ADMA concentrations in Day 4 isograft recipients. In addition to PRMT1, other enzymes involved in protein methylation remain to be investigated. Changes in the methylation of several yet unidentified proteins were detected in western blots using antibodies binding to dimethylarginine–glycine repeats [42]. Methylation of proteins regulates their function [28], which is probably the most important role of PRMT. For instance, cytokine gene expression by effector T lymphocytes critically depends on methylation of the signalling molecule NIP45 [56]. Interestingly, methylation of several proteins was almost absent in Day 42 allografts.

(iv) Dimethylarginines are released upon degradation of methylated proteins [49]. It can be predicted that tissue damage during surgery results in increased protein degradation. Accordingly, ADMA and SDMA levels are increased early after transplantation. To clarify the impact of tissue damage on dimethylarginine levels, an additional group of sham-operated animals could be included. We refrained from these experiments as the data of isograft recipients, which do not develop nephropathy, suggest that the initial increase in dimethylarginine levels is not responsible for allograft nephropathy. Furthermore, allograft rejection should result in an increased turnover of leukocytes, another mechanism which may increase dimethylarginine levels.

Interestingly, ADMA and SDMA plasma levels, as well as PRMT1 and eNOS protein concentrations, were increased in isograft recipients in comparison to controls. Several factors may contribute to these differences: ischaemia/reperfusion injury caused by transplantation [46], reduced renal function due to total recipient nephrectomy or due to renal growth induced by a reduction of the total renal mass in recipient rats.

In conclusion, our data do not support the concept that the fate of renal allografts during acute rejection is decided by dimethylarginines. Dimethylarginines may play a role in triggering chronic rejection, but a contribution to the process of vascular remodelling itself is improbable. In contrast, differential arginine methylation by PRMT1 may be involved in the pathogenesis of acute and chronic rejection.

Supplementary data
Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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