Dichotomous effects of rosiglitazone in transplantation-induced systemic vasodilator dysfunction in rats

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Abstract

Background. Transplantation-induced systemic endothelial dysfunction causes severe cardio-vascular morbidity and mortality after transplantation. Interventions that improve systemic endothelial function after transplantation and furthermore reduce intragraft vascular dysfunction might improve graft and patient survival. Treatment with the PPARγ agonist rosiglitazone is an intervention that potentially fulfills these criteria. In this study we determined the effect of rosiglitazone treatment on transplantation-induced endothelial dysfunction and vasomotor activity in an experimental model for chronic transplant dysfunction in rats.

Methods. Lewis abdominal aortic allografts were orthotopically transplanted into Brown Norway recipients that received either regular chow or chow containing rosiglitazone (~4.2 mg/day). Endothelium-dependent (response to metacholine) and total (response to sodium nitrite) vasodilatory responses were determined in autologous thoracic aortic rings using an ex vivo organ bath setup. Measurements were performed 8 weeks after transplantation.

Results. Aortic allografting induced systemic endothelial dysfunction as measured by reduced endothelium-dependent vasodilation in the recipient's vascular system. Rosiglitazone treatment restored endothelium-dependent vasodilatory responses to pre-transplantation levels. However, rosiglitazone treatment reduced the total dilatory response despite normalized endothelial function, indicating impairment of vascular smooth muscle cell vasomotor activity.

Conclusions. Rosiglitazone treatment after allogeneic transplantation restores endothelial function but impairs vascular smooth muscle cell vasomotor activity. This dichotomous effect of rosiglitazone might impede use of rosiglitazone after organ transplantation since this potentially increases cardiovascular risk despite improved endothelial cell function.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>ME</td>
<td>metacholine</td>
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<tr>
<td>PE</td>
<td>phenylephrine</td>
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<tr>
<td>PPAR-γ</td>
<td>peroxisome proliferator-activated receptor-γ</td>
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<tr>
<td>RSG</td>
<td>rosiglitazone</td>
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<tr>
<td>SED</td>
<td>systemic endothelial dysfunction</td>
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<td>SN</td>
<td>sodium nitrite</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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Introduction

After solid organ transplantation vascular dysfunction is an important problem, both within and outside the transplanted organ. Within the transplanted organ, transplant arteriosclerosis is a main contributor to transplant dysfunction, as has been shown for the heart\(^1\) and the kidney.\(^2\) The development of transplant arteriosclerosis is supposed to cause downstream ischemic tissue damage resulting in deterioration of graft function and graft loss eventually. Outside the transplanted organ, transplant-associated systemic endothelial dysfunction (SED) occurs, which is a main contributor to the elevated cardiovascular risk in transplant recipients. In renal transplantation, recipients are at increased risk to develop cardiovascular disease (CVD) which is associated with reduced patient survival independent of graft dysfunction.\(^3\) CVD in this population is multifactorial. By the time of transplantation most patients have established cardiovascular damage, related to pre-existent CVD as well as to the complex of factors associated with uremia.\(^4\) After transplantation uremia-related cardiovascular risk factors improve, but factors related to the transplanted state emerge. Among the latter, an important factor is new-onset diabetes mellitus, which is characterized by insulin-resistance and SED.\(^5\)\(^6\) SED in transplant recipients is associated with significant morbidity and mortality.\(^6\)\(^7\) SED after transplantation develops not only as a result of the pre-existent clinical condition, but also as a result of the systemic inflammatory burden caused by the ongoing, subclinical, rejection response.\(^9\)

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor family that modulate expression of genes involved in lipid and glucose metabolism.\(^10\) Thiazolidinediones, such as rosiglitazone (RSG), are synthetic agonists of PPAR\(_\gamma\), a specific subfamily of PPARs. Thiazolidinediones are prescribed to Type 2 diabetics as anti-hyperglycaemic drugs by virtue of their insulin-sensitizing effects.\(^11\) Since PPAR\(_\gamma\) is also expressed on vascular smooth muscle cells (VSMC’s) and endothelial cells (EC’s)\(^11\)\(^12\), treatment with PPAR\(_\gamma\) agonists modulate vascular biological processes.\(^13\) PPAR\(_\gamma\) agonists have been shown to improve vasodilator function under conditions associated with SED such as hypertension\(^14\)\(^15\), diabetes\(^16\), and metabolic syndrome.\(^17\)\(^18\) Furthermore, PPAR\(_\gamma\) agonists have been shown to attenuate intima hyperplasia (restenosis) after coronary stenting in both diabetic and non-diabetic patients.\(^19\)\(^22\) Reduced VSMC migration and proliferation might be the underlying mechanism of reduced neointima formation following treatment with PPAR\(_\gamma\) agonists.\(^23\)

These clinical data have been confirmed in animal experimental models of restenosis in (non) diabetic rodents.\(^24\)\(^26\) Since VSMC’s are the main constituents of neointimal lesions in both restenosis and transplant arteriosclerosis, we recently tested the efficacy of RSG to attenuate the development of transplant arteriosclerosis after experimental aorta transplantation in rats.\(^27\) Our data demonstrate that RSG is indeed a very effective drug to reduce the development of transplant arteriosclerosis after allogeneic transplantation. Mechanisms involved include suppression of the alloreactive immune response as well as direct antiproliferative effects on VSMC’s.\(^27\) Similar results were reported after treatment
with RSG and pioglitazone after experimental heart transplantation in rats and mice, respectively.\textsuperscript{28,29} 

Treatment with RSG thus attenuates development of transplant arteriosclerosis and furthermore has been shown to improve insulin resistance after renal transplantation.\textsuperscript{30,31} We now propose that RSG might have potential to additionally improve SED after transplantation independent of improving insulin resistance. Together these effects of RSG can be anticipated to reduce cardiovascular risk and thereby patient morbidity and mortality. However, a recent widely publicized meta-analysis published by Nissen \textit{et al.} concluded that treatment with RSG significantly increased risk for myocardial infarction and cardiovascular death.\textsuperscript{32} Although this study has some limitations\textsuperscript{33}, increased risk of myocardial infarction and heart failure in Type 2 diabetics treated with rosiglitazone was confirmed by a meta-analysis performed by Singh \textit{et al.}\textsuperscript{34} However, others could not confirm these data\textsuperscript{35,36} and therefore the precise effect of RSG on cardiovascular risk needs as yet to be established.

The potential hazard of RSG on increasing cardiovascular risk led us to test the effect of RSG on transplantation-induced SED and vasomotor activity. To this end we determined the endothelial (EC)-dependent and total vasomotor activity (that comprises an endothelium-dependent and an endothelium-independent VSMC response) in an experimental model of chronic transplant dysfunction and transplant arteriosclerosis \textit{i.e.} aorta allografting in rats. We show that long-term treatment with RSG in transplanted rats causes an overall net improvement of vasodilator function outside the transplant. Although RSG treatment restored EC function to pre-transplantation levels, RSG also has direct deleterious effects on VSMC contractility and dilatory capacity. These dichotomous effects of RSG on EC function and VSMC vasomotor activity might impede use of RSG in transplantation as a treatment for SED, CVD and long-term allograft loss.

\section*{Methods}

\textit{Rats}

Specified pathogen free male Lewis (Lew, RT-1\textsuperscript{l}) and Brown Norway (BN, RT-1\textsuperscript{n}) rats were obtained from Harlan (Horst, The Netherlands). Rats were 13 weeks of age and were maintained under clean conventional conditions. Animals were housed and treated in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and institutional guidelines.

\textit{Surgical procedures}

Under anesthesia (2\% halothane, 0.4 L/min O\textsubscript{2} and 0.4 L/min N\textsubscript{2}O) Lew aortic allografts were transplanted to BN recipients as described previously.\textsuperscript{37} Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor, perfused with saline and subsequently orthotopically transplanted into the recipient via end-to-end anastomosis. Total cold and warm ischemic time was consistently less than 25 minutes. Recipient rats did not receive anti-rejection therapy after transplantation.
**Rosiglitazone (RSG) treatment**

Recipient rats were maintained on standard rat chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) formulated with or without RSG (Avandia, GlaxoSmithKline, Zeist, The Netherlands). RSG was admixed in the chow to a final concentration of 0.026% wt/wt. In this study we aimed at treating the rats (having a mean BW of ~250 gr during the treatment period) with a concentration of ~15 mg RSG/day/kg BW *i.e.* ~3.75 mg RSG/rat/day. This concentration was chosen since it was shown previously that treatment with 10-30 mg RSG/day/kg BW is biologically active and results in normalization of HbA1c levels and significant lowering of plasma glucose and triglyceride levels in Zucker diabetic fatty (ZDF) rats treated with RSG for 8 weeks. Based on a measured average food intake of ~16 g/day/rat, the average RSG intake in our experiments was ~4.2 mg/day/rat which was close to our target concentration. Food intake between RSG-treated and non-treated rats did not differ throughout the duration of the experiment. Treatment with RSG started 1 wk before transplantation and was continued throughout the duration of the experiment (8 weeks). Using this protocol RSG plasma levels obtained were 2.2±0.3 μg/ml in treated animals and below detection levels in non-treated rats. Although the dose RSG per kg BW given to the rats was ~200x higher than the dose given to Type 2 diabetic patients (generally 4-8 mg/day), the peak plasma levels of RSG obtained in our experiments were ‘only’ 19x higher than the peak levels observed in healthy control subjects given a single oral dose of 8 mg RSG (peak levels: 117 ng/mL; http://www.gsk.com/products/prescription_medicines/us/medicines-ae.htm; accessed November 6, 2007) indicating a difference in pharmacokinetics of RSG between rats and humans.

**Measurement of vascular function**

During sacrifice, the thoracic aorta was carefully removed for further preparation *ex vivo*. Peri-aortic tissue was removed from the thoracic aorta and rings of approximately 2 mm length were cut. The rings were connected to an isotonic displacement transducer at a preload of 14 mN in an organ bath with Krebs solution (pH 7.5) containing (mM): NaCl (120.4), KCl (5.9), CaCl₂ (2.5), MgCl₂ (1.2), NaH₂PO₄ (1.2), glucose (11.5), NaHCO₃ (25.0), at 37°C and continuously gassed with 95% O₂ and 5% CO₂. After stabilization, during which regular washing was performed, rings were checked for viability by stimulation with phenylephrine (PE: 10⁻⁶ M). After viability check, the rings were washed and restabilized. Sets of rings were then contracted with cumulative doses of PE (10⁻⁹ to 10⁻⁶ M). After reaching the maximal contraction on PE, the EC-dependent vasodilation was assessed by cumulative doses of metacholine (ME: 10⁻⁸ to 10⁻⁴ M). Subsequently, the total dilatory response (that comprises an endothelium-dependent and an endothelium-independent VSMC response) was assessed by measuring the response to sodium nitrite (SN: 10 mM). PE and ME were purchased from Sigma-Aldrich (Steinheim, Germany). Salts and carbohydrates were purchased from Merck (Darmstadt, Germany). From each rat the responses of two aorta rings were measured and results obtained were averaged. No major differences were observed between the responses of the two rings.
**Statistics**

Vascular responses were expressed either in μm or in percentage from maximal contraction to PE or SN. The SN response in μm was calculated as maximal contraction to PE minus dilation to 10 mM SN. SN responses were also expressed as percentage of maximal PE response. $E_{\text{max}}$ is the maximal response to PE or SN. Data are expressed as mean value ± standard error of the mean (SEM). Statistical analysis between dose-response curves were tested by General Linear Model (GLM) for Repeated Measures (and, where appropriate, corrected for sphericity: Greenhouse-Geisser correction). Single factor data were compared by Student's $t$-test. Values of $p<0.05$ were considered statistically significant. For statistical analysis SPSS 12.0.2 for Windows (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 4 for Windows (GraphPad Software, Inc., San Diego, CA, USA) was used.

**Results**

**Rosiglitazone attenuates neointima formation after aortic allografting**

Aortic allografts transplanted to BN recipients were characterized by severe neointima formation 8 weeks after transplantation (Figure 1A,B). RSG treatment resulted in a marked reduction in the surface neointima in aortic allografts (Figure 1C,D) as we showed previously.\(^{27}\)

**Rosiglitazone impairs PE-induced vasoconstriction**

To assess whether abdominal aortic allografting with RSG treatment modulated the peripheral vascular response to a sympathetic stimulus, contraction in recipient thoracic aorta rings in response to phenylephrine (PE) was measured. As shown in Figure 2, aortic allografting without RSG treatment (open circles) did not change the contractive response to PE compared with age-matched non-treated, non-transplanted controls (open triangles). However, RSG treatment in transplanted rats induced a moderate but significant decrease in the maximal contractive response to PE ($p<0.05$ vs. non-treated but transplanted rats).

**Rosiglitazone improves EC-dependent vasodilator function**

To determine whether abdominal aortic allografting in rats causes SED, EC function was measured in thoracic aorta rings of transplanted and non-transplanted rats. To this end, EC-dependent relaxation of medial VSMC’s was measured as the dilatory response to metacholine (ME). The net vasodilator response depends on the release of EC-derived relaxing factors and the responsiveness of medial VSMC’s to these factors. As shown in Figure 3A, abdominal aortic allografting without further treatment (open circles) caused a more than 50% reduction of $E_{\text{max}}$ of the EC-dependent vasodilation to cumulative dosages of ME, 8 weeks post-transplantation compared with age-matched non-treated, non-transplanted control rats (open triangles) (Figure 3A, $p<0.01$). RSG treatment of transplanted rats improved but did not normalize the net EC-dependent vasodilator response to cumulative dosages of ME (filled circles) (Figure 3A, $p<0.05$ vs. non-treated but transplanted rats). These data indicate that RSG has a beneficial effect on EC function. When expressing the
Figure 1. Rosiglitazone attenuates neointima formation in aortic allografts. BN recipient rats were treated with rosiglitazone or left untreated as described in Methods. (A) Transplant arteriosclerosis in non-treated recipients is characterized by disruption of the elastic laminae in the media and severe neointima formation. (B) Higher power magnification of the framed area shown in A (magnification x100). (C) Treatment with rosiglitazone resulted in reduced neointima formation compared with the non-treated control group. (D) Higher power magnification of the framed area shown in C (magnification x100). Staining performed on the sections shown in A-D: Verhoeff’s elastin staining. Abbreviation: NI: neointima.

Figure 2. Rosiglitazone (RSG) treatment after transplantation reduces VSMC contractive responses to PE. Aortic allografting without RSG treatment (Tx, □, n=8) does not change the contractive response of VSMC’s to PE compared with non-treated, non-transplanted controls (no Tx, ▽, n=4). Treatment with RSG after transplantation (Tx + RSG, □, n=8) significantly lowers (*, p<0.05) the maximal contractive response to PE compared with transplantation without RSG treatment (Tx, □, n=8). (Data are presented as mean ± SEM, GLM for Repeated Measures).
dilatory response as a percentage of the maximal contractive response thereby correcting for the PE pre-contraction values, RSG was indeed shown to significantly improve EC-dependent vasodilation (filled circles) compared with non-treated but transplanted rats (open circles) (Figure 3B, p<0.05) to a level that was not statistically different from age-matched non-treated, non-transplanted controls (open triangles).

**Rosiglitazone impairs VSMC vasodilator function**

To determine the total (EC-dependent and independent) dilatory response in non-transplanted and transplanted rats (with or without RSG treatment), thoracic aortic rings were stimulated with sodium nitrite (SN) as an exogenous NO source. As shown in the left part of Figure 4A, aortic transplantation (white bar) resulted in a significantly reduced dilatory response to SN compared with age-matched non-treated, non-transplanted controls (grey bar, p<0.001). RSG treatment (black bar) further reduced the responses to SN (p<0.01 vs. non-treated but transplanted). This effect of RSG on the total dilatory response occurred independently of RSG-induced reduction of VSMC contractility (as shown in Figure 2) since similar effects were observed when expressing the total dilatory response as a percentage of pre-contraction values to PE (Figure 4A, right part). Since the EC-dependent vasodilatory response was improved (as shown in Figure 3) but the total vasodilatory response to SN was impaired (as shown in Figure 4A) after RSG treatment, we corrected the ME responses for the response to SN. This corrected response determines the ability of the endothelium to release relaxing factors upon RSG treatment. ME responses corrected for the response to SN are shown in Figure 4B. Aortic allografting (open circles) resulted in a significantly reduced dilatory response (p<0.01 vs. age-matched non-treated, non-transplanted controls). Data are presented as mean ± SEM, GLM for Repeated Measures.

**Figure 3. Rosiglitazone (RSG) improves EC-dependent vasodilator function after aorta transplantation.**

(A) Aortic allografting (Tx, n=8) resulted in significantly reduced vasodilation in response to metacholine (ME) (as determined 8 weeks after transplantation) compared with age-matched non-treated, non-transplanted controls (no Tx, n=4) (**p<0.01). Treatment with RSG (Tx + RSG, n=8) improved vasodilator function compared with non-treated, transplanted rats (Tx, n=8) (* p<0.05) (B) When dilatory responses were expressed as a percentage of maximal contraction to phenylephrine (PE), similar results were obtained (* p<0.05). RSG treatment restored transplantation-induced systemic endothelial dysfunction to levels that did not statistically differ from age-matched non-treated, non-transplanted controls. (Data are presented as mean ± SEM, GLM for Repeated Measures).
non-treated, non-transplanted rats, open triangles) reflecting reduced release of relaxing factors by EC’s after transplantation. The ability of EC’s to release relaxing factors was fully restored after treatment with RSG (filled circles) (p<0.01 vs. non-treated but transplanted rats) even showing a trend towards improved EC function as compared to age-matched non-treated, non-transplanted controls (open triangles).

**Figure 4. Rosiglitazone (RSG) reduces total dilatory responses to SN.** (A) Aortic allografting results in reduced vasodilation (response to 10 mM sodium nitrite [SN]) compared with age-matched non-transplanted controls. RSG-treatment further reduced the dilatory response. Responses are expressed either in μm (left panel) or as a percentage of maximal contraction to phenylephrine (PE) (right panel). (Data are expressed as mean ± SEM, Students’ t-test, ** p<0.01, *** p<0.001) (B) After correction of the ME responses for the responses to SN, data indicate that RSG treatment (Tx + RSG, ○, n=8) reestablishes the ability of endothelium to release relaxing factors compared with aortic allografting in non-treated rats (Tx, ○, n=8) (* p<0.01). (Data are presented as mean ± SEM, GLM for Repeated Measures).

**Discussion**

Development of CVD after transplantation is characterized by insulin-resistance and SED and is associated with significant morbidity and mortality. Since PPARγ agonists like RSG have been shown to improve EC function in diabetes and hypertension we tested the hypothesis that RSG also improves transplantation-induced SED in addition to its beneficial effect on the development of transplant arteriosclerosis. Our data indicate that aorta transplantation induces SED which is restored to pre-transplantation levels after treatment with RSG. On the other hand, RSG induces VSMC dysfunction as demonstrated by a moderate reduction in VSMC contractility and a pronounced decrease in VSMC dilatory capacity.

After aortic transplantation in rats SED developed which was characterized by reduced EC-dependent vasodilation. Experimental and clinical data suggest a strong relation between inflammation and endothelial dysfunction. Therefore, transplantation-induced SED in our model is most likely the result of a chronic systemic inflammatory state of the allo-condition which parallels our previous observations after experimental renal transplantation and stenting in rats.
RSG treatment after transplantation restored EC-dependent vasodilation which was most likely due to improved ability of EC’s to release vasodilating factors. However, we observed that in vivo exposure to RSG induced a reduction in VSMC contraction in response to a sympathetic (PE) stimulus. More importantly, despite complete restoration of EC-dependent dilator function, the total dilatory response (that comprises an endothelium-dependent and an endothelium-independent VSMC response) was reduced. These data indicate that the favorable effect of RSG on EC function after transplantation is counteracted by direct EC-independent deleterious effects on VSMC dilatory potential, as witnessed by the reduced response to exogenous nitric oxide (response to sodium nitrite).

As anti-inflammatory treatment strategies are known to improve EC function, the anti-inflammatory properties of RSG might have contributed to preservation of EC function after aortic transplantation. Alternatively, or together with EC preservation, RSG treatment might have facilitated endothelial repair mediated by endothelial progenitor cells (EPC’s). EPC’s are involved in maintaining endothelial integrity under stressful conditions. The number of circulating EPC’s correlates with EC-dependent vasodilation and EPC-mediated repair of vascular injury is associated with normalization of endothelial function at the site of injury. RSG has been shown to facilitate EPC differentiation and function and to enhance EPC-mediated reendothelialization. Presence of EPC-mediated systemic vascular repair after transplantation is currently under investigation.

In our study, RSG was shown to reduce the total dilatory response and, to a lesser extent, PE-induced VSMC contraction. The effect of RSG on EC-independent vasodilation tended to counteract the gain of endothelial function. In line with our data, RSG-induced reduction of VSMC contraction in response to sodium fluoride or thromboxane A₂ was recently described although in this study RSG did not affect PE-induced contraction. These findings show that impaired contractility is not limited to adrenergic stimuli. Furthermore, RSG-induced reduction of VSMC contraction in response to PE in our transplantation model might reflect an adaptive response of VSMC’s to their transplantation-induced reduction of dilator capacity.

Taking in consideration that EC function is effectively conserved by RSG after transplantation, the repressing effects on VSMC contraction and dilation stand out all the more. In addition to a relatively mild impairment of contractile potential, RSG treatment in transplant recipients leads to a pronounced decrease in VSMC dilator capacity. The mechanism remains to be explored, and might involve both morphological as well as pharmacological changes. Morphological changes such as deposition of extracellular matrix and VSMC hypertrophy might increase stiffness of the vessel wall and thus results in both decreased contractile as well as dilator function. The group of pharmacological changes comprises a large variety of putative mechanisms, including changes in receptor expression, second messenger activation (Ca²⁺ mobilization, cGMP production), decrease of nitric oxide (NO) function e.g. by changes in NO production or scavenging by reactive oxygen species, loss of actin and myosin function. Within the limitations of the current study our data clearly demonstrate that the pronounced effect of RSG on vasomotor function is not due to its impact on EC function but a matter of functional changes in VSMC’s.
However, from our study no conclusions can be drawn on the mechanism(s) involved in RSG-induced VSMC dysfunction after transplantation and more comprehensive studies need to be performed to address this issue.

Given the pro-inflammatory and disturbed metabolic status (characterized by insulin resistance and SED) after clinical transplantation, transplanted subjects would benefit from a therapy that is immunosuppressive (to reduce rejection and transplant arteriosclerosis) but simultaneously improves insulin-sensitivity and EC function. Based on our previous results and data reported in this study RSG may fulfill these criteria. Exposing patients eligible for transplantation to RSG should however be considered with caution, especially in the view of current debate on the effect of RSG on increasing cardiovascular risk in Type 2 diabetics. Although our data clearly indicate that RSG restores transplantation-induced endothelial dysfunction, also a clear EC-independent deleterious effect on VSMC contraction and relaxation was observed. These dichotomous effects of RSG might impede use of RSG in the transplantation setting since this can potentially increase the risk for CVD despite improving EC function.

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References

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