Chapter 3

Costimulation blockade followed by a 12-week period of Cyclosporine A facilitates prolonged drug-free survival of rhesus monkey kidney allografts

Krista G. Haanstra1, Ella A. Sick1, Jan Ringers2, Jacqueline A.M. Wubben1, Eva-Maria Kuhn3, Louis Boon4, and Margreet Jonker1

*Transplantation* 2005; 79(11): 1623-1626

1Biomedical Primate Research Centre, Rijswijk, The Netherlands.
2Department of Surgery, Academic Hospital, Leiden, The Netherlands.
3Intervet International BV, Boxmeer, the Netherlands.
4PanGenetics BV, Utrecht, The Netherlands.
Chapter 3

Abstract

Costimulation blockade as a single immunosuppressive treatment modality is not sufficient to prevent graft rejection. Here, we report an induction therapy using antagonistic antibodies against CD40 and CD86, given twice weekly from day -1 until day 56, followed by a delayed 12-week course of low-dose Cyclosporin A (CsA) treatment in the rhesus monkey kidney allograft model. Low-dose CsA treatment was initiated on day 42 and tapered until total cessation of all treatment on day 126. Treatment with anti-CD40/86 alone resulted in graft survival of 61, 71, 75, 78, and 116 days. Costimulation blockade followed by CsA resulted in more than 3-year drug-free survival in two of four animals. None of the animals developed donor-specific alloantibodies. Transforming growth factor (TGF)-β producing cells are present in early as well as in late kidney graft biopsies and could play a role in the observed long-term drug-free graft survival.
Costimulation blockade followed by CsA

Introduction

In a previous study, we showed that it is possible to prevent rhesus monkey kidney allograft rejection by antagonising the costimulatory molecules CD40 and CD86 or CD40 alone [1]. Costimulation blockade will be combined with standard immunosuppressive drugs (ISD) in clinically applicable treatment protocols. However, conventional ISD have been reported to counteract the immunoregulatory properties of costimulation blockade. Adverse effects of simultaneous CsA and costimulation blockade treatment (CTLA4-Ig + anti-CD40L) have been demonstrated in mice [2] and in non-human primate (NHP) studies [3].

Although effective in prolongation of graft survival, high doses of calcineurin inhibitors (CI) are associated with nephrotoxicity. The induction therapy with the anti-CD40 and anti-CD86 monoclonal antibodies (mAbs) has an advantage because it circumvents the use of CIs during the immediate posttransplantation period.

The mechanism by which costimulation blockade induces long-term survival is thought to involve regulatory T-cells (Tregs) [4], and ISD are likely to prevent the induction of Tregs. This report is, to our knowledge, the first report of a NHP study where the effects of an induction treatment with costimulation blockade, followed by a standard ISD, CsA are studied. The results were compared with animals treated with anti-CD40 with and without anti-CD86 that were reported previously [1].

Biopsies of transplanted kidneys in rhesus monkeys with long-term stable graft function without immunosuppression were shown to stain for latent TGF-β positive cells in their grafts [5]. This finding suggested that TGF-β plays a crucial role in the maintenance of stable graft function. We therefore investigated the presence of latent TGF-β positive cells in biopsies early after transplantation.

Materials and methods

Animals (n=4; group 3; Table 3.1) were selected to undergo heterotopic kidney allograft transplantation with bilateral nephrectomy, as described previously [1, 6, 7]. Immunosuppressive treatment with antagonistic chimeric mAb against anti-CD40 (ch5D12) and anti-CD86 (chFun-1) (PanGenetics BV, Utrecht, The Netherlands) was given intravenously as described previously [1]. Tapering dosages of antibodies were given twice weekly from day -1 until day 56. Determination of therapeutic antibody levels, rhesus anti-chimeric antibody (RACA) levels, and anti-donor antibodies were determined as described previously [1]. In animals treated with anti-CD40 and anti-CD86 alone for 56 days posttransplantation, signs of rejection became apparent at the end of the 56-day treatment period, but they did not show significant signs of rejection on day 42, therefore CsA (Sandimmune, Novartis) treatment was initiated on day 42. CsA was given once daily by intramuscular injection from day 42 onward, targeting trough levels of 300 ng/ml serum until day 70, 200 ng/ml until day 98, and 100 ng/ml until day 126, where after CsA was discontinued. Twenty-four hour trough levels were monitored twice weekly. The CsA trough levels in this study are not sufficient as a monotherapy to sustain long-term kidney graft survival.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Graft survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ch5D12, high dose</td>
<td>91, 134, 217</td>
</tr>
<tr>
<td>2</td>
<td>ch5D12 + chFun-1</td>
<td>61, 71, 75, 78, 116</td>
</tr>
<tr>
<td>3</td>
<td>ch5D12 + chFun-1 + CsA day 42 - 126</td>
<td>140, 231, &gt;1290, &gt;1320</td>
</tr>
<tr>
<td>4</td>
<td>CsA, high dose, day 0 - 35</td>
<td>25, 69, 74, 266, 312</td>
</tr>
<tr>
<td>5</td>
<td>CsA, low dose, day 0 - 35</td>
<td>26, 29, 33</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>5, 6, 6, 7</td>
</tr>
</tbody>
</table>

Table 3.1: Identification of groups and survival times of all animals.

in rhesus monkeys [8, 9] or in cynomolgus monkeys [10]. Graft function was monitored by serum creatinine and urea levels. In addition, histomorphologic evaluation of sequential biopsies was performed according to the Banff classification [11]. A rejection episode was not treated.

Latent TGF-β staining (mAb TB21, BioSource international, Camarillo, CA) [5] was assessed in sequential kidney biopsies of groups 1, 2 and 3. Survival times were compared and the log-rank test was used to determine statistical significance between the groups [12]. Antibody serum trough levels were compared between groups using the *t* test assuming unequal variances.

**Results**

Survival times from animals in group 3 (140, 231, >1290, and >1320 days; Table 3.1) were significantly longer than those of animals treated with only costimulation blockade in groups 1 and 2 (*p* = 0.00162), as previously described [1]. Results are compared with animals treated with anti-CD40 alone (group 1; Table 3.1), anti-CD40 plus anti-CD86 alone (group 2), CsA alone (group 4 and 5, high- and low-dose, respectively), or animals that did not receive any treatment (group 6). Animals that were treated with CsA after costimulation blockade did not reject during the treatment period. Figure 3.1 shows the histopathologic course of the individual monkeys of groups 2 and 3. Rejection is very limited in the biopsies during antibody treatment, even until just before the animals in group 2 are euthanized. From day 42 onward, signs of chronic allograft pathology (CAN) are present in animals of group 3. Two animals rejected after cessation of CsA treatment (day 140 and 231). Two animals are still alive, over three years after discontinuation of immunosuppressive treatment, both with good kidney function. One animal (survival >1290 days) remains rejection free. Repeated kidney biopsies after day 308 demonstrate foci of chronic fibrotic changes in one animal (survival >1320 days) (data not shown).

We have demonstrated previously that ch5D12 was immunogenic only to a very limited extend [1]. Low titers of anti-ch5D12 RACAs were detected in the serum of only one of four animals (survival > 1290 days), and these only developed after discontinuation of mAb treatment.
Costimulation blockade followed by CsA

Figure 3.1: Histopathological evaluation of kidney biopsies and at rejection. Indicated are acute rejection scores (n, normal, b, borderline). When CAN is present, the score is indicated after the slash. The necropsy sample from the animal that survived 231 days (group 3) was the only sample in which chronic vascular changes were detected. * No evaluation of biopsy possible.

High titers of anti-chFun1 RACAs developed during mAb treatment in only two animals (survival 231 and >1290 days), whereas the other two animals developed low anti-chFun1 RACA responses after discontinuation of mAb treatment. The CsA target levels were reached in all animals. Average CsA levels were 310 ng/ml, 239 ng/ml and 94 ng/ml in the three target level periods, respectively, and were undetectable within 18 days after discontinuation.

None of the animals of group 3 developed alloantibodies of the IgM or IgG isotype, in contrast with the animals treated with mAb alone (group 2). In this group, in two of the five animals alloantibodies of the IgM isotype could be demonstrated. But also in these animals, no alloantibodies of the IgG isotype were demonstrated (data not shown).

Latent TGF-β was found in biopsies of stable kidney graft recipients [5]. We analysed TGF-β staining during treatment and it reveals a changing staining pattern over time (Fig. 3.2A-F). In early biopsies, positive areas can be seen in the interstitium. In later biopsies the TGF-β staining appears to be more in and around single cells. The bar graph in Figure 3.2 shows the differences in latent TGF-β staining, expressed as positive areas per tubule, between the groups. Latent TGF-β is absent at the time of euthanasia. Biopsies taken during costimulation blockade also have only low amounts of latent TGF-β. CsA treatment appears to cause lower levels of TGF-β during treatment in animals of group 3, but after CsA is stopped, levels of TGF-β staining increased.
Figure 3.2: TGF-β immunohistochemistry. Kidney biopsies were immunolabelled with an antibody specific for latent TGF-β. The number of stained areas or cells were counted and expressed as number of positive cells per tubulus. The photos show latent TGF-β staining (Fuchsine) in sequential kidney biopsies. Representative biopsies of the two long-term surviving monkeys (>1290 and >1320 days) (magnification x200). (A) Day 21, (B) day 42, (C) day 70, (D) day 112, (E) day 308 and (F) day 726. The graph shows mean latent TGF-β staining/tubulus per group (±SEM).
Discussion

Clinically applicable protocols based on costimulation blockade as a method to prevent graft rejection will include the use of standard ISD. However, ISD have been reported to interfere with the anergy that is induced after costimulation blockade [3]. Combined use of ISD and costimulation blockade will have to be tested preclinically to yield an optimised protocol. The strategy of delayed administration of CI has a clear clinical advantage because it enables treatment without the use of nephrotoxic drugs during the initial posttransplantation period. It may allow for the induction of Tregs, and at later stages, these Tregs may be maintained in the presence of CsA.

Although long-term survival was observed using 35-day high-dose CsA treatment, this treatment protocol was unable to prevent the induction donor-specific alloantibodies [7]. This is in contrast with cases where CsA was used sequentially after using costimulation blockade in the current study, in which no alloantibodies were formed, even after discontinuation of CsA treatment. This is a unique capacity of the ch5D12 mAb. CD40 signalling is needed for isotype class switching in B-cells, but other anti-CD40 or anti-CD154 (CD40L) mAb have been reported to not have this capacity [3, 13, 14].

CAN was seen in all animals of group 3, either in day 42 or day 70 biopsies. Later biopsies did not reveal the presence of CAN, probably because of the small biopsy size, by which local areas of fibrosis and tubular atrophy can be missed. A remarkable observation during this study was that the borderline and grade IA acute rejection seen in three animals on day 70 had resolved by day 112. This indicates that CsA is compatible with costimulation blockade induced unresponsiveness. CsA trough levels were too low to maintain stable kidney function when given as a monotherapy starting at the day of transplantation [9], but the animals did not reject while being on low-level CsA treatment.

TGF-β is correlated with normal or borderline graft function and histology in long-term drug free kidney graft survivors [5]. However, the presence of TGF-β did not predict absence of subsequent rejection. The same observation was made in our study, in which large interstitial TGF-β positive areas could be found in all four animals in biopsies taken at day 112, but in spite of this, two animals rejected, whereas two did not. The presence of TGF-β indicates that active downregulation of immune reactivity may be one of the mechanisms by which graft rejection is prevented.

Acknowledgements

The authors thank the veterinary staff and personnel of the BPRC for excellent care taking of the animals. The authors are indebted to Henk van Westbroek for help in preparing the figures, to Ed Remarque for help with the statistical analysis, and to Ivanela Kondova for help in evaluation of the histopathology results. The authors thank Bert ’t Hart and Michel Vierboom for helpful discussions.
References


