Original Article

Expressional time phase of leukocyte molecules induced by allogenic cardiac antigen and cyclosporin A in rats’ in vitro model

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Abstract: The immunosuppressive agent cyclosporin A has been proven to reduce the rejection rate and prolong the survival time of transplanted hearts. But some reports showed that cyclosporine A did not completely suppress the rejection. We performed in vitro studies to model a time course to observe the effect of cyclosporin A. Methods: The experiment was divided into a control group (group I), an antigen group (group II), a cyclosporin A group (group III) and an antigen + cyclosporin A group (group IV). After transplantation, at 2 h, 6 h, 12 h, 24 h, 48 h and 72 h, leukocyte molecules were monitored. Results: The expression of IL-2R peaked at 12 h in group II and at 6 h in group III. There was a gradual decline in the expression of the P59 gene in group I, positive expression at 2 h and between 12 h and 24 h in group II, in group IV, there was a decrease at 48 h. The expression of the CD4 gene was lowest at 2 h in group I and at 6 h in group II. CD4 expression then quickly increased to a maximum at 48 h in group III, at 2 h in group IV. There was a minimal expression was reached at 12 h in group I and IV and at 6 h in group III in the expression of the CD8 gene. Conclusions: Alloantigen induced lymphocytes to release IL-2R and P59 and stimulated the induction of the CD4 gene’ transcription for 6 h. Cyclosporin A stimulated the release of IL-2R for 2 h. These results provide an in vitro basis for describing the time phases of rejection inhibited by cyclosporin A.

Keywords: Transplantation rejection model in vitro, early expression’ time phase, cyclosporin A

Introduction

Transplantation rejection is associated with lymphocyte activation, which is regulated by multiple genes and signal transduction pathways [1-4]. The immunosuppressive agent cyclosporin A has been proven to reduce the rejection rate and prolong the survival time of transplanted hearts [5-8]. However, the use of cyclosporin A for organ transplantation rejection alters peripheral blood mononuclear cells and caused them to express cytokines, the major histocompatibility complex (MHC) and allostimulatory molecular markers. There are several tens of genes that participate in activation and expression. The genes can be divided into three categories: immediate genes, which are expressed 15–30 min after stimulation, early genes, which are expressed 0.5–24 h after stimulation and late genes, which are expressed several days after stimulation.

Our previous rat homogenic transplantation experiment showed that in spite of the interference by cyclosporin A, there were pathological rejection changes in the same time-frame as the control group. This suggested that cyclosporin A could not completely block the rejection paths, and its mechanism needed to be explored further.

To observe early stage changes after the induction of lymphocyte surface protein markers and genes and to explore the exact inhibitory effect on the immunological system of cyclosporin A in effective concentrations, we compared the
expression timeframe of lymphocyte genes and surface markers groups treated with or without cyclosporin A, thus providing a basis for detection, prevention and cure.

We performed in vitro studies to model a time course to observe the effect of cyclosporin A.

**Materials and methods**

**Animals and grouping**

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments of Anzhen Hospital, Capital Medical University. All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering. One male Sprague Dawley (SD) rat weighing 250 g was used as the allogenic myocardial antigen donor source and was provided by the Anzhen Hospital Animal Center.

Five Wistar rats weighing 250 g were used as the recipients of the splenic lymphocytes. Cyclosporin A (Neoral Sandimmune) was obtained from Novartis Pharma.

Recipient splenic lymphocytes were added to cell culture bottles. After an overnight culture, the cells were divided into four groups. Group I was the control blank group; group II was the antigen group, also termed the allogenic myocardial intervention group, which received 10 mL of a splenic lymphocyte suspension in addition to 200 μl of a myocardial tissue antigen homogenate; group III was the cyclosporin A intervention group, which received 10 mL of a solution of splenic lymphocyte suspension and cyclosporin A (final concentration 15 μg/mL); group IV was the allogenic myocardial antigen + cyclosporin A intervention group, which received 10 mL of a solution of splenic lymphocyte suspension in addition to 200 μl of a myocardial tissue antigen homogenate and cyclosporin A (final concentration 15 μg/mL). The blank control group was monitored at seven time points (0 h, 2 h, 6 h, 12 h, 24 h, 48 h and 72 h; the splenic lymphocyte suspension from each recipient rat was divided into six bottles).

**Materials**

RPMI1640 culture media (Gibco), newborn calf serum (NCS; Hyclone), CO₂ cell culture incubator (American Pyroelectricity) were used in this study.

**Myocardial tissue antigen and splenic lymphocyte preparation and culture**

The SD rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium at a dose of 45 mg/kg weight. The heart was removed, washed with physiological saline and placed in a tissue homogenizer obtain a myocardial homogenate. The myocardial homogenate was centrifuged for 10 min at a speed of 2000 rpm. The sediments were washed twice with RPMI1640 culture media containing 10% NCS. Next, 15 mL of 10% NCS-containing RPMI1640 culture media was added to the sediments to obtain a myocardial tissue antigen suspension, which was aliquoted into fractions of 1 mL/tube and stored at -20°C. The entire process was performed under sterile conditions.

The five Wister rats were anesthetized by intraperitoneal injection of a 3% pentobarbital sodium at a dose of 45 mg/kg weight. The spleens were removed and placed on a cellular sieve. The spleens were placed in culture media and softly ground. The cell suspensions were collected and centrifuged for 3 min at 1200 rpm. The supernatant was removed, and the cell pellet was resuspended in 4 mL of hemolysate for 4 min for hemolysis. The hemolyzed samples were centrifuged for 3 min at 1200 rpm. The supernatant was removed, and the cell suspension was aliquoted into fractions of 1 mL/tube and stored at -70°C. The entire process was performed under sterile conditions.

**Lymphocyte surface protein expression levels**

XL A27153 flow cytometer (American Culter Company) was used to monitor the percentage (%) of CD4, CD8, IL-2R, ICAM-I and MHC-II.
Expression levels of lymphocyte protein coding genes by real-time quantitative PCR

Total lymphocyte RNA was extracted using Trizol (Gibco). The expression levels of CD4, CD8, P59 and β-actin lymphocyte-coding genes were monitored in every group and at each time point by real-time quantitative PCR according to the manufacturer’s recommendations (Gibco).

Statistical analysis

SPSS statistical software was used to analyze the results in every time point in the four groups in relation to the 0 h time point in the respective group. All data are reported as x±SD. A t-test was used to detect significant differences among the results, using P<0.05 as an indicator of significance.

Results

Time course of MHC-II molecular expression

In all four groups, there was a gradual increase in the molecular expression of MHC-II. There was a significant deviation in the non-control groups compared with the control group (Figure 1).

Time course of CD4 molecular expression

In all four groups, the molecular expression of CD4 gradually decreased. There were not significant deviations in the non-control groups compared with the control group, although there was one plateau in the cyclosporine group between 2 h and 24 h.

Time course of CD8 molecular expression

In all four groups, the molecular expression of CD8 gradually decreased. There were significant deviations between all four groups. In the cyclosporine group, there was no plateau between 2 h and 24 h.

Time course of IL-2R molecular expression

In the antigen group, IL-2R expression was initially positive at 2 h, peaked at 12 h, reached a minimum at 24 h, and then gradually increased between 24 h and 72 h. In the cyclosporin A group, IL-2R expression was initially positive at 2 h, peaked at 6 h, reached a low point at 12 h,
and then quickly increased at 48 h. There were no statistically significant changes (Figure 2).

Time course of ICAM-I molecular expression

In the antigen group and the antigen + cyclosporine group, the expression of ICAM-1 was negative at 6 h following intervention, positive from 6 h to 12 h and then fluctuated from negative to positive, which is similar to the expression changes seen in the control group. In the cyclosporine group, the expression of ICAM-I peaked at 2 h after the intervention; at subsequent time points, ICAM-I expression was similar to that of the control group. There were no statistically significant differences.

Time course of P59 gene expression

In the antigen group, the expression of P59 was initially positive at 2 h and then gradually became negative between 2 h and 12 h, reaching a minimum at 12 h. The P59 level became positive again at 24 h and then returned to negative relative to the control group. P59 expression was positive at 2 h and between 12 h and 24 h in the antigen group. In the cyclosporin A group, there was no obvious peak in P59 gene expression. In the antigen + cyclosporine group, P59 gene expression reached a minimum at 48 h and then quickly increased during the 48 h-72 h period (Figure 3).

Time course of CD4 gene expression

In the cyclosporine group, CD4 gene expression was initially moderate, peaked at 48 h and was then negative compared with the control group. The CD4 expression was negative and reached a minimum at 2 h.

In the antigen + cyclosporine group, CD4 expression had a sharp positive peak at 2 h and then gradually became negative by 12 h compared to the control group. In the antigen group, CD4 expression gradually became negative and reached a minimum at 6 h after the intervention and then quickly increased (Figure 4).

Time course of CD8 gene expression

CD8 expression was negative in the four groups. CD8 expression reached a minimum at

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**Figure 2.** A time-course of IL-2R expression in rat lymphocytes. Compared to the levels prior to intervention in the same group; *P<0.05, **P<0.01.
12 h in the control and at 24 h in antigen + cyclosporine groups and at 6 h in the cyclosporine group and then quickly increased (Figure 5).

Discussion

Cyclosporin A (CsA) is an immunosuppressive agent that exerts its effects by interacting with the CsA receptor (cyclophilin, CyP) in the cytoplasm. CyP and CsA form the CyP/CsA compound, which forms the CyP/CsA/CN ternary complex with calcineurin. CyP/CsA/CN can block CN dephosphorylation and hinder Ca\(^{2+}\) influx to inhibit the T cell nuclear activation and transduction pathway. CyP/CsA/CN can also suppress the expression of IL-2R, IL-2 and early T cell-activated genes in T cells to finally suppress rejection [5-7]. Thus, CsA has been used clinically to prevent organ transplantation rejection.

The results of our clinical study showed that CsA could not block the early activation of T lymphocytes induced by allogenic antigens. Kang HG concluded that CsA does not suppress the positive expression of the IL-2 gene [5]. The results lead to the following questions: (1) What is the precise mechanism and outcome of a CsA defect [5-7]? (2) What is the effect of organ transplantation on lymphocyte surface molecules and gene expression levels? To address these questions, we performed in vitro studies to model a time course for the expression of rat lymphocyte surface proteins and coding genes induced by allo-myocardial tissue and observed the time-dependent effect of cyclosporin A.

Major histocompatibility complex II (MHC-II) plays a role in antigen presentation of antigen presenting cells (APC) [9]. In four groups, MHC-II expression was gradually increased. There was not a significant deviation in the other three group compared with the control group. These data showed that myocardial tissue antigen, CsA, antigen and CsA activation did not increase MHC-II expression.

Lymphocyte activation required a double signal, namely, a second signal (allostimulatory signal) in which allostimulatory molecules on APC bound the allostimulatory molecules on the lymphocytes. ICAM-1, a ligand of LFA-1 located on the surface of lymphocytes, induced
the lymphocyte activation and proliferation [10-13]. In vitro experiments showed that there were no significant changes in any of the four groups, although in the cyclosporine group, there was one positive peak at 2 h after the intervention, which indicated that ICAM-1 was released.

CD4 and CD8 are accessory receptors that bind the antigen peptide-MHC-II/I complex through the TCR-CD3 co-receptor. In vitro experiments showed that there was a gradual increase in the expression of CD4 and CD8 in the four groups. These experiments indicted that myocardial tissue antigen, CsA, antigen and CsA activation did not directly affect the positive expression of CD4 and CD8 in lymphocytes in the absence of positive MHC-II expression. In the antigen and CsA activation group, there were no changes in IL-2R expression. However, in the CsA group, IL-2R molecules were released.

The P56 and P59 genes are the principal members of the tyrosine kinase family. Due to signal transduction and the corresponding tyrosine phosphorylation, lymphocytes are activated and proliferate in response to CD4, CD8 and IL-2 lymphocyte surface marker expression. In another study, CD45 was identified as the key molecule in early stage T lymphocyte activation. Cytoplasm containing CD45 had increased tyrosine phosphatase activity, which eliminated the phosphate group on negative tyrosine residues in lck and fyn to promote the tyrosine phosphorylation of other substrates and the activation of T cells [13]. In the control group, negative expression occurred in a gradual, multistep process. There were two instances of positive expression in the antigen group, at 2 h and between 12 h and 24 h. This signified that the allogenic antigen induced the start of lymphocyte activation at 2 h. However, the negative expression of the P59 gene between 2 h and 12 h showed that the start point of the lymphocyte signal transduction pathway (phosphate tyrosine kinase activation) was suppressed. In the cyclosporin A group, there was positive expression of the P59 gene between 6 h and 12 h, a 6 h delay compared to the antigen group. In the antigen + cyclosporine group, P59 expression was positive between 2 h and 6 h, which is a 2 h delay following intervention compared to the antigen group. These results indi-
h-12 h period. This indicates that allogenic myocardial tissues may induce temporary transcription of the CD4 gene through a P59 signal transduction pathway. In the cyclosporine group, CD4 expression was positive, which is in contrast to the negative expression of P59 at 6 h following intervention. This indicates that CsA may induce CD4 gene transcription through the same pathway. In the antigen + cyclosporine group, the positive expression of the P59 gene was 2 h delayed compared to the time of positive CD4 gene expression. These data may explain the observation that when the transcription factor began to modulate CD4 gene transcription, P59 transcription was quickly suppressed. Within 2 h after the intervention, CD4 expression was positive and P59 expression was negative.

There was negative expression in all four groups. The control and antigen + cyclosporine groups reached minimum values at 12 h, and the cyclosporine group reached a minimum value at 6 h; the expression then quickly decreased.
increased in these three groups. These data indicate that the translation process was modulated by the CD8 gene. Myocardial antigen did not directly induce CD8 gene transcription and expression. In the cyclosporine and cyclosporine + antigen groups, CD8 expression levels were lower than that in the control group. In the cyclosporine + antigen group, the positive expression of CD8 occurred 18 h after the cyclosporine group, indicating that CsA only suppressed early transcription of the CD8 gene after antigen intervention and did not suppress the middle-late CD8 gene transcription; CsA did, however, suppress translation of CD8.

In the cyclosporine group, there was a gradual decrease in expression in lymphocyte CD4 and CD8 proteins, which showed that the activity of the lymphocytes was in the silence state. The expression of CD4 and CD8 proteins did not correlate with CD4 and CD8 at the transcriptional level, which indicated that changes in CD4 and CD8 transcription did not alter the translation of lymphocyte surface proteins. There was a gradual increase in expression in the CD4 gene at 48 h following the intervention, which demonstrated that CsA can induce CD4 gene transcription to a certain degree [14-16]. However, CD4 transcription and protein expression did not correlate, indicating that CsA may not be sufficient to stimulate translation of lymphocyte CD4 surface proteins and CD4+T lymphocytes. Mechanisms other than the calcineurin pathway are still unclear and require further study.

In conclusion, it is clear from this study that alloantigen induced lymphocytes to release IL-2R and P59 and stimulated the induction of the CD4 gene transcription for 6 h. Cyclosporin A stimulated the release of IL-2R for 2 h. These results provide an in vitro basis for describing the time phases of rejection inhibited by cyclosporin A.

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Conflict of interest

None.

Abbreviations

APC, antigen presenting cells; CN, calcineurin; CsA, Cyclosporin A; CyP, cyclophilin; MHC, major histocompatibility complex; NCS, Newborn Calf Serum; SD rat, Sprague Dawley.

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