Repression of allo-cell transplant rejection through CIITA ribonuclease P⁺ hepatocyte

Rong Guo, Ping Zou, Hua-Hua Fan, Feng Gao, Qing-Xin Shang, Yi-Lin Cao, Hua-Zhong Lu

AIM: Allo-cell transplant rejection and autoimmune responses were associated with the presence of class II major histocompatibility complex (MHCII) molecules on cells. This paper studied the effect of Ribonuclease P (RNase P) against CIITA, which was a major regulator of MHCII molecules, on repressing the expression of MHCII molecules on hepatocyte.

METHODS: M1-RNA is the catalytic RNA subunit of RNase P from Escherichia coli. It was constructed that M1-RNA has guide sequences (GS) recognizing the 452, 3408 site (psNAV-M1-452-GS, psNAV-M1-3408-GS) respectively. The target mRNA could be used as a tool to cleave target RNA that formed a base pair with the GS. Stable transfectants of hepatocyte cell line with psNAV-M1-3408-GS were tested for expression of class II MHC through PCR for the level of IL-2 mRNA on T cell by mixed lymphocyte transfectants of hepatocyte cell line with psNAV-M1-3408-GS and its target RNA (psNAV-M1-3408-GS) respectively. The donor shortage has become the major restriction on liver transplantation with the increasing demands for it. Now people are trying to produce artificial liver with hepatocytes and various biological materials, but are faced with the challenge of rejection in allo-hepatocyte transplantation. Allo-transplant rejection, was associated with the presence of class II major histocompatibility complex (MHCII) on the tissues and organs. MHCII played a critical role in the induction of immune responses by presenting fragments of alloantigenic peptides to CD4⁺ T lymphocytes, resulting in the activation of CD8⁺ T lymphocytes. So it was more important for compatibility of MHCII in allo-transplantation. Moreover, the abnormal expression of MHCII molecules was associated with autoimmune disease too. There are codominance and multiple allele for MHCII molecules which lead to their complicated polymorphism, so it is difficult to repress every MHCII molecules expression directly. MHC class II transactivator (CIITA) was the major rate-limiting factor for both constitutive and inducible MHCII expression. There was no rejection in allo-skin grafted into the future application of M1-3408-GS as a new nucleic acid drug against allo-transplantation rejection and autoimmune diseases.

CONCLUSION: The Ribonuclease P against CIITA-M1-3408-GS could effectively induce antigen-specific tolerance through cleaving CIITA. These results provided insight into the future application of M1-3408-GS as a new nucleic acid drug against allo-transplantation rejection and autoimmune diseases.

INTRODUCTION

The donor shortage has become the major restriction on liver transplantation with the increasing demands for it. Now people are trying to produce artificial liver with hepatocytes and various biological materials, but are faced with the challenge of rejection in allo-hepatocyte transplantation. Allo-transplant rejection, was associated with the presence of class II major histocompatibility complex (MHCII) on the tissues and organs. MHCII played a critical role in the induction of immune responses by presenting fragments of alloantigenic peptides to CD4⁺ T lymphocytes, resulting in the activation of CD8⁺ T lymphocytes. So it was more important for compatibility of MHCII in allo-transplantation. Moreover, the abnormal expression of MHCII molecules was associated with autoimmune disease too. There are codominance and multiple allele for MHCII molecules which lead to their complicated polymorphism, so it is difficult to repress every MHCII molecules expression directly. MHC class II transactivator (CIITA) was the major rate-limiting factor for both constitutive and inducible MHCII expression and with rare exceptions, its expression parallels to that of MHCII transcripts. There was no rejection in allo-skin grafted into the future application of M1-3408-GS as a new nucleic acid drug against allo-transplantation rejection and autoimmune diseases.

RESULTS: When induced with recombinant human interferon-gamma (IFN-γ), the expression of HLA-DR, DP, -DQ on psNAV-M1-3408-GS⁺ hepatocyte was reduced 83.27 %, 88.93 %, 58.82 % respectively, the mRNA contents of CIITA, HLA-DR, DP, -DQ and I decreased significantly. While T cell expressed less IL-2 mRNA in the case of psNAV-M1-3408-GS⁺ hepatocyte.
MATERIALS AND METHODS

Plasmids

PTK117, a pUC19 derivative in which the DNA sequence coding for E.coli M1RNA is under the control of the T7 RNA polymerase promoter, was provided by Dr Chen B[16], pGEM-7zf(+) vector was purchased from Shanghai BioEngineering Company, adeno-associated virus vector (psNAV) was provided by Dr Lu HZ.

Enzymes and chemicals

T4 DNA ligase and restriction endonucleases EcoRI, SalI, BglIII, XhoI were purchased from MBI; mouse anti-human isotype control (IgG2a)-FITC, HLA-DR (IgG2a, k) -DQ (IgG2a, k) monoclonal antibody and recombinant human IFN-γ were obtained from PharMingen, mouse anti-HLA-DP (IgG2b, BRA-FB6) from CYMBUS, GENETICIN (G418) from Gibco, TRIZOL® from GibcoBRL, TITANIUM® one-step RT-PCR kit from Clonetch.

Cell culture

Cell line used included L-02 cell line (from human fetal liver) and Raji cell line (human B lymphoma), all purchased from Cell Bank of Shanghai Academy of Science. Cells were maintained in RPMI-1640 (GIBCOBRL) medium supplemented with 15% fetal calf serum (HyClone).

In vitro cleavage of anti-CIITA RNSaeP

RNaseP construction The M1-RNA with the anti-CIITA-directed GS (M1-452-GS or M1-3408-GS) were constructed by the polymerase chain reaction (PCR) with the gene for M1-RNA as found in plasmid pTK117 as a template. The 5′' primer, OliT7: 5′-gcgggaattcGAACATGCCTGTCCAGAGC-3′, annealing with the T7 promoter and providing a 5′ EcoRI site for cloning. The 3′' primers contained the appropriate GS and sequence. The two different PCR products were annealed either acrylamide/urea or agarose electrophoresis. RNaseP production system-T7 (Promega), then transcribed ribozyme in vitro from the above cells in the 6-well plate.

Construction of artificial substrate The CIITA (3176-3560) mould RNA was obtained from Raji cell line by RT-PCR, according to instructions of TRIZOL® (pGM-3176). The CIITA (3176-3560) mould RNA was linearized with XhoI and cloned into pGEM-7zf (+) vector. The CIITA mRNA was under the control of the T7 RNA polymerase promoter, and Raji cell line (human B lymphoma), all purchased from Cell Bank of Shanghai Academy of Science. Cells were maintained in RPMI-1640 (GIBCOBRL) medium supplemented with 15% fetal calf serum (HyClone).

In vitro transcribed mould plate RNA of unrelated CIITA

Cleavage point (452)

5′UCUUCCAGGACUCCAGGUGAGGGCCUGAAGGACA3′

In vitro transcribed mould plate RNA of pGEM-3176 in CIITA

Cleavage point (3408)

5′GACGUGCCUGGACGCCUGAAGGUGGUCCUAUGGA3′

Figure 1 Cleavage of CIITA substrate by M1-1 RNA in vitro (A): The mould plate RNA of CIITA pGEM-3176 (3176-3560) include the cutting point of only M1-3408-GS, not M1-452-GS. (B): Schematic representation of targeting the CIITA mRNA by M1-RNA; GS encoding 12 or 11 nucleotides complementary to CIITA was covalently linked to the 3′ end of M1-452-GS or M1-3408-GS. So M1-452-GS and M1-3408-GS specify cut CIITA on the site of 452 and 3408 respectively. (C): Sequence-specific cleavage of pGEM-3176 substrate by M1-452-GS: autoradiograph of transcripts of M1-452-GS (lane 1), M1-452-GS (lane 2) and pGEM-3176 (lane 3), and pGEM-3176 substrate was incubated either with M1-3408-GS (lane 4), or with M1-452-GS (lane 5). So only M1-3408-GS (not M1-452-GS) could cleave pGEM-3176.

Cell transfection through nanometer vector

Hepatocytes were transfected with 0.4 µg psNAV-M1-3408-GS by nanometer vector. According to Effectene (QIAGEN) kit’s instructions, seeded 2.5×10⁴ cells/well the day before transfection. The cell number seeded should produce 40-80 % confluence on the day of transfection. psNAV-M1-3408-GS were diluted in 100 µl EC buffer, mixed with Enhancer 3.2 µl, incubating 2-4 min at RT, then adding Effectene 10 µl, at RT 7-8 min, mixed with 600 µl medium containing serum and antibiotics, and immediately transferred the total volume to the above cells in the 6-well plate.
The expression of MHCII antigens on hepatocyte by FCM
Hepatocytes were collected and washed with 1.5 g/L MPBS buffer (10 g/L BSA and 1 g/L Na3) once at the density of 1x10^6/ml, adding IgG2a, HLA-DR, DP, DQ 10 µl respectively, incubating at 4 °C for 30 min, detecting the expression of MHCII molecules by Flow cytometry (COULTER, EPICSXL).

RNA analysis
RT-PCR was done according to the instructions of TRIZOL® and TITANIUM® one-step RT-PCR kit. In a total 50 µl volume, 50 °C 1 h, 94 °C 5 min, 94 °C 30 s, 65 °C 30 s, 68 °C 1 min, 30 cycles, 72 °C extending 7 min. Primers sequences (synthesized by Shanghai Bioengineering Company) referred to Table 1.

| Primer          | Sequence                        | Length |
|-----------------|---------------------------------|--------|
| CIITA mould     | L 5’-CCGCTGAGAGCCTAAGCTGTTGAA-3’| 349bp  |
| CIITA           | R 5’-GGGATGACACCTTTGGTGGTA-3’    | 349bp  |
| M1-452-GS       | L 5’-GGCGAATTCATATACGACTCACTATAG-3’| 454bp  |
| HLA-DR          | R 5’-GCGGAAATTCAGAATGCCTGTCCAGAGC-3’| 446bp  |
| M1-3408-GS      | L 5’-CCGCGAGCTCGAGTTGTTCCAGGACTGACCGATGACCGACTGACCGACCGACG-3’| 454bp  |
| HLA-DR          | R 5’-GGAGCTGTGGAATTCCTTTGGTGGTA-3’| 454bp  |
| HLA-DQ          | R 5’-ACCGCGAGCTCGAGTTGAGGCCATGACCGACG-3’| 454bp  |
| Ii              | R 5’-CCCGCTCACTGAGTTGAGGCCATGACCGACG-3’| 454bp  |
| β-actin         | R 5’-ATCGGACTGACAGACTCCTGG-3’    | 310bp  |
| CIITA mold      | L 5’-CATCTCGACTCGTTAGCTGCATTGCAAGCTTGC-3’| 714bp  |
| β-actin         | R 5’-GCTGGAATTCAGAATGCCTGTCCAGAGC-3’| 454bp  |

Mixed lymphocyte reaction (MLR)
It were incubated at 37 °C, 5% CO2 (keeping away light) after adding mitocin-C (25 µg/ml, Sigma) into IFN-γ induced psNAV-M1-3408-GS hepatocyte (1x10^6/ml), and washed with RPMI1640 twice, plated at a density of 1x10^6/well as well as stimulating cells. Then added blood mono-nucleated cells (PBMNC, 1x10^6) from healthy donors into above stimulating cells. The level of IL-2 mRNA from PBMNC after 48 h incubation was Detected through RT-PCR[17].

RESULTS
The expression of MHCII molecules on hepatocyte
Hepatocyte without IFN-γ induction The expression of HLA-DR, DP, DQ on hepatocyte was low, (0.14±0.04) %, (26.76±5.26) %, (2.12±0.56) % respectively. Hepatocyte after IFN-γ induction The expression of HLA-DR, DP, DQ on hepatocyte with IFN-γ (40 ng/ml) induction for 3 d increased significantly, (18.68±2.94) %, (41.78±4.90) %, (4.78±1.26) % respectively.

Nrnase P down-modulating MHCII expression on hepatocyte
The expression of MHCII on psNAV-M1-3408-GS hepatocyte after the induction of IFN-γ (40 ng/ml) for 3 d was repressed. Compared with void-vector hepatocyte, the expression of HLA-DR, DP, and DQ on psNAV-M1-3408-GS hepatocyte was inhibited 83.27 %, 88.93 %, and 58.82 % respectively.

Hepatocyte inducing mixed lymphocyte reaction
The secretion of IL-2 from PBMNC stimulated by psNAV-M1-3408-GS hepatocyte with IFN-γ (40 ng/ml) induction was repressed. In allo-cell transplantation or autoimmune diseases, some cytokines such as IFN-γ induced some low/non MHCII antigen expressing cells to express these molecules highly[18,19]. We selected IFN-γ to induce hepatocyte for 3 d, the expression

DISCUSSION
In allo-cell transplantation or autoimmune diseases, some cytokines such as IFN-γ induced some low/non MHCII antigen expressing cells to express these molecules highly[18,19]. We selected IFN-γ to induce hepatocyte for 3 d, the expression...
of HLA-DR, DP, DQ antigens increased exactly, while the expression of HLA-DP was most apparent.

CIITA regulated the transcription of MHCII gene by interacting with the trans-acting factors such as RXF, X2BP and NFY. The expression of CIITA paralleled to that of MHCII molecules and appeared only in the MHCII-positive cells [10,11]. In the hepatocyte detected by us, the expression of CIITA was consistent with that of MHCII molecules: without IFN-γ induction, all hepatocytes didn’t express MHCII molecules and CIITA gene; following IFN-γ induction, these cells expressed MHCII molecules and CIITA gene simultaneously; in the case of anti-CIITA psNAV-M1-3408-GS positive hepatocytes, the induced MHCII expression on their surface was nearly completely lost, and their CIITA mRNA detected by RT-PCR was also absent, perhaps the latter was the direct reason that MHCII expression didn’t react to IFN-γ induction. This view was coincidence with that of Luber et al [18,20]. TOXO plasma gondi parasite lowered the MHCII expression by inhibiting its induced CIITA expression. Moreover, HMG-CoA reductase inhibitors, cyclosporine and phosphatidylethanolamine-linked hyaluronic acid (HYPE) could completely repress MHCII expression of human microvascular endothelial cells by reducing its induced CIITA mRNA contents ex vitro [19,21,22].

Gene blocking techniques were mainly made up of anti-sense oligonucleotide, anti-sense RNA, ribozyme and RNA interference (RNAi), and so on. Anti-sense oligonucleotide referred to a small fragment of single-strand deoxyribonucleic acid (14-23 bases) synthesized artificially, which could hybridize with target DNA or mRNA. However, there were still some problems of stability and efficiency of entering cell in vivo about it. Anti-sense RNA, ribozyme and RNAi all took action on target mRNA, namely anti-sense complementation, cutting and interference respectively. The novel RNAi technique was the double-strand RNA connected by anti-sense RNA and sense RNA in essence, and was more efficient than single anti-sense RNA [24,25]. The mechanism of which was still not clear and might be related to activating ribonucleaseap to degrade target mRNA. But when it is larger than 30bp, the action of it was not specific [24]. Compared with above-mentioned gene blocking techniques, ribozyme not only sealed mRNA, but also cut mRNA with specificity. Moreover, ribozyme could be used repeatedly, so it had higher efficiency. Both hammerhead ribozyme and hairpin ribozyme require GUC sequence to identify in target sequence. However, RibospP was not limited to this and could aim at any site in the target sequence, so it had wider selective range [25]. There was no report on RnaseP yet at home. According to human RNA sequences published in the NCBI Gene Bank, our experiment selected 452, 3408 site and 3408 site in the CIITA gene as target sites of M1-RNA after eliminating the possibility of their homology. PST and LRR regions initiated by 452, 3408 site were very essential to the transcription activation of CIITA. Moreover, the secondary structure around them was relatively simple and accessible. The GS of M1-3408-GS and M1-452-GS were programmed as 11 and 12 nucleotides respectively, to fit for the combination with their own substrate, the disconnection of cutting products with the ribozyme, and the specificity of the ribozyme. The 5’-terminal of M1-RNA had a TATA box (T7 promoter), and M1-RNA was cloned into the psNAV vector (without T7 promoter), then the transcription was gone on owing to T7 promoter in the ribozyme itself. This could avoid supplementary sequence of the psNAV vector and objectively reflect the cutting activity of the Rnase P. The result of our experiment revealed the expected cutting stripes in the electrophoresis of cutting products of M1-3408-GS and CIITA mould plate ex vitro.

The reason why our experiment used nanometer-vector to mediate the transfection of M1-RNA into hepatocyte was that nanomater had the advantages of both virus vector and non-virus vector [26,27]. For instance, adenovirus vector [28,29] or retroviral vectors [30] could cause too strong immunological reaction of body to fit for the study of inhibiting the immunological rejection in our experiment; Especially, nanometer-vector could mediate exogenous gene to integrate into the chromosome DNA of host cell so that the long-term and stable expression of transgene could be obtained. In our experiment using the novel nanometer vector Effectene to transfect human hepatocyte, the rate was about 11 %, and it could rise to 60-80 % after the screening with G418 for 1 wk. Nanometer vector, however, has just begun to be used in the field of gene therapy. So far, internationally, there has no report on it used in the gene therapy of clinical or pre-clinical study.

Moreover, hepatocytes induced by IFN-γ could stimulate the secretion of IL-2 mRNA from exogenous T cell, while psNAV-M1-3408-GS+ hepatocyte after IFN-γ induction lost this ability. Therefore, M1-3408-GS inhibited CIITA mRNA and thus the family of MHCII molecules regulated by CIITA, then down-regulated the ability of stimulating mixed lymphocyte reaction. In conclusion, our research will have important theoretical and practical meaning on the study of transplantation immune in the whole hepatocyte tissue engineering and the therapy of autoimmune diseases.

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