T Cell-specific Immunosuppression Using Tautomycetin or PTD-conjugated Protein Drugs

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IMMUNE RESPONSES

The immune response is the collective and coordinated body response to infectious pathogens as well as noninfectious foreign substances by cells and molecules responsible for immunity. However, the immune system that normally protects individuals from infection and eliminates foreign substances is itself capable of causing tissue injury and autoimmune disease in some situations.

Physiological mechanisms for defending the host against foreign invaders are present in some form in all multicellular organisms and these mechanisms constitute innate immunity. Innate immunity consists of mechanisms that exist before infection, are capable of rapid responses to microbes, and react in essentially the same way upon repeated infection. In contrast to this primitive or primary defense against microbes, a system of more highly evolved defense mechanisms is present in vertebrates.

This is called adaptive immunity and is composed of two types of immune reaction: "humoral immunity" and "cell-mediated immunity" (Fig. 1). The major component of the former is antibodies which are secreted by plasma B cells, while that of the latter is usually mediated by T cells. However, the innate and adaptive immunity systems are not separated from each other but rather they react in an integrated manner against infection.

There are two features of this coordinated relationship. First, the innate immune response to microbes stimulates the adaptive immune responses and influences their essential characteristics of adaptive immune response. Second, the adaptive immune responses employ various effector mechanisms of innate immunity to eliminate microbes, and they often function by enhancing the antimicrobial activities of the defense mechanisms of innate immunity.

Together with this response-based categorization, the immune responses in our body represent two characteristics in most of their modes of action. One is specificity, which means the specific immune responses and their cellular context and components for a given antigenic peptide or pathogen. The other is memory, which prevents the recurrence of infection for foreign pathogens. To provide an efficient way of eliminating infectious pathogens, secondary lymphoid organs and the circulation system of the body cooperates to present possible and potential antigens for T lymphocytes which are the major component of regulating immune responses. These T lymphocytes include not only conventional CD4 and CD8 T cells for given pathogens but also regulatory T cells and γδ T cells for specialized function and regulatory functions.

From an anatomical view, our secondary lymphoid organs have an efficient microenvironment to capture pathogens and thereby presenting antigenic peptide by APCs (Antigen Presenting Cells) such as dendritic cells (DCs), B cells and macrophages. Very few T lymphocytes in the circulating blood recognize the given APCs, and initiate immune responses. The removal of patho-
gens is subsequently followed by the contraction of the number of activated and proliferated immune cells, which is primarily studied in T cells, to maintain the “immunological homeostasis”. The breakdown of homeostasis is caused by the deficiency or mutation of essential genes for regulating complex immune responses and this is why more than one gene usually contributes to the autoimmune diseases.

With these common features of the immune system, it should be noted that there is a paradox for discerning self and non-self antigens for immune responses. In fact, the autoimmune diseases themselves are the functional outcome of the recognition of self antigens as foreign ones. Here we will discuss organ transplantation and autoimmune diseases as the major challenge for overcoming self-nonself problems and the breakdown of immunological homeostasis in T cells. A newly discovered immunosuppressor, taumomycetin (TMC), which specifically acts on activated T cells, will be introduced, along with its in vitro and in vivo immunosuppressive activity and the mechanism of its action. In addition to the small chemical compound-based immunosuppressor suppression of specific immune response by delivery of cytoplasmic signal mediator or transcriptional factor involved in immune receptor-mediated signal transduction pathways is possible using the Protein Transduction Domain (PTD). This protein transduction technology will be useful not only in functional verification of genes or proteins identified through genomic or proteomic approaches but also in the development of therapeutic protein drugs for the treatment of various immunological diseases in the post-genomic era.

ORGAN TRANSPLANTATION AND CURRENT THERAPEUTIC APPROACHES

Transplantation is the process of taking cells, tissues or organs, called a graft, from one individual and placing them into a different individual. In clinical practice, transplantation is used to overcome a functional or anatomic deficit in the recipient. A major limitation in the success of transplantation is the immune response of the recipient to the donor tissue. Most transplantation is done between two genetically different individuals of the same species called allogeneic graft (allograft). The immune response to alloantigens is mainly T-cell mediated although antibodies might contribute to this process. For almost all strong and rapid rejection reactions are based on the major histocompatibility antigen complex (MHC) molecules. This strong and rapid rejection is based on as many as 2% of an individual’s T cells which are capable of directly recognizing and responding to a single foreign MHC molecule (Fig. 2 and 3).

As for the time interval of rejection, three types of rejection are present. The first is hyperacute rejection which is characterized by hemorrhage and thrombotic occlusion of the graft vasculature that begins within minutes to hours after host blood vessels are anastomosed to graft vessels. This rejection is mediated by preexisting IgM alloantibodies in the host circulation that bind to donor endothelial antigens. The ABO antigens are well known examples of these alloantibodies. More importantly, hyperacute rejection of allografts, when it occurs, is usually mediated by IgG antibodies directed against protein alloantigens, such as foreign MHC molecules, or against less well-defined alloantigens expressed on vascular endothelial cells. Such antibodies generally arise as a result of prior exposure to alloantigens through blood transfusion, prior transplantation, or multiple pregnancies.

The second is acute rejection which is a process of vascular and parenchymal injury mediated by T cells, macrophages and antibodies that usually begins after the first week of transplantation. Both CD4 and CD8 T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which cause necrosis.

The third is chronic rejection, which is characterized by fibrosis with loss of normal organ structures occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than that of acute rejection. In many cases, graft arterial occlusion occurs as a result of the proliferation of intimal smooth muscle cells.
Fig. 1. Cellular and Humoral Immunity. Overview of the humoral and cell-mediated branches of the immune system. The humoral response involves the interaction of B cells with antigen (Ag) and their differentiation into antibody-secreting plasma cells. The secreted antibody (Ab) binds to the antigen and facilitates its clearance from the body. The cell-mediated responses involve various subpopulations of T cells that recognize the antigen presented on self-cells. TH cells respond to antigen by producing cytokines. TC cells respond to antigen by developing into cytotoxic T lymphocytes (CTLs), which mediate killing of altered self-cells (e.g., virus-infected cells).

Fig. 2. Mechanisms of direct allorcognition. The interactions between donor MHC molecules and recipient T-cell receptors (TCRs) can be viewed as lying along a spectrum, in which the associated peptide contributes increasingly more energy to the overall binding affinity as the donor and recipient alleles converge. At one end of the spectrum, the peptide has minimal input ('high-density determinant' model), whereas at the other end of the spectrum, the peptide has a crucial role ('multiple binary complex' model). Most interactions between donor antigen-presenting cells (APCs) and recipient T cells are thought to lie somewhere in the middle of the spectrum.

Fig. 3. Cellular reactions characterizing the allorcresponse. A. The role of antigen-presenting cells (APCs) in the allorcresponse. Donor APCs trafficking through the draining lymph nodes of the recipient elicit a direct allorcresponse. Furthermore, these APCs provide a vehicle for the supply of donor histocompatibility antigens to recipient APCs. Migrant recipient APCs trafficking through the graft also have the opportunity to capture alloantigens, before egressing back to the lymph nodes. Such alloantigens are then able to evoke an indirect allorcresponse, involving CD4+ T cells interacting with APCs through MHC class II molecules and, to a lesser extent, CD8+ T cells interacting with cross-presenting APCs through MHC class I molecules. The unique ability of dendritic cells (DCs) to prime naive T cells and to mediate cross-presentation places them at the centre-stage of the indirect allorcresponse, which is thought to contribute mainly to chronic rejection (16). B. Integration of the allorcresponse. Interaction of the direct and indirect pathways of allorcognition mediates graft rejection. Apart from the help that is given by CD4+ T cells to CD8+ cytotoxic T cells (a), helper T cells might also elicit a delayed-type hypersensitivity response mediated by macrophages activated by the secretion of interferon-γ (IFN-γ) (6). Furthermore, there is evidence to indicate that CD4+ T cells might mediate direct cytotoxicity through their expression of FAS ligand (CD95L; CD178). Eosinophilic inflammation elicited by T helper 2 (TH2) CD4+ T cells through interleukin-5 (IL-5) might also damage the graft (c). Note the central role of recipient CD4+ T cells.
This process is called accelerated or graft arteriosclerosis. This symptom is frequently seen in failed cardiac and renal allografts and can develop in any vascularized organ transplant within 6 months to a year after transplantation.\textsuperscript{34,36}

Immunosuppression is the major approach for the prevention and management of transplant rejection. The several methods of immunosuppression that are commonly used fall into two categories. The first is chemical-based immunosuppressors and this includes cyclosporin A (CsA), FK506, rapamycin, mycophenolate mofetil and corticosteroids. While corticosteroids act to block macrophage cytokine production, rapamycin blocks IL-2 signaling so that it causes T cell cycle arrest. Similarly, CsA and FK-506 block NFAT activation in T cells and transcription of IL-2 and other cytokine genes. The second is monoclonal antibodies including anti-CD3 and anti-CD25. Anti-CD3 enhances the clearance of T cells or blocks surface CD3 function, and anti-CD25 blocks IL-2 activation of T cells so that it clears activated T cells. Also the use of regulatory T cells to prevent this allograft rejection has been performed experimentally in murine models.\textsuperscript{37,38}

AUTOIMMUNE DISEASES AND THEIR CURRENT TREATMENT APPROACHES

A common cause of autoimmunity is failure of self-tolerance. The potential for autoimmunity exists in all individuals because during their development lymphocytes may express receptors specific for self antigens and many self antigens are readily accessible to the immune system. The features of autoimmune diseases may be either systemic or organ specific, and various effector mechanisms are responsible for tissue injury in different autoimmune diseases. In addition to this, multiple interacting factors contribute to the development of autoimmunity. These factors include immunologic abnormalities affecting lymphocytes and APCs, genes that predispose to autoimmunity, microbial infections, and tissue injury. As combinations of these factors may be operative in different disorders, autoimmune diseases are heterogeneous.\textsuperscript{39}

The effector mechanisms of autoimmune diseas-

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\textsuperscript{37,38}  Another type of autoimmune diseases is caused by T cells and T cells play a major role in tissue injury either by triggering delayed type hypersensitivity reaction or by directly killing target cells. Th1 type of CD4 and CD8 T cells are both activated in this process, thereby activating macrophages and induce inflammation. Insulin-dependent diabetes mellitus, experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis (RA) are categorized to these T cell-mediated autoimmune diseases. The common causes of autoimmune diseases by T cells are subject to at least 4 categories. First, aberrant expression of costimulators such as B7-1 and B7-2 in tissues may result in a breakdown of T cell anergy and tissue specific autoimmune reactions. Second, T cell anergy may also fail because of defects in molecules such as CTLA-4 that normally inactivate these cells. Third, mutations that interfere with the apoptotic death of mature lymphocytes may result in autoimmune diseases, especially for Fas and Fas Ligand deficiency. Fourth, defective T cell-mediated suppression contributes autoimmunity. Recently, several animal models have supported the idea that the loss or deficiency of regulatory T cells, whether from naturally arising CD4+ CD25+ or IL-10 secreting regulatory T cells, is the major cause of autoimmune diseases. Strategies for treating immune-mediated diseases are similar to those used to prevent graft rejection. The mainstay of therapy for autoimmune diseases is anti-inflammatory drugs such as corticosteroids. Monoclonal antibodies against cytokines (e.g.
anti-TNF alpha antibody for RA treatment) are also used. Such drugs are targeted at reducing the effector phase of tissue injury (Fig. 4). 20

NOVEL T CELL-SPECIFIC IMMUNOSUPPRESSOR, TAUTOMYCETIN

The systematic study of products from bacteria and fungi has led to the development of immunosuppressive drugs such as CsA, FK506 (tacrolimus), and rapamycin. CsA and FK506 block T cell activation by preventing the induction of IL-2 gene expression, whereas rapamycin blocks the signaling pathway triggered by IL-2 receptor. They exert their pharmacological effects by binding to the immunophilins, and the immunophilin and drug complex binds and inhibits the SeThr phosphatase calcineurin, which is activated when intracellular calcium ion level rises on T cell activation. Rapamycin has a different mode of action from either CsA or FK506. However, the

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Fig. 4. The normal events that occur during the lifetime of a T cell are outlined (black arrows), as well as the circumstances that might disrupt these events and lead to autoimmunity (red arrows). a. Weak T-cell stimulation by self-peptide-MHC generally leads to the survival and maintenance of the naive T cell (black arrows). An event that alters T-cell survival or homoeostasis might lead to splenomegaly and lymphadenopathy (lymphoproliferative disorders). Further interactions, such as CD40L-CD40 interactions, between these partially activated T cells (red) and B cells (green) might lead to systemic autoimmunity (red arrows). b. If a T cell encounters a self-antigen that is able to stimulate the T cell, tolerance occurs by deletion or anergy (black arrows). Defects in apoptosis (defective deletion) might lead to impaired tolerance induction and susceptibility to autoimmunity (red arrows). Alternatively, interactions between T cells and inappropriately mature DCs might lead to the activation of T cells that are specific for self-antigens and autoimmunity (red arrows). c. After activation by pathogens, the responding T-cell population will include various different clones. Some T cells might have a weak cross-reaction with self-antigen and might cause transient autoimmunity. Other cells might strongly cross-react with self-antigen and cause autoimmunity (red arrows). However, most cells will be pathogen-specific and contribute to effective immunity (black arrows). Systemic autoimmunity has been associated with model a, and organ-specific autoimmunity has been associated with models b and c, but it is also possible that systemic autoimmunity might arise through pathways b and c, and vice versa. It is not yet clear which situations will be modified by regulatory T cells; however, regulatory cells are likely to be important in some of these circumstances.
Fig. 6. Inhibition of proliferation and activation of T cells by TMC. (A) MLR was set up by culturing splenocytes of Balb/c mouse with mitomycin C-treated allogenic spleen cells of mouse C57BL/6 in the presence of TMC (●), CsA (○) or cremophor only (□). (B) Spleen cells from Balb/c mouse were stimulated with Con A (Sigma) for 48 h in the presence of TMC (CsA (○) or cremophor only (□) and then [H] Thymidine incorporation was measured. (C) Human primary T cells (1.0 × 10^6) were pretreated with TMC (1 μg/ml), CsA (1 μg/ml, ○), or cremophor only (□), and then stimulated with OKT3 mAb (10 μg/ml) and anti-CD28 mAb (10 ng/ml) (Sigma) at 37°C for 24 h. Supernatants were harvested and IL-2 ELISA was performed using detection antibody and HRP-conjugated avidin. Wells were washed with 7 times and TMB substrate was added to each well. 50 μl of stop solution was added and absorbance was measured at 450 nm within 30 min.

The inhibitory effect of TMC on T cell proliferation was confirmed in the mixed lymphocyte reaction (MLR) between splenocytes of C57BL/6 and mitomycin C-treated splenocytes of Balb/c mouse in the presence of serial dilutions of TMC or CsA, the most frequently used immunosuppressor. The level of inhibition of MLR by TMC was 100-fold higher than that by CsA, and the IC_{50} of TMC and CsA was 7.8 nM and 417 nM, respectively (Fig. 6A). Similar levels of proliferation inhibition were observed in mouse splenocytes stimulated with the mitogen Concanavalin A (ConA) (Fig. 6B). In addition, similar kinetics of inhibition by TMC or CsA were observed in rat splenocytes. Induction of IL-2 gene expression, CD69 and IL-2Rα chain surface expression has been well documented as valuable markers for TcR-distal activation events. The potency of TMC in the IL-2 secretion assay in human primary T cells is similar to CsA, in contrast to its more potent inhibitory activity than that of CsA in
murine MLR and murine spleen cell proliferation in response to Con A. This demonstrated that TMC possesses immunosuppressive activity to inhibit the proliferation of T cells by inhibition of IL-2 secretion (Fig. 6C). In contrast to cells pretreated with CsA, the surface expression of CD69 or IL-2R α chain was not induced in Jurkat T cells pretreated with TMC after TcR stimulation (data not shown). These results indicated that TMC has the capacity to inhibit the intracellular signaling pathway leading to T cell activation and proliferation, and that its mechanism of action might be different from that of CsA.

To identify the signaling event targeted by TMC in the TcR-mediated signaling pathway, the inhibition of tyrosine phosphorylation of various intracellular substrates by TMC was examined in primary human T cells. TMC blocked the phosphorylation of tyrosine residues on several specific cellular proteins in T cells stimulated by immobilized OKT3 mAb (Fig. 7A). This result raises the possibility that TMC may block the tyrosine phosphorylation of intracellular signal mediators like genistein or herbimycin. To rule out this possibility, the induction of tyrosine phosphorylation of intracellular proteins was examined in the human primary B cells following B cell receptor (BcR) stimulation by anti-BcR mAb.

As shown in Fig. 7B, TMC, CsA or solubilizing reagent cremophor did not affect the BcR-induced tyrosine phosphorylation of intracellular signaling molecules. The specificity of TMC on activated T cells was further confirmed in Fig. 8B where activated or resting human primary T and B cells were used. Therefore, TMC specifically inhibits the tyrosine phosphorylation of T cell specific intracellular signal mediators involved in the T cell activation signaling pathway.

To identify the target molecule affected by TMC we examined the tyrosine phosphorylation induction of several key molecules participating in TcR-proximal or distal signaling events in the presence of TMC using the CD8-ζ Jurkat transfectant. In the presence of TMC, the intracellular domain of ζ chain, ZAP-70 tyrosine kinase, immune-specific adapters such as LAT and SLP76, ubiquitous adapter c-Cbl, PLC-γ, Vav and MAP kinase ERK were not tyrosine phosphorylated which have been characterized to be downstream of Lck and Fyn in TcR signaling cascade (Fig. 7C). Surprisingly, the tyrosine phosphorylation of Lck and Fyn and their kinase activity were not influenced, suggesting that TMC targets a signal mediator between ZAP-70 and Lck or Fyn tyrosine kinase in TcR-mediated signal transduction cascade. However, Lyn tyrosine kinase specifically

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Fig. 7. T cell-specific inhibition of tyrosine phosphorylation on intercellular signal mediators by TMC. (A) Human primary T cells were pretreated with TMC (1 μg/ml) or CsA (1 μg/ml) for 5 h. Th cells were then stimulated with immobilized OKT3 mAb (10 μg/ml) and rabbit anti-mouse IgG (10 μg/ml) for the indicated times. (B) Human primary B cells were pretreated with TMC (1 μg/ml), CsA (1 μg/ml) or solubilizing reagent (cremophor) for 5 h and then stimulated with goat anti-human IgM F(ab')2 (Sigma) for the indicated times. The cell lysates were subjected to 10% SDS-PAGE under reducing conditions, and then immunoblotted with anti-phosphotyrosine Ab 4G10 (Upstate Biotechnology). (C) CD8-ζ Jurkat T transfectants or primary human B cells were pretreated for 5 h with or without TMC (1 μg/ml) and then stimulated with OKT3 mAb (10 μg/ml) and rabbit anti-mouse IgG (10 μg/ml) or goat anti-human IgM F(ab')2 (Sigma) (10 μg/ml) for 5 min respectively. Immunoprecipitates or total lysates were resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. Treatment of cells with these drugs for 5 h did not induce cell death. An equal amount of protein was loaded in each well and this was confirmed by Ponceau S staining and immunoblotting with anti-ZAP-70 mAb.
induced upon BcR stimulation was not influenced by TMC.

TMC induced DNA fragmentation of primary T cells at concentrations as low as 1.7 nM. Primary B cells showed minimal cell death at high TMC concentrations and apoptosis was not observed in HeLa cells in the presence of TMC (Fig. 8A). The purified, activated, human T cells showed much higher sensitivity to TMC than resting T cells, activated or resting B cells (Fig. 8B). This result is consistent with the mechanism of action of TMC which inhibits the tyrosine phosphorylation induction of T cell-specific intracellular signal mediators. To investigate the molecular mechanism of apoptosis induction by TMC, the cleavage of downstream effectors caspases and their key substrates was examined (Fig. 8C). Bcl-2, which was known to bind to mitochondria and inhibit the release of cytochrome c, was cleaved as a consequence of TMC treatment in a dose-dependent fashion and overexpression of Bcl-2 in Jurkat TAg cell line abrogated the apoptotic effect of TMC (Fig. 8D). This finding suggested the involvement of caspase-8 in TMC-mediated apoptosis induction via mitochondria. These results indicated that the T cell specific induction of apoptosis by TMC is in part mediated by Bcl-2 cleavage leading to the release of cytochrome c which facilitates the binding of Apaf to caspase-9 in its presence, and subsequent activation of caspase-8 and caspase-3 in apoptosis induction signaling pathways.

In recent studies, the serine/threonine kinase Akt has emerged as a key molecule involved in regulating cell survival in a variety of models. To investigate the involvement of PI-3 kinase, Akt and BAD in T cell-specific apoptosis induction by TMC, the induction of the phosphorylation of PI-3 kinase, Akt and BAD was examined in the presence of TMC using the primary T cells stimulated by immobilized OKT3 mAb. As shown in Fig. 8E, TMC significantly inhibited the phosphorylation of Akt and BAD, suggesting that these three molecules might be responsible for the possible functional crosstalk between the inhibition of T cell activation and the induction of T cell-specific apoptosis by TMC (Fig. 9).

To assess the in vivo immunosuppressive effect of TMC, we measured allograft survival in rats treated with either TMC or CsA following heterotopic cardiac transplantation. The mean graft survival without administration of any immunosuppressor or solubilizing reagent was only 9.5 days, compared with more than 100 days for an isograft. The recipient was treated with CsA (5 mg/kg of rat weight) optimally formulated in Cremophor-EL solution for 40 days or with several different doses of TMC solubilized in PBS or in microemulsion form for 30 days after heterotopic cardiac transplantation. As shown in Table 1, the graft survival was prolonged for more than 100 days in the CsA-treated group. The grafted hearts survived for more than 160 days in the TMC-treated group using a dose as low as

<table>
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<tr>
<th>Drug</th>
<th>Concentration (mg/kg)</th>
<th>Injection</th>
<th>Survival days</th>
<th>No. of Rats</th>
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<tr>
<td>Cremophor only</td>
<td>5</td>
<td>I.P.</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>CsA/cremophor</td>
<td>5</td>
<td>I.P.</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Tautomycin/PBS</td>
<td>0.05</td>
<td>I.P.</td>
<td>&gt;100</td>
<td>2</td>
</tr>
<tr>
<td>TMC/PBS</td>
<td>0.05</td>
<td>I.P.</td>
<td>&gt;160</td>
<td>12</td>
</tr>
<tr>
<td>TMC/ME</td>
<td>0.03</td>
<td>I.V.</td>
<td>&gt;160</td>
<td>8</td>
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Hearts from Lewis rats were transplanted within the abdominal cavity of Wistar rats by microsurgical anastomosis. Following the heterotopic heart transplantation, the recipients were administered with CsA (5 mg/kg of rat weight and optimally formulated in cremophor-EL solution) for 40 days, tautomycin (0.05 mg/kg of rat weight and solubilized in PBS) or TMC (0.03-0.05 mg/kg of rat weight and solubilized in PBS or in microemulsion form (ME)) for 30 days. Graft survival was monitored by examining the heartbeat through the abdominal wall and anatomical examination was done on the 100th or 160th day after heterotopic cardiac transplantation.
Fig. 8. T cell-specific induction of apoptosis by TMC. (A) The primary T lymphocytes and B lymphocytes and Hela cells were incubated for 10 h with the medium alone or various concentrations of TMC. Cells were lysed and DNA fragmentation was analyzed by electrophoresis. (B) The primary human T lymphocytes were pre-incubated without (○) or with (●) immobilized OKT3 mAb (10μg/ml) and B lymphocytes were pre-incubated without (◇) or with (▼) goat anti-human IgM F(ab')2 for 3 h, and then treated with TMC (1μg/ml) for 5 h. Cell viability was determined by staining with propidium iodide (PI) and measured by flow cytometry. (C) Jurkat T cells were incubated with various concentrations of TMC for 5 h. Cell lysates were subjected to SDS-PAGE under reducing condition (anti-PARP blot, anti-caspase-3 blot, anti-caspase-9 and anti-Bcl-2 blot), and native PAGE under non-reducing conditions (anti-caspase-1 blot), and then immunoblotted with the indicated antibodies. As positive controls for the cleavage of these molecules cells were stimulated with NIH3T3 transfectant stably expressing human Fas-L, PARP Ab (H-250) and Bcl-2 Ab (100) (Santa Cruz Biotechnology), CPTP32 Ab (Transduction Labs), Caspase-9 Ab (PharMingen), ICE (Upstate Biotechnology)]. (D) Jurkat T cells (3×10⁶) stably expressing high level of SV40T antigen were transfected using Superfect transfection reagent (Qiagen) with null vector (Gray) or bcl-2 expression vector (Black). Cells were treated for 5 h with TMC (1 or 10μg/ml) after transfection and then analyzed by PI staining assay. (E) The primary human T lymphocytes purified from peripheral blood using Ficoll were pretreated with TMC (1μg/ml) for 5 h. Cells were then stimulated with immobilized OKT3 mAb (10μg/ml) for 5 min. Lysates were immunoprecipitated with anti-p–Tyr-conjugated agarose beads and the precipitates were immunoblotted with anti-PI-3K/p85 α mAb. For anti-phospho-Akt, anti-phospho-Bad and anti-PI-3K/p85 blots the lysates were immunoblotted with the indicated antibodies. An equal amount of protein was added in each lane, which was confirmed by Ponceau S staining and immunoblotting with the anti-PI-3K/p85.
0.03 mg/kg and the level of graft survival was dose-dependent. Upon histological examination on the 160th day after transplantation, the cardiac muscle cells, vascular architecture and renal tubes were well preserved and T cell infiltration was minimal in the recipient rats. Biochemical analysis of GOP, GTP, glucose and creatine levels in blood following administration of TMC up to 5 mg/kg in rats for 30 days did not show any significant liver or kidney toxicity. In contrast to TMC tautomycin did not show any immunosuppressive effect in in vivo heart allograft transplantation. These results demonstrated that the in vivo immunosuppressive activity of TMC is as effective as CsA in an in vivo organ transplantation model and that the immunosuppressive effect of TMC can be further improved if the pharmacological formulation of TMC is optimized.

We are currently identifying the specific target molecules of TMC in the signal transduction pathway of T cell activation, as well as its chemical modification and optimal drug formulation for efficient clinical administration.

The concept of PTDs originally comes from HIV viral Tat peptide which has a length of 11 amino acids (Table 2). This arginine-rich peptide showed a potent delivery capability when it is conjugated with macro-size molecules up to 120 kDa protein of beta-galactosidase into living cells and in vivo murine models.

It was suggested that this membrane penetrating peptide transverses the plasma membrane by interacting with heparan sulfate on the cell surface. Other studies have suggested the possible existence of a kind of cargo molecule to transport PTD-conjugated macromolecules. However, the exact macromolecule delivery mechanism for PTD remains controversial. In more practical terms, these types of arginine-rich peptides are found in the nuclear localization signals of transcriptional factors for interacting with DNA sequences by positively charged amino acids. However, the
Table 2. Commonly Used Protein Transduction Domain (PTDs)

<table>
<thead>
<tr>
<th>Protein</th>
<th>PTD amino acid sequence</th>
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<tr>
<td>Cationic</td>
<td></td>
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<tr>
<td>HIV-1 TAT(47-57)</td>
<td>YGRKKRRQRRR</td>
</tr>
<tr>
<td>Drosophila Antennapedia(43-58) (ANTP)</td>
<td>RQIKIWFQRRMKWKK</td>
</tr>
<tr>
<td>Poly-arginine (R7) [synthetic]</td>
<td>RRRRRRR</td>
</tr>
<tr>
<td>PTD-5 [synthetic]</td>
<td>RRQQRTSKLMKR</td>
</tr>
<tr>
<td>Amphipathic</td>
<td></td>
</tr>
<tr>
<td>Transportan [chimeric, galanin fragment plus mastoparan]</td>
<td>GWTLNSAGYLGKINLKALALAKKIL</td>
</tr>
<tr>
<td>KALA [synthetic]</td>
<td>WEAKLAKALAKALKHLKALAKALKACEA</td>
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Number of arginine residues is not the sole factor for determining the delivery efficiency as previously shown in studies, describing that there is a certain limit to the arginine residue number for the efficient delivery of macromolecules. Moreover, other PTDs have no apparent arginine residues although still they perform efficient macromolecule delivery. Therefore, it should be noted that no definite rules in the primary amino acids sequences have yet been found to characterize PTDs (Fig. 10).

Among various PTDs, VP22 from the herpes simplex virus protein is also well known for its delivery capacity although it does not contain continuous arginine-rich motifs. As PTDs can be conjugated to any macromolecules, nucleic acids (DNA/RNA) are coupled to PTD for delivery. So far, the DNA delivery has been done with DNA/PTD peptide mixture as an alternative to the lipid-based transfection method in vitro culture system.

Recently we have identified two novel PTDs,
one of each human (Sim-2: 9 amino acids) and mouse (Mph-1: 11 amino acids) origin. The in vivo and in vitro transduction efficiencies of the two PTDs were better than those of HIV Tat. These PTDs can deliver target proteins up to 120 kDa within 4-6 hours at the nM range of concentration in vivo. By conjugating the important functional domain of intracellular signal mediators (PTEN, Tbet, FLIP), the cytoplasmic domain of CTLA-4 or TcR zeta chain, or chemical compounds with a marked degree of toxicity or that were undeliverable due to their large molecular weight, we successfully delivered these target molecules and confirmed their functional and therapeutic effect in various animal models of immunological disease. Importantly, these PTD can facilitate the delivery of the conjugated protein drugs through local administration routes such as the eye, mucosal airway or skin, and can thereby enhance the specificity of target proteins. The development of various immunotherapeutic protein drugs is being undertaken.

APPLICATION OF PTD TECHNOLOGY FOR DEVELOPMENT OF IMMUNOTHERAPEUTIC PROTEIN DRUGS

Most of the identified PTDs have shown their capacity in vitro. Meanwhile, in vivo studies using murine models have been performed mainly with reporter systems such as beta-galactosidase, CreloxP system to investigate beta-galactosidase expression and prove their in vivo delivery efficiency. Although there are a few studies which uses PTD for conjugating therapeutic chemicals to overcome the major membrane penetration problem, the delivery of therapeutic proteins was not intensively studied until now, although it has potent therapeutic activity in vitro systems. Recently, several studies have been performed for cell cycle regulators (e.g. p27 Kip1) on cancer cells while other studies have also been done on the manipulation of bone marrow stem cell transplantation. Besides these protein-based approaches, a previous study utilized PTD to link with siRNA in genetically blocking the function of the target protein in vivo system. As reported in previous studies, while intraperitoneal injection of PTD-target protein conjugate requires more than 4 hours to reach the target organs, the intravenous injection of PTD-conjugated compounds or proteins usually reaches the target site within 30 minutes. The route of administration should therefore be considered carefully. Another factor influencing the design these therapeutic proteins is their half-life time, which can be less than 2 hours in certain experimental situations. In addition, the delivery of proteins or other macromolecules can be adjusted to either systemic or local symptoms. With these experimental points in mind, the development of immunotherapeutic protein drugs for autoimmune diseases and transplantation should be designed.

CONCLUDING REMARKS

The major players in immune reactions are lymphocytes which can function in both adaptive immunity and innate immunity. In most autoimmune diseases and transplantation, a large body of research has confirmed that CD4 and CD8 T cells are crucial populations to perform immune responses. To circumvent the problems of current immunosuppressors in the treatment of autoimmune diseases and organ transplantation, we developed a novel compound TMC, which can inhibit T cell activation 100-fold more than CsA with less cytotoxicity in vitro and in vivo. In addition to chemical-based immunosuppressors immunotherapeutic protein transduction technology has been utilized to deliver various signal mediator or transcriptional factors for their functional verification and therapeutic effect in vitro and in vivo. The utilization of various PTDs for developing target-based immunotherapeutic protein drugs is now being undertaken.

REFERENCES

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