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Immunosuppressive Activity of 4-*O*-Methylascochlorin

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Abstract A major strategy for suppressing immune responses is the elimination of antigen-reactive lymphocytes through apoptosis. 4-*O*-Methylascochlorin (MAC) is a methylated derivative of a prenyl-phenol antibiotic, ascochlorin. MAC induces apoptosis in various lymphocyte cell lines. We found that MAC strongly suppressed killer T-cell activity induced by allogenic skin grafts. MAC did not suppress the killer T-cell activity induced by intraperitoneal injection of live allogenic tumor cells bearing both class I and II MHC. MAC suppressed IL-2 production of splenocytes from allogenic skin-implanted mice when induced by specific spleen adherent cells, but not by antibodies for T-cell receptor ε. These results suggest that MAC suppresses the antigen presentation process of alloantigen that is mediated by professional antigen presenting cells. MAC significantly increased the survival time of allogenic skin implanted on the flank mice. These results suggest that MAC may be clinically useful as an immunosuppressant that targets the antigen presentation process.

Keywords ascochlorin, MAC, CTL, immunosuppressant, graft rejection

Introduction

Immunosuppressants affect T lymphocytes by two general mechanisms, namely by transcription suppression and by direct toxicity. Some of them suppress the transcription of T-cell lymphokines, including interleukin-2 (IL-2) and interferon-γ. Cyclosporin A (CsA) and FK506 inhibit lymphokine transcription through the formation of drug-binding protein complexes that suppress cytoplasmic calcineurin, a protein phosphatase required for nuclear translocation of the regulatory domain of the nuclear factor of activated T cells, a major transcription factor for T-cell lymphokines [1~5]. Other immunosuppressants eliminate activated lymphocytes by direct toxicity. Methotrexate and cyclophosphamide induce apoptosis of T lymphocytes [6]. Azathiopurine and mycophenolic acid inhibit DNA synthesis by inhibiting the purine synthesis pathway [7, 8]. Glucocorticoid hormones and FTY720 induce apoptosis of lymphocytes [9, 10]. Rapamycin suppresses lymphocyte proliferation by interfering with the target of rapamycin signal transduction pathway of growth factors [11].

Ascochlorin (Fig. 1) is a prenyl-phenol antibiotic that was originally isolated as an antiviral agent produced by an incomplete fungus, *Ascochyta visiae* [12, 13], and synthesized in racemic and optically active form [14]. Ascochlorin and its derivatives exhibit various physiological activities including hypolipidemic activity [15, 16], suppression of hypertension [17], amelioration of type I and II diabetes [18, 19], immunomodulation [20, 21], and anti-tumor activity [21, 22]. Ascochlorin and its derivative,

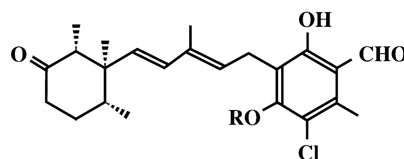

Fig. 1 Structures of ascochlorin and 4-*O*-methy-ascochlorin.

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ascofuranone, inhibit oxidative phosphorylation by inhibiting ubiquinone-dependent electron transport in isolated mitochondria [23～25]. It has been suggested that the anti-viral activity of ascochlorin and the macrophage activation by ascofuranone are caused by the inhibition of mitochondria respiration [23, 24, 26]. Some ascochlorin derivatives also modulate the activity of nuclear hormone receptors. AS-6, an ascochlorin derivative, activates peroxisome proliferator activated receptor-γ, and ascochlorin activates the estrogen receptor [27, 28]. Thus, mechanisms other than those involving the respiratory chain may contribute to the physiological activities of these drugs. In human renal carcinoma cells, ascochlorin selectively suppresses AP-1 and its downstream targets, such as the matrix metalloproteinase-9 promoter [29].

We recently found that 4-*O*-methylascochlorin (MAC, Fig. 1) strongly induced apoptosis in human T-lymphocyte cell lines [30]. Similar to Fas-stimulation, MAC, but not actinomycin D or staurosporine, induced apoptosis in a human B-cell line, SKW6.4, in which apoptosis is strongly dependent on the death-inducing signaling complex. On the other hand, MAC-induced apoptosis is suppressed by Bcl-2 overexpression, but not by a deficiency of death-inducing signal complex including caspase-8 and Fas-associated death domain. These results suggest that MAC-induced apoptosis is dependent on a death-inducing pathway involving mitochondria but not caspase-8 or the Fas-associated death domain. Thus, MAC induces apoptosis of lymphocytes through a mechanism different from that of conventional apoptosis inducers. In the present study, we describe the immunosuppressive activity of MAC *in vivo* using mice transplanted with allogenic skin, and find that MAC significantly suppresses antigen-specific immune responses different from known immunosuppressive agents.

Materials and Methods

Mice

Six-week old female mice were purchased from Sankyo Laboratory Service (Tokyo, Japan). The animal studies were conducted in accordance with institutional guidelines for laboratory animal care under approved protocols.

Cell Culture

Mouse cell lines P815 and EL-4 were kind gifts from Ayako Hino (Tokyo Medical Institute, University of Tokyo, Tokyo, Japan). P388D1 was purchased from Dai-Nippon Pharmaceutical Company (Osaka, Japan). All cells were cultured and maintained in DMEM supplemented with 5% fetal bovine serum, 50 μM 2-mercaptoethanol, 8 μg/ml tylosin tartrate, 50 μg/ml kanamycin, 0.1 mM MEM non-essential amino acid solution (Invitrogen, Grand island, NY, U.S.A.), and 1 mM sodium pyruvate (Invitrogen).

Chemicals

MAC was supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). For *in vivo* administration, MAC was dissolved in DMSO and then suspended in phosphate buffered saline (pH 7.4) containing 0.5% Tween 80. CsA was purchased from Novartis Pharmaceutical (Tokyo, Japan) and dissolved with sesame oil (Sigma, St. Louis, MO, U.S.A.) for intraperitoneal administration.

Skin Transplantation and Induction of Cytotoxic T Cells

Cytotoxic T cells were induced in C57BL/6 mice by intraperitoneal injection of 2×10^7 P815 or P388D1 cells or by allogenic skin graft. Skin grafting was performed as described previously [31] with slight modifications. The recipients of the transplantation were anesthetized by intraperitoneal injection of 1.5 mg sodium pentobarbital (Dai-Nippon Pharmaceutical). Tail skin was removed from Balb/c mice, processed to about 50 mm^2, and engrafted to the left flank of the recipients.

Cytotoxic T Lymphocyte (CTL) Assay

Spleens pooled from recipients (3 mice per group) were removed and teased to make single cell suspensions. The suspensions were then incubated with ^{51}Cr-labeled target cells (1×10^4 cells/well) for 4 hours at 37°C. The isotope released in the culture supernatant was measured. Percent lysis was calculated by the formula:

$$\text{Percent lysis}=100 \times(\text{experimental cpm}-\text{spontaneous cpm})/(\text{maximum cpm}-\text{spontaneous cpm})$$

where spontaneous cpm and maximum cpm are radioactivities released in the absence of effector splenocytes and in the presence of 1% SDS, respectively.

IL-2 Induction

Splenocytes (5×10^6/ml) pooled from three mice per group were cultured with or without a hamster monoclonal anti-CD3 antibody (145-2C-11, 1 μg/ml, eBiosciences, San Diego, CA, U.S.A.), or allogenic spleen adherent cells (SAC) prepared as described previously [32]. After 20 hours of culture, the IL-2 concentration in the culture supernatant was determined by ELISA (Endogen, Woburn, MA, U.S.A.).

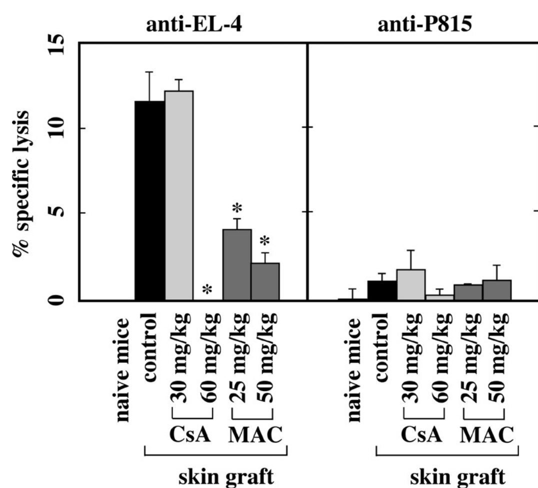

Fig. 2 MAC suppresses CTL activity induced by allogenic skin grafts.

Balb/c mice received allogenic tail skin grafts from C57BL/6 mice. Ten days after transplantation, CTL activity in spleens from 3 mice was determined at an effector-to-target ratio of 1 : 100, using target cells bearing syngenic MHC (P815) or specific allogenic MHC (EL-4). CsA or MAC was intraperitoneally injected once a day for 9 consecutive days from 24 hours after the implantation. Values represent mean±S.D. of triplicate cultures. * Indicates a statistically significant difference when compared to control cultures ($p<0.05$, double-sided t-test).

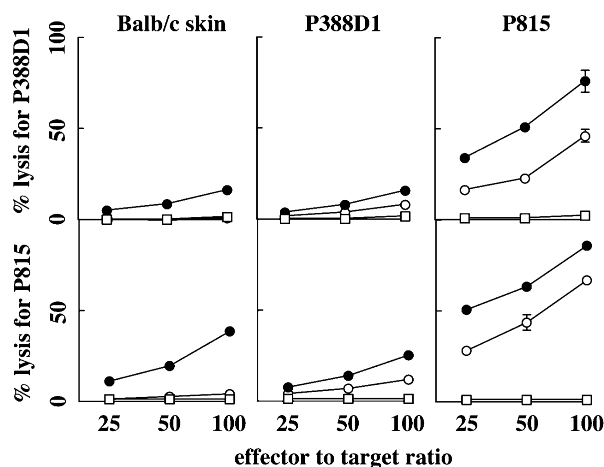

Fig. 3 MAC suppresses induction of CTL activity induced by allogenic skin but not by live allogenic tumor cells.

C57BL/6 mice were immunized with implantation of allogenic tail skin from Balb/c mice (left panels) or with intraperitoneal injection of allogenic tumor cells, P815 (right panels) or P388 D1 (center panels). Ten days after the immunization, CTL activity in the spleens from mice treated with vehicle (closed circles) or MAC (open circles) was determined at the indicated effector-to-target ratios, using P388D1 cells (top panels) or P815 cells (bottom panels) as target cells. Open squares indicate the percentage lysis by naive spleen cells. MAC was intraperitoneally injected once a day for 9 consecutive days from 24 hours after the immunization. Values represent mean±S.D. of triplicate cultures.

Statistics

Difference between given two experimental groups were statistically evaluated using double-sided t-test. Difference giving p values less than 0.05, are considered statistically significant.

Results

Cytotoxic activity was induced in spleen cells of Balb/c mice (H-2^d) by implanting C57BL/6 (H-2^b) tail skin on their flank. Ten days after the transplantation, significant cytotoxic activity against EL-4, a mouse T-cell line with the same major histocompatibility antigen complex (MHC) haplotype (H-2^b) as the skin graft, was detected in recipient spleen (Fig. 2). This cytotoxic activity is specific for graft MHC because the killing activity against target cells with recipient MHC (P815, H-2^d) was much less than the killing activity against EL-4. The cytotoxic activity is due to typical class-I restricted CTLs because the activity is completely diminished by treatment with anti-CD8 antibody and complement (data not shown). Intraperitoneal treatment with MAC (50 and 25 mg/kg once a day after the transplantation) significantly reduced the CTL activity. Similar treatment with 60 mg/kg of CsA completely eliminated CTL activity.

Recipient C57BL/6 mice received an intraperitoneal immunization of P815 cells, and the CTL activity in their splenocytes was determined 10 days after the immunization. With this protocol, much stronger CTL activity was detected, as compared to skin transplantation (Fig. 3). MAC treatment only partially suppressed the CTL activity induced by P815 immunization. Similarly, MAC only partially suppressed CTL activity when P388D1, an inducer of CTLs that is as weak as allogenic skin grafts, was used for the immunization. These results suggest that the antigen presentation process specific for skin grafts, but not for tumor cells, is crucial for the suppression by MAC. These results also demonstrate that the expression of class II MHC on the cells for immunization is not essential for MAC-mediated suppression of CTL activity because P388D1 expresses both class I and class II MHC.

Because our results suggested that MAC does not directly suppress the killing activity of CTLs, we examined IL-2 production by T cells from the immunized mice (Fig. 4). Mice transplanted with allogenic skin received intraperitoneal MAC or CsA treatments once a day. The IL-2 production of the spleen was determined 10 days after the implantation by stimulating T cells with anti-CD3 antibody

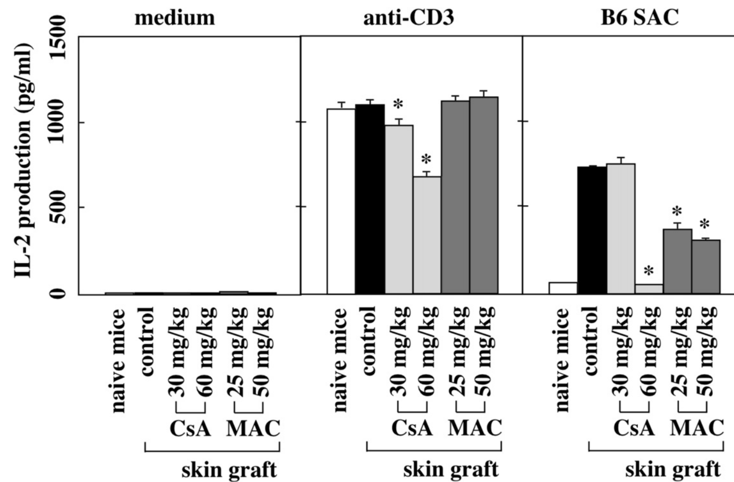

Fig. 4 MAC suppresses IL-2 production of splenocytes from allogenic skin graft stimulated with SAC bearing specific allogenic MHC.

Balb/c mice were implanted with allogenic tail skin from C57BL/6 mice. Ten days after the implantation, splenocytes were cultured with medium alone (left panel), an anti-CD3 antibody (center panel) or allogenic SAC (right panel). IL-2 activity in the culture supernatant was determined by ELISA 20 hours after the initiation of the culture. CsA or MAC was intraperitoneally injected once a day for 9 consecutive days from 24 hours after the implantation. Values represent mean±S.D. of triplicate cultures. * Indicates a statistically significant difference when compared to the control culture ($p<0.05$, double-sided t-test).

or with SAC bearing specific alloantigen. Although the spleen cells only negligibly secreted IL-2 in the medium without stimulation *in vitro*, the stimulated spleen cells produced a significant amount of IL-2. Anti-CD-3 antibody induced the same level of IL-2 production in naïve mice and transplanted mice. However, specific alloantigen induced IL-2 more efficiently in transplanted mice than in naïve mice, suggesting that allogenic SACs specifically stimulate anti-allogenic helper T cells stimulated with the skin transplantation. MAC treatment significantly suppressed IL-2 production induced by anti-allogenic SAC without affecting the IL-2 production induced by anti-CD-3 antibody, while CsA treatment significantly suppressed the IL-2 production induced by both stimulators. These results suggest that MAC, but not CsA, suppresses IL-2 production by interfering with the antigen presentation process specific for allogenic SAC.

We evaluated the immunosuppressive activity of MAC with a skin transplantation experiment (Fig. 5). Tail skin was removed from Balb/c mice and engrafted to the left flank of recipient C57BL/6 mice. The rejection of allogenic skin began 8 days after the transplantation, and the skin grafts from all mice were rejected by 9 days after the transplantation. The mean graft survival time in control mice was 8.6±0.5 days. Daily MAC treatments starting 24 hours after implantation significantly elongated graft survival to 10.2±1.17 days ($p<0.05$). A greater suppressive effect occurred in CsA-treated mice. Half of the mice that

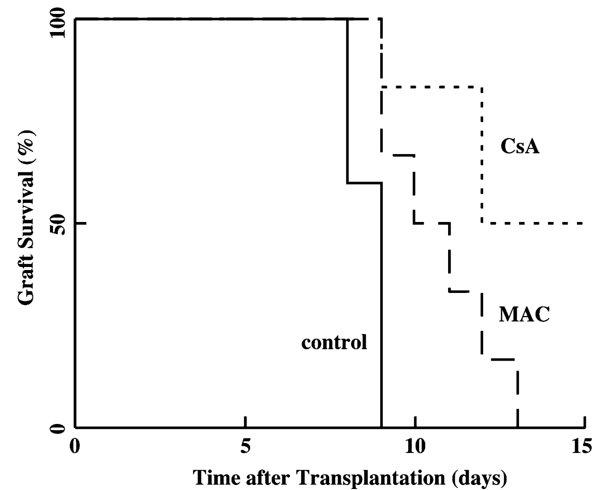

Fig. 5 MAC increases the survival time of allogenic skin grafts.

Balb/c mice were implanted with allogenic tail skin from C57BL/6 mice, and the graft survival time was followed for 15 days. Vehicle (—), 60 mg/kg of CsA (----), or 50 mg/kg of MAC (– –) was intraperitoneally injected consecutively once a day from 24 hours after the implantation. Mean survival time at day 15 was 8.6±0.49 days for the control group, 13.0±2.24 days for the CsA group, and 10.2±1.17 days for the MAC group. There was a statistically significant increase in the survival times of the CsA-treated group and MAC-treated group when compared to the control group ($p<0.05$, double-sided t-test).

received 60 mg/kg of intraperitoneal CsA had skin grafts that survived for more than 15 days.

Discussion

Elimination of reactive lymphocytes through the induction of apoptosis is a major mechanism engaged by immunosuppressants. MAC, a methylated derivative of the fungal metabolite ascochlorin, induces apoptosis of human lymphocytes [30]. MAC induces apoptosis of leukemia cell lines that are resistant to conventional apoptosis inducers, such as staurosporine, cycloheximide, and anti-Fas IgM. This observation led us to study the immunosuppressive activity of MAC in a murine skin graft model. As expected, MAC significantly suppressed CTL activity against specific alloantigen and increased the survival time of implanted skin grafts. However, MAC did not suppress the CTL activity induced by immunization with live allogenic tumor cells that were intraperitoneally injected. The lack of suppression was not caused by the lack of class II antigen on the tumor cells, because the resistance of CTL induction against MAC was observed with tumor cells bearing both class I and II MHC, and because MAC failed to suppress CTL activity against target cells bearing both class I and II MHC. Moreover, MAC did not suppress the killing activity of activated CTLs (data not shown), an immunosuppressive property distinct for prodigiosin 25-C or concanamycin A, which directly inhibit the cytotoxic machinery of CTLs by preventing acidification of their cytotoxic granules [33, 34]. These results suggest that MAC does not directly suppress cytotoxic T cells induced by allogenic antigen.

Another class of immunosuppressants, including FK506 and CsA, targets the IL-2 production of helper T cells. These immunosuppressants specifically bind to their own cytoplasmic binding proteins, which subsequently bind to and inhibit calcineurin, a phosphatase responsible for the nuclear transport of the regulatory domain of nuclear factor of activated T cells, a major transcription factor required to induce IL-2 in response to helper T-cell stimulation [1∼5]. We found that MAC significantly suppresses the IL-2 production of splenocytes from mice transplanted with allogenic skin when stimulated with SAC from specific allogenic mice. However, MAC did not suppress the IL-2 production stimulated with anti-CD3 antibody, which nonspecifically stimulates helper T cells. In contrast, CsA inhibited the IL-2 production induced by both stimulators. In addition, MAC did not inhibit IL-2 transcription stimulated by T-cell activators *in vitro* (data not shown). These results, together with the observation that MAC suppresses CTL activity induced by allogenic skin but not by allogenic tumor cells, suggest that MAC suppresses T-cell responses evoked by allogenic antigen by interfering with the antigen presentation process of professional APCs in the spleen.

According to a licensing model proposed by Guerder and Matzinger [35], helper T cells deliver a signal that activates APCs. The licensed APC can then directly stimulate killer T cells. The molecules responsible for the interaction are CD40 on helper T cells and CD40 ligand (CD40L) on APCs [36∼38]. Thus, licensed APCs can activate killer T cells without the aid of helper T cells. Instead of an interaction with helper T cells mediated through the CD40/CD40L interaction, inflammation or a viral infection activates the APCs to be in a licensed state [36, 37, 39]. Intraperitoneal injection with live tumor cells, which was conducted in the present study, might evoke inflammatory responses in the peritoneal cavity, leading APCs into a licensed state without T-cell help. Thus, intraperitoneal injection of P815 cells induces killer T cells in the absence of $CD4^{+}$ cells [40]. It is possible that MAC, which suppresses killer T cells induced by allogenic skin but not those induced by live allogenic tumor cells, inhibits the process required for APC licensing by helper T cells. The antigen presentation process regulates properties of the immunological network [39, 41], and the modulation of this process by specific antibodies is a recently developed strategy for immunosuppression. The present results demonstrate that MAC suppresses the induction of killer T cells induced by alloantigen through a mechanism different from those of immunosuppressants such as CsA, FK-506, prodigiosin 25-C, and FTY720. Given MAC uses a mechanism different from these immunosuppressants, MAC might supplement their clinical effects. We also expect that additional studies of MAC will elucidate a novel mechanism for APC licensing by helper T cells.

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