Polyamine compound deoxyspergualin inhibits heat shock protein-induced activation of immature dendritic cells

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Received: 22 February 2008 / Revised: 26 June 2008 / Accepted: 1 July 2008 / Published online: 7 August 2008
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Abstract Polyamine compound deoxyspergualin (DSG) is a potent immunosuppressive agent that has been applied clinically for protecting graft rejection and treatment of Wegener's granulomatosis. Though DSG can bind to heat-shock proteins (HSPs) in cells, its mechanism of immunosuppressive action remains unknown. It is widely accepted that extracellular HSPs are capable of stimulating dendritic cells (DC) through cell surface receptors, leading to DC activation and cytokine release. In this study, we examined if DSG analogs could inhibit HSP70-induced DC activation. Bone marrow derived immature mouse DCs and peripheral blood mononuclear cell-derived immature human DCs were generated and incubated with Alexa 488-labeled Hsp70 in the presence of methoxyDSG (Gus-1) that had comparable HSP70-binding affinity to DSG or DSG analog

GUS-7, which had much more reduced binding affinity for HSP70. The binding of HSP70 to immature DCs was analyzed by laser microscopy and flow cytometry. HSP70-induced DC activation was assessed by TNF- α release by enzyme-linked immunosorbent assay. Binding of Hsp70 to the cell surface of immature DCs was inhibited under the presence of Gus-1, but not under the presence of Gus-7. Immature DCs were activated and released TNF- α by the stimulation with HSP70 for 12 hours; however, the HSP70-induced TNF- α release was suppressed under the presence of Gus-1, and partially suppressed under the presence of Gus-7. Similar results were observed when immature human DCs were stimulated under the same conditions. Immunosuppressive mechanism of DSG may be explained, at least in part, by the inhibition of extracellular HSP70-DC interaction and HSP70-induced activation of immature DCs.

Electronic supplementary material The online version of this article (doi:10.1007/s12192-008-0064-y) contains supplementary material, which is available to authorized users.

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Keywords HSP70 · Deoxyspergualin · Dendritic cells ·
Immunosuppressant

Introduction

Heat-shock proteins (HSPs) are proteins whose expression is increased when the cells are exposed to elevated temperatures or other stress. They are known to play an important role in intracellular protein quality control since they serve to assist folding, sorting, and degradation of cellular proteins, thus preventing intracellular accumulation of degenerated proteins. Recently, it was revealed that extracellular HSPs could serve as a danger signal, which activate inflammatory response and natural immunity in response to cellular injury (Manjili et al. 2005; Asea 2006;

Calderwood et al. 2007). 70 kD HSP (HSP70) and 96 kD HSP (gp96) have been well documented for the mechanism of activation (Doody et al. 2004; Massa et al. 2005; Li et al. 2006; Bendz et al. 2007). HSPs are released into extracellular fluid during cell death or injury, and then bind to the surface of immune cells through cell surface receptors, such as toll-like receptor (TLR) 2, TLR4, CD91, CD40, CCR5, LOX-1, and Scavenger receptor A (Arnold-Schild et al. 1999; Delneste et al. 2002; Roelofs et al. 2006; Warger et al. 2006; Facciponte et al. 2007; Pido-Lopez et al. 2007). The extracellular HSPs can elicit cytokine releases from dendritic cells, macrophages and lymphocytes, leading to activation of innate immunity (Todryk et al. 1999; Asea et al. 2000; Asea 2006). In addition, HSPs are capable of facilitating antigen cross-presentation in dendritic cells, i.e., presentation of extracellular antigens to major histocompatibility complex (MHC) class I pathway, therefore leading to activation of adaptive immunity as well (SenGupta et al. 2004; Ueda et al. 2004; Facciponte et al. 2005; Enomoto et al. 2006; Bendz et al. 2007; Kurotaki et al. 2007). Such an unique immunopotentiative character of HSPs are now applied to vaccine adjuvants, especially in the field of cancer immunotherapy (Srivastava and Udono 1994; Tamura et al. 1997; Noessner et al. 2002; Hauser et al. 2004; Ueda et al. 2004; Wang et al. 2005).

On the other hand, immunosuppressive therapy is required in the field of organ transplantation and autoimmune diseases. Deoxyspergualine (DSG) is one of such immunosuppressive agents, which has been used after renal transplantation and for the treatment of glomerulonephritis (Amemiya 1996; Hotta et al. 1999; Kozaki et al. 1999; Amada et al. 2005; Lorenz et al. 2005). Recently, it was revealed that DSG was effective in the treatment of refractory Wegener's granulomatosis (Schmitt et al. 2005; Erickson and Hwang 2007). Disease improvement during treatment with DSG was achieved in 70% of cases (Birck et al. 2003). In spite of the clinical efficacy of DSG, molecular mechanism of the immunosuppressive action has been still enigmatic. Nadler et al. demonstrated previously that DSG could bind to HSP70 and HSP90 (Nadler et al. 1992, 1998; Nadeau et al. 1994). They showed that DSG can suppress NF- κ B signal indirectly by binding to intracellular HSP70 (Nadler et al. 1995). We have found that DSG inhibited the association of HSP70 to TAP, thus leading to inhibition of MHC class I antigen presentation (Kamiguchi et al. 2008). In the present study, we focused on the effect of DSG to immunopotentiative action of extracellular HSPs. Binding of HSP70 to the cell surface and subsequent cytokine release from dendritic cells were assessed in the presence or absence of DSG analogs that have a distinct binding affinity to HSP70. We demonstrate that immunosuppressive action of DSG is mediated, at least in part, through blocking of danger signals of HSP70.

Materials and methods

Reagents

Two distinct DSG analogs, Gus-1 (methoxyDSG) and Gus-7 (Fig. 1), were provided by Nippon Kayaku (Tokyo, Japan). DSG and Gus-1 have a high binding affinity for HSP70 ($K_d=7\ \mu\text{M}$ and $4\ \mu\text{M}$, respectively), whereas Gus-7 has much reduced affinity for HSP70 ($K_d=250\ \mu\text{M}$) (Nadeau et al. 1994). Purified human Hsp70 and recombinant HSP90 were purchased from StressGen Biotech (Ann Arbor, MI). Bovine serum albumin (BSA) and phosphorylase B were purchased from Sigma-Aldrich (St. Louis, MO).

Generation of bone marrow derived dendritic cells

Bone marrow derived immature mouse DCs were generated from the femurs and tibia of 5- to 6-week-old C57BL/6 mice (CLEA Japan, Tokyo, Japan). Bone marrow cells (1×10^5 /well in a 24-well plate) were incubated in a complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (GIBCO/Invitrogen, Carlsbad, CA) and 20 ng/ml GM-CSF (Endogen, Woburn, MA) for 5 days. GM-CSF-containing medium was gently replaced on day 2 and day 4.

Immature human DCs were generated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers after obtaining informed consent. Briefly, PBMCs were isolated by using Lymphoprep (Nycomed, Oslo, Norway), and then separated into CD14⁺ cells and CD14⁻ cells by using MACS separation system and anti-CD14 monoclonal antibody-coupled magnetic microbeads (Miltenyi Biotech, Bergish Blabach, Germany) according to the manufacturer's

DSG

Gus-1

Gus-7.

Fig. 1 Structure of DSG and two distinct DSG analogs, Gus-1 and Gus-7. Gus-1 (methoxyDSG) has high binding affinity for HSP70 ($K_d=4\ \mu\text{M}$), whereas Gus-7 has reduced binding affinity for HSP70 ($K_d=250\ \mu\text{M}$) as compared to DSG and Gus-1

instruction. Immature DCs were generated from CD14⁺ cells in the plastic flask by culturing in AIM-V medium (GIBCO-Invitrogen Japan) supplemented with 10% human serum, HEPES (10 mmol/L), 2-mercaptoethanol (50 μ mol/L), granulocyte macrophage colony-stimulating factor (100 ng/mL), and IL-4 (1,000 units/mL) for 7 days.

Detection of fluorescence-labeled Hsp70 on the cell surface of DCs

HSP70 and phosphorylase B were labeled with Alexa Fluor 488 by using Alexa Fluor 488 Protein Labeling Kit (Invitrogen, Japan) according to the manufacturer's instruction (Kurotaki et al. 2007). DCs were incubated with 10 μ g/ml of Alexa-labeled Hsp70 or Alexa-labeled phosphorylase B in the presence of Gus-1 or Gus-7 (10 μ g/ml). DCs were then washed twice with ice-cold phosphate-buffered saline (PBS), fixed with ice-cold 1% formaldehyde PBS, and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) or laser microscopy (Olympus, Japan).

TNF- α release assay

Dendritic cells were pulsed with the indicated concentrations of Hsp70 in the presence of Gus-1 or Gus-7 (10 μ g/ml). After incubation for 12 hours, the concentration of TNF- α released into the culture supernatant was quantified by using mouse TNF ELISA kit (Endogen, Woburn, fMA).

Results

DSG inhibits the binding of HSP70 to the cell surface of immature DCs

Purified HSP70 and phosphorylase B were labeled with Alexa Fluor 488 and then incubated with immature mouse DCs for 30 min on ice. Laser microscopic analysis revealed that HSP70 was bound to the cell surface of DCs with a dot-like pattern, whereas phosphorylase B was not (Fig. 2A). Then, the fluorescence intensity was analyzed by flow cytometer in the presence or absence of Gus-1 or Gus-7 (10 μ g/mL). As shown in Fig. 2B, the fluorescence intensity of HSP70-pulsed DCs was not changed between PBS and Gus-7 (left panel), whereas it was decreased in the presence of Gus-1 (right panel). These data indicate that Gus-1, but not Gus-7 could inhibit the binding of HSP70 to the cell surface of DCs.

HSP70 stimulation induces TNF- α release of immature DCs

Next, we examined if HSP70 can activate immature DCs. As an activation index, the level of TNF- α release was assessed by ELISA. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (1×10^5 cells/well), and TNF- α concentration of culture supernatant was quantified after 12-hour incubation. Stimulation with

Fig. 2 DSG inhibits the binding of HSP70 to the cell surface of immature DCs. Purified HSP70 and phosphorylase B were labeled with Alexa Fluor 488 and then incubated with immature DCs for 30 min on ice. After washing twice with ice-cold PBS, cells were fixed in 1% formaldehyde PBS and subjected to laser microscopic examination (A) or flow cytometry (B). In B, fluorescence intensity was analyzed after incubation with or without Gus-1 (*right panel*) or Gus-7 (*left panel*). P-B indicates fluorescence intensity after incubation with Alexa 488-labeled phosphorylase B

HSP70 clearly induced the release of TNF- α from DCs, while BSA failed under the same condition (Fig. 3A). Time course of TNF- α release indicated that it reached to almost the peak level after 12 hours incubation (supplemental figure). We also examined if HSP90 was capable of stimulating human DCs. As shown in Fig. 3B, HSP90 has just minimal effect on the immature human DCs. As a positive control, the same numbers of DCs were stimulated with the indicated concentration of lipopolysaccharide (LPS). Examination of the LPS concentration in the HSP70 used in this study revealed approximately 25 pg/ml (Mitsubishi Chemical Medience, Japan). These data indicate that HSP70 may have higher stimulatory activity to immature DCs as compared with HSP90, as far as TNF- α release is assessed as an activation index. IL-12 release assay resulted in the similar results (data not shown).

DSG inhibits the HSP70-induced TNF- α release of immature DCs

We then examined if DSG analogs could inhibit the HSP70-induced activation of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (5×10^4 cells/well) in the presence or absence of

Gus-1 or Gus-7 (10 μ g/ml). Following incubation for 12 hours, TNF- α concentration of culture supernatant was quantified. It was shown that TNF- α release was inhibited to almost the half level of PBS control in the presence of Gus-1, whereas it was partially inhibited in the presence of Gus-7 (Fig. 4A). Similar results were obtained when immature human DCs (1×10^5 cell /well) were pulsed with HSP70 in the presence or absence of Gus-1 or Gus-7 (10 μ g/ml), though the significant difference was observed only in the case of 10 μ g/ml pulsation of HSP70 (Fig. 4B).

In order to rule out the possibility that the suppressive action of DSG might be mediated through intracellular molecules, such as intracellular HSP70 and HSP90, immature mouse DCs were pre-incubated for 2 hours with Gus-1 or Gus-7 (10 μ g/ml), then washed twice and pulsed with 5 μ g/ml of HSP70 in the presence or absence of Gus-1 (10 μ g/ml). The data show that pre-incubation did not have any effect on the HSP70-induced TNF- α release, indicating that the suppressive action of DGS might be resulted from the binding of DSG to extracellular HSP70 (Fig. 4C).

DSG inhibits the HSP70-induced maturation of DCs

We next examined if DSG analogs could affect the HSP70-induced maturation of immature DCs. Immature mouse DCs were incubated for 12 hours in the presence or absence of 10 μ g/ml of HSP70. In some cases, Gus-1 or Gus-7 (10 μ g/ml) was added into the culture. Following incubation for 12 hours, cell surface level of CD80 was analyzed by flow cytometry as a DC maturation index. It was shown that incubation of immature DCs with HSP70 increased the CD80 levels, indicating that HSP70 induced DC maturation. The HSP70-induced DC maturation was suppressed in the presence of Gus-1 (Fig. 5A), but not in the presence of Gus-7 (Fig. 5B). The other maturation markers such as CD86 and CD40 were not changed after the stimulation with HSP70. The data imply that HSP70-induced partial maturation of immature DCs detected by CD80 expression was inhibited in the presence of DSG.

Discussion

In the present study, we demonstrated for the first time that DSG analogs had suppressive activity to the so-called “danger signals.” A variety of cell injuries damage the cell membrane, leading to the release of intracellular HSPs into extracellular fluids. The released HSPs are bound to immunostimulatory molecules, such as TLRs (TLR2 and 4), CD40 and CCR5, or captured by scavenger receptor families, such as CD91, LOX-1 and SR-A (Arnold-Schild et al. 1999; Delneste et al. 2002; Lipsker et al. 2002; Roelofs et al. 2006; Warger et al. 2006; Facciponte et al.

Fig. 3 HSP70 stimulation induces TNF- α release of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (50,000 cells/well), and TNF- α concentration of culture supernatant was quantified after 12 hours (A). The indicated concentrations of HSP90 or BSA were pulsed to immature human DCs (1×10^5 cells/well), and TNF- α concentration of culture supernatant was quantified after 12 hours (B). The data represent the mean values of triplicated samples and standard deviations. As a positive control, the indicated concentration of LPS was pulsed to the same numbers of immature mouse DCs

Fig. 4 DSG inhibits the HSP70-induced TNF- α release of immature DCs. The indicated concentrations of HSP70 or BSA were pulsed to immature mouse DCs (5×10^4 cells/well) in the presence or absence of Gus-1 or Gus-7 (10 μ g/ml) for 12 hours. TNF- α concentration of culture supernatant was quantified (A). The indicated concentrations of HSP70 or BSA were pulsed to immature human DCs (1×10^5 cells/well) in the presence or absence of Gus-1 or Gus-7 (10 μ g/ml) for

12 hours. TNF- α concentration of culture supernatant was quantified (B). Immature mouse DCs (50,000 cells/well) were pre-incubated for 2 hours with Gus-1 or Gus-7 (10 μ g/ml), then washed twice and pulsed with 5 μ g/ml of HSP70 in the presence or absence of Gus-1 (10 μ g/ml), followed by TNF- α quantification (C). The data represent the mean values of triplicated samples and standard deviations

2007; Pido-Lopez et al. 2007). The former stimulation induces release of inflammatory cytokines, thus activating innate immune responses (Asea et al. 2000). On the other hand, the latter stimulation induces endocytosis of HSPs with HSP-bound antigenic proteins or peptides, then facilitating the cross-presentation of the HSP-bound antigens and acquired immunity (Noessner et al. 2002; SenGupta et al. 2004). Though the mechanism of the

HSP-mediated efficient cross-presentation remains largely unknown, we have reported previously that HSP70 and HSP90 could facilitate the transfer of endosomal antigens to recycled endosomes that contain empty MHC class I molecules (Ueda et al. 2004; Kurotaki et al. 2007). In addition, HSP90 could also assist the transfer of endosomal antigens into cytosol by unknown mechanism, and facilitate the proteasomal degradation of the antigens (Kurotaki et al.

Fig. 5 HSP70 stimulation induces up-regulation CD80 level of immature DCs. Immature mouse DC were stimulated with 10 μ g/ml of HSP70 or BSA, cultured for 12 hours and examined for the cell surface level of CD80 by flow cytometry. In A and B, Gus-1 or Gus-7 (10 μ g/ml) was included in the culture, respectively

2007). In the present study, we clearly demonstrate that DSG was capable of inhibiting the binding of extracellular HSP70 to the cell surface of DCs. Therefore, it is highly likely that DSG inhibits not only innate immune responses but also acquired immune responses.

Deoxyspergualin has been used clinically as an immunosuppressive agent after organ transplantation (Nomura et al. 1998). Moreover, most recent clinical trials in Germany demonstrated that it is effective to refractory autoimmune vasculitis including Wegener's granulomatosis (Schmitt et al. 2005; Erickson and Hwang 2007). Concerning the mechanism of immunosuppressive and anti-inflammatory actions of DSG, there are several reports that explain the molecular mechanism (Amemiya 1996). Nadler et al. demonstrated that the intracellular targets of DSG were HSP70 and HSP90 and suggested that suppression of NF- κ B signal might be involved in the anti-inflammatory reaction (Nadler et al. 1995). However, there are some controversial data as to the hypothesis. Kawada et al. showed that DSG inhibits AKT signals and phosphatidylcholine synthesis (Kawada et al. 2002). Waaga et al. reported that DSG could down-regulate the expression of MHC class II molecules, though the precise mechanism remained unknown (Waaga et al. 1996). Our group showed that intracellular HSP70 might be a target of DSG, and the binding of DSG to HSP70 disrupted the association of cytosolic antigen-bound HSP70 with TAP, leading to inhibition of TAP-mediated peptide transfer into the endoplasmic reticulum and down-regulation of cell surface MHC class I levels (Kamiguchi et al. 2008). In the present study, we demonstrated that the target molecules of DSG might be not only intracellular HSPs but also extracellular HSPs, which mediate the so-called "danger signals". It is expected that DSG might be effective in the treatment of other refractory inflammatory or autoimmune diseases, such as Behcet disease, inflammatory bowel diseases, glomerulonephritis, and systemic inflammatory response syndrome, which are considered to be broken out by dysregulated activation of danger signals.

Acknowledgment We are grateful to Dr. Hisami Ikeda of Hokkaido Red Cross Blood Center for his generous help in our study. This study was supported in part by a grant-aid for Clinical Cancer Research from the Ministry of Health, Labor and Welfare of Japan and a grant-aid from Ministry of Education, Culture, Sports, Science and Technology of Japan.

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