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Immunosuppressant Deoxyspergualin-induced Inhibition of Cell Proliferation is Accompanied with an Enhanced Reduction of Tetrazolium Salt

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Deoxyspergualin (DSG) has both antitumor and immunosuppressive activities. We explored the mechanism of DSG activities using an aqueous soluble analogue, methyldeoxyspergualin (MeDSG) for *in vitro* culture studies. It is known that DSG has inhibitory effects on cell proliferation, and we also observed that MeDSG inhibited [^{3}H]-thymidine incorporation by rapidly dividing murine T cell hybridomas. However, when tetrazolium (MTT) colorimetric assay was adopted to evaluate its inhibitory effects on cell proliferation, MeDSG induced an enhanced MTT reduction. When we examined whether these results were applicable to the actively dividing cells of other origins than T cells, similar effects were seen with Raji cells, J774.1 cells and NIH3T3 cells. N-30, another analogue which was capable of suppressing anti-SRBC antibody production *in vivo*, also induced inhibition of cell growth and an enhanced MTT reduction. In contrast, the analogue which failed to prevent the antibody production, neither enhanced MTT reduction nor inhibited cell proliferation. Our results demonstrated that the ability to generate MTT formazan in dividing cells is a common property among DSG analogue with the immunosuppressive and antiproliferative activities.

The immunosuppresant deoxyspergualin (DSG) is a synthetic derivative of spergualin, which is a natural product isolated from *Bacillus laterosporus*. Spergualin was originally described as an antitumor agent[1], and subsequently found to possess immunosuppressive properties in experimental animal transplantation[2]. In the additional studies, DSG has demonstrated immunosuppressive activity in many animal models of transplant rejection including those of kidney, heart, liver, pancreas, pancreatic islets and bone marrow. These include transplants of various organs in diversed donor-host as either allo- or xeno-disparities[3~5]. The safety and effectiveness of the DSG treatment in human transplantation were proven in kidney graft recipients[6,7]. Moreover, *in vivo* studies demonstrated a potent immunosuppressive effect in autoimmune models[8].

Several studies have compared the immunosuppressive activity of DSG to the popular immunosuppressive agents, cyclosporin A (CsA) and FK506[9~11]. DSG is frequently more effective than other immunosuppressive drugs, and the side effects of DSG both in animals and patients appear to be minimal and reversible at the cessation of treatment[7,14,15].

A number of studies have described that DSG is shown to modulate various immune responses such as T cell proliferation[14~16] and differentiation[17], CTL generation[15,18,19], B cell maturation[17,20], antibody production[21~23], and antigen presentation[24]. DSG was found to interact intracellularly with Hsc 70, the constitutively expressed member of the heat shock protein 70 (Hsp 70) family[25]. However, the precise relationship between Hsc 70 and DSG is uncertain at the present time.

Although it is clear that DSG has strong immune suppressive activity, little is known about the mechanism of action of DSG[26]. As described above, DSG is known to inhibit the growth of a number of tumor cell lines. During investigations on the antiproliferative effects of MeDSG which is methylated in the hydroxyl group at position 15 of DSG, we observed that rapidly growing cells are more sensitive to MeDSG than resting cells. To define the mechanism of action of DSG, we chose to use

different kinds of dividing cells in terms of origin.

In the present study, we found that the cells treated with MeDSG exhibited a remarkable discrepancy between cell number and reduction of tetrazolium salt, *i.e.*, the inhibition of cell growth was accompanied with an enhanced formazan generation. Furthermore, the discrepancy was observed in the cells treated with another analogue which possessed immunosuppressive activity. On the other hand, the analogue which failed to suppress antibody production, did not possess such biological properties in *in vitro* experimental studies.

Materials and Methods

Reagents

Deoxyspergualin (DSG) and its analogues were synthesized in our laboratory. These reagents were dissolved in PBS and used for the experiments.

Mice

8 wk-old female Balb/c mice were obtained from Clea Japan Inc. (Tokyo, Japan).

Cells

Murine T cell hybridomas 2-45-12 and N3-6-71 were described previously[27]. Burkitt lymphoma Raji cells were a gift of Dr. D. BORASCHI (Dompe Research Center, Italy). J774.1 is a murine macrophage-like cell line. These cells and murine fibroblast NIH3T3 cells were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10 % heat-inactivated FCS (Hyclone Laboratories, Logan NY), 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. In the experiments for evaluation of the influence of MeDSG or its analogues on cell cultures, FCS was replaced by human AB serum since FCS contains large amounts of polymamine oxidase and may hydrolyze DSG and interfere with its activity[28].

Determination of Cell Proliferation

T cell hybridomas were incubated in the presence or absence of MeDSG for 24 hours or 48 hours, followed by a 2-hour pulse with 1 μCi of [methyl-^{3}H]-thymidine. Cells were then harvested on glass-filter paper, and the rate of [^{3}H]-thymidine uptake was quaintitated by liquid scintillation counting. Alternatively, after cells were treated with indicated agents, cell number was determined by a Coulter Counter model ZM (Coulter Electronics Inc., Beds, U.K.).

MTT assay

After cells were cultured in the presence or absence of MeDSG, N-30 or N-353 in 96-well microtiter plate for the indicated periods. MTT colorimertic assay was performed by using Celltiter 96™ aqueous non-radioactive cell proliferation assay kit (Promega, Madison, WI), as following the supplies' instructions. The absorbance was determined at 492 nm in a microtiter plate reader, titertek miltiskan MCC/340 MKII (ICN Pharmaceuticals, Inc., Costa Mesa, CA).

Plaque-forming Cell (PFC) Assay

Balb/c mice received an intravenous injection of sheep red blood cells (SRBC) (1×10^8) on the day 0. DSG or its analogues were intraperiteneally administered once a day for 3 days starting one day after the immunization. On 4 days after the SRBC immunization, spleens were removed from these mice and assayed for the plaque-forming cells-producing anti-SRBC antibodies. Control mice received saline in stead of these drugs under the same condition. Each experimental group consisted of 5 to 7 mice. The percentage of inhibition was calculated by the following formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{mean of numbers of PFC/spleen in drug-injected group}}{\text{mean of numbers of PFC/spleen in control group}}\right) \times 100$$

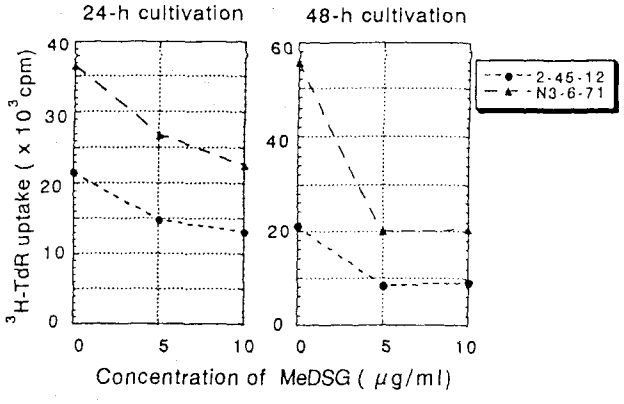
Results

MeDSG Inhibits [^{3}H]-Thymidine Incorporation by Murine T Cell Hybridomas

Methyldeoxyspergualin (MeDSG) is a DSG analogue, which has a hydroxyl group at position 15 of DSG methylated. We have used MeDSG for *in vitro* culture studies because it is more resistant to hydrolysis in *in vitro* culture than DSG but still possesses similar immunosuppressive activity. Although a number of studies reported DSG-mediated inhibition of immune responses *in vitro* at concentrations greater than 50~200 μg/ml, we used MeDSG at concentrations of 5 to 20 μg/ml, which are thought to be achievable in *in vivo* animal and human pharmacokinetic studies.

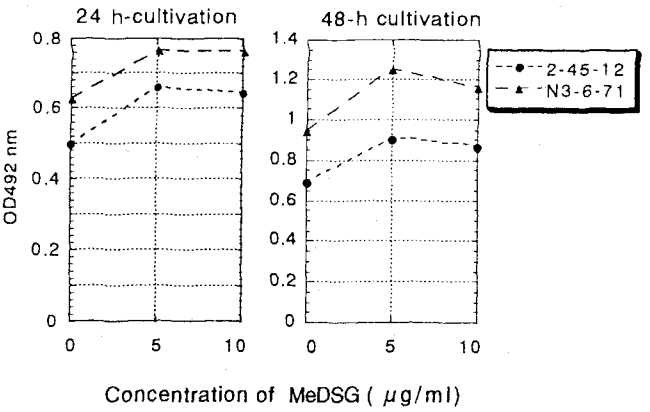
We examined the effects of MeDSG on proliferation of murine T cell hybridomas by [^{3}H]-thymidine incorporation. As shown in Fig. 1, when 2-45-12 cells or N-3-6-71 cells were treated with MeDSG for 24 hours,

Fig. 1. Inhibition of cell proliferation in MeDSG-treated murine T cell hybridomas.



Murine T cell hybridoma 2-45-12 or N3-6-71 were cultured in the presence or absence of MeDSG. The cell numbers in 96-well microtiter plate for 24 h-cultivation and 48 h-cultivation were 5×10^3 cells and 2×10^3 cells, respectively. After 24 hours or 48 hours, cells were pulsed with [^{3}H]-thymidine, and harvested after an additional 2 hours. Incorporation of [^{3}H]-thymidine into cellular DNA was measured in a scintillation counter. The results are expressed as the mean of triplicate cultures and the standard deviations are less than 10% of the mean.

Fig. 2. Enhancement of MTT reduction in MeDSG-treated murine T cell hybridomas.



2-45-12 or N3-6-71 were cultured in the presence or absence of MeDSG. The cell numbers in 96-well microtiter plate for 24 h-cultivation and 48 h-cultivation were 5×10^3 cells and 2×10^3 cells, respectively. Twenty-four or forty-eight hours later, MTT reagent was added, and the amount of formazan production was measured an additional 2 hours later as described in Materials and Methods. The results are expressed as the means of triplicate cultures and the standard deviations are less than 10 % of the mean.

a decrease in [^{3}H]-thymidine incorporation was observed in a dose-dependent manner. At 48-hour cultivation, the inhibition of cell proliferation was more significant in N3-6-71 cells treated with MeDSG.

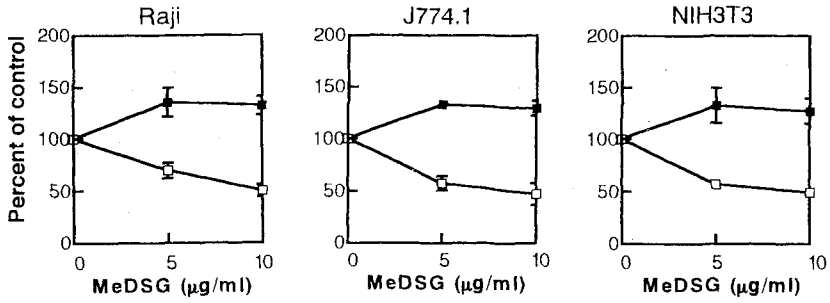
MeDSG Induces an Enhanced MTT Reduction in Murine T Cell Hybridomas

Tetrazolium salts such as MTT are metabolized by mitochondorial dehydrogenases to form a blue formazan dye and therefore is commonly employed as indicators of cell number and viability. MTT assay has also been proposed as a valid alternative to [^{3}H]-thymidine uptake method for analyzing cell proliferation [29]. Therefore, we used MTT assay to evaluate the inhibitory effect of MeDSG on cell proliferation. After 2-45-12 or N-3-6-71 cells were incubated in the presence or absence of MeDSG, the amount of MTT formazan generation was measured at 24-hour or 48-hour cultivation. The results are shown in Fig. 2. Surprisingly, MeDSG induced an enhanced reduction of MTT to formazan at 24-h incubation, at which time the cell proliferation was markedly inhibited, when evaluated by [^{3}H]-thymidine incorporation. At 48-hour cultivation, a remarkable increase in MTT formazan production was observed in MeDSG-treated cells. Thus, MeDSG caused a significant enhancement of MTT reduction. Similar finding was observed in MeDSG-treated human leukemia cell line Jurkat (data not shown). However, MeDSG itself did not exert any effect on MTT formazan generation in the absence of cells (data not shown).

MeDSG Induces the Inhibition of Cell Growth and an Enhanced MTT Reduction in Dividing Cells of Other Origins than T Cells

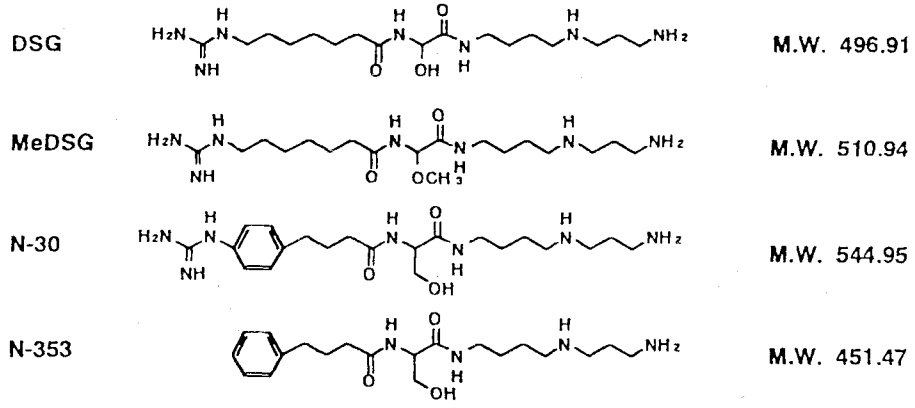
We investigated whether the effects of MeDSG on the production of MTT formazan would be restricted to T cells. Therefore, the effects of MeDSG on cell growth and MTT reduction were examined using human Burkitt lymphoma Raji cells, murine macrophage-like J774.1 cells, or murine fibroblast NIH3T3 cells. After these cells were cultured with or without MeDSG (5 or 10 μg/ml) for 72 hours, cell growth was evaluated by direct cell counting and MTT assay was performed. The results are presented in Fig. 3. When Raji cells, J774.1 cells, or NIH3T3 cells were treated with MeDSG for 72 hours, cell growth was markedly inhibited. In contrast, MeDSG enhanced the MTT reduction in these cells. Thus, a remarkable discrepancy between cell number and MTT

Fig. 3. Effects of MeDSG on cell growth and MTT reduction in Raji cells, J774.1 cells or NIH3T3 cells.



Raji cells, J774.1 cells or NIH 3T3 cells were prepared at 2×10^3 cells/well in 96-well microtiter plate and cultured in the presence or absence of MeDSG for 72 hours. Cell number was evaluated by direct counting. Results are expressed as percentage of the cell number in the absence of MeDSG and represent the mean ± SD of triplicate cultures (□). MTT assay was performed as described in Materials and Methods and absorbance was determined at 492 nm in a microplate reader. Results are expressed as percentage of the absorbance in the absence of MeDSG and represent the mean ± SD of triplicate cultures (■).

Fig. 4. The structure of deoxyspergualin (DSG) and its synthesized analogues.



reduction in the MeDSG treatment was also applicable to the actively dividing cells of other origins than T cells.

Effects of DSG Analogues on *In Vivo* Antibody Production and on *In Vitro* Cell Culture

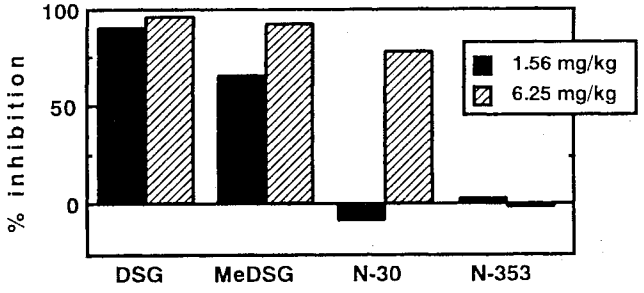
DSG consists of three portions of the 7-guanidinoheptanoic acid, α-hydroxyglycine, and spermidine moieties. These portions are postulated to play an important role in the manifestation of biological activities of DSG[30]. The structures of four synthesized analogues are represented in Fig. 4. N-30 is reported to retain immunosuppressive activity[30]. Since N-353 possesses a modification of guanidine in N-30, we predicted that N-353 resulted in loss of immunosuppressive activity.

The ability of these four analogues to prevent anti-SRBC antibody production was evaluated by plaque-forming cell (PFC) assay. The results are shown in Fig. 5. DSG showed excellent effects at the two doses used in this experiments, while MeDSG exhibited a slightly weaker effect at a dose of 1.56 mg/kg than DSG and was as effective as DSG at a dose of 6.25 mg/kg. As demonstrated previously[30], N-30 revealed a strong suppression of antibody formation at a dose of 6.25 mg/kg, but was not effective at lower concentration. Administration of N-353 failed to suppress anti-SRBC antibody formation.

Next, the effects of the above analogues except DSG on cell growth and MTT reduction were examined using T cell hybridomas 2-45-12. The concentrations of three analogues used for this experiments were lower than those in the experiments of Fig. 1 and 2. After T cells were treated with MeDSG, N-30 or N-353 for 48 hours, cell growth was evaluated by direct cell counting (Fig. 6).

MeDSG induced cell growth inhibition and the enhancement of MTT reduction as indicated in Fig. 1 and 2. N-30, which possessed immunosuppressive activity, inhibited cell growth in a dose-dependent manner. Additionally, an enhanced formazan production was detected in N-30-treated cells. Thus, a marked discrepancy between cell number and MTT formazan production was observed even in N-30-treated cells. On the other hand, N-353 which was unable to suppress antibody production *in vivo* affected neither cell proliferation nor MTT reduction.

Fig. 5. Immunosuppressive activity of deoxyspergualin (DSG) and its synthesized analogues in anti-SRBC antibody formation.



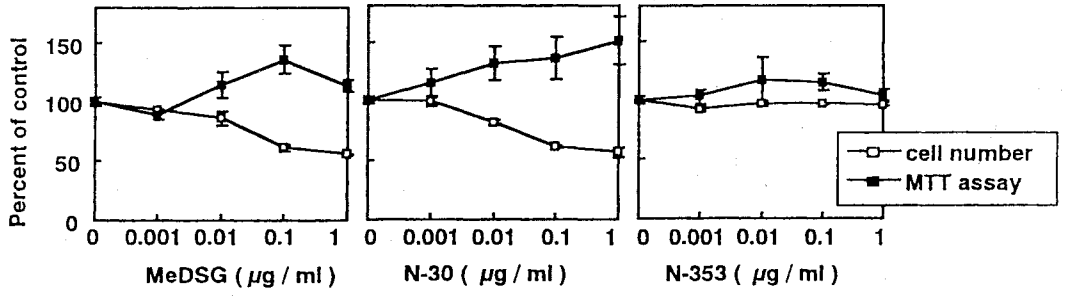
Balb/c mice received an intravenous injection of sheep red blood cells (SRBC) (1×10^8) on the day 0. Each compound was administered i.p. at a dose of 1.56 mg/ml or 6.25 mg/ml once a day for 3 days starting one day after the immunization. On 4 days after SRBC immunization, the spleens were removed from mice and assayed for plaque-forming cell-producing anti-SRBC antibodies. The percentage of inhibition of anti-SRBC antibody formation was calculated as described in Materials and Methods.

Discussion

In this study, we observed that MeDSG inhibited the cell growth in murine T cell hybridomas, Raji cells, J774.1 cells and NIH3T3 cells. DSG was originally described as an antitumor agent and showed the activities in non-solid tumors, such as erythromyeloid tumors and leukemia cell lines. It was demonstrated that MTT reduction to formazan was proportional to the number of metabolically viable cells in culture. Therefore, the MTT colorimetric assay is currently used as the method for estimating cell growth. When the MTT assay was adopted to evaluate the inhibitory effects of MeDSG on cell proliferation, we found that MeDSG enhanced MTT reduction in all the cells which we tested. Thus, MeDSG appears to induce the inhibition of cell growth and an enhanced MTT reduction in multiple cell types.

Additionally, N-30, another analogue which possessed immunosuppressive activity induced the enhancement of MTT reduction whereas it significantly inhibited cell growth when cell number was evaluated by direct cell counting. In contrast, N-353 which failed to suppress anti-SRBC antibody production did not have such biological properties. Our results demonstrated that the ability to enhance production of MTT in dividing cells is a common biological property among DSG analogue with immunosuppressive and antiproliferative activities. Since MeDSG itself did not exert any effect on formazan generation in MTT assay and the capacity of long-term treated cells to generate MTT formazan was clearly increased, we could rule out the possibility of chemical interaction between MeDSG and MTT in the reduction process.

Fig. 6. Influence of the DSG analogues on cell proliferation and MTT reduction in murine T cell hybridoma 2-45-12.



2-45-12 cells were prepared at 1×10^3 cells/well in 96-well microtiterplate and cultured in the presence or absence of the indicated analogues for 48 hours. Cell number was evaluated by direct cell counting. Results are expressed as percentage of the cell number in the absence of the indicated analogues and represent the mean ± SD of triplicate cultures. MTT assay was performed as described in Materials and Methods and the absorbance was determined at 492 nm in a microplate reader. Results are expressed as percentage of the absorbance in the absence of MeDSG and represent the mean ± SD of triplicate cultures.

It has been reported that MTT is reduced at the ubiquinone and cytochrome b and c sites of mitochondrial electron transport system[31]. However, recent studies demonstrated that MTT reduction occurs at multiple cellular sites, mostly at extramitochondrial sites[32]. It was reported that a number of factors, such as medium pH, glucose concentrations in medium and age of cultures, affect formazan generation[33]. Further experiments will be necessary to investigate the involvement of these factors in our experiments. Genistein, a specific inhibitor of tyrosine specific protein kinases, is reported to show the same discrepancy between the real number of viable cells and the amount of formazan generation[34]. It was concluded that tumor cells arrested in G_2/M cell cycle phase by genistein show an enhanced MTT reduction due to an increase of mitochondrial activity. When distribution of cell cycle in MeDSG-treated T cell hybridomas was analyzed, it revealed a progressive accumulation of cells in the G_1 phase of the cycle (data not shown).

As previously shown, DSG has inhibitory effects on *in vitro* T cell proliferation provoked by stimuli such as PHA and Con A[9,18]. When DSG is added to an *in vitro* culture at anytime within 48 hours after the initiation of the culture, it is equally effective at suppressing lymphocyte proliferation. *In vivo* transplant studies have demonstrated that DSG acts most effectively during the induction phase of the immune response at a later time than CsA. DSG is capable of blocking late rejection or even reverse ongoing rejection in situation which other immunosuppressive drugs were not effective[7,11]. We have observed that rapidly growing cells were more sensitive to MeDSG than slowly growing cells, and that quiescent cells were resistant to MeDSG (data not shown). Although the mechanism of enhancement of MTT reduction in MeDSG-treated cells is not yet clear, MeDSG may influence mitochondrial respiratory function. Rapidly dividing cells in culture continuously require energy and therefore may be susceptible to DSG. Further experiments will be necessary to examine the effect of MeDSG on mitochondrial respiratory function.

Although we can not define the mechanism by which MeDSG or N-30 induce an increase of MTT reduction in growing cells, our finding will help to elucidate the mechanism of action of DSG.

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