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Immunosuppressant Deoxyspergualin Affects Mitochondrial Respiratory Function in Growing Cells

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The immunosuppressant deoxyspergualin (DSG) is a synthetic derivative of spergualin that is a natural product isolated from *Bacillus laterosporus*[1]. It showed antitumor activities against some nonsolid tumors[1], and was subsequently found to possess immunosuppressive properties[2]. Although DSG has been shown to inhibit various immune responses such as T cell proliferation, CTL generation, T cell differentiation, B cell differentiation, antibody production, antigen presentation, and monocyte/macrophage function[3], there are a number of conflicting reports on its effects in these *in vitro* experiments. DSG has been found to interact intracelluarly with Hsc70, a constitutively expressed member of the heat shock protein 70 (Hsp 70) family[4], but the mechanism of action of DSG still requires further explanation.

We have used methyldeoxyspergualin (MeDSG) for *in vitro* culture studies of DSG, because it is more stable to hydrolysis than DSG but still possesses a similar immunosuppressive activity. Recently, we found that rapidly dividing cells are more sensitive to MeDSG than slowly growing cells and that MeDSG inhibits cell growth, followed by apoptosis. Quiescent cells are resistant to MeDSG[5]. Tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and therefore are commonly employed as indicators of cell number and viability. We have also observed that an enhanced MTT reduction precedes apoptosis in MeDSG-treated cells[6].

The aim of this study was to explore the cellular events related to the action of MeDSG, particularly focusing on its effect on mitochondrial respiratory function in dividing cells. We first investigated the influence of oxygen on the action of MeDSG. Murine T cell hybridoma 2-45-12 cells were chosen for this study since they are rapidly dividing and extremely sensitive to MeDSG. T cell hybridomas were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with heat-inactivated 10% fetal calf serum (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, and incubated at 37°C in 5% CO_2 humidified air atmosphere. When 2-45-12 cells were treated with MeDSG, FCS was replaced by human AB^+ serum since FCS contains large amounts of polyamine oxidase and may hydrolyze DSG and interfere with its activity. Under 0.1% oxygen concentration, cells were cultured in DULBECCO's modified EAGLE's medium containing 4.5 mg/ml of glucose, 2% human serum, 10 mM HEPES, 1 mM sodium pyruvate, 5×10^{-5} M 2ME and 100 μg/ml uridine, to prevent glucose shortage.

Although a number of studies have reported DSG-mediated inhibition of immune responses *in vitro* at concentrations greater than 50~200 μg/ml, we have used MeDSG at concentrations of 5 to 20 μg/ml, which are thought to be achievable in *in vivo* animal and human pharmacokinetic studies. In our previous studies, when 2-45-12 cells were exposed to 10 μg/ml MeDSG, the cells showed a decline of [^{3}H]-thymidine uptake after 24-hour incubation. Thereafter, the cell viability was gradually reduced and 48 hours later, approximately 60% of the cells underwent cell death *via* apoptosis[5,6]. The concentrations of MeDSG used in this study ranged from 0.0025 to 25 μg/ml, which were lower than those used in our previous studies[5,6]. Cell growth was evaluated by counting the cell number, using a Coulter Counter model ZM (Coulter Electronics Inc., Beds, U.K.).

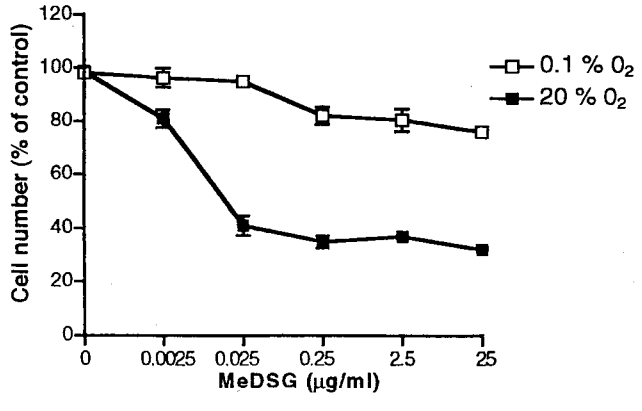
As shown in Fig. 1, when T cell hybridomas were treated with MeDSG under 5% CO_2/95% air for 72 hours, MeDSG inhibited cell growth in a dose-dependent manner. 0.025 μg/ml MeDSG inhibited cell growth by approximately 60%. By contrast, when cells were treated with MeDSG under 5% CO_2/0.1% O_2/94.9% N_2 for 72 hours, the cells exhibited a marked resistance to MeDSG-induced cell growth inhibition. Inhibition of cell growth was hardly detectable in the presence of 0.025 μg/ml MeDSG. Thus, the effects of MeDSG were diminished in the cells cultured under lower oxygen concentrations. The results imply that the action of MeDSG may be dependent on the function of

mitochondrial respiratory chain. However, when T cells were cultured under low oxygen concentrations for 72 hours, the cells exhibited relatively slow growth compared with T cells cultured under the normal condition. Therefore, we cannot exclude the possibility that T cells may become resistant to MeDSG under low oxygen concentrations due to the lack of the rapid growth.

Next, we investigated whether the effects of DSG are dependent on the mitochondrial genome. To examine this, we utilized the cell lines carrying mitochondrial DNA (mtDNA) (ρ^+) and its derivative lacking mtDNA (ρ^0). 143BTK$^-$ was a thymidine kinase-deficient human osteosarcoma cells, and ρ^0 206 was derived from 143BTK$^-$ cells[7]. 701.2.8c was obtained by the transformation of a human fibroblast strain GM701 with the simian virus 40, and ρ^0 701.2a was derived from 701.2.8c[8]. 143BTK$^-$ and 701.2.8c cells were maintained in DULBECCO's modified EAGLE's medium containing 4.5 mg/ml of glucose and 10% FCS. On the other hand, ρ^0 cells depleted of mtDNA were grown in the same medium supplemented with 100 μg/ml uridine and 1 mM sodium pyruvate in order to prevent cell death through a decline in intracellular ATP level and to prevent a reduction of pyrimidine biosynthesis by inhibition of the mitochondrial dihydro-orotase dehydrogenase complex. When these cell lines were treated with MeDSG, FCS was replaced by horse serum. In our previous studies, MeDSG enhanced mitochondrial reduction of MTT during apoptosis in growing cells[6]. Therefore, the effects of MeDSG on cell growth and MTT reduction were examined using cell lines carrying mtDNA (ρ^+) and its derivative lacking mtDNA (ρ^0). Since the osteosarcoma cells and the fibroblast cells were grown slowly, the concentrations of MeDSG used for these cells were higher than those in the experiments of Fig. 1, and the evaluation was carried out at 120 hours of cultivation. The cell growth was determined as described in the experiments of Fig. 1, and MTT colorimertic assay was performed as described previously[6].

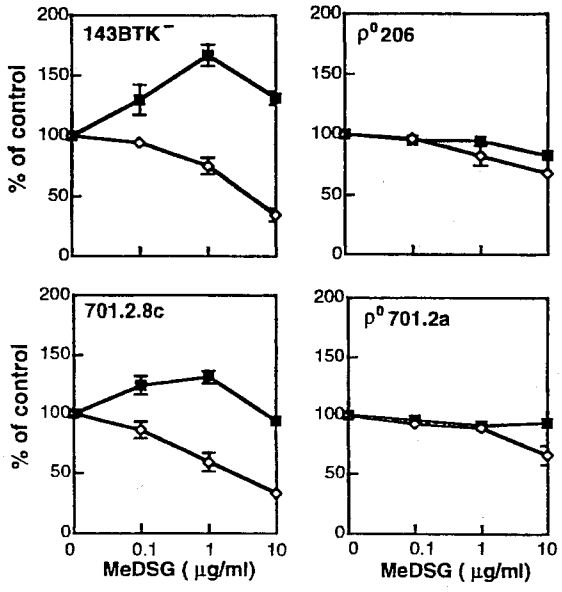
The results are presented in Fig. 2. When parental cells, 143BTK$^-$ or 701.2.8c were treated with MeDSG for 120 hours, these growth were markedly inhibited. Moreover, an

Fig. 1. The influence of oxygen concentrations on the efficacy of MeDSG.



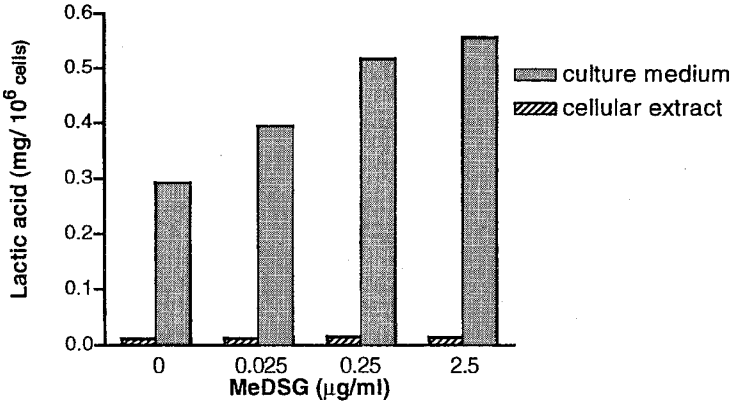
Murine T cell hybridoma 2-45-12 cells (5×10^3 cells/ml) were cultured in assay medium and incubated under 5% CO_2/95% air or 5% CO_2/0.1 % O_2/94.9% N_2 with or without MeDSG for 72 hours. Cell number was evaluated by direct counting. Results are expressed as percentage of the cell number in the absence of MeDSG and represent the mean±SD of triplicate cultures.

Fig. 2. Susceptibility of mtDNA-containing cells and mtDNA-deficient cells to the exposure of MeDSG.



MtDNA-containing cells (143BTK$^-$ and 701.2.8c) or mtDNA deficient cells (ρ^0 206 and ρ^0 701.2a) were seeded in 24-well culture plates at a density of 5×10^3 cells/well for the determination of cell proliferation, and in 96-well culture plates at a density of 1×10^3 cells/well for MTT colorimertic assay, and then these cells were cultured in the presence or absence of MeDSG for 120 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 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. 3. Lactic acid production in MeDSG-treated murine T cell hybridoma 2-45-12 cells.



2-45-12 cells were prepared at concentration of 5×10^4 cells/ml in assay medium and incubated under 5% CO_2 humidified air atmosphere in the presence or absence of MeDSG. 24 hours later, the content of lactic acid in the cellular extract and culture medium was determined. The amounts of lactic acid are expressed as mg/10^6 cells. Data are shown as the mean of triplicate cultures and the SD are less than 10% of the mean.

enhanced formazan generation was observed in MeDSG-treated ρ^+ cells in a dose-dependent manner. On the other hand, when mtDNA-lacking cell lines, ρ^0 206 or ρ^0 701.2a were treated with MeDSG, these cell growth were similar to that of untreated cells. Additionally, MeDSG failed to affect MTT reduction in ρ^0 cells, compared with ρ^+ cells. These results showed that ρ^0 cells lacking functional mitochondrial respiratory chain were resistant to MeDSG, unlike the parental wild-type ρ^+ cell lines. These findings suggest that MeDSG leads to impairment of mitochondrial respiratory function in growing cells.

If MeDSG induced dysfunction in mitochondrial respiratory chain, the energy metabolism in MeDSG-treated cells might be affected. Therefore, we examined the content of lactic acid in MeDSG-treated T cell hybridomas. 2-45-12 T cells (2×10^5 cells/ml) were plated into 24-well culture clusters and treated with MeDSG in 5% CO_2 humidified air atmosphere. After 24 hours-incubation, the cells were harvested, washed with PBS for 3 times, and suspended in PBS (4×10^6 cells/ml). The cell suspension was exposed to 3 cycles of rapid freezing/thawing, centrifuged at 10,000 rpm and the supernatant was used as cellular extract. This extract or its culture medium was mixed with an equal volume of 0.6 M perchloric acid, placed on ice for 10 minutes, and then centrifuged. The supernatant was neutralized by the addition of potassium hydroxide, placed on ice for 15 minutes, and then filtered. The content of L-lactic acid in the cellular extract or culture medium was measured using a lactic acid assay kit (Boerhinger, Mannheim, Germany)[9].

When 2-45-12 cells were treated with MeDSG for 24 hours, cell growth was inhibited at the concentrations greater than 0.25 μg/ml MeDSG in a dose-dependent manner. However, cell death was not detected within 24 hours even when treated with 10 μg/ml MeDSG (data not shown). Fig. 3 shows that MeDSG induced a significant increase of lactic acid production in the culture medium of 2-45-12 cells in a dose-dependent manner. Intracellular accumulation of lactic acid was undetectable in MeDSG-treated T cells. This indicated that MeDSG-treated cells may release lactic acid into culture medium. An apparent enhancement in lactic acid production was detected even after the exposure of 0.025 μg/ml MeDSG at 24 hours of incubation, at which time the cell proliferation was not different from that of the control cultures, as assessed by [^{3}H]-thymidine uptake (data not shown). We conclude that MeDSG-treated cells produced lactic acid before the cells revealed growth inhibition or apoptosis. Although an antitumor activity of DSG was discovered using mouse leukemia cell line L1210[1], the release of large amounts of lactic acid was detected in the culture medium of L1210 cells exposed to MeDSG for 24 hours (data not shown).

It is reported that mitochondrial respiratory chain inhibitors such as rotenone, antimycin A, and oligomycin induce apoptosis[10]. Unlike tumor cells, quiescent cells are resistant to these reagents[11]. Furthermore, these

mitochondrial respiratory inhibitors do not induce apoptosis in ρ^0 cells that are depleted of functional mitochondrial respiratory chain[10]. Together, the susceptibility of cells to the action of MeDSG appears to resemble the behavior of these mitochondrial respiratory chain inhibitors. MeDSG-mediated apoptosis may be due to inhibition of mitochondrial respiratory function. Since rapidly dividing cells in culture continuously require energy, they may be susceptible to the mitochondrial respiratory chain inhibitors. In MeDSG-treated cells, an enhanced MTT reduction precedes MeDSG-induced apoptosis[5,6]. It has been suggested that MTT is reduced at the ubiquinone and cytochrome b and c` sites of mitochondrial electron transport system[12]. However, recent studies have demonstrated that MTT reduction occurs at multiple cellular sites, mostly at extramitochondria[13]. Since we observed that ρ^0 cells were resistant to MeDSG, dehydrogenases responsible for formazan generation in MeDSG-treated ρ^+ cells may be located in mitochondria. Rotenone, antimycin A or oligomycin inhibits MTT reduction during cell death. Azide that inhibits cytochrome a in mitochondrial complex IV also induces apoptosis, but it also induced a slight enhancement of MTT reduction (ODAKA, C., unpublished data). Although, at present, we are not able to define the mechanism by which MeDSG induces an increase of MTT reduction in growing cells, our findings suggest that MeDSG interferes with the mitochondrial respiratory function in growing cells. Our observations will help to elucidate the mechanism of action of DSG, but further experiments will be necessary to determine how DSG interferes with particular molecule(s) in the complex of mitochondrial respiratory chain.

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References

1) TAKEUCHI, T.; H. IINUMA, S. KUNIMOTO, T. MASUDA, M. ISHIZUKA, M. TAKEUCHI, M. HAMADA, H. NAKAGAWA, S. KONDO & H. UMEZASA: A new antitumor antibiotic, spergualin: isolation and antitumor activity. J. Antibiotics 34: 1619~1621, 1981
2) UMEZAWA, H.; M. ISHIZUKA, T. TAKEUCHI, F. ABE, K. NEMOTO & K. SHIBUYA: Suppression of tissue graft rejection by spergualin. J Antibiotics 38: 283~284, 1985
3) TEPPER, M. A.; S. G. MILNER, C. MAZZUCCO, C. SINGH & S. L. KELLRY: 15-Deoxyspergualin, a novel immunosuppressive drug: studies of the mechanism of action. Ann. N. Y. Acad. Sci. 685: 136~147, 1993
4) NADLER, S. G.; M. A. TEPPER, B. SCHACTER & C. E. MAZZUCCO: Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp 70 family of heat shock proteins. Science 258: 484~486, 1992
5) ODAKA, C.; E. TOYODA & K. NEMOTO: Immunosuppressant deoxyspergualin induces apoptotic cell death in dividing cells. Immunology 95: 370~376, 1998
6) ODAKA, C.; E. TOYODA & K. NEMOTO: Immunosuppressant deoxyspergualin-induced apoptosis is accompanied with an enhanced reduction of tetrazolium salts. J. Antibiotics 52: 92~99, 1999
7) KING, M. P. & G. ATTRARDI: Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 246: 500~503, 1989
8) YONEDA, M.; K. KATSUMATA, M. HAYAKAWA, M. TANAKA & T. OZAWA: Oxygen stress induces an apoptotic cell death associated with fragmentation of mitochondrial genome. Biochem. Biophy. Res. Comm. 209: 723~729, 1995
9) GUTMANN, I. & A.W. WAHLEFELD: Carbohydrates. *In* Methods of Enzymatic Analysis. *Ed.*, H. U. BREGMEYER, pp. 1464~1468, Weinheim/Academic Press Inc., New York and Lodon, 1974
10) WOLVETANG, E. J.; K. L. JOHNSON, K. KRAUER, S. J. RALPH & A. W. LINNANE: Mitochondrial respiratory chain inhibitors induce apoptosis. FEBS. Lett. 339: 40~44, 1994
11) STEFANELLI, C.; F. BONAVITA, I. STANIC, G. FARRUGGIA, E. FALCIERI, L. ROBUFFO, C. PIGNATTI, C. MUSCARI, C. ROSSONI, C. GUARNIERI & C. M. CALDARERA: ATP depletion inhibits glucocorticoid-induced thymocytes apoptosis. Biochem. J. 322: 909~917, 1997
12) SLATER, T. F.; B. SAWYER & U. ATRULI: Studies on succinate-tetrazolium reductase systemIII. Points of couling of four diffrent tetrazolium salts. Biochim. Biophys. Acta 77: 383~393, 1963
13) BERRIDGE, M. V. & A. S. TAN: Characterization of the cellular reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch. Biochem. Biophy. 303: 474~482, 1993