Enhanced Metastatic Potential of Cloned Low-Metastatic Lewis Lung Carcinoma Cells Treated in Vitro with Dimethyl Sulfoxide

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ABSTRACT

Treatment of low-metastatic Lewis lung carcinoma cells (P-29) with dimethyl sulfoxide in vitro enhanced their lung-colonizing ability. The concentration of dimethyl sulfoxide used delayed the in vitro growth of P-29 cells but was not cytotoxic. The arrest and retention in the lung of untreated and dimethyl sulfoxide-treated P-29 cells labeled with 5-3H[125I]iodo-2'-deoxyuridine after injecting them into a tail vein of syngeneic mice were examined. Dimethyl sulfoxide-treated P-29 cells were trapped in the lungs more than untreated cells and were cleared from the lungs more slowly than untreated cells. Treatment of P-29 cells with dimethyl sulfoxide resulted in the increase in their homotypic aggregation and adhesion to plastic culture dishes, monolayers of endothelial cells, and a subendothelial extracellular matrix. This treatment also increased significantly their activities of degradative enzymes, such as glycosidases and cathepsin B, and their production of plasminogen activator. These results indicate that the enhanced lung-colonizing ability of P-29 cells treated with dimethyl sulfoxide is due to the increase in adhesiveness, resulting in arrest and retention of the cells in the lung of the host and in the increase in their degradative enzyme activities. The enhancing effect of dimethyl sulfoxide on the lung-colonizing ability of P-29 cells was found to be reversible.

INTRODUCTION

The process of metastasis involves the release of cells from a primary tumor, then their dissemination to distant sites, their arrest in the microcirculation in organs, and finally their survival and growth into new tumor colonies (12, 22). Both intrinsic factors in tumor cells and host factors are involved in these steps (22, 24). Various tumor models are now available for study of metastasis (22). Using these tumor models, the properties of low- and high-metastatic cells have been compared extensively (12, 22, 24). However, little is known about the initial cellular changes of tumor cells associated with the progression from a low- to a high-metastatic phenotype. For a study of the process by which low-metastatic tumor cells become high-metastatic ones, there is a need to develop a system in which low-metastatic cells become highly metastatic in vitro in a short time.

Dimethyl sulfoxide (DMSO) and other polar compounds have been shown to exert many biological effects on mammalian cells (3, 5, 6, 8, 9, 13, 15, 16). In studying the effect of DMSO on mammalian cells, I found that short-term treatment of cloned low-metastatic Lewis lung carcinoma cells (P-29) with DMSO enhanced their lung-colonizing ability. This paper describes the properties of DMSO-treated P-29 cells. This cell line should prove useful for studies on the changes of tumor cells associated with progression from a low- to a high-metastatic phenotype.

MATERIALS AND METHODS

Reagents. [3H]IdUrd (5 Ci/mg) was purchased from the Radiochemical Centre, Amersham, England. Benzoylcarbonyl-phenylalanylanil-nine-4-methyl-7-coumarylamide was obtained from the Peptide Research Foundation, Osaka, Japan. DMSO was supplied by Wako Pure Chemical Industries, Ltd., Osaka, Japan. Human urokinase was purchased from The Green Cross Corp., Osaka, Japan. All p-nitrophenyl derivatives (p-nitrophenylphosphatase, p-nitrophenol-β-D-mannopyranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-glucuronide, and p-nitrophenyl-2-acetamide-2-deoxy-β-D-glucopyranoside) were supplied by Sigma Chemical Co., St. Louis, MO. Other chemicals were of the highest purity available.

Mice. Six- to 8-week-old inbred C57BL/6 mice were obtained from Shizuoka Agricultural Association for Laboratory Animals, Shizuoka, Japan. They were housed 5 animals to a cage and given food and water ad libitum.

Tumor Cell Line and Cell Culture. Lewis lung carcinoma (3LL) was routinely maintained in C57BL/6 mice by s.c. transfer every 3 to 4 weeks. A primary tumor cell line was established from a single-cell suspension prepared from a s.c. tumor transplant as follows. The tumor tissue was minced with scissors and passed through an 80 mesh stainless steel wire screen. The cells were washed by low-speed centrifugation; seeded in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml); and cultured at 37° in a humidified atmosphere of 5% CO2 in air.

Five days after seeding, the loosely adhering tumor cells were isolated by brief exposure to 0.01% trypsin as described previously (23). The cancer of the primary tumor cells was confirmed by grafting the cells into C57BL/6 mice. The cultured primary tumor cells were doubly cloned in microriters plates (Falcon No. 3040), and one clone (named P-29) was used in this study. When P-29 cells were injected s.c. into syngeneic mice, they grew as well as cultured primary tumor cells, but they showed less spontaneous metastatic ability. They also showed lower lung-colonizing ability than did the primary tumor cells when injected i.v. into syngeneic mice.

Assay of Lung-colonizing Ability. P-29 cells were detached from culture dishes by 10-min treatment with 2 mM EDTA in PBS at 37°. Single-cell suspensions of the cells (1 to 2 x 10^5 cells/0.2 ml HBSS/mouse) were injected into the lateral tail vein of age-matched male C57BL/6 mice. All mice were killed 17 days later, and their lungs were removed, rinsed in water, and fixed overnight in Bouin's solution. The number of lung nodules was determined by counting parietal nodules under a dissecting microscope.

Assay of Arrest and Retention of Tumor Cells in the Lung. P-29 cells were cultured for 5 days in medium with or without 2% DMSO (v/v). In the last 20 hr, the cells were labeled by addition of 0.5 µCi [3H]-IdUrd/ml medium. The cultures were washed with serum-free medium, and the radiolabeled cells were harvested by 10 min treatment with 2 mM EDTA in PBS, washed 3 times with HBSS to remove unbound radiolabel, and resuspended in HBSS. Single viable cells were determined...
by the trypan blue dye exclusion test and adjusted to 5 \times 10^5 cells/ml HBSS. The radiolabeled untreated and DMSO-treated P-29 cells were injected as single-cell suspensions in 0.2 ml HBSS into the lateral tail vein of age-matched male C57BL/6 mice. After 10, 60, and 180 min and 24 and 72 hr, the animals were killed, and their lungs were removed. The lungs were placed in 70% ethanol, which was replaced once daily for 3 days to remove essentially all ethanol-soluble [3H]. Then, radioactivity was counted (14).

Enzyme Assays. For lysosomal enzyme assays, P-29 cells were cultured for 5 days in medium with or without 2% DMSO (v/v). The untreated and DMSO-treated P-29 cells were soaked off the dishes with a rubber policeman and washed 3 times with chilled PBS. Then, they were resuspended in a small amount of PBS, rapidly frozen at −20°C, and used as an enzyme source. The activities of acid phosphatase, β-o-galactosidase, β-o-glucuronidase, α-o-mannosidase, and N-acetyl-β-o-glucosaminidase were determined as described previously (10) after addition of p-nitrophenyl derivatives as substrates. β-Nitrophenol was used as a standard. Cathepsin B activity was determined by fluorometric assay using benzoylloxycarbamylphenylalanine-glycine-4-methyl-7-coumarylamide as a substrate (2). One unit of activity is defined as the quantity releasing 1 nmol of 7-amino-4-methylcoumarin/min. Protein was determined by the method of Lowry et al. (19) with crystalline bovine serum albumin as a standard.

For plasminogen activator, P-29 cells were cultured in medium with or without 2% DMSO (v/v) for 5 days. The untreated and DMSO-treated P-29 cells were further cultured in serum-free medium for 24 hr. Plasminogen activator secreted by the untreated or DMSO-treated cells into serum-free medium was measured as described previously (27, 28). Briefly, the samples (10 μl) were applied to wells cut in opaque casein-containing agarose (1%) gels with (2 Mg/ml) and without plasminogen. A human urokinase preparation was used as a standard. Sodium dodecyl sulfate (0.3%) was added to the samples to destroy possible inhibitors of plasminogen activator. Plasminogen-dependent activity was calculated by subtraction of degradation without plasminogen.

Assay of Homotypic Aggregation. Homotypic aggregation of the cells was measured by incubating single cell suspensions (5 \times 10^5 cells/ml) at 37°C in serum-free medium. The cell suspensions were gently agitated, duplicate samples were collected at specific times, and the number of single cells was determined in a hemocytometer (17).

Culture of Endothelial Cells and Preparation of Dishes Coated with Subendothelial Matrix. Bovine pulmonary arterial endothelial cells were obtained from the American Type Culture Collection, Rockville, MD. The cells were cultured in a humidified atmosphere of 5% CO2 in air in Dulbecco’s modified Eagle’s minimal essential medium containing 20% fetal bovine serum. The cells were maintained in monolayer culture and subcultured weekly. Subendothelial extracellular matrix was prepared as described previously (35). Briefly, the confluent cultures were washed once with PBS and exposed to 0.5% Triton X-100 in PBS (v/v) for 30 min with gentle shaking at room temperature. Remaining nuclei and cytoskeletons were removed by 2-3 min exposure to 0.025 N NaOH followed by 4 washes with PBS.

Assay of Adhesion. P-29 cells were cultured for 5 days in medium with or without 2% DMSO (v/v). In the last 20 hr, the cells were radiolabeled by addition of 0.5 μCi [125I]ldUrd/ml medium. The cells were detached from culture dishes by 10-min treatment with 2 mM EDTA, washed 3 times with PBS, and resuspended in complete medium. They (5 \times 10^5 cells/2 ml medium) were then introduced onto completely confluent monolayers of bovine pulmonary arterial endothelial cells, a subendothelial extracellular matrix, or plastic culture dishes (35-mm diameter) and allowed to adhere without agitation at 37°C. At the indicated times, the unattached cells were carefully removed by 3 washings with 2 ml of warm PBS, and the remaining adherent cells were lysed with 1 ml of 0.1 N NaOH. The lysate was collected, and its radioactivity was measured.

**Effect of DMSO on Metastatic Potential**

**Detachment Assay.** P-29 cells were treated with 2% DMSO (v/v) for 96 hr. Untreated and DMSO-treated P-29 cells (3 \times 10^5 cells) were introduced into 35-mm-diameter dishes (Falcon No. 3001) and allowed to become attached and grow for 24 hr in medium with or without 2% DMSO. Then, the plates were washed with serum-free medium and treated with 0.1% trypsin (Difco; 1:250). The plates were placed on an orbital shaker rotating at 60 rpm. At the indicated times, the cells released into the supernatant fluid were collected and counted in a Model 2B Coulter Counter. After 5 min, all the cells that were still attached were detached by vigorous pipetting and counted. The number of cells released at each time was calculated as a percentage of the total cell number per culture dish.

**RESULTS**

**Effects of DMSO Treatment on Lung-colonizing Ability of P-29 Cells.** I examined the lung-colonizing ability of P-29 cells after treatment with DMSO in vitro. For this, I treated the cells with 2% DMSO (v/v) for 1, 3, 5, and 7 days; injected them i.v. into syngeneic mice at a concentration of 2 \times 10^5 cells/mouse; and examined lung nodules 17 days later. As shown in Chart 1a, untreated P-29 cells formed a few lung nodules, while DMSO-treated P-29 cells formed many, their number depending on the duration of treatment. Furthermore, when P-29 cells were treated with various concentrations of DMSO for 5 days before injection into mice, the number of lung nodules increased markedly with the dose of DMSO used (Chart 1b). DMSO (2%) delayed the *in vitro* growth of P-29 cells but was not cytotoxic (Chart 2).

**Arrest and Retention of Radiolabeled Untreated and DMSO-treated P-29 Cells in the Lungs.** Next, I examined the arrest and retention in the lung of DMSO-treated and untreated P-29 cells labeled with [125I]ldUrd after injecting them into a tail vein. Chart 3 shows that DMSO-treated P-29 cells were trapped in the lung more than untreated cells. Therefore, they were cleared from the lungs slower than untreated cells; 3 hr after injection, the percentages of DMSO-treated and untreated cells retained in the lungs were 24.5 ± 1.8% (S.D.) and 1.2 ± 0.5%, respectively, and at all times up to 72 hr after their injection, the percentage of retention of DMSO-treated P-29 cells was higher than that of untreated cells.

**Adhesiveness of Untreated and DMSO-treated P-29 Cells.** Tumor cell arrest or attachment to the vascular endothelium is a critical step in blood-borne metastasis (22, 24, 26). Homotypic aggregation may facilitate the initial arrest of tumor cells in a target organ. In fact, it has been shown that cells with high metastatic potential form more and larger aggregates than do cells with a low metastatic potential (22, 24, 26). It has also been shown that increased tumor cell adhesion to endothelial cells and to noncellular foreign surfaces *in vitro* is correlated with an increased metastatic potential (22, 24, 26). The results in Chart 3 showing increased initial arrest and subsequent retention of DMSO-treated P-29 cells suggest increased adhesiveness of the cells. Therefore, I next compared the degrees of homotypic aggregation and adhesion of untreated and DMSO-treated P-29 cells to monolayers of endothelial cells, a subendothelial extracellular matrix, and plastic culture dishes. Results showed that DMSO-treated P-29 cells aggregated more rapidly than did untreated cells (Chart 4) and adhered more rapidly to monolayers of endothelial cells (Chart 5a), a subendothelial extracellular matrix (Chart 5b), and plastic culture dishes (Chart 5c). I examined the firmness of their adhesion to culture dishes by trypsin-mediated detachment assay. Chart 6 shows that DMSO-treated
P-29 cells adhered more firmly than did untreated cells. Thus, DMSO-treated P-29 cells adhered both more rapidly and more firmly to foreign surfaces than did untreated cells.

**Effect of DMSO Treatment on Degradative Enzyme Activities of P-29 Cells.** A marked change occurring after neoplastic transformation is increase in degradative enzymes (22, 24, 26). These enzymes may facilitate invasion of malignant cells at primary and secondary sites by degrading the extracellular matrix. Then, I next measured the degradative enzyme activities of cell lysates of untreated and DMSO-treated P-29 cells. I also measured the production of plasminogen activator by untreated and DMSO-treated P-29 cells. The results in Table 1 show that treatment of P-29 cells with 2% DMSO (v/v) for 5 days resulted in significant increases in cellular acid phosphatase, ß-o-galactosidase, ß-o-glucuronidase, N-acetyl-ß-o-glucosaminidase, and cathepsin B activity, but not in ß-o-mannosidase activity. DMSO-treated P-29 cells also secreted much more plasminogen activator than did untreated cells.

**Effect of DMSO Treatment on Morphology of P-29 Cells.** The morphological change of P-29 cells after treatment with DMSO is shown in Fig. 1. Most untreated P-29 cells were round (Fig. 1a). When P-29 cells were treated with DMSO, they became flatter (Fig. 1b). This morphological change was apparent after 24-hr treatment with DMSO. However, after removal of DMSO, P-29 cells gradually became round again (Fig. 1c). Therefore, the effect of DMSO on the morphology of P-29 cells was reversible.
Bars. S.D. The level of significance of difference between values is shown.

For 24 hr in medium with or without 2% DMSO. Then, the plates were washed with serum-free medium and treated with 0.1% trypsin. O, untreated; •, DMSO treated.

Untreated and DMSO-treated cells (3x10^6 cells) were introduced into 35-mm-diameter dishes and allowed to become attached and grow in plastic culture dishes to detachment by trypsin. P-29 cells were treated with 2% DMSO (v/v) for 96 hr. Untreated and DMSO-treated cells with high lung-colonizing ability on treatment with DMSO seemed to be due to increases in adhesiveness, resulting in their arrest and retention in the lung of the host and increase in their degradative enzyme activities. Since the concentration of DMSO used in this study was not cytotoxic to P-29 cells, the increase of cells with high lung-colonizing ability on treatment with DMSO was not the result of selection of a subpopulation with high lung-colonizing ability by killing a subpopulation with low lung-colonizing ability.

Treatment of P-29 cells with DMSO increased their lung-colonizing ability. This increase seemed to be due to increases in adhesiveness, resulting in their arrest and retention in the lung of the host and increase in their degradative enzyme activities. Since the concentration of DMSO used in this study was not cytotoxic to P-29 cells, the increase of cells with high lung-colonizing ability on treatment with DMSO was not the result of selection of a subpopulation with high lung-colonizing ability by killing a subpopulation with low lung-colonizing ability.

Treatment of P-29 cells with DMSO resulted in significant increases in homotypic aggregation and adhesions to the surface of culture dishes, a subendothelial extracellular matrix, and monolayers of endothelial cells. The biochemical basis of the enhancing effect of DMSO on adhesiveness of P-29 cells is not clear at present. There are many reports that cell surface sialic acid (7, 11, 21), laminin (32, 33, 34), and glycosaminoglycans (22) affect cell adhesiveness. DMSO has been shown to influence the distribution of cell surface sialic acid (3) and glycosaminoglycans (6). Therefore, DMSO may alter the synthesis and distribution of these components of P-29 cells. Further studies are needed to clarify this possibility.

Treatment of P-29 cells with DMSO resulted in marked increases in degradative enzyme activities and in production of plasminogen activator. Glycosidases, cathepsin B, and plasminogen activator may contribute to the degradation of the subendothelial matrix (22). Cathepsin B can degrade pericellular protein (30) and collagen (4) at neutral pH. Plasmin can directly degrade fibronectin (1) and laminin (18). Recently, Wang et al. (36) observed a positive correlation between the production of plasminogen activator and the metastatic potential, and Sloane et al. (29) observed a positive correlation between cathepsin B activity and the metastatic potential in B16-mouse melanoma cell lines. Therefore, the increase in degradative enzyme activities of P-29 cells after treatment with DMSO may contribute to their enhanced lung-colonizing ability.

It should be stressed that DMSO-treated P-29 cells became more malignant (more metastatic) rather than more benign. DMSO and other polar compounds have been shown to cause alterations in the growth and morphology of mammalian cells (8, 9, 16), and the changes reported are relevant to the phenomenon of "reverse transformation," i.e., restoration of density-dependent growth inhibition, loss of growth in semisolid medium, and...
marked reduction in tumorigenicity. Moreover, DMSO has been reported to induce differentiation of mouse erythroleukemia cells (13), mouse neuroblastoma cells (15), and human promyelocytic leukemia HL-60 cells (5). Therefore, the effect of DMSO on P-29 cells is in the opposite direction.

The enhancing effect of DMSO on lung-colonizing ability of P-29 cells was reversible. Therefore, it is conceivable that the increased lung-colonizing potential after treatment of P-29 cells with DMSO is the consequence of epigenetic rather than genetic alterations. Polar solvents, such as DMSO, have cryoprotective effects (25). This had led to the suggestion that the primary target of action of DMSO may be the cell membrane (20). Another possibility is that this freely diffusible compound may act directly in the nucleus, somehow altering the conformation of DNA/chromatin complexes in such a way as to initiate transcription of genes (31).

Although the effect of DMSO on P-29 cells was reversible, DMSO-treated cells exhibited several phenotypes favorable for completing the complicated process of metastasis. These phenotypes have been shown to be positively correlated with metastatic ability in several cell systems (22). Therefore, investigating the present experimental model might enable the detection of initial cellular changes in tumorigenic cells associated with progression from a low- to a high-metastatic phenotype.

REFERENCES

Fig. 1. Morphological change of P-29 cells treated with DMSO. a, untreated P-29 cells. × 100. b, P-29 cells cultured in medium with 2% DMSO for 5 days. × 100. c, P-29 cells cultured in medium with 2% DMSO for 5 days and in regular medium for a further 5 days. × 100.
Enhanced Metastatic Potential of Cloned Low-Metastatic Lewis Lung Carcinoma Cells Treated \textit{in Vitro} with Dimethyl Sulfoxide

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