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Antitumor resistance induced by zinostatin stimalamer (ZSS), a polymer-conjugated neocarzinostatin (NCS) derivative

I. Meth A tumor eradication and tumor-neutralizing activity in mice pretreated with ZSS or NCS

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Abstract Zinostatin stimalamer (ZSS) is a new anticancer agent derived from neocarzinostatin (NCS), which is synthesized by conjugation of one molecule of NCS and two molecules of poly(styrene-co-maleic acid). ZSS exhibited potent in vitro and in vivo antitumor activity in preclinical experiments, and a clinical trial of the intra-arterial administration of ZSS with iodized oil on hepatocellular carcinoma showed potent antitumor activity. We investigated the effect of ZSS and NCS on antitumor resistance and found that pretreatment with either drug suppressed the growth of MethA tumors in Balb/c mice and induced tumor eradication when given separately by single administration at therapeutic doses between 1 day and 4 weeks before tumor transplantation. The findings that the cytotoxic activity of these drugs was not detected in vivo at the time of tumor transplantation and that tumor regression was preceded by a period of transient growth suggested that tumor regression was due to host-mediated antitumor activity induced by these drugs. Pretreatment with ZSS or NCS also suppressed the growth of Colon 26 carcinoma and Sarcoma 180. The finding that NCS showed the same effect as ZSS suggests that poly(styrene-co-maleic acid) is not essential for the induction of host-mediated antitumor activity. Furthermore, apo-ZSS, which lacks cytotoxic activity, did not induce antitumor activity. From this, it is suggested that the cytotoxic effect of ZSS involves the induction of host-mediated antitumor resistance. In athymic Balb/c nu/nu mice, pretreatment with ZSS or NCS did not induce tumor eradication, suggesting that mature T lymphocytes play an important role in tumor eradication. Challenging MethA was rejected without transient growth in mice that had been cured of MethA, but challenging Colon 26 was not, showing that anti-MethA resistance was augmented selectively in the MethA-eradicated mice. Splenocytes from MethA-bearing mice pretreated with the drug showed tumor-neutralizing activity beginning 14 days after tumor transplantation. Tumor-neutralizing activity was only induced after MethA transplantation. The effector cells of this tumor-neutralizing activity were Thy1.2+ T lymphocytes that had been passed through a nylon-wool column, but no significant augmentation of cell-mediated cytotoxic activity of splenocytes from MethA-eradicated mice was observed in vitro.

Key words Zinostatin stimalamer · Neocarzinostatin Antitumor resistance · Tumor-neutralizing activity MethA

Introduction

Zinostatin stimalamer (ZSS) is a polymer-conjugated drug, recently marketed as a chemotherapeutic agent for the treatment of liver cancer in Japan. ZSS (average molecular mass 15 kDa) was synthesized by conjugation with one molecule of neocarzinostatin (NCS), a proteinaceous antitumor agent (molecular mass 11753 Da) [10, 15], and two molecules of poly(styrene-co-maleic acid) (average molecular mass 2500 Da). ZSS has superior stability in plasma and tissues and shows more efficient accumulation in tumor tissue compared with NCS [16, 18]. ZSS exhibited potent in vitro and in vivo antitumor activity against various human and murine tumor cells by inhibiting DNA synthesis [26, 33]. ZSS has a narrower molecular mass distribution than SMANCS, a similar agent in early stages of development [17, 27].
Most anticancer chemotherapeutic agents induce immunosuppression by antiproliferative action. For the purpose of examining the effects of ZSS and NCS on antitumor defense, pretreatment with these drugs before tumor transplantation was conducted in order to exclude any direct cytotoxic effect. If these drugs did not augment antitumor resistance and depressed the host defense systems, tumor growth would have been accelerated in the pretreated mice compared with controls. However, we found that pretreatment with ZSS or NCS induced tumor growth inhibition and tumor eradication. This study was conducted to clarify the mechanism of the antitumor action of ZSS and NCS.

Materials and methods

Animals and tumor cells

Female Balb/c, male athymic Balb/c nu/nu, male ICR, and male and female C57BL/6, and male and female DBA mice, all aged 6–8 weeks, were purchased from Japan SLC (Shizuoka, Japan). MethA, a methylcholanthrene-induced fibrosarcoma, and Colon 26, an N-nitroso-N-methylurethane-induced undifferentiated adenocarcinoma, were maintained by intraperitoneal and subcutaneous passage, respectively, in Balb/c mice. Sarcoma 180, a mouse sarcoma, EL4, a 9,10-dimethyl-1,2-benzanthracene-induced lymphoma, and P815, a mastocytoma, were maintained by intraperitoneal passage in ICR, C57BL/6/N and DBA/2 mice respectively. YAC-1, a Moloney-leukemia virus-induced lymphoma, was maintained in culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Commonwealth Serum Lab., Melbourne). For the measurement of antitumor activity, MethA or Colon 26 cells were inoculated subcutaneously into Balb/c or Balb/c nu/nu mice, and Sarcoma 180 cells were inoculated subcutaneously into ICR mice in the flank. EL4, P815, YAC-1 and MethA were used as target cells in the cell-mediated cytotoxicity assay.

Drugs

Zinostatin stimalamer (ZSS, Yamanouchi Pharmaceutical, Tokyo) and neocarzinostatin (NCS, Kayaku, Tokyo) were dissolved in sterile phosphate-buffered saline (PBS) just before use. Preparation and intravenous administration of these drugs were performed under subdued lighting to prevent light inactivation. Apo-ZSS, which has no active prosthetic groups and no cytocidal activity, was subdued in ICR, C57BL/6N and DBA/2 mice respectively. YAC-1, a Moloney-leukemia virus-induced lymphoma, was maintained in culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Commonwealth Serum Lab., Melbourne). For the measurement of antitumor activity, MethA or Colon 26 cells were inoculated subcutaneously into Balb/c or Balb/c nu/nu mice, and Sarcoma 180 cells were inoculated subcutaneously into ICR mice in the flank. EL4, P815, YAC-1 and MethA were used as target cells in the cell-mediated cytotoxicity assay.

Antitumor resistance after tumor eradication

Balb/c mice were treated with 2.5 mg/kg ZSS or 1 mg/kg NCS 3 days before transplantation of MethA (7.5 × 10^5 cells, day 0). On day 29, cured mice were divided into two groups, and transplanted subcutaneously with MethA (7.5 × 10^5 cells) or Colon 26 (2 × 2 mm block) as tumor challenge studies. Tumor weights were determined as described above. Balb/c mice that had not been pretreated were transplanted with MethA or Colon 26 at the time of tumor challenge were used as a control.

Preparation and fractionation of spleen cells

Spleens were aseptically removed from normal mice, MethA-bearing mice or drug-pretreated MethA-bearing mice, and splenocytes were prepared as described previously [19]. The cells were treated with TRIS-buffered ammonium chloride (0.83%) to deplete erythrocytes and were washed three times with RPMI-1640 medium.

To remove T lymphocytes, splenocytes were treated with anti-Thyl.2 monoclonal antibody (Cedarlane Labs, Ontario) and Low-Tox-M rabbit complement (Cedarlane Labs) for 1 h at 37°C. Cells recovered in experiment 1 were examined by flow cytometry. This fraction contained 12.8% L3T4 dull-positive cells and 0.8% Lyt2.2-positive cells (Thyl.2-negative fraction).

For enrichment of T lymphocytes, splenocytes were treated with a nylon-wool column. Nylons were packed into a syringe (0.3 g) and autoclaved. Splenocytes (10^6 cells/ml in minimal essential medium, MEM, with 10% heat-inactivated FBS) were loaded on the nylon-wool column and incubated for 1 h at 37°C. Cells that passed through the column after addition of MEM medium with 10% heat-inactivated FBS were collected. L3T4-positive cells, Lyt2.2-positive cells and double-positive cells (L3T4 + Lyt2.2 + ) were collected as experiment 1 fraction represented 61.5%, 26.2% and 0.7% of cells in experiment 1, and 59.5%, 27.5% and 0.8% of cells in experiment 2 respectively.

For flow-cytometric analysis, cells were stained with fluorescein-isothiocyanate-conjugated anti-L3T4 monoclonal antibody and phycoerythrin-conjugated anti-L3T4 monoclonal antibody (Becton Dickinson, Mountain View, Calif.) for 15 min at 4°C. After washing, more than 5 × 10^6 cells were analyzed using an Epics Profile (Coulter Electronics, Hialeah, Fla.). The mean percentages of L3T4 +, Lyt2.2 + and double-positive cells (L3T4 +, Lyt2.2 +) in this fraction represented 61.5%, 26.2% and 0.7% of cells in experiment 1, and 59.5%, 27.5% and 0.8% of cells in experiment 2 respectively.

In vivo tumor neutralization assay (Winn assay)

A modified Winn assay [34] was used. Effector cells (splenocytes) were mixed with the target cells (5 × 10^6 MethA cells) at the effector:target ratios of 50:1 and 25:1, and inoculated into Balb/c or Balb/c nu/nu mice. Tumor diameters were measured and tumor weights were calculated as described above.

Cell-mediated cytotoxicity assay

Natural killer (NK) cell activity, lymphokine-activated-killer (LAK)-like activity and tumor-specific T cell activity were determined by a 4-h 51Cr-release assay using YAC-1, EL4 and P815, and MethA cells as the target respectively. Non-specific monocyte cytotoxicity was measured by an 18-h 51Cr-release assay using NCS or P815 cells as the target. Target cells for the cytotoxicity assay (1 × 10^6 cells) were labelled with 37 kBq or 74 kBq sodium [51Cr]chromate (Amersham Japan, Tokyo) for 1 h at 37°C and washed three times with RPMI-1640 medium with 10% heat-inactivated FBS. The cells were labelled with 37 kBq or 74 kBq sodium [51Cr]chromate (Amersham Japan, Tokyo) for 1 h at 37°C and washed three times with RPMI-1640 medium with 10% heat-inactivated FBS. The cells were labelled with 37 kBq or 74 kBq sodium [51Cr]chromate (Amersham Japan, Tokyo) for 1 h at 37°C and washed three times with RPMI-1640 medium with 10% heat-inactivated FBS.

Antimarial activity

Mice were treated with or without the anticancer drug once between 1 day and 4 weeks before tumor transplantation (day 0). Tumor diameters were measured twice a week with calipers. Tumor weights were estimated from their volume using the following formula [9]:

\[ \text{tumor volume (mm}^3) = \text{[short diameter (mm)]}^2 \times \text{long diameter (mm)/2} \]

At the end of some experiments, tumors were removed and weighed.

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