Anti-Cancer Drugs 1999, **10**, pp. 229–233

Research paper

Inhibitory effect of ND2001 on spontaneous multiple metastasis of NC 65 tumors derived from human renal cancer cells intradermally transplanted into nude mice

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The NC 65 tumor cell line derived from human renal cell carcinoma was selected from among nine human cell lines by determining the inhibition of invasion by ND2001 (sodium D-glucaro-δ-lactam) *in vitro*. The efficacy of this agent against these tumor cells was investigated in an experimental metastatic model of human tumors *in vivo*. Although ND2001 did not inhibit growth of NC 65 cells intradermally transplanted into male KSN mice (*nu/nu*), this agent inhibited multiple spontaneous metastasis. [© 1999 Lippincott Williams & Wilkins.]

Key words: Antimetastic agents, NC 65, ND2001, nude mice, renal cell carcinoma.

Introduction

Advances in cancer treatment have contributed to an increase in the survival rate of patients by 5 years, but the effectiveness of treatment in patients with advanced tumors is still low, mainly due to the development of distant metastases and failure of local control. We have constructed metastatic models using various human-derived tumors by means of intradermal transplantation to avoid artificial dissemination of the tumor in tissues and to observe the growth rate of tumors as well as the mechanism of metastasis.[1] The models are considered to reflect actual clinical metastasis of advanced-stage cancers more accurately than widely used metastatic models that employ tumors derived from animals of which the growth rate is much more rapid than that of human tumors.

ND2001 (sodium D-glucaro-δ-lactam) showed inhibitory effects on pulmonary metastasis in some animal models without exhibiting cytotoxicity.[2–4] In screening programs for antitumor agents, there are many cases in which some agents show little activity against human-derived tumors though they exhibit strong action against animal tumors. In this study, we investigated the effects of ND2001 on our above-mentioned models. On the other hand, a correlation between the metastasis inhibitory effect of ND2001 and its inhibitory effect on animal tumor cells invasion in the Boyden chamber system has been reported.[2,4]

We also examined this correlation in human-derived tumors.

Materials and methods

Cell lines and culture

Detroit 562 cells (ATCC CCL-138) derived from head and neck carcinoma, KB cells (ATCC CCL-17) from head and neck carcinoma, ACHN cells (ATCC CRL-1611) from renal cell carcinoma (RCC), A-498 cells (ATCC HTB-44) from RCC, NC 65[5] cells from RCC, WiDr cells (ATCC CCL-218) from colon carcinoma, HT-1080 cells (ATCC CCL-121) from fibrosarcoma, G 361 cells (ATCC CRL-1424) from melanoma, and A 375 cells (ATCC CRL-8401) from melanoma were used. The NC 65 cell line was kindly supplied by Professor

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Ken-ichiro Okada (Fukui Medical University). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Flow, McLean, VA) was used as culture medium and the above-mentioned cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Invasion assay

Invasion by tumor cells was assayed using 24-well Transwell cell culture chambers (Costar, Cambridge, MA) according to a method previously reported.[2,6,7] The cultured tumor cells were harvested by treatment with 0.08% trisodium citrate in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}, washed twice with DMEM containing 0.1% bovine serum albumin (Sigma, St Louis, MO) and suspended in the medium. Then 5 μg of Matrigel (Collaborative Research, Bedford, MA) was applied to the upper and lower surfaces of the Nucleopore filter (8.0 μm pore size) of the Boyden chamber. Then 100 μl (10^5 cells) of the cell suspension was added to the upper chamber and 600 μl of 3T3-conditioned medium was added to the lower chamber for cell attraction. In some experiments, 10 μg of laminin (Collaborative Research) was applied to the lower surface as a chemoattractant. In this case, DMEM containing 0.1% bovine serum albumin was placed in the lower chamber. After the chambers were incubated at 37°C for 3–5 h in a humidified atmosphere containing 5% CO_2 and 95% air, the cells on the upper surface of the filter were removed and the filter was cut out. The cells on the lower surface of the filter were fixed, stained and counted under a microscope.

Animals and experimental metastasis

Four-week-old male KSN mice (*nu/nu*) inbred at FUB (Chiba, Japan) were used. NC 65 cells (1×10^5 cells) were intradermally transplanted in the right hind leg. Sixteen days later, the mice in which engraftment was confirmed were divided at random into various groups. From that day, 30, 100 or 300 mg/kg of ND2001, or 0.9% NaCl (vehicle), was administered

Table 1. Inhibition of invasion by human-derived tumor cell lines by ND2001[a]

Experiment	Cell line	Concentration (μg/ml)	Invasion		
			Time (h)	Cell no.[b]	%
1	NC 65	0	5	13.0	100.0
	NC 65	100	5	4.3	33.1
2	NC 65	0	5	19.7	100.0
	NC 65	100	5	9.0	45.7
3	HT-1080	0	4	322.3	100.0
	HT-1080	100	4	321.7	99.8
4	HT-1080	0	3	116.7	100.0
	HT-1080	100	3	129.0	110.5
5	G 361	0	5	14.3	100.0
	G 361	100	5	11.3	79.0
6	G 361	0	4	5.7	100.0
	G 361	100	4	5.0	87.7
7	G 361	0	5	29.3	100.0
	G 361	300	5	18.7	63.8
8	G 361	0	5	17.3	100.0
	G 361	300	5	7.3	42.2
9	ACHN	0	4	160.3	100.0
	ACHN	100	4	131.3	81.9
10	ACHN	0	5	112.7	100.0
	ACHN	100	5	99.0	87.8
11	ACHN	0	5	134.7	100.0
	ACHN	300	5	116.7	86.6
12	ACHN	0	5	143.7	100.0
	ACHN	300	5	88.0	61.2

[a]Refer to the text for the procedure. Laminin was used as the attractant in Experiment 6 and 3T3-conditioned medium in the other experiments. Tumor cells were treated with ND2001 for 2 days (Experiments 2 and 10) or for 3 days (the other experiments).
[b]Per mm^2.

via the tail vein for five consecutive days per week as a rule, excluding holidays. To examine metastasis to various organs, four mice per group were autopsied 29 days after the initiation of the administration (21 doses in total), i.e. 45 days after transplantation. ND2001 was continuously administered to the remaining mice until on 75 days after the initiation of the administration (52 doses in total, 91 days after transplantation). The survival period was determined 120 days after transplantation for five mice in each group.

Statistical analysis

Student's two-tail *t*-test was applied for the evaluation of p values. When a p value was less than 0.05, the difference was considered to be statistically significant.

Results

Inhibition of tumor cell invasion

The assay was feasible for four of the nine cell lines employed: ACHN, NC 65, G 361 and HT-1080. Little or no movement was observed in case of the remaining five cell lines. The cell lines for which the assay was feasible were treated with ND2001 to investigate the effect of ND2001 on their invasion activity. ND2001 showed no cytotoxicity, as previously reported,[2,3] on these four cell lines. As shown in Table 1, ND2001 inhibited invasion by NC 65 cells most strongly. Invasion of the HT-1080 cell line was not inhibited by ND2001. Although 100 μg/ml of ND2001 was insufficient to inhibit invasion of the G 361 cell line, inhibition was clearly observed with 300 μg/ml of ND2001. The inhibitory effect of ND2001 on invasion of ACHN cells was moderate, as in the case of G 361 cells. The inhibitory effect of ND2001 on invasion of human-derived cell lines was milder than that exerted on murine tumor cell lines.[2,3] This tendency was also observed for haptotaxis as previously reported.[8]

Antimetastatic activity

Based on the above-mentioned results, the effect of ND2001 on the development of metastasis by NC 65 cells was investigated. ND2001 did not affect body weight of the mice up to 29 days after the initiation of the administration (Figure 1). ND2001 did not affect tumor growth (Figure 2).

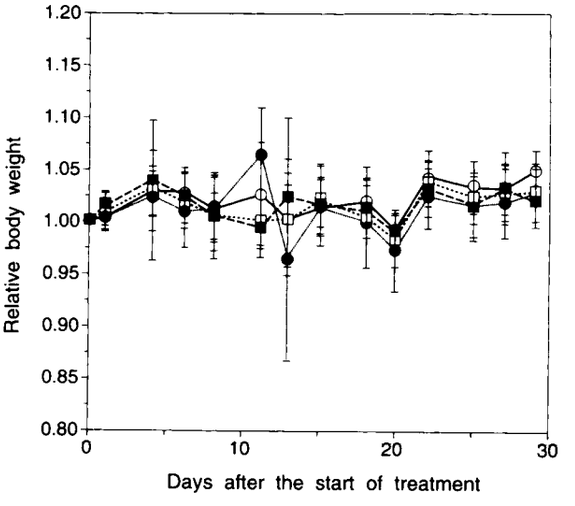

Figure 1. Effect of ND2001 on body weight of KSN nude mice transplanted with NC 65 cells. Treatment: ○, vehicle (0.9% NaCl); ●, 30 mg/kg ND2001; □, 100 mg/kg ND2001; ■, 300 mg/kg ND2001. Points, mean; bars, SD. There were no significant differences between the control (vehicle) group and the ND2001 administration groups.

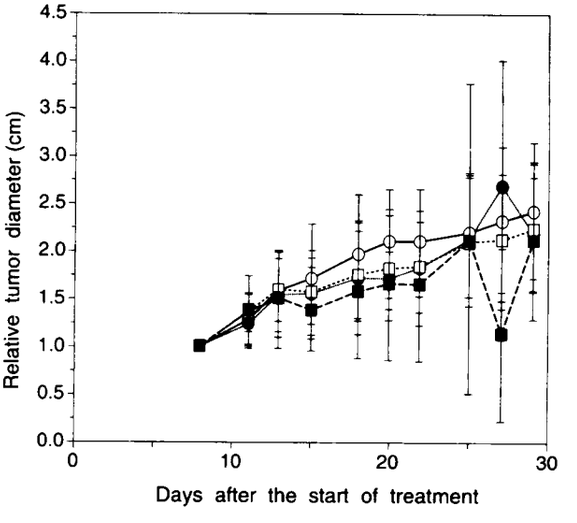

Figure 2. Effect of ND2001 on tumor growth in KSN nude mice transplanted with NC 65 cells. Treatment: ○, vehicle (0.9% NaCl); ●, 30 mg/kg ND2001; □, 100 mg/kg ND2001; ■, 300 mg/kg ND2001. Points, mean; bars, SD. There were no significant differences between the control (vehicle) group and the ND2001 administration groups.

Pulmonary metastases were observed in all mice in the control group but the number of mice with pulmonary metastases was reduced in the 100 and 300 mg/kg ND2001 administration groups (Table 2).

Table 2. Effect of ND2001 on the development of spontaneous metastases from NC 65 tumors after intradermal transplantation into the right hind legs of KSN nude mice

Treatment (mg/kg)	No. of metastasized mice/no. of mice used					Inguinal lymph nodes (mg)
	Lungs	Spleen	Kidneys	Liver	Inguinal lymph nodes	
0	4/4	0/4	0/4	0/4	3/4	2.3
30	3/4	0/4	0/4	0/4	0/4*	2.0
100	1/4*	0/4	0/4	0/4	0/4*	>1.5
300	1/4*	0/4	0/4	0/4	0/4*	0.5

*$p<0.05$, compared with the control group (Student's two-tailed *t*-test).

No clear difference was observed, however, in the average number of pulmonary metastases. On the other hand, metastases to the inguinal lymph nodes were observed in three of the four mice in the control group but not in the ND2001 administration groups (Table 2). No metastases to the spleen, kidneys or liver were observed in the control group or the drug administration groups.

Two of the five mice in the control group died by 120 days after transplantation, while only one mouse died in the ND2001 administration group. One mouse died by accident in the 300 mg/kg administration group.

Since ND2001 did not inhibit tumor growth (Figure 2) but did inhibit the development of multiple metastases (Table 2), we consider that the increased survival rate after administration of ND2001 was not caused by its inhibitory effect on tumor growth, but by its inhibitory effect on the development of metastases.

Discussion

Many reports have been published concerning the mechanisms of cancer metastases.[9-11] Various strategies against metastasis have been tested using mouse tumors which show rather rapid growth resulting in a short lifespan of the host mice or human cancer xenografts obtained through orthotopic transplantation. In these systems, the original phenotypes are well preserved and the type of metastasis is similar to that of the original cancers. However, tumor growth is not easy to observe and mechanical procedures may induce tumor growth and metastases. Some inhibitors of metastasis have been shown to suppress tumor growth as well as metastasis. In this respect, the ectopic transplantation model may be useful to evaluate the activity of metastasis inhibitors which may also suppress tumor growth.

The NC 65 tumor cell line was selected from among nine human cell lines by determining the inhibition of invasion by ND2001 and the efficacy of this agent against these tumor cells was investigated in an experimental metastatic model of human tumors. There are two types of tumors, those which are sensitive to ND2001 and those which are not.[2,4] As the inhibitory effect of ND2001 on metastasis was shown in the nude mouse model, the invasion assay could be a useful predictive method to assess the sensitivity of cells to this agent. However, the invasive activity could not be determined in four cell lines in this study, because it is difficult to prepare cells for the assay. More convenient methods might be necessary when clinical application is considered.

RCC cells have been shown to be highly metastatic, especially via the hematogenous route. Despite various attempts to improve treatment for RCC, such as interferon or LAK plus interleukin-2, the 5-year survival rate after multimodel therapy for patients with RCC in the advanced stages of the disease is low. The most important prognostic factor of RCC is its invasive or metastatic potential.[12] RCC is also very radioresistant irrespective of its hypervascularity.

It is considered that ND2001 inhibits the step of extravasation in the metastatic process, which begins with the release of tumor cells from the primary tumor.[2,8] MMP-2 and MMP-9 play important roles in tumor cell invasion.[13,14] It was revealed by gelatin zymograms of sections of NC 65 tumors extirpated from KSN nude mice after 2 weeks of administration of 800 mg/kg/day ND2001, that this agent did not suppress the activity of these enzymes (data not shown). Thus, ND2001 may not modulate the activity or expression of MMP which contributes to the invasive process.

Although the inhibitory effect of ND2001 on the development of metastases could be demonstrated in our nude mouse model using human-derived tumor

cell lines, various therapies, including drugs with different mechanisms of action, and different modes of treatments such as radiation therapy, seem to be required in clinical practice to prevent the development of metastases and to treat them.[15] It appears to be important to investigate the effects of the above-mentioned combination therapies using this model in the future.

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(Received 6 October 1998; accepted 13 October 1998)